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Patterns of presence and concentration of pesticides in fish and waters of the Júcar River (Eastern Spain).

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Abstract

The Júcar River, in a typical Mediterranean Basin, is expected to suffer a decline in water quality and quantity as a consequence of the climate change. This study is focused on the presence and distribution of pesticides in water and fish, using the first extensive optimization and application of the QuEChERS method to determine pesticides in river fish. Majority pesticides in water –in terms of presence and concentration- were dichlofenthion, chlorfenvinphos, imazalil, pyriproxyfen and prochloraz (associated with a frequent use in farming activities), as well as buprofezin, chlopyriphos and hexythiaxoz. In fish, the main compounds were azinphos-ethyl, chlorpyriphos, diazinon, dimethoate and ethion. The analysis of bio-concentration in fish indicated differences by species. The maximum average concentration was detected in European eel (a critically endangered fish species). The wide presence of pesticides in water and fish suggests potential severe effects on fish populations and other biota in future scenarios of climate change, in a river basin with several endemic and endangered fish species. The potential effects of pesticides in combination with multiple stressors require further research to prioritize the management of specific chemicals and suggest effective restoration actions at the basin scale.

Key words:

Organophosphorus compounds; Bioaccumulation; River water; Fish condition; QuEChERS; LC-MS/MS; Mediterranean Rivers

1. Introduction

Rivers around the world are threatened by socioeconomic drivers that degrade environmental conditions by altering land use and climate, thereby affecting hydrology and water quality [1, 2]. Climate change and human use both pose threats to the flow regime of water ecosystems, and altered flow regimes can have a high impact on the ecological and chemical status of waters [3]. In order to repair this situation, the European Parliament established the Water Framework Directive in 2000. Its ultimate objective is to achieve “good ecological and chemical status” for all Community waters by 2015. For this, priority substances (some of them pesticides) to be monitored and their limits have been established to control the pollution in surface waters [4].

However, the first round of the River Basin Management Plans in the EU show that more than half of Europe's surface water bodies are in less than good ecological status, and the reports about the Habitat Directive indicate that over two thirds of all river and lake habitats and inland water species are in unfavourable conservation status [3]. Furthermore, some regions of the EU are at risk of water scarcity, and the water ecosystems services upon which society depends may become more vulnerable to extreme events such as floods and droughts [5].

In the Júcar River basin (Spain), the last nationwide report on climate change estimated a 10–25% reduction of the mean annual flow [6], which indicates potential notable effects on water availability. Therefore, a reduction of water quality, which would produce severe risks for the ecosystem integrity, is probable [7]. Von der Ohe et al. [8] analyzed waters in four European rivers (including the Llobregat River in Spain), reporting that most of the high and very high risk substances detected were pesticides (74%). They reported that pollution with organic chemicals is a Europe-wide problem.

In a previous study on contaminants in Spain, different pesticides were detected, in the Duero, Ebro and Miño River basins (in decreasing order of quantity and concentrations) [9]. However, a review on the monitoring programs indicated that the analytical methods for most compounds were not sufficiently developed to consistently detect their often very low concentrations in the environment [10]. This lack of unified sample preparation and analytical methods in environmental matrices other than water and in particular in biota has been widely remarked in several reviews [11, 12]. As a quick, easy, cheap, effective, rugged and safe sample preparation method, the QuEChERS method has attracted great attention for pesticide residue determination in fruit and vegetables. Recently, QuEChERS method was also applied on fish to detect pyrethrin and pyrethroid pesticides [13], as well as for the most commonly applied pesticides for cereals and oleaginous crops in France [14]. However, complementary research is needed to determine a wider range of pesticides in fish.

In this context, the aims of this study were: i) to test the effectiveness of the QuEChERS method for determining the presence and concentration of pesticides in freshwater fish; ii) to establish general patterns of presence and concentration of pesticides in water and fish along the Júcar River; and iii) to assess the potential risk for the health of freshwater fish species, based on bio-concentration and fish condition. This is to our knowledge the first study that simultaneously monitors a large number of pesticides in both water and fish.

2. Materials and methods

2.1.Study area and sampling

The Júcar River is 497.5 km long and its mean annual flow is 10 m^3/s; it flows through three provinces (Teruel, Cuenca and Valencia) in Eastern Spain, under a typical

Mediterranean climate. Sampling was performed at five sites distributed along the main stream of the Júcar River (Fig.1) in October 2010. The site (JUC-I) is located at the basin headwaters, showing the natural flow regime. In the other sites, a great percentage of flat lands is dedicated to agriculture and the river flow is regulated by small and large dams.

The sampling was carried out, as much as possible, following the Environmental Quality Standards Directive 2008/105/EC (EQSD) [15]. October was the month selected for several reasons, (i) it coincides with the end of the growing season period, which is the appropriate for monitoring of fish, and (ii) there are not very recent applications of pesticides, which allow to establish what pesticides are constantly present in the environment because its capacity of accumulation and/or its persistence.

Physical and chemical characteristics of water (temperature, pH, total soluble salts, dissolved O_2 and redox potential) were recorded at the sampling sites using a Multiparameter Eutech Instrument CyberScan PCD 650 (Thermo Fisher Scientific, Basel, Switzerland). Water samples were collected in glass bottles (2.5 L) and transferred immediately to the laboratory for analysis. The samples were stored at 4 °C for no more than 10 days before analysis. Five hundred millilitres of water samples were filtered to remove any floating or insoluble materials.

Fish were sampled using electrofishing for approximately one hour at each site, with standard equipment, following the recommendations of the Norm UNE-EN 14011:2003 regarding sampling of fish with electricity. This norm states that in general the sampling should take place at the end of the growth period, when the juveniles are large enough to be captured by electrofishing. In this river, the best time approximately corresponds to October, although water temperature differs from the upper to lower study sites. Accordingly, a sampling campaign was carried out by the Water Authority

of the Jucar River Basin in October 2010, in order to monitor pesticides concentration in fish; such data allowed the comparison of results. The sampling in water was performed in the same month to show potential relations between concentrations in fish and water.

According to the aforementioned European norm, the weight (g) and fork length (mm) of each fish were measured in the field. In total, one-hundred-seventy-two individuals belonging to nine fish species were collected. The different fish species were distributed as follows. In JUC-I: Iberian gudgeon (n=8) and brown trout (n=9); in JUC-II, Iberian gudgeon (n=24), brown trout (n=2) and Iberian nase (n=6); in JUC-III, Iberian gudgeon (n=28) and largemouth bass (n=6); in JUC-IV, European eel (n=3), bleak (n=4), pumpkinseed (n=1), Iberian gudgeon (n=14), Eastern Iberian barbel (n=1) and largemouth bass (n=5); in JUC-V, Iberian gudgeon (n=7), pumpkinseed (n=1), bleak (n=27), northern pike (n=2), largemouth bass (n=2), European eel (n=13) and Eastern Iberian barbel (n=6).

The collected fish samples were transported to the laboratory in a cool-box and classified depending on the site and species. Then, the entire fishes were grinded using a Oster BPST02-B00 (London, United Kingdom). The wet weights were recorded and fish samples then stored in aluminium wrappers, freeze-dried at -80°C and lyophilized.

2.2.Extraction procedures

The full list of chemicals and reagents used, as well as the pesticides selected as target compounds are provided in the Supplementary Material (Table S1). Very briefly, water samples were extracted by solid-phase extraction (SPE) with Oasis HLB cartridge using a previously published procedure [16]. The limits of detection (LODs) and quantification (LOQ) ranged from 0.1 to 2 ng/L and from 0.3 to 6 ng/L, respectively. depending on the pesticides. Calibrations curves were linear in the concentration range

of 10 ng/L to 10 µg/L and the matrix effect was always ≤ 20 %. Recoveries varied from 48.50% to 70% and precision was below 20% for all pesticide.

The fish samples were prepared with the modified QuEChERS method. Two grams of lyophilized fish were placed in a 50 mL Falcon tube and added with 8 ml of H_2O MiliQ and 15 ml of acetonitrile and shaken vigorously for 30 s. Six g of magnesium sulphate ($MgSO_4$) and 1.5 g of sodium chloride (NaCl) were then added and the tube was shaken again for 1 min. The tube was centrifuged for 4 min using a centrifuge 5810 R (Eppendorf AG, Hamburg, Germany) at 4000 rpm. Two ml from the resulting supernatant were transferred to a 15 ml Falcon tube and cleaned-up with 0.3 g of $MgSO_4$, 0.1 g of PSA, 0.1 g of C_{18} and 0.015 g of activated charcoal. The 15 ml Falcon tube was shaken for 30 s and centrifuged at 4000 rpm for 4 min. The supernatant was transferred to an auto-sampler vial for LC-MS analysis through a MR PTFE Syringe filter (0.22 µm). All samples were analysed in triplicate. The results presented are the average of the three values.

2.3.LC-MS/MS analysis

Pesticides were determined by liquid chromatography tandem mass spectrometry (LC-MS/MS) using an Agilent 1260 Infinity system (Agilent Technologies, Palo Alto, California, USA) equipped with a binary pump, an automatic injector, a mass spectrophotometer Agilent 6410 triple Quad LC/MS System connected by an ESI source and software Mass Hunter Workstation version B.04.00/Buil 4.0.225.19. The analytical column was a Luna 18 (150 x 2.0 mm, 3 µm) from Phenomenex (Paris, France). The mobile phase (A) was 10 mM of formic acid in methanol and the mobile phase (B) was 10 mM of formic acid in water. The initial conditions were 50% B, increased to 83% B in 10 min, and then, increased to 98% B in 2.5 min and maintained for 3 min. The stabilization time was 12 minutes, therefore the

155 total run time was 27.5 min. The temperature of the column was 30 ºC, flow-rate, 0.4
156 $mL \cdot min^{-1}$ and injection volume, 5 µL. The source parameters were ionization voltage of
157 4000 V; nebulizer gas 15 psi; and source temperature 300 ºC.

158 The ionization and fragmentation of the study compounds was optimized by
159 injecting the solutions of each analyte without column using the Optimizer program.
160 Optimum fragmentor voltages were between 10 and 150 V, and collision energy
161 between 10 and 100 V (detailed conditions are outlined in Table S2, Supplementary
162 material).

163 **2.4.Validation**

164 Compound recoveries were determined using a hake (*Merluccius merluccius*)
165 from a Spanish market. Before, it was tested for the presence of any of the selected
166 pesticides that could interfere in the results. The hake sample was spiked with 150 µl of
167 a mixture of all pesticides of interest (at a concentration 5 µg/ml of each). As the spiked
168 volume was low, special care was taken to ensure a proper distribution of the pesticide
169 within the sample. The 150 µL were added using a GC syringe of 200 µL incrementally
170 to the sample. Each increment was carried out by spreading 50 µL of the solution as
171 much as possible in the Falcon tube containing 1 g of lyophilized samples that was then
172 vortexed at 1300 rpm for 2 min. This was repeated three time until all 150 µL were
173 added. The spiked samples were left to stand at room temperature for 20 min to ensure
174 the evaporation of the organic solvent a more homogeneous distribution. The recoveries
175 were determined in quintuplicate (n=5) comparing the pesticide areas of the sample
176 extract spiked before QuEChERS extraction to those of standards prepared, at the same
177 concentration, in blank fish extracts. Precision was calculated as the relative standard
178 deviation, % RSD of five samples analyzed in the same day, spiked with a standard 50
179 ng/ml, to provide a measure of intra-day accuracy.

Matrix effect was established by comparing the response of a standard (50 µg/g) prepared in acetonitrile to that of a blank hake extract spiked with the same concentration as the standard (matrix matched standard). Matrix effect was calculated according to the following equation:

$$\text{Signal value} = \left(\frac{A_{fish} - A_s}{A_s}\right) \times 100$$

Where: A_s= standard area; and A_{fish}= spiked matrix area.

Linearity of the method was established with eight calibration points at concentrations of 0.1, 1, 5, 10, 20, 30, 40 and 50 ng/ml. Results were adjusted to a simple linear regression not forced to go through the origin.

LOD was determined as the pesticide concentration that produces a signal-to-noise ratio (S/N) of 3 and LOQ as the lowest validated spike level meeting the method performance acceptability criteria (mean recoveries for each representative commodity in the range 70-120%, with an RSDr ≤ 20%) in the accordance with the European Union Guideliness [17, 18].

2.5.Data analysis

The presence and concentration patterns of pesticides in water and fish samples were initially analysed separately to determine the most affected sites. According to occurrence and concentration, the pesticides were classified in three groups: i) those with regular presence along the river (four or more consecutive sites) at concentrations > 30 ng/L; ii) those with regular presence concentrations ≤ 30 ng/L, or less frequent (three sites) with concentrations > 30 ng/L; and iii) those detected randomly in the river (in three sites or less) at concentrations ≤ 30 ng/L.

An analysis of variance (ANOVA) with a linear regression was carried out to assess the correlation between the concentration in water and in fish (1-α = 95%), and between the concentration in fish and the fish condition. The indices of fish condition

are indicators of the weight-to-length relationship, thus the well-being of a fish or population; the most common measure is the Fulton condition factor ($K = W \cdot 100/L^3$), where K = fish condition index, W = weight and L = length [19], ($1-\alpha = 95\%$). Regarding the analysis of fish condition, only the pesticides with a relevant presence in fish were considered, chlorpyriphos, diazinon and ethion. Statistical analyses were performed in the program Statgraphics 5.1.

3. Results and Discussion

3.1.Optimization of the extraction procedure

The QuEChERS protocol has two major steps: a salting-out extraction and a dispersive SPE (dSPE) clean-up including many possible permutations to allow adaptation to analyte and matrix. For the extraction, citrate-buffered and unbuffered versions of the method, as well as acetonitrile with or without 1% of acetic acid were tested. dSPE clean-up was adapted to the matrix and analytes studied because it could retain the compounds of interest or react with them or be not enough effective to eliminate matrix interferences.

3.2.Validation of the extraction procedure

Figure 2 shows the performance of the chromatographic determination. In the left part, a chromatogram of a QuEChERS extract of an fish sample spiked with the 41 pesticides analyzed by LC-MS/MS is presented. The chromatogram shows complete separation of most of the compounds (Fig. 2, up), with the exception of those that co-elute between min 10-11 and 14-15. The separation of all compounds took 15 min. The right side shows the extracted ion chromatograms (EIC) corresponding to pesticide detected in fish samples and compares them to those of the analytical standards. Peaks were clear with similar areas and retention times in samples and standards.

Recoveries range between 70 and 100% (Fig. 3). Only diuron, imazalil and omethoate showed recoveries below 70% and only fenthion sulfone, fenoxon sulfoxide and carbofuran provided recoveries over 100% (see full results in the supplementary material, Fig. S1). A possible explanation for these high recoveries could be the degradation of parent or other precursor compounds (fenthion, fenoxon or fenthion sulfoxide) to fenoxon sulfoxide and fenthion sulfone or the presence of a pesticide as degradation product of others non-targets present in the blank samples (carbosulfan). The first hypothesis was checked spiking the samples with the parent pesticides. However, it was dismissed because no degradation was observed. The second was tested searching for other "possible" precursor non-target pesticides in the blank samples that were not detected.

RSDs were lower than 20% for all pesticides for concentrations between 10 and 100 ng/g. These results almost met the limits recommended by the European Guidelines aforementioned. If any of the pesticides that does not fulfil the criterion on recoveries was detected, the sample was reanalysed using standard additions as alternative approach according to the European Guidelines. This procedure is designed to determine the content of an analyte in a sample, inherently taking into account the recovery of the analytical procedure and also compensating for any matrix effect.

A low matrix effect was observed (1-20%) with the exception of simazine and carbofuran-3-hydroxy (See Supplementary material Fig. S2). Therefore, it did not require correction, even though matrix-matched standards were used to calculate and quantify recoveries. The matrix effects were negative (lower response compared to the standard) for almost all the pesticides, with the exception of buprofezin, carbofuran, diazinon, metholachlor and omethoate, which showed an increase in the response. Matrix effect depends on the combination matrix and compound. Comparing to other

similar studies, Lazartigues et al. [20] found a matrix effect higher than 50% for isoproturon in fish (changing 8 units between three species of fish).

The linear equations and correlation coefficients are listed in the supplementary material in Table S3. It shows that most R^2 are higher than 0.99, except for methiocarb. A linear analysis is therefore shown to be acceptable for calculating compounds concentrations in fish.

Table 1 outlined the LODs and LOQs obtained for the studied compounds. LODs were between 0.01 and 0.5 ng/g, with the exception of simazine. There is not any official maximum limit of pesticide residue established for fish non-intended for human consumption. Table S4 in the supplementary material shows a comparison of the LODs and LOQs obtained in other studies. The limits obtained allow us to determine concentrations of environmental relevance.

3.3.Patterns of pesticides in water

Table 2 and figure 4 present the pesticide concentrations measured in water and fish. All pesticides were classified in three groups depending on the spatial distribution (regular/irregular presence in the study sites) and the concentrations, as follows:

1. Regular presence in the river at concentrations >30 ng/L. Some pesticides were found at all sampling points along the river (mean value for 5 sites in brackets): pyriproxyfen (89.66 ng/L), a banned substance in the EU since 22/9/2010, prochloraz (76.04 ng/L) and dichlofenthion (42.54 ng/L). Imazalil (126.38 ng/L) and chlorfenvinphos (70.23 ng/L, forbidden in EU since 22/9/2010) were at JUC-II and all other sites downstream. The first pesticides rapidly degrade in the aquatic ecosystem (less than one month); chlorfenvinphos and imazalil need between 4 and 5 months.

2. Regular presence at concentrations ≤ 30 ng/L or less frequent (three sites) with concentrations > 30 ng/L. Chlorpyriphos (18.87 ng/L), hexytiazox (17.94 ng/L), buprofezin (12.98 ng/L), diazinon (7.23 ng/L) and ethion (4.5 ng/L) were in all sampling points. From JUC-III to downstream, parathion ethyl (19.72 ng/L) as well as atrazine (JUC-III, 7.97 ng/L) and its metabolite atrazine desethyl (4.79 ng/L) were detected. Atrazine and parathion ethyl are very persistent in groundwater, but they rapidly degrade in surface waters by photolysis. Buprofezin is very stable (half live up to ten months), ethion and chlorpyriphos have moderate persistence (between 1 and 4 months), and the others pesticides degrade rapidly (< 1 month). Four of these eight compounds are forbidden in the EU (buprofezin and ethion since 22/9/2010; atrazine and parathion ethyl since 2005).
3. Irregular presence at concentrations ≤ 30 ng/L: dimethoate, fenoxon-sulfoxide, malathion and tolclofos methyl. All of them are pesticides that degrade rapidly. Only malathion is forbidden in the EU since 2005.

Pesticide concentration in water can be mainly influenced by: (i) Degradation (half-life); persistence that can vary between soil and water (Supplementary material Table S5). (ii) Drainage area and land uses that affect the quantity of pesticides from non-point sources, such as air, runoff or infiltration. There is a large variety of crops in the floodplain, and many fields irrigated by sprinklers (2 meters high or more) are only five or ten metres far from the river, with the separation of a small band of riparian trees or shrubs. (iii) River flow and physico-chemical parameters of water (e.g. water temperature and pH) that affect dilution and degradation. (iv) Reservoirs along the river, whose volume determines the water residence time and consequently concentrations of dissolved contaminants. These reservoirs increase the time for the degradation of pesticides. (v) Season, which is related to the type of crops (thus chemical treatments)

and atmospheric conditions. Most of the crops in this area are sowed in the early spring and harvested at the end of summer whereas few of them are year-round cultivations. Treatments with herbicides, insecticides and fungicides are applied during crop growth until few days before the harvesting. Thus, many pesticides could be applied throughout the year.

Table 3 summarizes the environmental information related to the abiotic degradation of the pesticides at each study site and between consecutive sites. The dissolved oxygen levels in the water samples (> 7.3 mg/L) were not detrimental to the development of aquatic microorganisms. The large volume of the Alarcon reservoir produces a hyperannual flow regulation. In 2010, the residence time was long (1640 days) because the reservoir had accumulated water and released very little flow; the reservoir was recovering of a long drought. Therefore, in this period any pesticide entering the reservoir was degraded with very high probability. For a general perspective, the residence time in this reservoir, annually estimated from March 2012 to February 2013, was 423 days.

The spatial distribution of pesticides was different depending on compounds, but the general patterns can be related to the land use and other factors aforementioned. The relation of crops in the different locations to the pesticides applied and their persistence were analysed (see table S5 in supplementary material). This information was obtained from the handbook of pesticides and nutritional products of Spain [21], the material supplied by the local offices of agricultural development (Ministry of Agriculture, Government of Spain) and our own field observations.

The first site (JUC-I) is located in the headwaters of the Jucar River; this is a natural area with little anthropogenic influence and also little river flow (less dilution); this site presents the lowest number of pesticides. Although the landscape was dominated by

329 natural vegetation, small lands with potatoes, mushrooms, poplars, olive, almond and
330 onion are present, as well as livestock. These activities were the source of the few
331 pesticides detected at JUC-I; the main compounds were related to the treatments for
332 insects on olive trees (pyriproxyfen), for fungi on mushrooms and potato (prochloraz)
333 and also for insects on livestock (pyriproxyfen and diclofenthion) (Table S5). The
334 presence of diclofenthion, associated to livestock, was approximately regular in the five
335 study sites.

336 The second site (JUC-II) is at the city of Cuenca (56,472 habitants), where the
337 livestock and agricultural activities are more important (including almond, cereals,
338 garlic, grapevine, olive, onion, potato and sunflower). The main pesticides (i.e., of
339 highest concentrations) were related with treatments for insects on potato
340 (chlorfenvinphos, prochloraz), on garlic and wheat (prochloraz) and on olive trees
341 (pyriproxyfen); with treatments for fungi on almond (imazalil), and for insects on
342 livestock (chlorfenvinphos and pyriproxyfen) (Table S5). As an indicator, the
343 cultivation area of garlic, in the province of Cuenca, mean the 17 % of this plant in
344 Spain [22]. In 6 out of 8 compounds it was observed a reduction (24 % on average)
345 from JUC-I to JUC-II (see figure 4, Table 2). The reservoir of La Toba, located 35 km
346 upstream of this site, contributes to the degradation of the pesticides coming from JUC-
347 I; additionally, the stream flow increases (approximately multiplied by two) from the
348 first site. Therefore, we can assume these are the reasons to explain that reduction,
349 regardless of the ample use of pesticides in the area.

350 At the third site (JUC-III) the river travels through an important area of farming in
351 the province of Albacete, which explains the remarkable input of pesticides; this is the
352 sampling point where more pesticides were detected. The crops were the same as in
353 JUC-II, with the addition of soybeans, tomato and broccoli. The main pesticides were

related to the treatments for fungi on almond (imazalil), for insects on olive and broccoli (pyriproxyfen, chlorfenvinphos) and treatments on livestock (pyriproxyfen, chlorfenvinphos). Some of the detected pesticides can be extensive use because they are recommended for several crops. For example, prochloraz is used for barley, garlic, onion, oat, potato, tomato and wheat. The concentration of 7 of the 11 pesticides detected increases (315 % on average) from JUCII to this site (figure 4, Table 2). JUCIII is located in an area of intensive irrigated agriculture. As an indication of the intensive agricultural activities in Albacete, in terms of area, this province has the 30 % of the land dedicated to garlic in Spain, 28 % of the onion and 5 % of the barley [22]. Other studies also detect high levels of nitrates (over the legal limits) in the waters of this area associated with the intensive irrigated agriculture [23]

Upstream of JUC-III, the presence of Alarcón Dam (the largest reservoir with a residence time of 1640 days) indicates that all the pesticides reaching the river upstream are expected to degrade in the reservoir. However, some pesticides increased their concentrations notably. These results indicate that below Alarcón Dam the contribution of pesticides into the river is remarkable. Despite of the large increment of drainage area from JUC-II to JUC-III, the river flow was very similar during the sampling days (due to flow extraction for agriculture), thus there is no increase of dilution in this river segment.

Between JUC-III and JUC-IV, the concentration of 9 of the 13 pesticides detected dismished (20 % on average). On the contrary, the concentration of atrazine-desethyl, bupofrezine, ethion and fenoxon sulfoxide increased, that of the two last was almost duplicated (Fig.4-A, Table 2). The increase in the concentration of these two pesticides could be related to the vineyards and cereals extensions between these consecutive sites. The decrease can not be explained by the river flow, very similar between both sites

379 during the sampling days. Then, it could be related to the storage volume in the Moliner
380 reservoir and the short half live of these pesticides. One of the most persistent
381 compounds, ethion, increased gradually from JUC-I to JUC-IV; such increase is
382 coherent with the accumulation produced by its regular use in the entire basin and its
383 persistence. The application of ethion is recommended in vineyards and citrus, as well
384 as for livestock.

385 The last site (JUC-V) near the city of Valencia collects the water from several
386 important tributaries covering a larger surface area (14.674 Km^2). The watershed
387 includes large extensions of rice, citrus fruits and vegetables. However, in the three
388 large reservoirs upstream most pesticides already transported by the river are degradate
389 (due to the accumulated residence time). Therefore, there was a decrease in the
390 concentrations of 5 pesticides in comparison with JUC-IV (figure 4-A, Table 2),
391 especially in Atrazine-desethyl (56 %), Hexythiazox (16 %) and diclofenthion (9 %).
392 However, other 7 compounds showed higher concentrations, especially chlorpyriphos
393 (from 2.23 to 36.23 ng/L), commonly used against insects in the extensive cultivation of
394 citrus in the Valencia province, which produces (in tons) 41 % of the oranges and 50 %
395 of the tangerines of Spain [22].

396 As it was expected, some of the more persistent compounds showed relatively stable
397 values (specifically imazalil, chlorfenvinphos, ethion and buprofezine); the results
398 indicate that they were used a few months before the sampling dates, and their
399 application is distributed throughout the river basin, because the large reservoirs could
400 not retain all the inputs in the rivers. Additionally, some of the pesticides of low
401 persistence also showed a regular pattern (pyriproxyfen, prochloraz, diclofenthion,
402 hexythiazox) indicating their extensive use in the Júcar River Basin during September
403 and October. Pesticides are applied with the irrigation water by a sprinkler system, and

404 then can reach the river carried out by the wind or by run-off since the distance from the
405 crops to the river is small.
406 The comparison of results with the data of the river basin authority (Confederación
407 Hidrográfica del Júcar, hereafter CHJ) was limited to a few coincidences in the
408 pesticides detected, sampling dates and sites. Firstly, one sampled at Los Frailes-CHJ
409 (comparable with JUC-III) indicated 15 ng/L of atrazine four days after our sampling
410 (14/10/2010 and 18/10/2010); this is approximately double our result (7.79 ng/L), which
411 confirms our findings. On the contrary, chlorpyriphos and chlorfenvinphos were not
412 detected by the CHJ, but we found concentrations of 32.14 ng/L and 83.07 ng/L,
413 respectively. This fact could be related to a rise of flow between sampling dates after
414 precipitation (flow increased from 1.847 m^3/s to 2.061 m^3/s) which diluted the atrazine
415 and helped to transport the other two pesticides downstream.
416
417 **3.4.Pesticides in fish and fish condition**
418 Most of the pesticides in waters were also detected in fish at low concentrations and
419 in isolated points. Only five compounds were present in fish taken at three or more sites
420 (Fig. S3; Table 2). Only Azinphos ethyl (a compound of high potential
421 bioaccumulation, KoW = 3.18) was detected in fish at high concentrations, but not in
422 water; this result can be explained by its low persistence in water, and indicates a
423 repeated use the intensive agricultural areas of Albacete, because the concentration in
424 fish was high in the two sites located in this province, i.e., 86.17 ng/L at JUC-III and
425 65.64 ng/L at JUC-IV. The declining or absence of a pesticide in water is not directly
426 related to the declining in fish. Similar results were observed in fish after the decline of
427 toxaphene in the Great Lakes [24]. Diazinon was detected in fish at the five sites, with a
428 maximum in the downstream site JUC-V (5.83 ng/L), probably due to its high capacity

429 of absorption by fish [25]. At JUC-III, which present the highest concentrations in
430 water, omethoate, chlorpyrifos and dimethoate were significantly accumulated, in
431 different species.

432 Regarding bio-concentration the species that presented relevant concentrations at the
433 different sampling points were one Iberian gudgeon at JUC-I (518.9 ng carbofuran/g;
434 Table 2); one largemouth bass at JUC-IV (86.17 ng azinphos ethyl/g; 78.82 ng
435 omethoate/g); one bleak at JUC-5 (65.64 ng azinphos ethyl/g); one Iberian nase and an
436 Iberian gudgeon, both at JUC-II (44.46 ng and 46.64 ng azinphos ethyl/g, respectively).
437 Although detected pesticides do not present the highest bio-concentration capacities (see
438 Kow in supplementary material Table S1), most of these fish species are benthic or
439 epibenthic, thus they live near the river bottom and feed on sediments, detritus, benthic
440 invertebrates or periphyton [26], which can also be a source of pesticides. Thus, the
441 accumulation of pesticides and other contaminants in sediments, biofilm and
442 periphyton, the base of the food web, require further research because it is fundamental
443 for the health of the aquatic ecosystems. Specifically, due to its sensitivity, fluvial
444 biofilms can be used as early warning systems for the detection of the effects of
445 toxicants on aquatic systems [27].

446 The comparison of concentrations in fish and water (Table 4) showed no clear
447 relationship in this river. The highest accumulation of chlorpyriphos was detected in
448 largemouth bass; the highest accumulation of ethion and diazinon was in the European
449 eel. The eel is omnivorous and the bass is mainly a predatory species, thus both species
450 feed on fish [26]. Therefore, we hypothesize that it is the position in the food web the
451 key factor that explains the highest pesticides concentrations in these fish species. It is
452 known that predators may bioaccumulate pesticides, PCBs, and metals from the
453 surrounding water or from feeding on other fish, which may result in the

biomagnification of these compounds in their tissues [28]. Accordingly, a study based on extensive sampling across the USA also found significant differences of contaminant levels in bottom feeding and predatory fish [29]. Additionally, the European eel tends to bio-concentrate more than the other species due to the high percentage of lipids in its body [30].

In accordance with the ample presence of pesticides in fish, when the samples from all the fish species were aggregated a significant relationship between the Fulton condition factor and diazinon concentration was observed ($p < 0.01$). Then, in the analysis by species, the relation of this compound with the condition of the Eastern Iberian barbel was significant ($p < 0.05$). This relation is not very robust, because the data corresponded to 17 fish but they were pooled in 3 independent samples. Nevertheless, this result is indicative of the potential effects of pesticides on fish growth in the Júcar River, and a stronger relationship can be expected if the data were more abundant and better distributed, as it was found in other studies.

Previous research showed that the exposure of pesticides produced a significant impact on fish health and growth. A study on the Australian catfish (*T. tandanus*) demonstrated that, in a short exposure, concentration of chlorpyriphos from 2 to 10 µg·L-1 affects the fish growth, with low FCR (Food Conversion Ratio) and PER (Protein Efficiency Ratio) [31]. Accordingly, another study indicated that diazinon reduces the growth of the snakehead fish (*Channa striata*) [32]. Without specifying the type of compound, a study along the Ebro River for several species revealed a significant decrease in fish condition at the polluted areas and that the responses to the pollutant were species-specific [33].

4. Conclusions

QuEChERS is a suitable method for the extraction of wide variety pesticides in fish and provides selective and sensitive results, combined with LC-MS/MS. This method was primarily designed to be an easy, economical and effective approach with high sample throughput for a large number of pesticides in fruits and vegetables and now, has demonstrated its efficacy for fish samples. The regular spatial pattern of some pesticides suggests a permanent or frequent supply of these compounds along the Júcar River Basin, including some components forbidden in the EU [34]. The use of the pesticides in different cultivations was related to the spatial patterns observed in the Júcar River. Concentrations in fish are not lethal [25], but the relationships between bio-concentration and fish condition requires further research due to the importance of some of the fish species, e.g. the brown trout (population in decline) and the European eel (critically endangered species) from the economic and ecological perspectives. The wide presence of pesticides in fish suggests potential severe effects on fish populations and other biota in future scenarios of climate change, due to the presence of endemic and endangered fish species in this river basin. Future research on the relevance of these factors, in combination with multiple stressors, will help to improve the fish populations and the resilience of the Mediterranean river ecosystems facing a future of water scarcity. Furthermore, this research is necessary to prioritize the management of specific chemicals and to suggest effective restoration actions at the basin scale.

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References

[1] S. Sabater, R. J. Stevenson, Foreword: Global change and river ecosystems–implications for structure, functions and ecosystem services, Hydrobiology 657 (2010) 1-2.

[2] C. Nilsson, C. A. Reidy, M. Dynesius, C. Revenga, Fragmentation and Flow Regulation of the World's Large River Systems, Science 308 (2005) 405-408.

[3] European Environment Agency, European Waters – Current Status and Future Challenges. Synthesis, EEA, Copenhagen, 2012.

[4] European Commission, Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy, 2000.

[5] European Commission, Communication from the commission to the European parliament, the council, the European economic and social committee and the committee of the regions. A Blueprint to Safeguard Europe's Water Resources, 2012.

[6] CEDEX, Evaluación del impacto del cambio climático en los recursos hídricos en régimen natural, Resumen ejecutivo, Madrid: Environmental Ministry, CEDEX, 2011. http://marm.es/es/agua/formación, 2011.

[7] M. Petrovic, A. Ginebreda, V. Acuña, R. J. Batalla, A. Elosegi, H Guash, et al., Combined scenarios of chemical and ecological quality under water scarcity in Mediterranean rivers, Trends Anal. Chem. 30 (2011) 1269-1278.

[8] P. C. von der Ohe, V. Dublio, J. Slobodnik, E. D. Deckere, R. Kühne, R.-U. Ebert, A. Ginebreda, W. D. Cooman, G. Shüürmann, W. Brack, New risk assessment approach for the prioritization of 500 classical and emerging organic microcontaminants as potential river basin specific river pollutants under the European Water Framework Directive, Sci. Total Environ. 409 (2011) 2064-2077.

[9] A. Hildebrandt, M. Guillamón, S. Lacorte, R. Tauler, D. Barceló, Analysis of monitoring programmes and their suitability for ecotoxicological risk assessment in four Spanish basins, J. Hazard. Matr. 42 (2008) 3315-3326.

[10] J. C. López-Doval, N. de Castro-Català, I. Andrés-Doménech, J. Blasco, A. Ginebreda, I. Muñoz, Analysis of monitoring programs and their suitability for ecotoxicological risk assessment in four Spanish basins, Sci. Total Environ. 440 (2012) 194-203.

[11] V. Andreu, Y. Picó, Determination of currently used pesticides in biota, Anal. Bioanal. Chem. 404(9) (2012) 2659-81.

[12] B. Huerta, S. Rodríguez-Mozaz, D. Barceló, Pharmaceuticals in biota in the aquatic environment: analytical methods and environmental implications, Anal. Bioanal. Chem. 404(9) (2012) 2611-24.

[13] D. F. K. Rawn, J. Judge, V. Roscoe, Application of the QuEChERS method for the analysis of pyrethrins and pyrethroids in fish tissues, Anal. Bioanal. Chem. 397 (2010) 2525–2531.

[14] A. Lazartigues, L. Wiest, R. Baudot, M. Thomas, C. Feidt, C. Cren-Olivé Multiresidue method to quantify pesticides in fish muscle by QuEChERS-based extraction and LC-MS/MS, Anal. Bioanal. Chem. 400 (2011) 2185-2193.

[15] European Parliament and Council, Directive 2008/105/EC of the European Parliament and of the Council of 16 December 2008 on environmental quality

554 standards in the field of water policy, amending and subsequently repealing
555 Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC,
556 86/280/EEC and amending Directive 2000/60/EC of the European Parliament and
557 of the Council, Off. J. Eur. Comm. 348 (2008) 84.
558 [16] A. Masiá, M. Ibáñez, C. Blasco, J.V. Sancho, Y. Picó, F. Hernández, Combined
559 use of liquid chromatography triple quadrupole mass spectrometry and liquid
560 chromatography quadrupole time-of-flight mass spectrometry in systematic
561 screening of pesticides and other contaminants in water samples, Anal. Chem.
562 Acta, 761 (25) (2013) 117-127.
563 [17] Method Validation and Quality Control procedures for Pesticide Residues
564 SANCO/12495/2011; implemented by 01/01/12, updated 06/02/12.
565 [18] Residue analytical methods - for post-registration control and enforcement
566 SANCO/825/00 rev. 8.1, updated 16/11/2010.
567 [19] R. O. Anderson, R. M. Neumann, Length, weight, and associated structural
568 índices, In: B. R. Murphy, D. W. Willis (Ed.), Fisheries techniques, 2nd edition,
569 American Fisheries Society, Bethesda, 1996, pp. 447-482.
570 [20] A. Lazartigues, M. Thomas, D. Banas, J. Brun-Bellut, C. Crean-Olivé, C. Feidt,
571 Accumulation and half-lives of 13 pesticides in muscle tissue of freshwater fishes
572 through food exposure, Chemosphere 91 (2013) 530-535.
573 [21] C. de Liñan Vicente, Valdemecum de productos fitosanitarios y nutricionales,
574 Ediciones Agrotécnicas, S.L., Madrid, 1999.
575 [22] Ministerio de Agricultura, Alimentación y Medio Ambiente (MAGRAMA,
576 Ministry of Agriculture, Food and Environment), Anuario de estadística Agraria,
577 MAGRAMA, Madrid, 2010.

578 [23] A. Moratalla, J. J. Gómez-Alday, J. De las Heras, D. Sanz, S. Castaño, Nitrate in
579 the Water-Supply Wells in the Mancha Oriental Hydrogeological System (SE
580 Spain), Water Res. Manag. 23 (2009) 1621-1640.
581 [24] Confederación Hidrográfica del Júcar, Red de control de sustancias peligrosas,
582 2010. http://www.chj.gob.es/Redesdecalidad/subred_pel_Difuso.aspx, 2012.
583 [25] C. de Liñan Vicente, Farmacología vegetal, Ediciones Agrotécnicas, S.L., Madrid,
584 1997.
585 [26] C. J. Schmitt, J. L. Zajicek, P. H. Peterman, National contaminant biomonitoring
586 program: Residues of organochlorine chemicals in U.S. Freshwater Fish, 1976–
587 1984, Arch. Environ. Contam. Toxicol. 19 (1990) 748-781.
588 [27] I. Doadrio, Origen y Evolución de la Ictiofauna Continental Española, In: Atlas y
589 Libro Rojo de los Peces Continentales de España, 2nd Ed., CSIC y Ministerio del
590 Medio Ambiente, Madrid, 2002.
591 [28] S. Sabater, H. Guasch, M. Ricart, A. Romaní, G. Vidal, C. Klünder, M. Schmitt-
592 Jansen, Monitoring the effect of chemicals on biological communities. The
593 biofilm as an interface, Anal. Bioanal. Chem. 387 (2007)1425–1434.
594 [29] J. M. Kidwell, L. J. Phillips, G. F. Birchard, Comparative analyses of contaminant
595 levels in bottom feeding and predatory fish using the national contaminant
596 biomonitoring program data, Bull. Environ. Contam. Toxicol. 54 (1995) 919-923.
597 [30] E. Sancho, M. D. Fernándo, C. Lleó, E. Andreu-Moliner, Pesticide toxicokinetics
598 in fish: Accumulation and Elimination, Ecotox. Envir. Safety, 41 (1998), 245-250.
599 [31] H. P. V. Huynh, D. Nugegoda, Effects of chlorpyriphos exposure on growth and
600 food utilization in Australian Catfish, *Tandanus tandanus*. Bull. Envir. Contam.
601 Toxicol. 88 (2012) 25-29.

602 [32] N. V. Cong, N. T. Phuong, M. Bayley, Effects of repeated exposure of diazinon
603 on cholinesterase activity and growth in snakehead fish (Channa striata), Ecotox.
604 Envir. Safety 72 (3) (2009) 699-703.
605 [33] L. Benejam, J. Benito, E. García-Berthou, Decreases in condition and fecundity of
606 freshwater fishes in a Highly polluted reservoir, Water Air Soil Pollut., 210
607 (2010) 231-242.
608 [34] Pesticide Action Network UK. 2011. Lista de Plaguicidas Prohibidos. RAP
609 Internacional. http://www.rap-al.org/index.php?seccion=3&f=edicion.php&id
610 _publicacion=7&id_edicion=151, 2012.
611

Table 1. LODs and LOQs of analyzed pesticides.

Pesticides	LOD (ng/g)	LOQ (ng/g)
Acetochlor	3.8	11.25
Alachlor	1	3
Atrazine	0.1	0.3
Atrazine desethyl	0.1	0.3
Atrazine desisopropyl	0.5	1.5
Azinphos ethyl	0.1	0.3
Azinphos methyl	1	3
Buprofezin	0.1	0.3
Carbofuran	0.1	0.3
Carbofuran-3-hydroxy	0.1	0.3
Chlorfenvinphos	0.1	0.3
Chlorpyriphos	0.01	0.03
Diazinon	0.1	0.3
Dichlofenthion	0.5	1.5
Dimethoate	0.01	0.03
Diuron	0.1	0.3
Ethion	0.01	0.03
Fenitrothion	1	3
Fenoxon sulfoxide	0.5	1.5
Fenoxon sulfone	0.1	0.3
Fenthion sulfoxide	0.1	0.3
Fenthion sulfone	0.01	0.03
Hexytiazox	0.01	0.03
Imazalil	0.1	0.3
Imidacloprid	0.01	0.03
Isoproturon	0.01	0.03
Malathion	0.5	1.5
Methiocarb	0.1	0.3
Metoalachlor	0.1	0.3
Molinate	0.5	1.5
Omethoate	0.01	0.03
Parathion ethyl	0.1	0.3
Parathion methyl	1	3
Prochloraz	0.1	0.3
Propanil	0.5	1.5
Propazine	0.1	0.3
Pyriproxyphen	0.1	0.3
Simazine	5	1.5
Terbutryn	0.1	0.3
Tolclofos methyl	0.5	1.5

Table 2. Concentrations in water (W, in ng/L) and fish (F, in ng/g) by study site in the Júcar River (from JUC-I to JUC-V) corresponding to the 23 pesticides detected.

Site / Pesticide	JUC-I		JUC-II		JUC-III		JUC-IV		JUC-V	
	W	F	W	F	W	F	W	F	W	F
Atrazine					7.97					
Atrazine-desethyl					8.65		10.61		4.68	
Atrazine desisopropyl						21.34-39.39		23.83		
Azinphos ethyl		2.52		2.36-46.63		86.17		65.64		
Buprofezine	14.07		13.06		11.68		13.27		12.82	Trace
Carbofuran		518.9		Trace						
Chlorfenvinphos			93.34		83.07		78.08		96.68	
Chlorpyriphos	6.84	Trace	16.99	Trace	32.14	24.42	2.23		36.23	7.13
Diazinon	11.94	0.92-3.53	0.44	1.04-2.31	8.59	0.37-2.36	6.31	1.33	8.87	0.87-5.83
Dichlofenthion	44.08		35.11		50.85		43.22		39.43	
Dimethoate		0.18		Trace	1.64	9.87		Trace		Trace
Ethion	0.09	Trace	2.45	Trace	7.07		12.9	13.76		0.48
Fenoxon sulfoxide					25.52		48.94		50.66	
Hexythiazox	17.71		17.5		20.65		18.38		15.45	0.38
Imazalil			166.7	6.39	171.5		152.5		141.3	
Malathion			12.62		10.72				8.75	
Methoalachlor										4.32
Omethoate						78.82		0.95		
Parathion ethyl					32.47		31.9		34.25	
Prochloraz	79.9		73.85		82.79		66.99		76.69	
Propazine				1.42						
Pyriproxyfen	99.59		89.95		87.4		82.92		88.43	Trace
Tolclofos methyl	28.64			12.63					27.57	

Trace: the value is below LOQ

*Where the minimum was 0.00, this value was omitted, showing only the maximum one.

Table 3. Summary of the main factors influencing the abiotic degradation of pesticides in the study sites. The volume in reservoirs corresponds to the dams between a study site and the previous one upstream. River flow previous month (m^3/s) was calculated as the averaged flow from the 21rst September to the 20th October, 2010. Mean river flow corresponds to the average mean annual river flow for the 10 hydrological years before the sampling. Elevation of the site is shown in meters above sea level. Storage volume is the total volume in the large reservoirs (hm^3) located between each study site and the previous one upstream.

	T^a (^0C)	River flow *(m^3/s)	River flow prev. Month (m3/s)	Mean river flow (m^3/s)	Elevation (m.asl)	Drainage basin (km^2)	Reservoir upstream of site	Storage volume (Hm^3)	Residence time (days)
Juc I	10	1.021	1.058	2.465	1170	250	-	-	-
Juc II	13.8	1.893	3.652	6.900	916	984	La Toba	4.3	12
Juc III	13.6	2.061	2.25	4.753	616	5403	Alarcon	570.5	1640
Juc IV	12.2	1.978	2.794	4.192	348	8122	Molinar	1.65	7
Juc V	19	1.007	8.393	5.508	37	14674	Cortes II, Naranjero , Tous	235.6	89 14 99

*Date corresponding to the sampling day.

Table 4. Comparative of concentrations in fish (F) and water (W) at the same location, only observed in three compounds for six fish species. The European eel is considered as a critically endangered species.

Species	**F (ng/kg)**	**W (ng/kg)**
Chlorpyrifos		
Largemouth bass (JUC-III)	$24.42 \cdot 10^{-3}$	32,14
Northern pike (JUC-V)	$7.13 \cdot 10^{-3}$	36,23
Ethion		
European eel (JUC-IV)	$24.42 \cdot 10^{-3}$	12,9
Iberian gudgeon (JUC-IV)	$0.04 \cdot 10^{-3}$	12,9
Eastern iberian barbel (JUC-IV)	$7.13 \cdot 10^{-3}$	12,9
Diazinon		
European eel (JUC-IV)	$24.42 \cdot 10^{-3}$	6,31
Iberian gudgeon (JUC-IV)	$0.04 \cdot 10^{-3}$	11,94
Pumpkinseed (JUC-V)	$1.12 \cdot 10^{-3}$	8,87
Largemouth bass (JUC-IV)	$7.13 \cdot 10^{-3}$	8,87

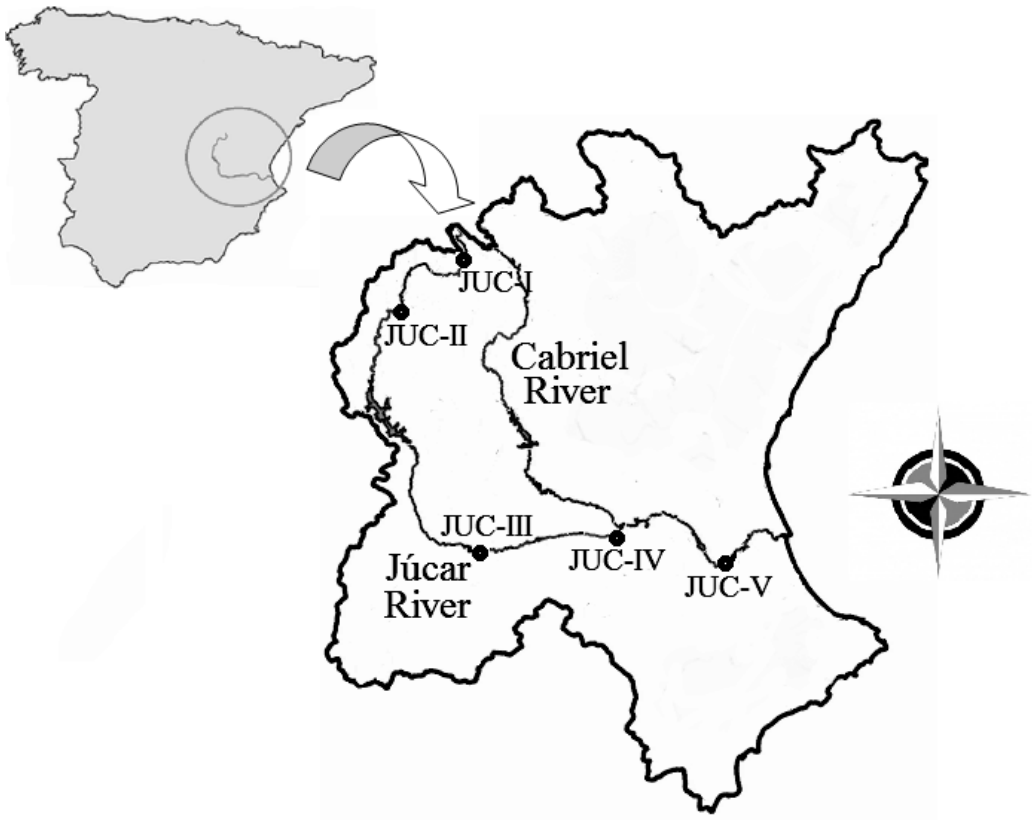

Figure 1. Location of the Júcar River Basin (Eastern Spain) and the five sampling sites along the Júcar River.

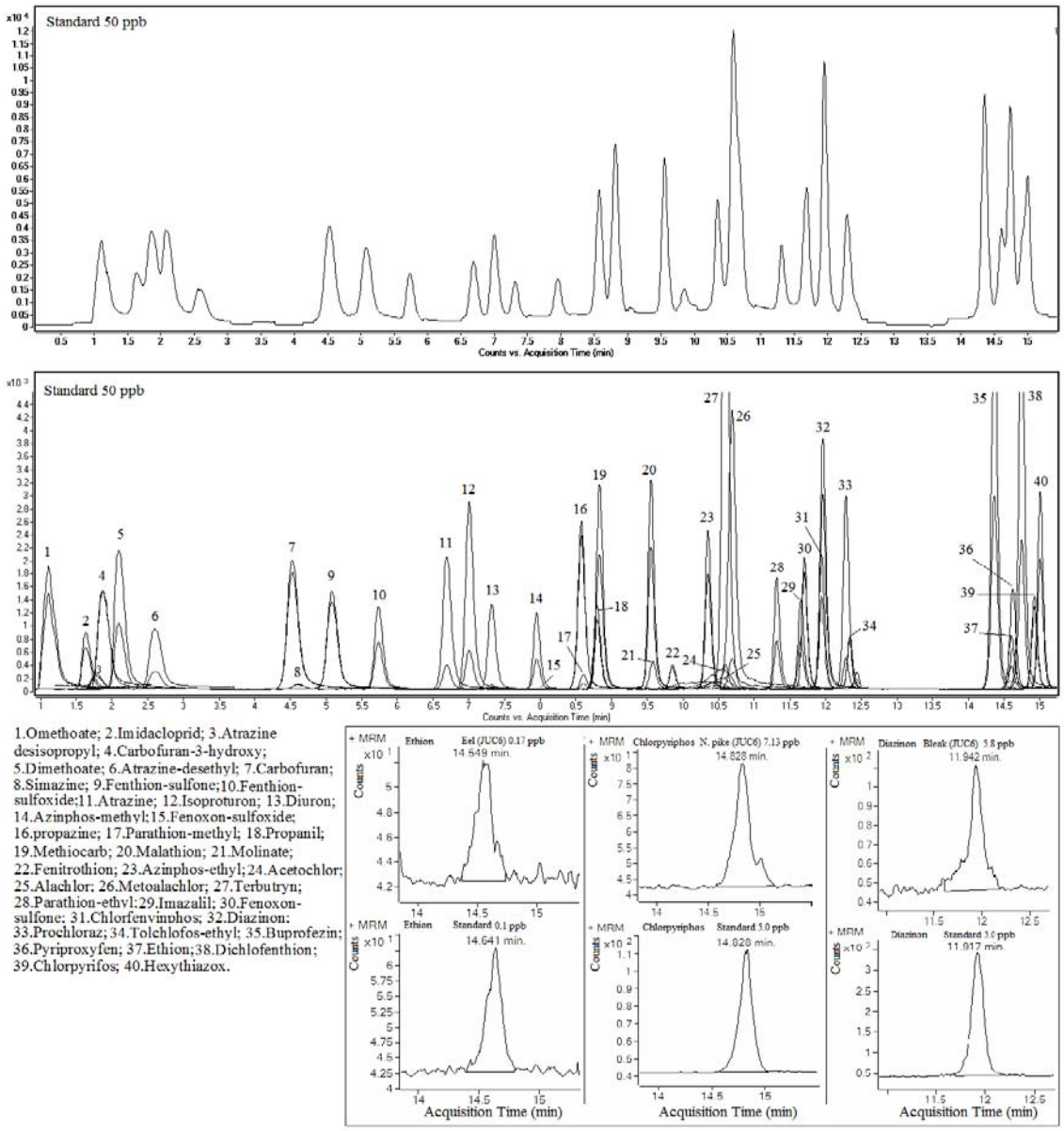

Figure 2. Chromatogram of analyzed compounds (up). Comparative between samples and standard chromatographs peaks (down).

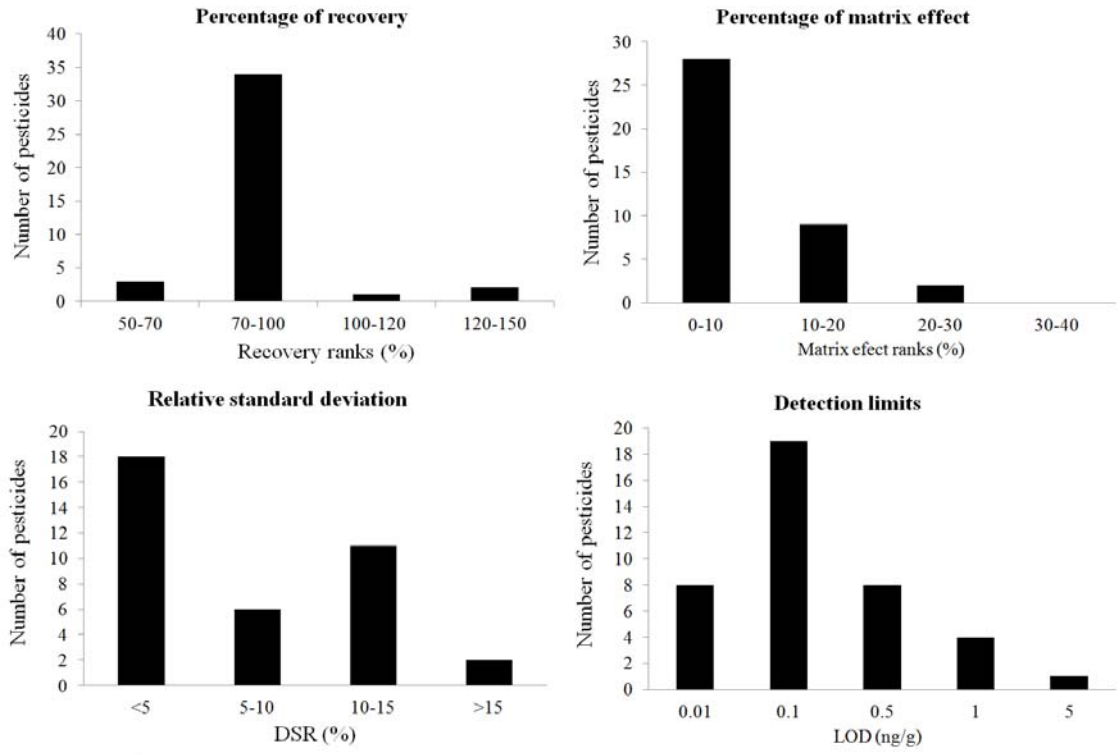

Figure 3. Frequency of pesticides regarding percentage of recovery and matrix effect (upper plots) as well as ranges of Relative standard deviation (DSR) on detection limits (lower plots).

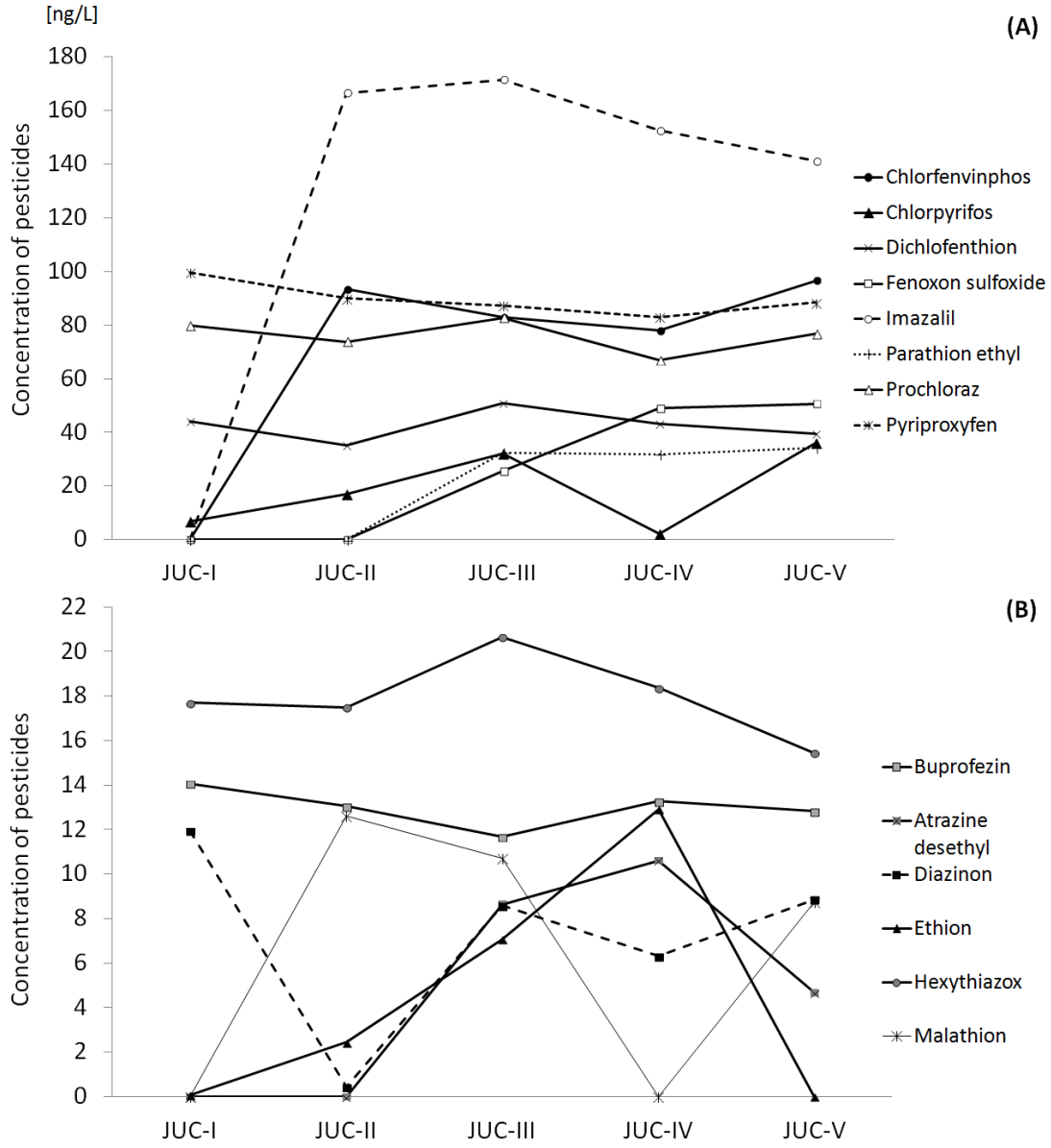

Figure 4. Pesticides concentration in water at the five sampling sites of the Júcar River; for a clear display, those with relatively high concentrations (A) and relatively low ones (B) are separated, and only those detected in three or more sites are shown.