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Predicting the aquatic risk of realistic pesticide mixtures to species assemblages in Portuguese river basins

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ABSTRACT

Although pesticide regulatory tools are mainly based on individual substances, aquatic ecosystems are usually exposed to multiple pesticides from their use on the variety of crops within the catchment of a river. This study estimated the impact of measured pesticide mixtures in surface waters from 2002 and 2008 within three important Portuguese river basins ('Mondego', 'Sado' and 'Tejo') on primary producers, arthropods and fish by toxic pressure calculation. Species sensitivity distributions (SSDs), in combination with mixture toxicity models, were applied. Considering the differences in the responses of the taxonomic groups as well as in the pesticide exposures that these organisms experience, variable acute multi-substance potentially affected fractions (msPAFs) were obtained. The median msPAF for primary producers and arthropods in surface waters of all river basins exceeded 5%, the cut-off value used in the prospective SSD approach for deriving individual environmental quality standards. A ranking procedure identified various photosystem II inhibiting herbicides, with oxadiazon having the relatively largest toxic effects on primary producers, while the organophosphorus insecticides, chlorfenvinphos and chlorpyrifos, and the organochloride endosulfan had the largest effects on arthropods and fish, respectively. These results ensure compliance with European legislation with regard to ecological risk assessment and management of pesticides in surface waters.

Introduction

Ecosystems are usually exposed to a cocktail of chemicals rather than one individual substance. This is particularly apparent in surface waters, where a multitude of potentially toxic substances enter the watercourse as a result of human activities throughout the drainage basin (Verro et al., 2009). Different agricultural practices can cause the presence of mixtures of pesticides in the aquatic environment, which can vary in terms of their complexity (Altenburger et al., 2014). As cumulative stress of toxicants may be identified as a main pressure affecting ecological status, mixture risks have to be evaluated and reduced (Brock, 2013).

The component-based approach, an option for regulatory mixture ecotoxicity assessment, calculates the expectable joint toxicity from toxicity data for individual mixture components by applying corresponding models, in particular those based on the reference models of concentration addition (CA), response addition (RA), and so-called mixed-model (Altenburger et al., 2014). The summation of PEC/PNEC (predicted environmental concentration/predicted no effect concentration) ratios and the summation of toxic units are examples of CA-based approaches

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used in the authorization procedure for technical mixtures of several active substances and their co-formulants (EC, 2009a; EFSA, 2013) and in the derivation of environmental quality standards for well-defined mixtures, *i.e.*, those with a well-defined qualitative and quantitative composition (*e.g.*, PCBs, dioxins) (EC, 2011). However, the sum of these ratios has no toxicological meaning, *i.e.*, if two substances have the same ratio > 1, their environmental impacts may be quite different (Traas et al., 2002).

As an alternative, this study proposes to use species sensitivity distributions (SSDs) based on laboratory toxicity data to derive a measure of effects that can be used in ecological risk assessment. This method is an improvement over current quotient methods, since it encompasses the often non-observed nonlinearity of species sensitivity and, especially, it allows for comparisons of (aggregated) ecological risk over compounds in a mixture, between *taxa*, and with other stressors (in very specific conditions) (Traas et al., 2002). The combi-potentially affected fraction (PAF) concept was developed by Hamers et al. (1996) and assumes that only compounds exerting narcotic effects are addressed by CA, while all other compounds are handled by RA (mixed-model approach). When the mixture contains compounds with highly specific toxic modes of action (TMoA) that differ among species groups, such as pesticides, it is also possible to generate mixed-model (CA and RA) multi-substance potentially affected fraction (msPAF) values for the individual taxonomic groups (Traas et al., 2002; De Zwart and Posthuma, 2005).

To our knowledge, only few studies (Pérez, 2013; Silva et al., 2012b) have been conducted on the risk assessment of realistic pesticide mixtures in Portuguese freshwaters for individual species by applying whole mixture and component-based approaches. Given also the need for quantitative data on mixture toxicity risks for other relevant assessment endpoints, as species assemblages, *i.e.*, on a higher tier level, toxic pressures (quantified as msPAFs) were calculated (1) to estimate the overall impacts for primary producers, arthropods and fish of measured pesticide mixtures in surface waters of three important Portuguese river basins ('Mondego', 'Sado' and 'Tejo'); and (2) to rank the relative contribution of individual pesticide compounds (or groups of pesticides with the same TMoA) *per* taxonomic group and river basin. The findings from this study will allow the derivation of optimized programs of measures to reduce ecological risks of pesticides in surface waters and evaluation of the control measures for this aquatic compartment. These ensure compliance with the prospective and retrospective risk assessment and management procedures for pesticides in surface waters as laid down in European legislation (EC, 2000, 2009a,b).

1. Experimental

1.1. Study area

In terms of water resources, 'Tejo', 'Sado' and 'Mondego' belong to the largest hydrological basins of continental Portugal occupying 25,666, 12,149 and 6659 km^2, respectively (APA, 2014). Several studies related to surface and ground water contamination have been performed in these basins, since they are located in some of the main Portuguese agricultural areas and, therefore, are potentially at risk. In the 'Médio Tejo' and 'Lezíria do Tejo' regions, located in the 'Tejo' river basin, there are some important irrigated crops such as maize, tomato for industry, rice, sugar beet, open-air horticultural crops and potato, as well as wheat and vines (RGA, 2001a). Some of these crops are also found in the 'Baixo Mondego' area, particularly maize, rice and potato, which occupy an important part of the agricultural area of this region (RGA, 2001b). Concerning the 'Sado' river basin, the agricultural area is mainly occupied by paddy rice (RGA, 2001c).

1.2. Pesticide compounds selected for the study and their TMoA

Twenty one herbicides, five insecticides and three metabolites were selected in this study due to their inclusion in the list of priority substances in the field of water policy (EC, 2013), the amount sold in Portugal (DGAV, 2014) since 2002, their approval for use on the main crops of the studied agricultural areas (see Section 1.1), their detection in previous studies performed in Portugal (Batista, 2003; Batista et al., 2001, 2002; Cerejeira et al., 2000, 2003; Pereira, 2003; Silva et al., 2006, 2011, 2012a, 2012b), and/or their inclusion in the priority list defined in the European project 'Optimization and evaluation of multiresidue methods for priority pesticides in drinking and related waters' (Jaskulké et al., 1999).

Taking into account the TMoA of these pesticide compounds and the presence or absence of specific target sites of toxic action in three important taxonomic groups of the freshwater environment (primary producers, arthropods and fish), nine specific TMoAs were distinguished, *i.e.*, seven groups of herbicides (and their metabolites) with the same specific TMoA for primary producers, and two for arthropods and fish. The organisms that lack the target receptor are not sensitive to pesticide exposure and will experience narcotic baseline toxicity or a secondary level of toxicity (Table 1).

1.3. Risk analysis

1.3.1. Exposure data

The exposure data used in this study correspond to 281 surface water samples collected at 43 sampling sites chosen to give a general environmental status of the 'Mondego', 'Sado' and 'Tejo' river basins during the main period of agricultural practices from 2002 to 2008, both in terms of pesticide application and irrigation. The pesticides alachlor, atrazine, chlorfenvinphos, chlorpyrifos, cyanazine, dichlobenil, endosulfan, ethofumesate, lindane, metolachlor, metribuzin, molinate, oxadiazon, pendimethalin, pirimicarb, prometryn, propanil, propazine, simazine, terbuthylazine, terbutryn, trifluralin, and the metabolites 3,4-dichloroaniline (3,4-DCA), desethylatrazine (DEA) and desisopropylatrazine (DIA) were extracted by solid-phase microextraction (SPME) followed by qualitative and quantitative analysis by gas chromatography (Varian ChromPack CP-3800, Walnut Creek, CA, USA) with mass spectrometric detection (Varian ChromPack Saturn 2000 ion trap MS, Walnut Creek, CA, USA) (Silva et al., 2012a,b), while the pesticides cycloxydim, MCPA, profoxydim and triclopyr by solid-phase extraction (SPE) followed by liquid chromatography

Table 1 – Primary toxic mode of action (TMoA) of the 29 pesticide compounds selected for the study for primary producers, arthropods and fish.

Pesticide compound	Primary producers	Arthropods	Fish
Herbicide			
Alachlor	InhMCellDiv	Narcosis	Narcosis
Atrazine	InhPhoto	Narcosis	Narcosis
Cyanazine	InhPhoto	Narcosis	Narcosis
Cycloxydim	InhACCase	Narcosis	Narcosis
Dichlobenil	InhCellWall	Narcosis	Narcosis
Ethofumesate	InhLS(notA)	Narcosis	Narcosis
MCPA	GrowReg	Narcosis	Narcosis
Metolachlor	InhMCellDiv	Narcosis	Narcosis
Metribuzin	InhPhoto	Narcosis	Narcosis
Molinate	InhLS(notA)	Narcosis	Narcosis
Oxadiazon	InhProto	Narcosis	Narcosis
Pendimethalin	InhMCellDiv	Narcosis	Narcosis
Profoxydim	InhACCase	Narcosis	Narcosis
Prometryn	InhPhoto	Narcosis	Narcosis
Propanil	InhPhoto	Narcosis	Narcosis
Propazine	InhPhoto	Narcosis	Narcosis
Simazine	InhPhoto	Narcosis	Narcosis
Terbuthylazine	InhPhoto	Narcosis	Narcosis
Terbutryn	InhPhoto	Narcosis	Narcosis
Triclopyr	GrowReg	Narcosis	Narcosis
Trifluralin	InhMCellDiv		
Metabolite			
3,4-DCA	InhPhoto	Narcosis	Narcosis
DEA	InhPhoto	Narcosis	Narcosis
DIA	InhPhoto	Narcosis	Narcosis
Insecticide			
Chlorfenvinphos	Narcosis	InhAChE	InhAChE
Chlorpyrifos	Narcosis	InhAChE	InhAChE
Endosulfan	Narcosis	InhGABA	InhGABA
Lindane	Narcosis	InhGABA	InhGABA
Pirimicarb	Narcosis	InhAChE	InhAChE

InhMCellDiv: mitosis and cell division inhibitor; InhPhoto: photosynthesis (photosystem II) inhibitor; InhACCase: fatty acid synthesis (ACCase inhibitor) inhibitor; InhCellWall: cell wall synthesis inhibitor; InhLS(notA): lipid synthesis (not A) inhibitor; GrowReg: synthetic auxin (also plant growth modifier); InhProto: protoporphyrinogen oxidase, leading to irreversible cell membrane damage inhibitor; InhAChE: acetylcholinesterase (AChE) inhibitor; InhGABA: GABA-gated chloride channel antagonist; Narcosis: narcosis or baseline toxicity.

with mass spectrometric detection (LC–MS) with an Agilent Series HP1100 (Palo Alto, CA, USA) (Silva et al., 2006).

Table 2 presents the mean and median concentration and detection frequency values for the pesticides compounds analyzed in surface waters of the 'Mondego', 'Sado' and 'Tejo' river basins. Molinate, 3,4-DCA, metolachlor and atrazine were the most frequently detected pesticides in surface waters of the 'Mondego' river basin with 62%, 58%, 56% and 53% detection, respectively. In surface waters of the 'Sado' river basin, molinate, 3,4-DCA and oxadiazon were the most frequently detected pesticide compounds, with 77%, 61% and 52% detection, respectively. Atrazine and alachlor were the most frequently detected pesticides in surface waters of the 'Tejo' river basin, with 44% and 33% detection, respectively. These pesticides also had the highest mean and median concentrations.

1.3.2. Laboratory toxicity data mining

In order to compute the SSDs, acute toxicity data were primarily extracted from the ECOTOX database (US EPA, 2014). In cases where median lethal (effective) concentrations ($L(E)C_{50}$) were not available for at least two species in each of the three taxonomic groups (*i.e.*, primary producers, arthropods and fish), the database was complemented with a variety of other data sources: draft assessment reports (EFSA, 2014), EU review reports (EC, 2014), the FOOTPRINT pesticide database (FOOTPRINT, 2014), (Tomlin, 2006), and the open literature. Only laboratory data fulfilling the selection criteria as set in Van den Brink et al. (2006) were included in the analysis. Since recent studies have demonstrated that toxicity data for freshwater and saltwater organisms may in principle be pooled for pesticides (EC, 2011; Klok et al., 2012), data for saltwater organisms were accepted unless they may be expected to have a clearly different life-form or feeding strategy than freshwater organisms (*e.g.*, macroalgae and crustaceans like crabs; EC, 2011). In accordance with EC (2011), geometric means of multiple comparable toxicity values for the same species and the same (considered acceptable) endpoint were calculated if available. Subsequently, the geometric mean of the most sensitive endpoint was used in that case.

Limited toxicity data were available for the herbicides profoxydim, propazine, the insecticide pirimicarb, and the metabolites DEA and DIA. Of this group, pirimicarb and propazine were not detected in the surface water samples, while DEA and profoxydim were only detected in four and one samples in the 'Tejo' and 'Sado' river basins, respectively.

1.3.3. Species sensitivity distributions

SSDs with acute toxicity/species sensitivity data sets segregated into primary producers, arthropods and fish were constructed for each pesticide compound as described by Daam et al. (2010). In brief, log-normal distributions of the toxicity values were derived using the ETX computer program version 2.0 (Van Vlaardingen et al., 2004). If log-normality was not accepted by the Anderson–Darling Test included in the ETX software package, the BurrliOZ program (Campbell et al., 2000) was used to fit a Burr type III distribution that best fitted the available data (log-logistic, log-normal, log-triangular, Weibull). BurrliOZ does not include software to indicate how well the datapoints fit the curves. Hence, in accordance with Daam et al. (2010), r^2 values were calculated by applying linear regression in Microsoft Excel on PAF values indicated by the curve and actual PAF values of the individual toxicity values as a measure of how well the curve fitted the datapoints.

For each pesticide compound and species group, and after log-transforming the respective acute toxicity values, the normal distribution parameters, mean (μ) and standard deviation (σ), were estimated (Table 3) and used as input for the toxic pressure calculation (msPAF) applied to each taxonomic group.

1.4. Toxic pressure calculation for pesticide mixtures

The combined toxic risk (msPAF) per taxonomic group of all 24 pesticide compounds was evaluated by sequentially applying

Table 2 – Mean, median concentration and detection frequency values for the pesticide compounds analyzed in 281 surface water samples collected at 43 sampling sites in the 'Mondego', 'Sado' and 'Tejo' river basins from 2002 to 2008.

Pesticide compound	Mean-median (μg/L)			Detection frequency (%)		
	'Mondego' river basin	'Sado' river basin	'Tejo' river basin	'Mondego' river basin	'Sado' river basin	'Tejo' river basin
Herbicide						
Alachlor	0.002–<DL	–	<DL–1.15	5	–	33
Atrazine	0.16–<0.05	–	<DL–0.57	53	–	44
Cyanazine	–	–	<DL–<DL	–	–	0
Cycloxydim	–	<DL–<DL	–	–	0	–
Dichlobenil	–	–	<DL–<DL	–	–	0
Ethofumesate	–	–	<DL–0.04	–	–	20
MCPA	–	0.02–<DL	–	–	10	–
Metolachlor	0.06–<0.05	–	<DL–0.07	56	–	20
Metribuzin	–	–	<DL–0.007	–	–	2
Molinate	1.03–0.06	5.4–0.14	–	62	77	–
Oxadiazon	0.006– < DL	0.15– < 0.05	–	12	52	–
Pendimethalin	–	–	<DL–0.004	–	–	2
Profoxydim	–	0.0002–<DL	–	–	2	–
Prometryn	–	–	<DL–<DL	–	–	0
Propanil	0.007–<DL	0.01–<DL	<DL–0.16	18	14	7
Simazine	0.08–<DL	–	<DL–0.08	47	–	23
Terbuthylazine	–	–	<DL–0.09	–	–	24
Terbutryn	–	–	<DL–0.006	–	–	10
Triclopyr	–	0.07–<DL	–	–	17	–
Trifluralin	–	–	<DL–<DL	–	–	0
Metabolite						
3,4-DCA	0.33–<0.05	0.39–<0.05	<DL–0.35	58	61	7
DEA	–	–	<DL–0.005	–	–	3
DIA	–	–	<DL–<DL	–	–	0
Insecticide						
Chlorfenvinphos	0.03–<EDL	0.05–<DL	<DL–0.007	9	18	5
Chlorpyrifos	–	–	<DL–0.02	–	–	16
Endosulfan	0.0008–<DL	0.01–<DL	<DL–0.02	3	7	7
Lindane	–	–	<DL–<DL	–	–	0
Pirimicarb	–	–	<DL–<DL	–	–	0
At least one of the pesticide compounds				93	49	82

–: No data.
<DL: below the detection limit.

the mixture toxicity mixed-model (Traas et al., 2002; De Zwart and Posthuma, 2005). First, the concentration addition $msPAF_{CA}$ values were calculated for pesticide compounds sharing the same TMoA by applying the function NORMDIST(log(ΣHU_{TMoA}), 0, Average(σ_{TMoA}), 1), where Average(σ_{TMoA}) is the average of the standard deviation (σ) for pesticide compounds within the same TMoA, and ΣHU_{TMoA} is the summation of their hazard units calculated by the following equation:

$$\sum_{i=1}^{n} HU_{TMoA} = \sum_{i=1}^{n} \frac{C_{TMoA,i}}{10^{\mu i}} \quad (1)$$

where, $C_{TMoA,i}$ is the concentration of pesticide compound i measured at a specific site and on a specific date and μ_i is the median of the respective pesticide compound. For algae, there were eight groups of pesticide compounds showing within-group concentration addition (InhMCellDiv, InhPhoto, InhACCase, InhCellWall, InhLS(notA), GrowReg, InhProto, Narcosis, see Table 1), while for arthropods and fish there were three (InhAChE, InhGABA, Narcosis, see Table 1).

For groups of pesticide compounds with different TMoA, the response addition model was then used and the final msPAF values were calculated as: $msPAF = 1-\Pi(1 - msPAF_i)$, where $msPAF_i$ stands for $msPAF_{CA}$ (for a group of pesticides with the same TMoA) or PAF (for a single pesticide with unique TMoA). It was assumed that aquatic species do not share a significant correlation in their sensitivity to different toxicants, i.e., $r = 0$.

2. Results and discussion

2.1. Toxic pressure for pesticide mixtures

The toxic pressure, quantified as msPAF, was calculated in 281 samples considering the given concentration of fourteen herbicides (alachlor, atrazine, ethofumesate, MCPA, metolachlor,

Table 3 – Parameters of the normal distribution fitted to the logarithm of acute toxicity values for 24 pesticide compounds in surface waters of the 'Mondego', 'Sado' and 'Tejo' river basins.

Pesticide compound	Primary producers		Arthropods		Fish	
	μ [a]	σ [b]	μ [a]	σ [b]	μ [a]	σ [b]
Herbicide						
Alachlor	−0.10	1.00	0.78	1.07	0.06	0.97
Atrazine	−0.76	0.76	0.43	1.13	1.08	0.61
Cyanazine	−1.27	0.54	1.17	0.71	1.00	0.29
Cycloxydim	1.41	0.62	1.99	0.19	2.17	0.24
Dichlobenil	−0.01	0.75	1.00	0.58	1.24	0.78
Ethofumesate	0.57	0.19	1.43	1.00	1.41	0.25
MCPA	0.94	0.95	2.31	0.08	1.56	1.29
Metolachlor	−0.01	0.75	0.73	0.31	0.74	0.59
Metribuzin	−1.37	0.56	1.65	0.32	1.83	0.38
Molinate	0.64	0.65	0.66	0.53	1.22	0.37
Oxadiazon	−1.59	0.62	0.26	0.88	0.43	0.69
Pendimethalin	−0.09	1.31	0.45	1.09	0.00	0.25
Prometryn	−1.93	0.54	−0.08	1.47	0.65	0.24
Propanil	−0.66	0.89	0.85	0.57	0.74	0.26
Simazine	−0.73	0.52	1.56	0.75	1.54	0.59
Terbuthylazine	−1.16	0.50	0.07	1.46	1.03	0.49
Terbutryn	−0.48	2.22	−0.31	1.91	0.58	0.18
Triclopyr	0.64	0.74	1.94	0.25	1.08	0.54
Trifluralin	−0.24	1.16	−0.13	0.96	−0.74	1.01
Metabolite						
3,4-DCA	0.60	0.43	0.13	0.91	0.41	0.55
Insecticide						
Chlorfenvinphos	0.16	0.06	−1.66	1.10	−0.65	0.77
Chlorpyrifos	−0.58	0.34	−2.57	1.15	−1.35	0.91
Endosulfan	−0.02	0.50	−1.64	1.33	−2.42	0.86
Lindane	−0.03	0.87	−1.29	0.97	−0.88	0.85

[a] Mean of the log-transformed acute toxicity values.
[b] Standard deviation of the log-transformed acute toxicity values.

metribuzin, molinate, oxadiazon, pendimethalin, propanil, simazine, terbuthylazine, terbutryn and triclopyr), three insecticides (chlorfenvinphos, chlorpyrifos and endosulfan) and one metabolite (3,4-DCA) detected at least once in surface waters of the 'Mondego', 'Sado' and 'Tejo' river basins.

Fig. 1 shows the distribution of median, box (25th and 75th percentiles) and whisker (5th and 95th percentiles) toxic pressure calculated for primary producers, arthropods and fish for measured pesticide mixtures in surface waters of the 'Mondego', 'Sado' and 'Tejo' river basins, assuming concentration addition within TMoAs and response addition between TMoAs. The highest median msPAF was found for primary producers (49%) in surface waters of the 'Sado' river basin, followed by the same taxonomic group in surface waters of the 'Tejo' (40%) and 'Mondego' (39%) river basins. For arthropods, the median msPAF also exceeded 5%, the threshold percentile value used in the prospective SSD approach for deriving environmental quality standards (EQSs) (EC, 2011).

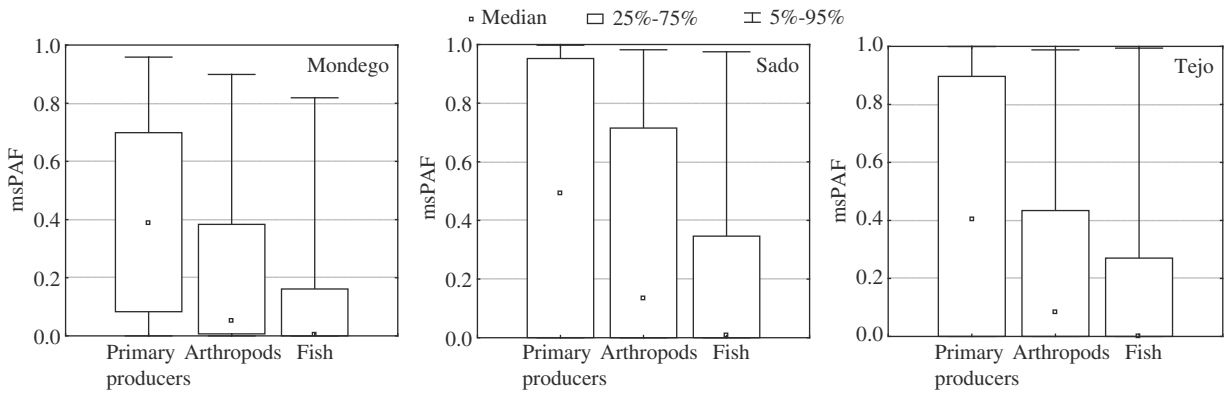

Fig. 1 – Box plots of toxic pressure (msPAF) for primary producers, arthropods and fish on measured pesticide mixtures in surface waters of the 'Mondego', 'Sado' and 'Tejo' river basins.

The overall EC_{50}-based toxic pressure for primary producers ranged from 0 (5th percentile) to 100% (95th percentile), followed by arthropods (0–99%) and fish (0–99%) (Fig. 1). Although the pesticide mixture types and their ratios were not shown in each sample, the variability in the msPAF results may be explained by the high pesticide detection frequency encountered in the samples, *i.e.*, in about 80% of them at least one pesticide compound was detected, wherein the herbicide compounds were the most frequently detected type. In addition, there were also differences regarding the detection frequency and concentration values of each individual pesticide compound.

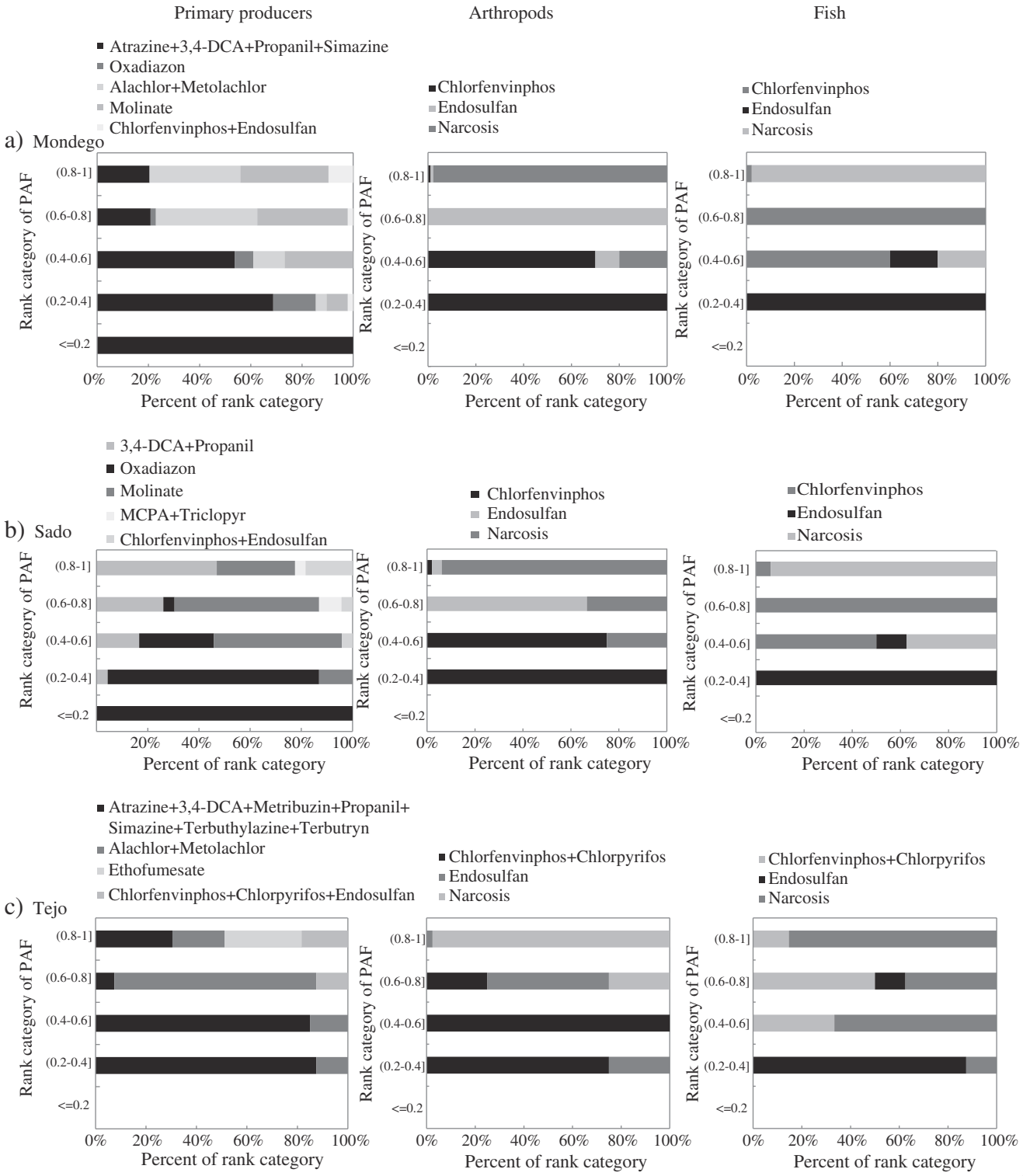

Fig. 2 – Influence of individual pesticides (or groups of pesticides with the same TMoA) on predicted risks for primary producers, arthropods and fish in surface waters of the 'Mondego' (a), 'Sado' (b) and 'Tejo'(c) river basins.

When high msPAFs for direct toxic effects are obtained for a certain taxonomic group, some possible indirect effects may be anticipated. For example, a reduction of the primary producers can lead to a decrease in the herbivore populations due to food limitation and/or habitat loss (Schäfer et al., 2011). Future studies could shed light on the implications associated with increased levels of toxic risk.

Four other case studies, which also used msPAFs posed by the pesticide mixtures as a measure of ecological risk at the local/river basin scale, were analyzed. However, caution is required when comparing the results with those of our study, because they differ in terms of methodological issues. First, only herbicide mixtures were analyzed in the four studies, and in three of these the msPAFs were calculated from the SSDs of the target *taxa* (primary producers) (Gregorio et al., 2012; Jesenska et al., 2013; Schuler and Rand, 2008), and one from the SSDs of all taxonomic groups (Faggiano et al., 2010). With the exception of one study (Jesenska et al., 2013), the msPAF sets were derived using acute SSD. However, taking into account these factors and others like the exposure profile and the influence of different data validation approaches on the msPAFs, the maximum msPAFs reached in our study were higher than in these previous studies.

2.2. Relative contributions of individual pesticide compounds (or groups of pesticides with the same TMoA) on the overall msPAF per taxonomic group

The influence of individual pesticide compounds (or groups of pesticides with the same TMoA) on the overall msPAF per taxonomic group was investigated. The approach followed by Jesenska et al. (2013) was adopted in this study. For each sample, the PAFs of individual pesticides (or class with the same TMoA) were ranked for the three species groups (the higher the PAF value, the bigger the influence of the respective pesticide compound on msPAF). The obtained rank values (o) were then weighted by dividing by the number of pesticides (n) that were actually determined in the specific sample. The final value (weighted rank = o/n) indicates the relative contribution of the individual pesticide compound (or TMoA class) to the total msPAF (the smaller the value of the weighted rank, the higher the relative importance of an individual pesticide compound; Fig. 2).

The inhibitors of photosystem II (atrazine, 3,4-DCA, propanil and simazine), followed by the protoporphyrinogen oxidase inhibitor oxadiazon, presented the highest percent distributions at the two lowest rank categories of PAF for primary producers in surface waters of the 'Mondego' river basin. For arthropods and fish, the corresponding pesticide compounds were the insecticides chlorfenvinphos and endosulfan, respectively (Fig. 2a). In surface waters of the 'Sado' river basin, the pesticide oxadiazon presented 100% distribution at the lowest rank category of PAF for primary producers, while chlorfenvinphos and endosulfan at the second lowest for arthropods and fish, respectively (Fig. 2b). The inhibitors of photosystem II (atrazine, 3,4-DCA, metribuzin, propanil, simazine, terbuthylazine and terbutryn) were the most problematic (very high percent fractions at the second and third lowest, i.e., most influential, weighted rank categories 0.2–0.4 and 0.4–0.6) for primary producers in surface waters of the 'Tejo' river basin. For the inhibitors of mitosis and cell division (alachlor and metolachlor), two peaks in these distributions of ranks were observed for primary producers, while the acetylcholinesterase (AChE) inhibitors (chlorfenvinphos and chlorpyrifos) and the GABA-gated chloride channel antagonist, endosulfan, appeared to be the most hazardous for arthropods and fish, respectively (Fig. 2c).

The results of msPAF for primary producers showed that the herbicide compounds investigated could provoke more effects in this species group, while for arthropods and fish the insecticides caused more effects. This difference is related to the highly specific TMoA and the low selective toxicity of herbicides to primary producers, and insecticides to arthropods. This was also expected, taking into account the predictive value of the SSDs for effects of herbicides and insecticides constructed in this study (Table 3) and others (Maltby et al., 2005; Van den Brink et al., 2006). In addition, several artificial ecosystem studies showed that primary producers and aquatic animals are the most sensitive groups for herbicides and insecticides, respectively (Brock et al., 2000a,b; Van Wijngaarden et al., 2005).

In relation to the TMoA, the photosystem II inhibitors were the pesticides with more pronounced predicted risks for primary producers in surface waters of the 'Mondego' and 'Tejo' river basins. Contributing factors were the large number of herbicides detected within that TMoA, their relatively high toxicity to primary producers, and measured concentration and detection frequency values. The herbicide oxadiazon mainly influenced the msPAFs for these *taxa* in surface waters of the 'Sado' river basin because it was more frequently detected than in surface waters of the 'Mondego' river basin, where it was also analyzed, and it has a specific TMoA that also acts as a photosynthesis inhibitor. The acetylcholinesterase (AChE) inhibitors, chlorfenvinphos and chlorpyrifos, were the pesticides with the most potential to affect the arthropods, whereas for fish this pesticide was the GABA-gated chloride channel antagonist, endosulfan, in surface waters of the three river basins. Although the two organophosphate insecticides were more frequently detected and with higher concentrations than the organochlorine insecticide, the greater toxicity of this compound to fish influenced the results (Table 3).

3. Conclusions

The present study estimated the impact of measured pesticide mixtures in 281 surface water samples from the 'Mondego', 'Sado' and 'Tejo' river basins on primary producers, arthropods and fish by toxic pressure calculation, quantified as msPAF. This multiple-species method of mixture risk prediction incorporates SSDs and mixture toxicity models into a single procedure, serving to underpin improved risk assessment.

The obtained acute msPAFs were variable between the river basins and the taxonomic groups. The median msPAF for primary producers and arthropods in surface waters of all river basins exceeded the threshold PAF of 5% used in the prospective SSD approach for deriving individual EQSs. A ranking procedure identified the inhibitors of photosystem II and oxadiazon as having the relatively largest toxic effects on primary producers, while the organophosphorus pesticides

chlorfenvinphos and chlorpyrifos, and the organochlorine endosulfan had the largest effects on arthropods and fish, respectively. This was influenced by a combination of factors, such as the number of detected pesticides with the same TMoA, the exposure data and the potential compound-specific effects on the aquatic species assemblages.

The data generated is of importance under the scope of European legislation for the derivation of optimized programs of measures, through the identification of the sites of the highest expected impacts, and the major pesticide compounds most likely contributing to the aquatic risks, as well as for the evaluation of risk mitigation measures.

In further research, the outcome of msPAF calculations should be confirmed and validated to improve ecological interpretation of the output by applying eco-epidemiological approaches or using other lines of evidence, such as toxicity testing and in-situ community analysis.

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