APPROACHES TO IMPROVE THE ECOLOGICAL RISK ASSESSMENT OF PESTICIDES IN FRESHWATERS

Tese apresentada para obtenção do grau de Doutor em Engenharia Agronómica

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List of Abbreviations and Acronyms

3,4-DCA 3,4-dichloroaniline
AA-EQS Annual Average-Environmental Quality Standard
AChE Acetylcholinesterase
ACR Acute to Chronic Ratio
ADI Acceptable Daily Intake
AF Application Factor, Adjustment Factor, Assessment Factor
AL Acceptable Level
CA Concentration (Dose) Addition
CIS Common Implementation Strategy
CL Confidence Limit
CW/DVB Carbowax Divinylbenzene
DEA Desethylatrazine
DIA Desisopropylatrazine
DL Detection Limit
DNEL Derived No Effect Level
EC$_{50}$ Median Effective Concentration
EI Electronic Impact
EL Exposure Level
EQS Environmental Quality Standard
EQSD Priority Substances Directive
EU European Union
GABA Gamma-AminoButyric Acid
GC-MS Gas Chromatography-Mass Spectrometry
GUS Groundwater Ubiquity Score
GWD Groundwater Directive
HC$_p$ Hazardous Concentration to p% of the species
HI Hazard Index
IA Independent Action
LC$_{50}$ Median Lethal Concentration
LC-MS Liquid-Cromatography-Mass Spectrometry
LOEL Lowest Observed Effect Level
MAC-EQS Maximum Allowable Concentration-Environmental Quality Standard
MACT Maximum Acceptable Toxicant Concentration
MCR Maximum Cumulative Ratio
MEC Measured Environmental Concentration
MS Member State
msPAF Multi-Substance Potentially Affected Fraction
NOAEL No Observed (Adverse) Effect Level
NOEC No Observed Effect Concentration
PAF Potentially Affected Fraction
PEC Predicted Environmental Concentration
PED Predicted Environmental Distribution
PICT Pollution-Induced Community Tolerance
PNEC Predicted No Effect Concentration
POD Point of Departure
PODI Point of Departure Index
PPP Plant Protection Product

RBSP River Basin Specific Pollutant
REACH Registration, Evaluation, Authorisation and Restriction of Chemicals
RQ Risk Quotient
SPE Solid-Phase Extraction
SPEAR SPEcies At Risk
SPME Solid-Phase Microextraction
SSD Species Sensitivity Distribution
STU Sum (summation) of Toxic Units (or TUS, Toxic Unit Summation)
TGD Technical Guidance Document
TMoA Toxic Mode of Action
TU Toxic Unit
TV Threshold Value
TVZ Tagus vulnerable zone
WFD Water Framework Directive
WOE Weight-of-Evidence

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Resumo

A utilização de pesticidas constitui um meio importante para melhorar a produção agrícola. No entanto, pode causar efeitos secundários nos ecossistemas aquáticos. De forma a alcançar um bom estado químico e ecológico das massas de água europeias, foram abordados os novos desafios da avaliação de risco. Foram desenvolvidas e aplicadas abordagens com base ecológica e específica a três importantes bacias hidrográficas portuguesas. Propuseram-se melhores critérios para a avaliação do estado ecológico e químico das massas de água calculando limiares para os pesticidas em águas subterrâneas e para outros específicos em águas superficiais. Obteve-se uma lista prioritária de misturas de pesticidas com potenciais riscos aquáticos através do cálculo de quocientes de risco, com base na adição da concentração, como primeira etapa da avaliação do risco de misturas de pesticidas, e da fração de espécies potencialmente afetada por misturas de substâncias, representando um nível superior com maior utilidade e relevância para gestão do risco. Para avaliação integrada do risco de pesticidas em águas doces, e com base numa abordagem mais holística, foram combinadas análises químicas com ferramentas de avaliação de efeitos, para relacionar a avaliação química com a ecológica. Os resultados contribuem para a redução dos riscos de pesticidas em águas doces.

Palavras-chave: avaliação de risco; pesticidas; misturas; água superficial; água subterrânea

Abstract

One of the most important ways of improving agricultural production is the use of plant protection products. As this type of pesticides is used within the catchment area of freshwaters there is a potential for side-effects to occur in aquatic ecosystems. New challenges for risk assessment were addressed to achieve good chemical and ecological status in European water bodies. Site-specific and ecologically-based approaches were developed and applied to three important Portuguese river basins. Better criteria for the assessment of the ecological and chemical status of water bodies was provided by calculating groundwater threshold values for pesticides and by focusing on river basin specific pollutants. A list of priority pesticide mixtures that might pose aquatic risks was obtained by employing risk quotients based on concentration addition as a ‘screening level’ risk assessment of pesticide mixtures, and the multi-substance potentially affected fraction, representing a higher tier, with more practical usefulness and relevancy for risk management. For an integrated assessment of pesticide stress in freshwaters, and moving toward a more holistic approach, chemical analysis were used in combination with effect-based tools, in order to provide a link between chemical and ecological assessments. The results contribute for reducing the risks of pesticides in freshwaters.

Key-words: risk assessment; pesticides; mixtures; surface water; groundwater

Chapter 1 Introduction

1. Introduction

1.1 Water Framework Directive and the retrospective risk assessment procedures for pesticides

In Europe, the largest land-cover category taken by urban and other artificial land development was agriculture land (EC, 2014a). One of the most important ways of protecting plants and plant products against harmful organisms, including weeds, and of improving agricultural production is the use of plant protection products (PPPs). However, this type of pesticides can also have non-beneficial effects on plant production. Their use may involve risks and hazards for human, animals and the environment, especially if incorrectly used. Because pesticides are used within the catchment area of freshwaters, contamination of surface and groundwater bodies is a well-recognized issue. Given the fact that freshwaters contain species taxonomically related to the target organisms of pesticides, there is a potential for undesirable side effects to occur in aquatic ecosystems. Several studies provided strong evidence that contamination with pesticides has a clear impact on aquatic communities and thus on the ecological status of a water body (Gregorio *et al.*, 2012; Leitão *et al.*, 2007; McClellan *et al.*, 2008; Ricart *et al.*, 2010; Vaj *et al.*, 2011).

Considering the risk from existing and/or past uses or releases of pesticides to the aquatic environment, retrospective risk assessments are needed making use of their measured concentrations or biological effects in exposed freshwater ecosystems and/or watersheds (Solomon *et al.*, 2008). Although this study focuses on the retrospective risk assessment of pesticides in freshwater ecosystems, it is recognized, however, that pesticides represent only one class of stressors that organisms may be exposed to in that environment. The Water Framework Directive (WFD) (EC, 2000) follows a retrospective approach and aims to improve the ecological and chemical status of water bodies in Europe.

The chemical status assessment is used alongside the ecological status assessment to determine the overall quality of a water body. Environmental quality standards (EQSs) are tools used for assessing the chemical status of water bodies. The new Directive

2013/39/EU (EC, 2013) amending Directives 2000/60/EC (EC, 2000) and 2008/105/EC (EC, 2008) as regards priority substances in the field of water policy establishes the maximum acceptable concentration (MAC) and/or annual average value (AA) for 45 priority (hazardous) substances which, if met, allows the chemical status of the water body to be described as ‘Good’.

In addition, the WFD establishes the principles to be applied by the Member States (MSs) to develop EQSs for specific pollutants that are ‘discharged in significant quantities’. In contrast to the EU-level priority (hazardous) substances the specific pollutants are established at local/river-basin/national. Compliance with EQSs for specific pollutants forms part of the assessment of ecological status. If the EQSs for these substances of concern are not met, the water body cannot be classified as either ‘Good’ or ‘High’ status, even if the biological quality is ‘Good’ or ‘High’. EQSs are therefore key tools in assessing and classifying chemical status and can therefore affect the overall classification of a water body under the WFD. The methodology for deriving EQSs for priority (hazardous) and river basin specific pollutants (RBSPs) is described in the new ‘Technical Guidance Document for Deriving EQSs’ (EC, 2011) for the WFD. In the effect assessment, the PPP Regulation (EC, 2009a) follows a tiered approach while the EQS derivation, according to the WFD, follows a weight-of-evidence (WOE) approach.

In October 2012, the Commission published a report with a comparison of specific pollutants and EQSs across the EU (EC, 2012). Based on their conclusions, it was evident 1) that different approaches have been used to identify RBSPs by different MSs and 2) that not all the EQSs values for RBSPs have been derived using the procedures specified in Annex V 1.2.6 of the WFD (EC, 2000) and defined in the Common Implementation Strategy (CIS) Guidance for Deriving EQSs (EC, 2011). It appears that for water bodies in many MSs a failure to achieve the EQS values for RBSPs in water bodies in river basins contributed to a failure to achieve good ecological status (i.e. was at least one cause of failure). In order to minimise the potential data gaps for RBSPs selected by MSs, the report pointed out that a harmonised approach to the selection of RBSPs should be adopted by all MSs to ensure that all relevant hazardous substances ‘released in significant quantities’ were identified. In addition, consistency in the derivation of RBSP could be improved by training based on the CIS Guidance and MS technical discussions on data exchange and data interpretation (EC, 2012).

Specific pollutants ‘released in significant quantities’ in Portugal were identified in collaboration with the Regional Coordination and Development Commissions. The list is of 126 specific pollutants, 21 of them do not have EQSs (INAG, 2009). Besides, the EQS values applied had been derived for previous legislation which may have been enacted before the WFD methodology was developed. The report on the comparison of specific pollutants and EQSs at the European level (EC, 2012) provided contradictory results, i.e. concluded that Portugal has not identified any RBSPs at the time of collection (i.e. up to August 2012). The presence in water bodies of substances ‘discharged in significant quantities’ and not identified as RBSPs may not be detected in the assessment of ecological status if no EQS values are applied. **Thus, defining candidate compounds for monitoring and prioritisation as RBSPS is a challenging task.** This research aims to meet this challenge by using an integrated approach, based on modelling, field and laboratory studies applied for site-specific risk assessment in freshwaters of three important Portuguese river basins (‘Mondego’, ‘Sado’ and ‘Tejo’).

For the purpose of the assessment of the chemical status of groundwater bodies or groups of groundwater bodies, Groundwater Directive (GWD) (EC, 2006) lays down criteria in the form of groundwater quality standards and groundwater threshold values.

The GWD defines groundwater quality standards for two pollutant types, nitrates and pesticides. For active substances in pesticides, including their relevant metabolites, degradation and reaction products the quality standard for individual substances is 0.1 μg/L and 0.5 μg/L for the sum of these substances.

However, if these groundwater quality standards are not adequate for achieving the environmental objectives set out in the WFD e.g. if concentrations in groundwater that are lower than quality standards are leading to (or are likely leading to) chemical and/or the ecological status failures in associated surface waters and/or significant damage to associated terrestrial ecosystems, then more stringent values have to be applied. These new values become threshold values (TVs) and their determination should be based on the extent of interactions between groundwater and associated aquatic and dependent terrestrial ecosystems, the interference with actual or potential legitimate uses or functions of groundwater and the hydro-geological characteristics including information on background levels and water balance.

GWD requires MSs to derive TVs for relevant parameters causing a groundwater body to be at risk of not meeting the WFD objectives. These TVs will become MS defined quality standards.

In March 2010, the Commission published a report with an accompanying working document, presenting these TVs as set by the MSs. Regarding pesticides, six MSs established TVs below the quality standard of 0.1 µg/L for 36 individual active substances in pesticides (respectively groups of active substances). The TVs range from 0.0001 µg/L to 0.1 µg/L. The number of TVs established by each MS according to pesticides varied between zero (Portugal) and 27 (United Kingdom). Portugal did not establish TVs so far at all as no groundwater body was identified as being at risk for pollutants other than nitrates (EC, 2010). However, several studies conducted over the past two decades have demonstrated groundwater contamination with pesticides in agricultural ecosystems of various Portuguese regions (Batista, 2003; Batista *et al.*, 2001, 2002; Cerejeira, 1993; Cerejeira *et al.*, 1995a,b, 2000, 2003; Silva *et al.*, 2006, 2011, 2012).

The TVs as set by the other MSs were generally based on i) environmental quality objectives for the protection of associated aquatic and dependent terrestrial ecosystems (e.g., EQS Directive 2008/105/EC); and ii) actual and potential legitimate uses of functions of groundwater (mostly based on drinking water standards) (EC, 2010).

From the discussed above, it may be concluded that TVs for groundwater ecosystems in the EU, as elsewhere, are not based on toxicity data for the biota of that ecosystem. **It thus seems imperative that an ecotoxicological evaluation of pesticides should be included in the ecological risk assessment of groundwater ecosystems. In the EU, this was acknowledged by the GWD, which sates in recital 20: *Research should be conducted in order to provide better criteria for ensuring groundwater ecosystem quality and protection*. For that reason, this important issue need to be covered.**

1.2 Cumulative stress of pesticides in freshwater ecosystems

Under the WFD, EQS derivation usually concerns a chemical per chemical approach. Only in exceptional cases they are derived for well-defined mixtures (e.g., biocide preparations, dioxins, PCBs), i.e. those with a well defined qualitative and quantitative composition (EC, 2011). Although compliance with good chemical status is primarily based on EQSs for individual substances, cumulative stress (including the toxicity of mixtures) may be identified as a main pressure affecting ecological status. In that case, the cumulative risks caused by pollutants have to be evaluated and reduced (Brock, 2013).

It has been demonstrated through various studies that aquatic organisms in their environment are typically confronted with mixtures of pesticides rather than that exposure is against individual compounds (Batista, 2003; Belden *et al.*, 2007b; Chèvre *et al.*, 2008; Gilliom, 2007; Gregorio and Chèvre, 2014; Hela *et al.*, 2005; Köck-Schulmeyer *et al.*, 2012; Palma *et al.*, 2014; Qu *et al.*, 2011; Reinert *et al.*, 2002; Schäfer *et al.*, 2011; Schuler and Rand, 2008; Silva *et al.*, 2011, 2012; Wang *et al.*, 2009). Also, experimental studies show that exposure against pesticide mixtures as present in the aquatic environment (Junghan *et al.*, 2006) or at low-effect concentrations of individual compounds (Faust *et al.*, 2001) may provoke combined effects and that ignoring these will underestimate resulting adverse biological outcomes. However, experimental investigation of every conceivable pesticide mixture for its adverse effects is not a viable option for risk assessment because of the very large number of possible mixtures (Altenburger and Greco, 2009) of pesticides that may occur in the aquatic environment as these are too variable as a consequence of different agricultural practices (simultaneous and/or serial application of the same active substance/different active substances on the same field/different fields). Thus, for risk considerations two principles routes in assessment may be followed: either a mixture typical for that of concern is considered as an entity which often is called a ‘whole mixture approach’ (*see 1.3 Linking the chemical and ecological status assessment*) or mixtures are inspected focussing on their relevant constituents which may be called ‘component-based approach’. Hence, it is essential to unambiguously define the mixture to which the assessment relates and use the correct relative proportions of mixture components in the calculation of the mixture toxicity by these last theorethical approaches (Altenburger et al., 2014). In this respect, there is a need to obtain data for mixtures by means of comprehensive monitoring as well as more complete spatially and temporally resolved data sets to investigate ecosystem variations of exposure (Di Guardo and Hermens, 2013).

Component-based approaches are based on the calculation of the expectable toxicity of a mixture of concern on the basis of the toxicity data for individual mixture components by applying appropriate concepts for predictive mixture toxicity assessments. As they do not require any further environmental system dependent information but rely on individual compound-biology interaction properties they can be considered as generic extrapolation tools suitable for application in ecological risk assessment. Three different assumptions are alternatively considered as a potential starting point: i) the assumption of a concentration-additive joint action (similar action) of all mixture components (CA), Table 1; ii) the assumption of a fully independent joint action (dissimilar action) of all mixture components (IA), Table 1; and iii) the assumption of an intermediate type of joint action (Altenburger *et al.*, 2014). The latter situation (option iii) may result from heterogeneous mixtures that do not fit into either of the ideal categories of strictly similar or strictly dissimilar action. For predictability of their joint toxicity Faust *et al.* (2003) assumed an intermediate toxicity, within a 'prediction window' defined by both concepts, or it may be modelled by means of a mixed-model combining the concepts of CA and IA (Olmstead and LeBlanc, 2005). Chemicals are assigned to groups based upon their presumed mechanisms of action. Chemicals having the same mechanism of action are assigned to the same group. The other chemicals all presumed to have different mechanisms of action and are assigned to separate groups. The joint toxicity of chemicals within a group is calculated using the CA approach, while the joint toxicity of different groups is calculated using the IA.

Extensive knowledge of the predictive capabilities of both reference models, CA and IA, is available. They have been found of reasonable predictivity for the vast majority of known pesticide mixture studies to date (Belden *et al.*, 2007a; Deener, 2000; Verbruggen *et al.*, 2010).

Table 1 Extrapolation concepts for mixture toxicity prediction (reference models) (adapted from Altenburger *et al.*, 2014)

	Concentration Addition (LOEWE Additivity)	Independent Action (BLISS Independence, Response Addition, Effect Multiplication)
Suggested for	Same site of action; similar mode of action	Different sites of action dissimilar modes of action
Formula		
Binary	$\frac{c_1}{EC_{x,1}} + \frac{c_2}{EC_{x,2}} = 1$	$E(c_{1,2}) = E(c_1) + E(c_2) - E(c_1)\,E(c_2)$
Multiple	$ECx_{mix} = (\sum_{i=1}^{n} \frac{p_i}{F_i^{-1}(x_i)})^{-1}$	$x\% = 1 - \prod_{i=1}^{n}(1 - F_i(p_i \times (ECx_{mix})))$

c_i concentration of substance i in the mixture (for CA at the EC_x of the mixture); EC_x effect concentration at the response level x; F function describing the relation between concentration and response for the individual component; p_i fraction of substance i in the mixture; x expected combined response; mix mixture

For the practical application of the concept of CA (option i) in a regulatory context, a number of different approaches have been suggested in the literature. For pragmatic reasons, these CA-based regulatory approaches (Table 2) usually include simplifying or additional assumptions, and hence they deviate more or less from the principal assumptions that are inherent to the original scientific concept of CA. As a result, such CA-based approaches may differ with respect to both the suitability for specific assessment purposes and the quantitative mixture toxicity estimates that are derived from their application (Altenburger *et al.*, 2014).

Table 2 Examples of pragmatic generalizations of CA for regulatory purposes (adapted from Altenburger *et al.*, 2014)

Approach	Assessment term
CA Concentration (Dose) Addition	$E_{mix} \leq x$ if $\sum_{i=1}^{n} TU_i = \sum_{i=1}^{n} \frac{c_i}{ECx_i} = \leq 1$
PODI Point of Departure Index	No significant effect if $\sum_{i=1}^{n} \frac{EL_i}{POD_i} \leq 1$, POD= LOEL, NOAEL, NOEC
HI Hazard Index	No reason for concern if $\sum_{i=1}^{n} \frac{EL_i}{AL_i} \leq 1$, AL=ADI, DNEL,...
PEC/PNEC Summation	No unacceptable risk if $\sum_{i=1}^{n} \frac{PEC_i}{PNEC_i} \leq 1$

c_i concentration of substance i in the mixture; EC_x effect concentration at the response level x; TU_i toxic unit of substance i in the mixture; EL exposure level; POD point of departure; LOEL lowest observed effect level, NOAEL no observed (adverse) effect level; NOEC no observed effect concentration; AL acceptable level; ADI acceptable daily intake; DNEL derived no effect level; PEC predicted environmental concentration; PNEC predicted no environmental concentration

Backhaus and Faust (2012) used the two well established mixture toxicity concepts (CA and IA) for providing a tiered outline for environmental risk assessment of mixtures, focusing on general industrial chemicals and assuming that the ‘base set’ of data (EC_{50}’s for algae, crustaceans, fish) is available. As mixture toxicities higher than predicted by CA are rare findings, they suggested applying CA as a precautious first tier, irrespective of the modes/mechanisms of action of the mixture components. Although the combination of PEC/PNEC ratios is less scientifically correct than the sum of toxic units (STU), they proved that the sum of PEC/PNECs is slightly more conservative and more easily applicable (in some cases) and therefore, for pragmatic reasons, may be used as a first-tier conservative approach. Finally, they suggested calling for mode-of-action driven analyses only if error estimations indicate the possibility for substantial differences between CA- and IA-based assessments.

The outlined scheme provides an approach for a general ‘screening level’ risk assessment of chemical mixtures that can be flexibility adapted and extended as needed. However, the actual consequences on the health of natural ecosystems produced by a risk quotient higher than one are unknown. It follows that effective risk management requires being able to calibrate changes in adverse effects with changes in chemical exposure, and assessments based on thresholds of effects are not helpful for risk management. This is particularly relevant if one considers that one of the aims of ecological risk assessment is to provide a basis for interventions that relate to effects that should be weighed against the costs of interventions (Forbes and Calow, 2013; Vighi and Villa, 2013).

Therefore, an ‘ecologically’-based ecological risk assessment, performed using more extensive information on ecologically relevant endpoints and ecologically realistic exposure assessment, should be based on a probabilistic assessment of the likelihood of a given adverse effect to occur, developing sound statistic approaches to quantify variability and uncertainty (Vighi and Villa, 2013). Ideally, it should provide information on the effect on the structural features of a community (species composition, biodiversity, etc.) and should allow some prediction on the functional features (primary productivity, biomass, etc.), by accounting for the role of 'key' species and food web changes (Vighi *et al.*, 2006). In this procedure, the risk characterization will not be expressed by a simple numerical threshold.

The component-based approach proposed by De Zwart and Postuma (2005) involves the use of both CA and IA models together (mixed-model approach) in combination with the probabilistic procedure, species sensitivity distribution (SSD), in ecological risk assessment by calculating the fraction of a set of species (that may be defined as a taxon, assemblage, or community) that is expected to be affected by the exposure to multiple substances (msPAF, multi-substance potentially affected fraction). Obviously, it requires much more information on a suitable number of species that should cover a substantial part of the range of variability of sensitivity to the considered chemicals. This approach also differs from the previous lower tier by using detailed information on the toxic mode of action (TMoA) for the different mixture components as well as full-curved-based modelling approaches.

The applications of PAF (potentially affected fraction) in risk assessment and management have some advantages over the use of risk quotients. It is powerful since it allows for comparisons of (aggregated) ecological risk over time and space, between *taxa*, and with other stressors. Furthermore, the PAF approach can be used not only diagnostically by using measured or interpolated environmental concentrations, but also prognostically by using predicted environmental concentrations generated by scenarios analyses. These features allow for comparison of the efficacy of different risk management scenarios for a specific location or for emission reduction measures on a local, regional, and national scale (Traas *et al.*, 2002).

Hence, the application of concepts and tools that address toxic effects of mixtures such as the approaches described above (De Zwart and Posthuma, 2005; Posthuma *et al.*, 2008; Traas *et al.*, 2002; Von der Ohe *et al.*, 2009) rather than an evaluation of single chemical concentrations using EQSs may help to increase realism in the assessment of toxic pollution.

Chemical monitoring data and model calculations reveal that in individual edge-of-field surface waters, usually a limited number of pesticides dominate the mixture in terms of toxic units (TUs), also when many are present (Belden *et al.*, 2007b; Finizio *et al.*, 2005; Schäfer *et al.*, 2007; Verro *et al.*, 2009). Consequently, when addressing cumulative stress of pesticides in ecological risk assessment and implementing restoration programmes, it seems cost-effective to focus on these in first instance. For this reason, it is an important research goal to construct exposure and effect databases for frequently occurring pesticide combinations that likely may dominate the potential for risk in water bodies of agricultural landscapes. This list of priority pesticide mixtures would serve as the bases for the evaluation of the current control measures to protect the aquatic environment that are mostly focused on single pure toxicants. **Thus, there is a need to respond to this demand by exploring the applicability of component-based approaches to this issue. Prioritisation should concern not only the mixture components that drive the risk on different scenarios, but also site-specific exposures and potentially affected species.** Some sites and species are likely to be more affected more than others, due to local conditions and specific species- and assemblage vulnerability characteristics.

1.3 Linking the chemical and ecological status assessment

The component-based approaches described above can only be used for pesticides that are monitored, and for substances for which the basic ecotoxicological data are available. It should also be pointed out that these approaches quantify only ‘known’ toxicity connected to pesticides which are measured, and do not account for possible (mixture) ecotoxicity of substances not included in chemical programmes. Not all toxicants that occur in aquatic ecosystems were and will be measured due to financial constraints, so that chemical monitoring programmes may underestimate the risks of cumulative stress (Brock, 2013).

There is a need to understand which are the real effects caused by the sum of the chemical substances in the aquatic environment (including emerging pollutants, metabolites and transformation products) and to link the observed effects with cost-effective management objectives. Furthermore, the substances present in the aquatic environment can form mixtures whose effects may not be predictable on the basis of chemical analyses alone. Aquatic effect-based monitoring tools, developed to respond to toxic substances, offer possibilities to overcome some of the limitations. The key advantage with monitoring also biological effects is that the overall response from co-exposure to multiple, bioavailable chemicals can be taken into account, at different levels of biological organisation, such as community, population, individual and/or suborganism levels. In this way, a more holistic approach is possible (EC, 2014b).

In the context of the WFD, the use of effect-based tools can be foreseen for the elaboration and implementation of monitoring programmes and could be used to support the assessment of water quality and provide a link between chemical and ecological assessments. *In vivo* bioassays are frequently performed using live organisms in more or less the same way as within chemicals testing. They have the capacity to provide an integrated response at organism level to contaminants in a sample. In general, highly relevant endpoints (such as survival, growth and mobility) are analysed (EC, 2014b).

Only chemical analysis approach does not provide any information on the quality of the risk assessment in terms of how much of the actual risk was really considered. **Thus, combined biological and chemical-analytical approaches provide an important progress towards an identification of those toxicants that are relevant for site-specific risks and towards an estimation of the portion of an effect that can**

be explained by the analysed chemicals. Approaches based on the correlation between concentrations of individual compounds and measurable effects in typically complex environmental mixtures generally do not provide reliable cause-effect relationships. In a study performed to evaluate the impact of pesticides on the rice environment, in some water samples was not possible to establish a correlation between the exposure level to individual pesticides and the observed acute toxicity, thus suggesting other factors (Pereira, 2003). In contrast, estimations of the contribution of chemically analysed compounds to the total measured effect of the mixture on the basis of compound-specific effect data and CA, as a concept for mixture toxicity, may be quite successful in estimating the contribution of selected compounds to the total effect (Brack *et al.*, 2007). **For an integrated assessment of pesticide stress in aquatic ecosystems, the combined use of multiple lines of evidence with a focus on the ecological status of the stressed ecosystem or watershed of concern is promising.**

Taking into account the issues addressed in this chapter and the need for research to improve the ecological risk assessment of pesticides in freshwater ecosystems, this thesis developed and applied approaches to reach this objective.

2. Thesis aims and structure

The general goal of this research is to improve the current retrospective ecological risk assessment procedures of pesticides in freshwaters addressing the issues given above in this Chapter 1 which were tackled with approaches developed and applied within this research. To this end, this study present and discuss them on basis of the following four scientific objectives: 1) to develop better criteria for ensuring groundwater ecosystem quality and protection, 2) to improve the classification of ecological status in surface waters in relation to RBSPs, 3) to apply new integrated approaches for the ecological risk assessment of pesticide mixtures in surface water and groundwater bodies, and 4) to provide evidence of pesticide stress in freshwater ecosystems. With the exception of 1), all the approaches were applied for a site-risk assessment in freshwaters of the ‘Mondego’, ‘Tejo’ and ‘Sado’ river basins where several relevant crops, on Portuguese agricultural land, are located within the catchment area of those rivers. In this Chapter 1, a synthesis is presented of a number of important issues and pointed out the need for research to improve the ecological risk assessment of pesticides in freshwaters and the tools that should be developed and applied to reach this objective. In Chapters 2 to 6 the research is detailed in a series of five manuscripts that contributed and provided data to meet the specific aims proposed in this study. Chapter 7 aims to draw several conclusions related to the specific aims of this thesis, outlined in this Chapter 1. Finally, Chapter 8 aims to provide areas for further research to improve ecological risk assessment of pesticides in freshwaters.

The specific aims of the research study presented in Chapters 2 to 6 were to accomplish the following:

- Provide a preliminary ecotoxicological evaluation of whether the current standard of 0.1 μg/L overprotects or underprotects the risks of PPPs to groundwater life (Chapter 2)
- Define candidate compounds for monitoring and prioritising as RBSPs in surface waters of three important Portuguese river basins (Chapter 3)
- Prioritise pesticide mixtures that might pose ecological risks in surface waters of three important Portuguese river basins, but also site-specific exposures and single-species/species assemblages, by applying component-based approaches (Chapter 4 and 5)
- Assess the role of pesticide stress for Portuguese freshwater ecosystems in relation to the impact of other stressors by integrating modelling approaches, chemical analysis, and effect-based tools (Chapter 6)

The information generated is of importance for reducing the risks of pesticide exposure in surface water and groundwater bodies which ensure compliance to European Regulations/Directives, such as Water Framework Directive (EC, 2000), Regulation 1107/2009/EC (marketing of PPPs) (EC, 2009a) and Directive 2009/128/EC (sustainable use of pesticides) (EC, 2009b). At the same time helps to strength the links between the three complementary European Regulations/Directives that aim to regulate and manage environmental risk of pesticides in water bodies.

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Chapter 2 Does the actual standard of 0.1 µg/L overestimate or underestimate the risk of plant protection products to groundwater ecosystems?

Based on the following manuscript:
Daam, M.A, Silva, E., Leitão, S., Trindade, M.J., Cerejeira, M.J. (2010) Does the actual standard of 0.1 µg/L overestimate or underestimate the risk of plant protection products to groundwater ecosystems? *Ecotoxicology and Environmental Safety* 73(5), pp.750-756

Abstract

The present groundwater standard of 0.1 µg/L for plant protection products (PPPs) has been under much debate because an ecotoxicological base is missing. In the present study, groundwater threshold values were calculated for all PPPs currently included in annex I to Directive 91/414/EEC using three different approaches: 1) first-tier (*Daphnia magna* and *Vibrio fisheri*); 2) species sensitivity distributions, constructed for surrogate freshwater organisms for the truncated groundwater biodiversity; 3) the case-based model PERPEST. For the majority of the PPPs, the trigger value of 0.1 µg/L appears to be sufficiently protective. However, it may not fully protect groundwater life from several insecticides. Implications for the environmental risk assessment of ground water and recommendations for future research are discussed.

Key-Words: Ground water; plant protection products; risk assessment; environmental quality standards

1. Introduction

Ground water has long been considered as an extreme environment inhabited by only a few specialized species. In the past decades, however, research into ground water has increased considerably (Figure 1) and several studies have shown that groundwater environments harbour relatively diverse communities of animals (e.g., Gibert *et al.*, 1994; Rouch and Danielopol, 1997; Galassi *et al.*, 2009; Gibert and Culver, 2009).

Subsequently, many authors started to dispute groundwater legislation for only considering ground water as a source of drinking water and not as an ecosystem (e.g., Gibert, 2001; Notenboom, 2001). In the EU, this was acknowledged with the implementation of a new Groundwater Directive in 2006, which states in recital 20: “Research should be conducted in order to provide better criteria for ensuring groundwater ecosystem quality and protection” (EU, 2006). However, such criteria have not been set to date (Steube *et al.*, 2009). The only groundwater standards that are currently applied for pesticides are 0.1 µg/L for any individual compound and 0.5 µg/L for the sum of all individual pesticides detected (EU, 2006). These threshold values are the same as those set in the Drinking Water Directive of 1980 (80/68/EEC; EU, 1980), and relate to the contemporary detection limits for pesticides (Notenboom *et al.*, 1999). Environmental risk assessment of plant protection products (PPPs) for aquatic ecosystems underpinned by ecotoxicological data is so far restricted to surface water, as laid down in the Uniform Principles (UP; Annex VI to Directive 91/414/EEC; EU 1997).

In the present study, groundwater criteria based on ecotoxicological data are established for the PPPs currently included in annex I to Directive 91/414/EEC (EU, 1997). In the absence of sufficient data for groundwater organisms, data for surface water taxa from taxonomic groups known to be well represented in ground water were used as surrogates. Three approaches were used to calculate predicted no effect concentrations (PNECs). As a first-tier approach corresponding with the method used in the UP, toxicity data for the crustacean *Daphnia magna* and the bacterium *Vibrio fisheri* were used. Secondly, for those PPPs for which sufficient toxicity data were available, species sensitivity distributions (SSDs) were constructed. Thirdly, for PPPs that were indicated in the previous two approaches to have a PNEC lower than 0.1 µg/L, the empirical model PERPEST was used to obtain an indication of the probability of potential effects on those taxonomic groups found in groundwater ecosystems. The aim of this study was to provide a preliminary ecotoxicological evaluation of whether the current standard of 0.1 µg/L overprotects or underprotects the risks of PPPs to groundwater life. Based on this analysis, substances with greatest potential risk to exert environmental side-effects below this standard are identified. Furthermore, available toxicity data for groundwater crustaceans are compared with those for *Daphnia magna*. This was done as to evaluate whether an additional assessment factor is needed when extrapolating surface water toxicity data to groundwater standards. Implications of study findings for the ecotoxicological risk evaluation of PPPs to groundwater ecosystems are discussed.

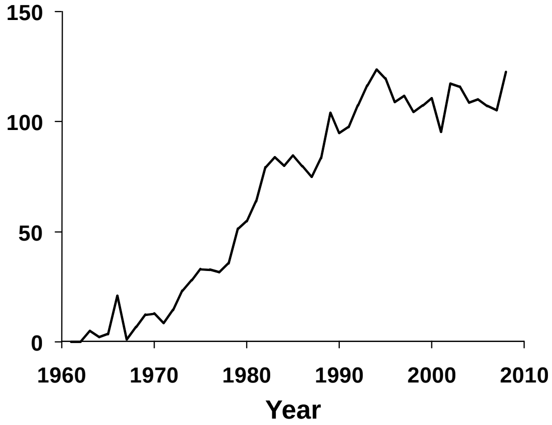


Fig. 1 Dynamics in publications on groundwater research over the past decades. The percentage of publications containing the word "groundwater" in the article title, abstract and/or keywords was calculated on a yearly basis. Subsequently, this percentage was divided by the percentage calculated for 1960. Source data: Scopus®

2. Materials and methods

2.1 Data selection

The use of groundwater species in routine toxicity testing has been reported to be hampered for various practical reasons, such as difficult accessibility of groundwater ecosystems, the patchy distribution of its fauna, the high number of endemic species therein with difficult taxonomy, the limited data on fauna and microbiology, difficult rearing in laboratory due to the very special life characteristics, and low reproductive rates (Notenboom *et al.*, 1999; Mösslacher *et al.*, 2001; Tomlinson *et al.*, 2007; Steube *et al.*, 2009). Due to the consequently low availability of ecotoxicological data for groundwater organisms, the use of ecotoxicological test results with surface water organisms seems to be generally recognized as an appropriate approximation (Notenboom *et al.*, 1999; Mösslacher and Notenboom, 2000; LAWA, 2004; Hose, 2005; Müller *et al.*, 2006). For example, Notenboom *et al.* (1999) and Hose (2005) estimated critical levels of pesticides encountered in monitoring programs conducted in respectively Australia and the Netherlands based on surface water toxicity data. Given the differences in ecosystem composition between surface waters and ground waters, only toxicity data for a reduced suite of taxa were considered in order to simulate the truncated biodiversity of groundwater ecosystems (Notenboom *et al.*, 1999; Hose, 2005). In the present study, a comparable approach was followed for all PPPs currently included in Annex I (available at: http://ec.europa.eu/sanco_pesticides/public/index.cfm; accessed on 9 July 2009) to obtain a holistic and up-to-date risk evaluation of all PPPs registered for use in the EU.

Crustacea and microorganisms have frequently been reported to be the most diversified and dominant or fundamental component of groundwater ecosystems, respectively (e.g., Danielopol *et al.*, 2000; Notenboom *et al.*, 1999; Pronk *et al.*, 2009). Accordingly, emphasis was put on these two groups of organisms in a "first-tier risk assessment approach". To this end, toxicity data for the cladoceran *Daphnia* spp and the aquatic bacterium *Vibrio fisheri* were collected as surrogates for the crustacean and microbial groundwater community, respectively. *Daphnia* spp were chosen because they are standard test organisms used for first-tier surface water risk assessments (EU, 2002) and are (subsequently) well studied. Results from Microtox® tests, single species test using *V. fisheri* with bioluminescence as endpoint, were considered since it is the most widely applied aqueous microbial test (Notenboom *et al.*, 1999).

Photosynthetic organisms do not occur in groundwater ecosystems and fish and insects are also rare or absent (Danielopol *et al.*, 2000; Gibert and Deharveng, 2002). Hose (2005) discussed that groundwater fauna includes crustaceans, rotifers, mites, oligochaetes and nematodes. Subsequently, SSDs were constructed for PPPs for which five of more toxicity data for these organisms were available.

Toxicity data were extracted from the US Environmental Protection Agency (US-EPA) ECOTOX database (http://cfpub.epa.gov/ecotox/). Only data that fulfilled the criteria set in Table 1 were used for further analysis. Since no data for *V. fisheri* are available in the ECOTOX database, the open literature was consulted to this end. When multiple data points were available for the same taxon, the geometric mean (gm) of those values was taken.

Table 1 Criteria used for the selection of short-term and long-term laboratory toxicity data

	Short-term toxicity data		Long-term toxicity data
Organisms	Surface dwelling aquatic crustaceans, rotifers, mites, oligochaetes and nematods	*Vibrio fisheri*	Surface dwelling aquatic crustaceans, rotifers, mites, oligochaetes and nematodes
Toxicity threshold	EC50, LC50	EC50	NOEC
Endpoint	Mortality, behaviour, intoxication	Bioluminescence	Mortality, behaviour, intoxication, reproduction
Test duration	1 - 4 days	5-30 minutes	> 4 days
Reference	Hose (2005); Van den Brink *et al.* (2003); Satapornvanit *et al.* (2004)	Notenboom *et al.* (1999)	Hose (2005); Van den Brink *et al.* (2003); Satapornvanit *et al.* (2004)

2.2 Species Sensitivity Distributions

Lognormal distributions of threshold values for those PPPs for which five or more toxicity data were available were constructed using the ETX computer program, version 2.0 (Van Vlaardingen *et al.*, 2004). The 5th and 50th percentile with their confidence limits were calculated with this software based on the methodology described by Aldenberg and Jaworska (2000). Since the model assumes a lognormal distribution of the data, lognormality was tested with the Anderson-Darling Test included in the ETX software package. If lognormality was not accepted at the 5% significance level, the BurrliOZ program (Campbell *et al.*, 2000) was used to fit a Burr type III distribution that best fitted the available data. The Burr type III distribution is a flexible three-parameter distribution that, depending on the value of those parameters, fits good approximations of the commonly used log-logistic, log-normal, log-triangular and Weibull distributions (Shao, 2000). The BurrliOZ software calculates confidence intervals for hazard concentration (HC) values using a bootstrap technique (Campbell *et al.*, 2000). As a result, confidence intervals may vary with subsequent re-runs (Hose, 2005). The lower HC5 estimate was determined by calculating the 95% percentile of the HC5. Each interval was estimated 10 times using 1000 permutations and the geometric mean of those 10 calculations was used as the best estimate of the lower bound of the HC5 (after Hose and Van den Brink, 2004). The BurrliOz program does not include a test to indicate how well the distributions fit the data. Hose (2005) derived r^2 values using the SPSS software. To this end, Hose (2005) applied non-linear regression with the distribution parameters from BurrliOz as the starting values. However, because SPSS software uses a different algorithm for the maximum likelihood estimates compared to BurrliOz, the resulting parameter estimates may differ (Hose, 2005). Therefore, a different approach was adopted in the present study. By applying the formula of the corresponding regression distribution to the concentration values of the EC50 values, we calculated the predicted affected fraction (PAF) as indicated by the regression curve. Subsequently, we calculated r^2 values by applying linear regression (Microsoft® Excel 2007) on these values and the actual PAF values of the individual EC50 points as a measure of how well the curve fitted the data points.

2.3 Assessment factors for PNEC calculations

In the first-tier approach, assessment factors were applied to the toxicity data of the crustacean *Daphnia* species and the bacterium *V. fisheri*. In accordance with the UP (EU, 1997), assessment factors of 100 and 10 were applied to the gmEC50 and the gmNOEC values for the most sensitive daphnid species, respectively. Tests with freshwater bacteria are not required in the UP so no assessment factors have been laid down for these organisms. Notenboom *et al.* (1999) set the EC10 value for *V. fisheri* as the critical concentration for groundwater microorganisms. To this end, they extrapolated the more common EC50 values to EC10 values by dividing EC50 values by a factor 3, a factor that is in accordance with that set by Van Beelen and Doelman (1997) for soil and sediment microorganisms (Notenboom *et al.,* 1999). In line with this, an assessment factor of 3 was applied to the EC50 values to calculate the PNEC for *V. fisheri* in the present study.

From the SSD curves, the concentration at which a specified proportion of species (*p*) is expected to suffer direct toxic effects, referring to as the hazardous concentration (HC) to *p*% of the species (HC*p*), may be calculated. Although SSDs have increasingly been used in the effect assessment of PPPs, no established guidance is yet provided regarding which HC*p* is appropriate for assessments under Directive 91/414/EEC. Brock *et al.* (2006) proposed to use the lower-limit hazardous concentration for 5% of the species (HC5) estimate based on acute EC50 values of a representative and sensitive taxonomic group of organisms. They based this on studies by Maltby *et al.* (2005) and Van den Brink *et al.* (2006), who concluded that the lower HC5 estimate was protective of adverse ecological effects of respectively insecticides and herbicides in freshwater (model) ecosystems (Brock *et al.*, 2006). In accordance with these findings, we used the lower HC5 estimate as the PNEC for the SSD approach.

Since only few NOEC values were available, SSDs were constructed with EC50 values. Given this lack of sufficient NOEC data, various studies used an acute to chronic ratio (ACR) to calculate long-term thresholds from EC50 data. For example, Hunt *et al.* (2009) and Notenboom *et al.* (1999) applied an ACR of 5 and 10, respectively, to convert acute data to estimates of chronic toxicity. In the Australian and New Zealand Water Quality Guidelines, the default ACR is 10 (ANZECC and ARMCANZ, 2000). Brock *et al.* (2006) indicated that an ACR of 10 may be used if the acute to chronic ratios of the 1st-tier standard test organisms support this. The average

EC50 to NOEC ratio that could be calculated for *Daphnia* species in the present study was 9.5, supporting the use of an ACR of 10 to calculate a PNEClong-term from the lower HC5 estimates.

2.4 PERPEST

The empirical PERPEST model was run for those PPPs for which a PNEC lower than 0.1 µg/L was calculated with one of the previous two approaches. PERPEST predicts the effects of a particular concentration of a pesticide on various (community) endpoints simultaneously based on a large database of aquatic model ecosystem experiments (Van den Brink, 2008). This results in a prediction showing the probability of classes of effects (no, slight, or clear effects) on the various grouped endpoints. The model was run with a concentration of 0.1 µg/L at default settings. For a detailed description of the model, the reader is referred to Van den Brink *et al.* (2002).

3. Results

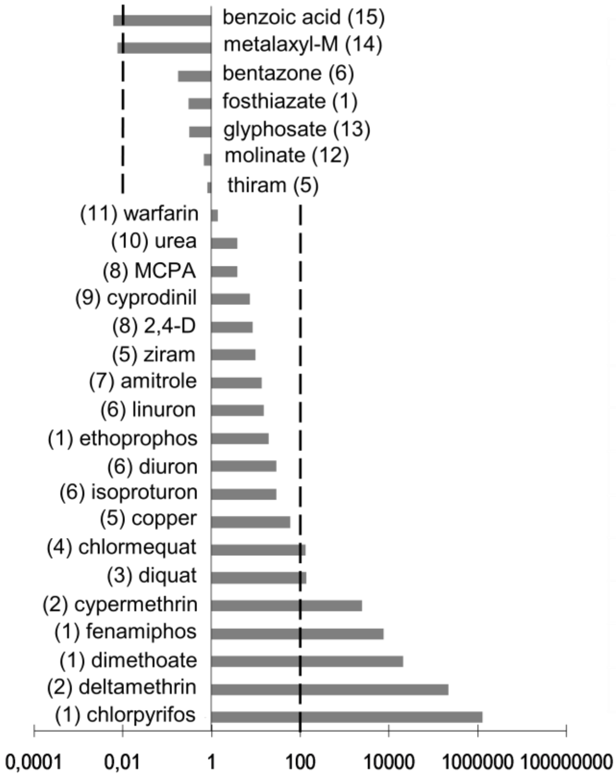
3.1 Data availability

On 9 July 2009, a total of 335 PPPs were included in Annex 1 to Directive 91/414/EEC. For a total of 118 compounds, EC50 data in accordance with the criteria set (Table 1) were available for *Daphnia* spp in the US EPA ECOTOX database. For 26 of these compounds, data meeting the criteria were also available for *V. fisheri*. For 12 PPPs, NOEC values considered valid were available for *Daphnia* spp. In addition, five or more EC50 values were available for 21 PPPs, for which SSDs were constructed.

3.2 Sensitivity of Daphnia spp versus V. fisheri

The relative sensitivity of *Daphnia* spp and *V.fisheri* to the selected PPPs is visualized in Figure 2. For the majority of the compounds, i.e., 19 out of 26, *Daphnia* spp were found to be more sensitive than *V. fisheri*. For the insecticides chlorpyrifos, deltamethrin, dimethoate, fenamiphos and cypermethrin, *Daphnia* spp were noted to be over 1000 times as sensitive as *V. fisheri*, whereas a factor of higher than 100 was calculated for the herbicide diquat (137) and the plant growth regulator chlormequat

(134). A clear greater sensitivity of *V. fisheri* was only found for metalaxyl-M (fungicide; 128) and benzoic acid (Insecticide/fungicide; 164) (Figure 2).



Nº	**Mode of action**
1	Cholinesterase inhibitor
2	Sodium channel modulator
3	Superoxide generator
4	Gibberellin biosynthesis inhibitor
5	Contact activity
6	Photosynthesis inhibitor
7	Chlorophyll formation inhibitor
8	Growth inhibitor
9	Inhibitor of methionine biosynthesis
10	Unknown
11	Anticoagulant
12	Germination inhibitor
13	EPSPS (enzyme) inhibitor
14	Protein synthesis inhibitor
15	Microtubule assembly inhibitor

Fig. 2 Relative sensitivity of *Vibrio fisheri* compared with *Daphnia magna* to selected PPPs. The vertical line at x = 0 represents an equal sensitivity of *D. magna* and *V. fisheri*. Horizontal bars at x > 0 indicate a greater vulnerability of *D. magna*, whereas those at x < 0 indicate a greater resilience as compared with *V. fisheri*. The mode of action for each PPP is indicated at the right side of the figure

3.3 PNECs

In Figure 3, the calculated PNECs are compared with the trigger value of 0.1 µg/L. In the first-tier approach, a PNEC lower than this trigger value was calculated for approximately 10% (11/118) and 15% (2/12) of the compounds for the short-term and long-term assessments, respectively. From the 21 SSDs that could be constructed, PNECs were below 0.1 µg/L in 8 (short-term) and 10 cases (long-term). The SSDs of the four PPPs for which the lowest PNECs were calculated (i.e., cypermethrin, dimethoate, fipronil and phosmet) are visualized in Figure 4. All threshold values for those compounds for which a PNEC lower than 0.1 µg/L was calculated with either the first-tier or SSD approach are listed in Table 2.

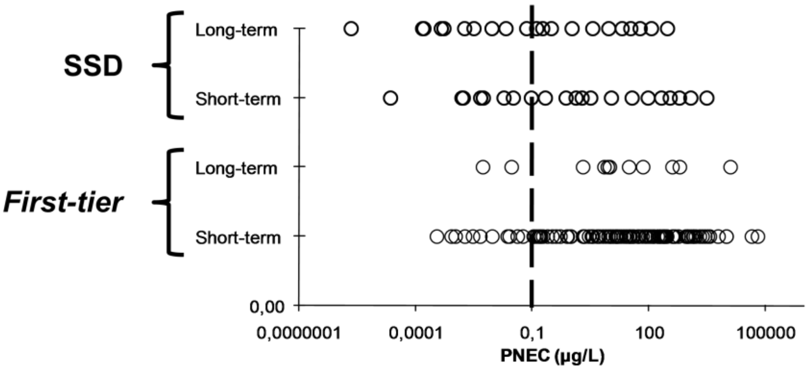


Fig. 3 Visualization of the Predicted-no-effect-concentrations (PNECs) as calculated using the first-tier and SSD approaches. Each open circle represents a PNEC of a PPP, whereas the dashed line indicates the actual environmental standard of 0.1 µg/L

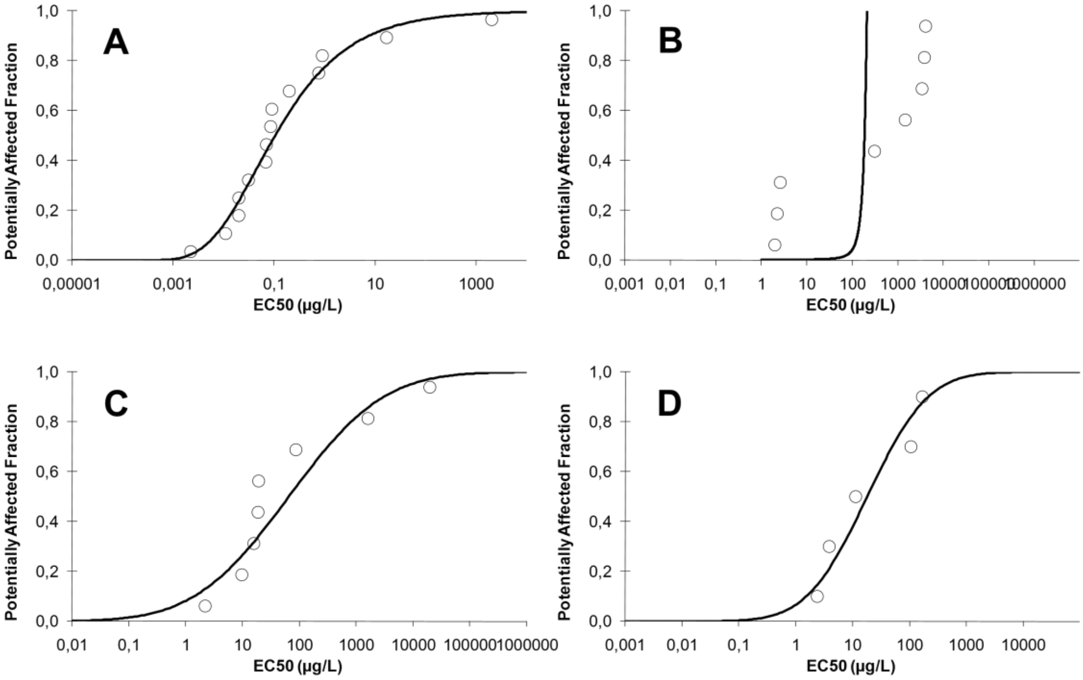


Fig. 4 Distribution curves of the four PPPs for which the lowest PNEC was calculated using the species sensitivity distribution (SSD) approach: cypermethrin (A), dimethoate (B), fipronil (C), and phosmet (D)

Table 2 Short-term and long-term Predicted No Effect Concentrations (PNECs) calculated with the first-tier or SSD approach to be lower than 0.1 µg/L. In addition, probabilities of effect class 3 (clear effect) occurring as predicted by PERPEST at 0.1 µg/L on macrocrustaceans, microcrustaceans, other macroinvertebrates and rotifers are presented. FU = fungicide; IN = insecticide; AC = acaricide; NE = nematicide

		First-tier		SSD		PERPEST			
Substance	Category	PNEC short-term	PNEC long-term	PNEC short-term	PNEC long-term	Macrocrustaceans	Microcrustaceans	Other macroinvertebrates	Rotifers
Chlorothalonil	FU	2.8	-	0.22	0.022	-	-	-	-
Chlorpyrifos	IN, AC	0.00035	0.0055	0.031[1]	0.0031[1]	73	47	8	25
Chlorpyrifos-methyl	IN, AC	0.0094	-	-	-	-	-	-	-
Cyfluthrin	IN, AC	0.00084	-	-	-	80	53	9	33
Cypermethrin	IN, AC	0.17	0.03	0.0038[1]	0.00038[1]	68[3]	32[3]	3[3]	12[3]
Deltamethrin	IN	0.0045	-	-	-	76	74	23	28
Diflubenzuron	IN	0.056	-	-	-	10	8	0	0
Dimethoate	IN, AC	0.026	7.3	0.00097[2]	0.000097[2]	-	-	-	-
Dinocap	FU, AC	0.042	-	-	-	-	-	-	-
Esfenvalerate	IN	0.0011	-	0.018	0.0018	75	57	8	27
Fenamiphos	NE	0.023	-	0.092	0.0092	-	-	-	-
Fipronil	IN	0.156	-	0.0046	0.00046	-	-	-	-
Lambda-Cyhalothrin	IN	0.0029	-	-	-	81	53	8	22
Pirimiphos-methyl	IN	0.0018	-	-	-	-	-	-	-
Phosmet	IN	0.11	-	0.0054	0.00054	-	-	-	-
Thiram	FU	2.1	-	0.033	0.0033	-	-	-	-

[1] SSD did not pass the Anderson-Darling test on log-normality; based on reciprocal weibull distribution: $r^2 = 0.984$ (chlorpyrifos); 0.965 (cypermethrin)

[2] SSD did not pass the Anderson-Darling test on log-normality; based on reciprocal pareto distribution: $r^2 = 0.913$ (dimethoate)

[3] See Figure 5

3.4 PERPEST

For those PPPs for which a PNEC lower than 0.1 µg/L was calculated with either the first-tier or SSD approach, the PERPEST model was consulted as to predict the probability of effects on different organism groups based on model ecosystem studies. Seven (out of 16) of these compounds were included in the PERPEST database, and the predicted chance on effect and its severity are summarized in Table 2. Furthermore, the output figure from PERPEST for cypermethrin is shown in Figure 5 as an example.

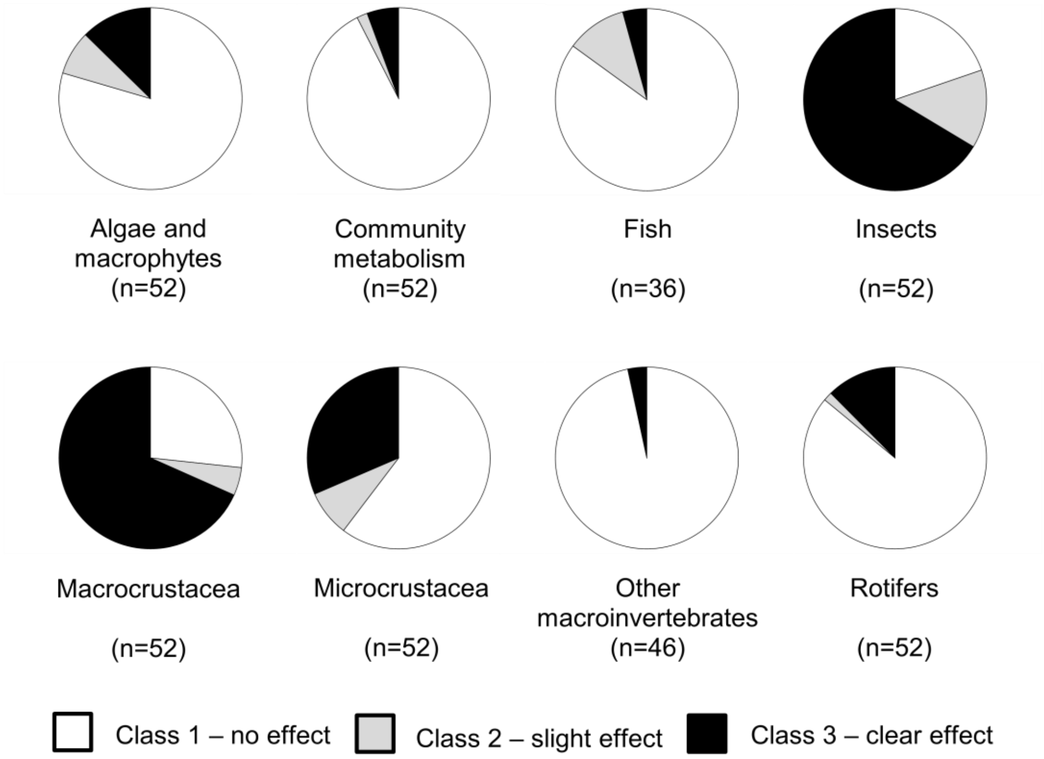


Fig. 5 Effects of 0.1 µg cypermethrin/L on different taxonomic groups as predicted by PERPEST. Effect classes (After Van den Brink *et al.*, 2002): class 1 = No effects demonstrated: no consistent adverse effects are observed as a result of the treatment - observed differences between treated test systems and controls do not show a clear causality; class 2 = Slight effects: confined responses on sensitive endpoints (e.g., partial reduction in abundance) - effects observed on individual sampling dates only and/or of very short duration directly after treatment; class 3 = Clear effects: convincing reductions on sensitive endpoints - effects observed on a sequence of sampling dates

4. Discussion

4.1 Ecotoxicological trigger values

For 85% to 90% of the PPPs included in the analysis, a threshold value greater than the actual trigger value of 0.1 µg/L was calculated using the first-tier approach (Figure

3). Based on this approach, the actual groundwater standard thus appears to be sufficiently protective for those PPPs. Trigger values obtained with the SSD approach were lower than 0.1 µg/L for a higher percentage of the PPPs (Figure 3). This may be partly due to a better estimation of the sensitivity of the aquatic community since more (potential sensitive) species are included in this approach. The methodology to calculate the PNEC may also have played a role. From the four PPPs for which the lowest PNEC was calculated, only the curve for cypermethrin indicates clear risks at 0.1 µg/L (Figure 4). EC50 values for approximately 50% of the surrogate groundwater community is indicated to be exceeded at a concentration of 0.1 µg cypermethrin/L, whereas the other three compounds do not have any data point below this level (Figure 4). In addition, the curve of dimethoate did not fit well with the data points (Figure 4B; Table 2). As pointed out in the materials and methods section, there is no guidance in the present legislation yet on how to calculate trigger values from SSDs. Hence, the recommendations by Brock *et al.* (2006) based on the information available at present were applied in the present study. Future studies are to indicate whether this may or not be too conservative for certain compounds.

None of the herbicides included in Annex I to Directive 91/414/EEC, had trigger values below 0.1 µg/L (Table 2). The majority of the PPPs indicating potential risks below this trigger values have mostly an insecticidal mode of action. Since the groundwater community is dominated by crustaceans and other (macro)invertebrates, the risk assessment was largely based on these biota. Consequently, these organisms may indeed be anticipated to be more vulnerable to insecticides than to herbicides, for which primary producers (absent in ground water) are evidently more sensitive. In line with this, Notenboom *et al.* (1999) also concluded that pesticides with potential risks at concentrations lower than 0.1 µg/L to groundwater life were insecticides.

4.2 Sensitivity of groundwater versus surface water organisms

Since hardly any ecotoxicological data is available for true groundwater organisms, data for surface water organisms within the truncated subterranean biodiversity were used. Although generally considered as the best available alternative at present (e.g., Notenboom *et al.*, 1999; LAWA, 2004; Hose, 2005; Müller *et al.*, 2006), the use of surface water toxicity data for groundwater risk assessments may be disputed for various reasons. Firstly, the groundwater community composition is vastly different from that of surface water fauna (Sket,1999). For example, Danielopol *et al.* (2000) reported that the aquatic stygobitic fauna is quite unique, compared with the fauna of surface waters, owing to the dominance of crustacean species (e.g., in Europe stygobitic species account for approximately 40% of the total crustacean fauna) and the virtual absence of Insecta (representing about 50% of the total European animal species living in surface waters). Ecosystems differing in species composition may be expected to have a different sensitivity towards toxic stress. In line with this, it has been demonstrated for insecticides that the taxonomic composition of the species assemblage used to construct an SSD has a significant influence on the threshold value assessment (Maltby *et al.*, 2005). Furthermore, different groundwater organisms display specific adaptations to survive in their peculiar environment characterized by the absence of light, generally restricted habitat diversity, low physic-chemical fluctuations and dependency on allochthonous energy supply. This sort of environmental “austerity”, together with the peculiar biogeographic processes of ground water colonization, led to a truncated groundwater functional biodiversity of food webs (Gibert and Deharveng, 2002). The functional redundancy, which is providential in case of water contamination due to the presence of more than one species having the same functional role in the community, is therefore almost lacking in groundwater communities (Notenboom *et al.*, 1999; Gibert and Deharveng, 2002).

Secondly, the metabolic and life history differences between surface water and groundwater dwelling taxa have been considered to imply a greater sensitivity for the latter taxa (Mösslacher, 2000). For example, long-life spans, low reproductive output, and the genetic isolation of groundwater organisms has been related with high ecotoxicological risk profiles (Posthuma and Van Straalen, 1993). On the other hand, the lower metabolic rates of hypogean fauna may necessitate a much longer exposure for toxic effects to be evident than for surface fauna (Plénet, 1999). According to Hose (2005), the differences between surface water and groundwater species make it difficult to conclude the relative sensitivity, but current evidence suggests any differences are likely to be toxicant dependant. At present, insufficient groundwater toxicity data is available to determine to what extent surface water toxicity data may be used for groundwater risk assessments. Hence, ecotoxicological studies evaluating true groundwater organisms are needed to validate this and to determine whether an assessment factor may be necessary when extrapolating toxicity data from surface water organisms to groundwater trigger values. This need is also acknowledged in the Water

Framework Directive by stating that assessments of groundwater community assemblages and toxicological surveys on autochthonous groundwater taxa are needed at least for groundwater bodies identified "at risk of achieving good groundwater chemical status" (Directive 2000/60/EC; EU, 2000). Given the long residence times and the relatively slow dynamics of ground water, emphasis should be given to ecotoxicity data on chronic effects (Müller *et al.*, 2006).

4.3 Sensitivity of Vibrio fisheri versus Daphnia magna

D. magna was equally or more sensitive as compared to *V. fisheri* for the majority of the PPPs (Figure 2). Only for benzoic acid and metalaxyl-M, PNEC values for *V. fisheri* were clearly lower than those *of D. magna* (Figure 2). In line with this, *V. fisheri* has been reported in various other studies to be relatively insensitive to pesticides compared to *D. magna* (Notenboom *et al.*, 1999; Gellert, 2000; Girotti *et al.*, 2008). However, this apparently low sensitivity of *V. fisheri* may be related with the mode of action of the PPPs under concern. For example, the five PPPs for which the greater vulnerability of *D. magna* was most apparent, are either cholinesterase inhibitors or sodium channel modulators (Figure 2). Since these are associated with the function of the animal nerve system, a relatively low sensitivity of *V. fisheri* may be anticipated.

The guidance document on aquatic ecotoxicology states that "whilst for most insecticides, *Daphnia* have been demonstrated to be representative, the toxicity of certain recent chemistries which have very specific receptor-mediated modes of action (e.g. neo-nicotinoids) may not be well represented by *Daphnia*" (EU, 2002). In the latter case, the guidance document requires tests with the non-biting midge *Chironomus riparus*. Van den Brink *et al.* (2007) discussed that many fungicidal pesticides affect a wide range of taxa, and that therefore the potential effects on microorganisms should be considered. However, these authors further stipulated that for those fungicidal compounds with a general mode of action, microorganisms are not expected to be the most sensitive taxonomic group (Van den Brink *et al.*, 2007). If the specific modes of action of benzoic acid (bactericide/fungicide; microtubule assembly inhibitor) and metalaxyl-M (fungicide; protein synthesis inhibitor) are associated with the observed greater sensitivity of *V. fisheri*, has to investigated. If this is the case, including microbial tests in a groundwater risk assessment may be especially relevant considering the discussed importance of microorganisms in the truncated biodiversity of groundwater ecosystems.

5. Conclusion

- Although the trigger value of 0.1 µg/L appears to be sufficiently protective for the majority of the PPPs presently included in Annex I, effects may ensue at this concentration for a number of PPPs, especially insecticides. Leachability to ground water and ways that groundwater organisms perceive pollutants to their environment will have to be evaluated for these compounds as to assess whether they may exert environmental side-effects to groundwater ecosystems.
- Ecotoxicological studies evaluating the sensitivity of true groundwater organisms are needed to validate whether and to what extent surface water toxicity data may be used for the risk assessment of groundwater life.
- The bacterium *V. fisheri* was more sensitive than the crustacean *D.magna* for two PPPs with specific modes of action (i.e., microtubule assembly inhibitor and protein synthesis inhibitor). Given the importance of microorganisms for groundwater ecosystems, the sole evaluation of the sensitivity of a crustacean may not be sufficient in such cases.

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Chapter 3 Aquatic risk assessment of priority and other river basin specific pesticides in surface waters of Mediterranean river basins

Based on the following manuscript:
Silva, E., Daam, M.A., Cerejeira, M.J. Aquatic risk assessment of priority and other river basin specific pesticides in surface waters of Mediterranean river basins. *Chemosphere* (submitted, editor assigned)

Abstract

To meet good chemical and ecological status, Member States are required to monitor priority substances and chemicals identified as substances of concern at European Union and local/river-basin/national level, respectively, in surface water bodies, and to report exceedances of the environmental quality standards (EQSs). Therefore, standards have to be set at national level for river basin specific pollutants. Pesticides used in dominant crops of several agricultural areas within the catchment of Mediterranean river basins (‘Mondego’, ‘Sado’ and ‘Tejo’, Portugal) and with detection in previous studies performed in Portugal were selected for monitoring, in addition to the pesticides included in priority lists defined in Europe. From the 29 pesticides and metabolites selected for the study, 20 were detected in surface waters of the river basins, seven of which were priority substances: alachlor, atrazine, chlorfenvinphos, chlorpyrifos, endosulfan, simazine and terbutryn, all of which exceeded their respective EQS values. EQSs for other specific pollutants were calculated using different extrapolation techniques (i.e. deterministic or probabilistic) based on the method described in view of the Water Framework Directive. Non-acceptable aquatic risks were revealed for molinate, oxadiazon, pendimethalin, propanil, terbuthylazine, and the metabolite DEA. Implications of these findings for the classification of the ecological status of surface water bodies in Portugal and at the European level are discussed.

Key-Words: Priority pesticides; river basin specific pesticides; environmental quality standards; risk assessment; Mediterranean surface waters

1. Introduction

Chemical pollution of surface water may present a threat to the aquatic environment with effects such as acute and chronic toxicity to aquatic organisms, accumulation in the ecosystem and losses of habitats and biodiversity, as well as a potential threat to human health (EC, 2008). European Union (EU) legislation provides for measures against chemical pollution of surface waters. There are two components - the selection and regulation of substances of EU-wide concern (the priority substances) and the selection by Member States (MS) of substances of national or local concern (river basin specific pollutants) for control at the relevant level (EC, 2014a). The first component constitutes the major part of the Union's strategy against the chemical pollution of surface waters. It is set out in Article 16 of the Water Framework Directive (WFD) (EC, 2000), and outlines the steps to be taken. The first step was to establish by way of Decision 2455/2001/EC (EC, 2001) a first list of priority substances to become Annex X of the WFD. These substances were selected from amongst those presenting a significant risk to or via the aquatic environment, using the approaches specified in Article 16 of the WFD.

This first list was replaced by Annex II of the Directive on environmental quality standards (EQSD) (EC, 2008), also known as the priority substances directive, which set environmental quality standards (EQSs) for the substances in surface waters (river, lake, transitional and coastal) and confirmed their designation as priority or priority hazardous substances, the latter being a subset of particular concern. As required by the WFD and EQSD, the Commission subsequently reviewed the list and in 2013 it put forward a directive amending the WFD and the EQSD as regards to priority substances in the field of water policy (EC, 2013). According to Annex V, point 1.4.3 of the WFD and Article 1 of the EQSD, good chemical status is reached for a water body when it complies with the EQSs for all the priority substances and other pollutants listed in Annex I of the EQSD (EC, 2014b).

In addition, the WFD (Annex V, section 1.2.6) establishes the principles to be applied to the MS to develop EQSs for specific pollutants that are ‘discharged in significant quantities’. These are also known as Annex VIII substances of the WFD. Compliance with EQSs for specific pollutants forms part of the assessment of ecological status. EQSs are therefore key tools in assessing and classifying chemical status and can thus affect the overall classification of a water body under the WFD. In addition, EQSs will be used to set discharge permits to waterbodies, so that chemical emissions do not lead to EQS exceedances within the receiving water (EC, 2011).

EQSs in view of the WFD should be derived according to the technical guidance document (TGD) on risk assessment (EC, 2003). A more detailed guidance was subsequently provided by Lepper (2005). At present, the new and existing substances regulation has been replaced by REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals), but the TGD is still into force for biocides. With respect to the aquatic ecosystem, the risk assessment methodology under REACH (ECHA, 2008) is basically the same as outlined in the TGD. The guidance of Lepper (2005) was revised recently, and an updated TGD for derivation of quality standards under the WFD was published in 2011 (EC, 2011).

In Portugal the substances adopted as river basin specific pollutants and the EQS values applied were derived for previous legislation which may have been enacted before the WFD methodology was developed. A report on the comparison of specific pollutants and EQSs at the European level (EC, 2012) provided contradictory results, i.e. concluded that Portugal has not identified any river basin specific pollutants at the time of collection (i.e. up to August 2012). The presence in water bodies of substances ‘discharged in significant quantities’ and not identified as RBSPs may not be detected in the assessment of ecological status if no EQS values are applied.

In this context, and in an attempt to improve the classification of ecological status of surface water bodies, the objectives of the present study were (i) to select priority (hazardous) substances and other substances of concern in relation to pesticide compounds to be analysed in surface waters of three Mediterranean river basins (‘Mondego’, ‘Sado’ and ‘Tejo’, Portugal); (ii) to calculate EQSs for short- and long-term exposure for them based on a simplified decision tree of the methodology for deriving water quality standards based on direct ecotoxicity as described in EC (2011); (iii) to assess the individual risk of priority and other substances of concern by comparing the measured concentrations with their respective EQSs values as set in this study or established under Community legislation for priority substances (EC, 2013); and (iv) to discuss the implications derived from the aquatic risk assessment findings for the classification of ecological status of surface water bodies in Portugal and at the European level.

2. Materials and methods

2.1 General characteristics of the studied agricultural areas

In terms of water resources, ‘Tejo’, ‘Sado’ and ‘Mondego’ belong to the largest hydrological basins of continental Portugal occupying 25666, 12149 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 like, maize, tomato for industry, rice, sugar beet, open-air horticultural crops and potato, as well as wheat and vine (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).

2.2 Pesticide compounds selected for the study

The herbicides alachlor, atrazine, simazine, terbutryn, trifluralin, and the insecticides chlorfenvinphos, chlorpyrifos, endosulfan and lindane were selected since they are indicated in the list of priority substances in the field of water policy (EC, 2013). The herbicides cyanazine, cycloxydim, dichlobenil, ethofumesate, MCPA, metolachlor, metribuzin, molinate, oxadiazon, pendimethalin, profoxydim, prometryn, propanil, propazine, terbuthylazine, triclopyr, the insecticide pirimicarb, and the metabolites 3,4-dichloroaniline (3,4-DCA), desethylatrazine (DEA) and desisopropylatrazine (DIA) were also included in the study due to the amount sold in Portugal (DGAV, 2014) since 2002, their approval for use in the main crops of the studied agricultural areas (see section 2.1), 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), 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).

2.3 Exposure assessment

2.3.1 Exposure prediction using a screening model

On the basis of a set of physicochemical and partition coefficient properties selected from different databases (FOOTPRINT, 2014; McBean, 2012), the environmental exposure was evaluated *a priori* through the Level I fugacity model (Mackay, 2001). The Level I calculation evaluates the equilibrium distribution of a fixed quantity of chemical (for example, 100000 kg) between the compartments in a closed evaluative or ‘unit world’ environment (Mackay *et al.*, 1996). It is a steady state calculation with no inflow, outflow, intermedia transport or degrading reactions. Output from the Level I calculation indicates the phases or media into which a chemical is likely to partition. It also provides equilibrium concentrations of the chemical in each compartment of the model environment and thus shows the media where concentrations are likely to be highest. Based on these calculations, Table 1 presents the predicted environmental distribution (PED) for water of each pesticide compound selected for the present study.

2.3.2 Surface water monitoring network

A total of 103 surface water samples were collected in five rivers (‘Arunca’, ‘Ega’, ‘Foja’, ‘Mondego’ and ‘Pranto’ rivers) in the ‘Mondego’ river basin from 2002 to 2004, during the main period of agricultural practices, both in terms of pesticide application and irrigation (Table 2). The following pesticide compounds were monitored: alachlor, atrazine, chlorfenvinphos, endosulfan, metolachlor, molinate, oxadiazon, propanil, simazine, and the metabolite 3,4-DCA.

Table 1 Predicted environmental distribution (PED) for water as calculated using the Level I fugacity model (Mackay, 2001) for the pesticide compounds selected in the present study

Pesticide compound	PED for water(%)[a]
Herbicide	
Alachlor	47.3 (AA)
Atrazine	77.7 (HA)
Cyanazine	89.8 (VHA)
Cycloxydim	98 (VHA)
Dichlobenil	59 (AA)
Ethofumesate	68.8 (HA)
MCPA	100 (VHA)
Metolachlor	58.1 (AA)
Metribuzin	96.5 (VHA)
Molinate	59.7 (AA)
Oxadiazon	1.34 (VLA)
Pendimethalin	0.69 (VLA)
Profoxydim	18 (VLA)
Prometryn	46.7 (AA)
Propanil	35.6 (LA)
Propazine	51.9 (AA)
Simazine	89.8 (VHA)
Terbuthylazine	30.5 (LA)
Terbutryn	19.8 (VLA)
Triclopyr	100 (VHA)
Trifluralin	1.56 (VLA)
Metabolite	
3,4-DCA	67 (HA)
DEA	-
DIA	-
Insecticide	
Z-chlorfenvinphos	13.5 (VLA)
E-chlorfenvinphos	6.23 (VLA)
Chlorpyrifos	2.15 (VLA)
α-endosulfan	1.96 (VLA)
β-endosulfan	1.75 (VLA)
Lindane	25.7 (LA)
Pirimicarb	95.7 (VHA)

[a] Mackay fugacity model (Level I, version 3.00, 2004, Trentu University, Canada) if $PED_W < 20\%$: Very Low Affinity (VLA); if $20\% \leq PED_W < 40\%$: Low Affinity (LA); if $40\% \leq PED_W < 60\%$: Average Affinity (AA); if $60\% \leq PED_W < 80\%$: High Affinity (HA); if $PED_W \geq 80\%$: Very High Affinity (VHA).
- No data.

Table 2 Number of sampling sites and surface water samples collected, in the period 2002-2008, in the ‘Tejo’, ‘Mondego’ and ‘Sado’ river basins

	2002		2003		2004		2005		2006		2008		Total	
	Sites	Samples	Sites	Samples	Sites	Samples	Sites	Samples	Sites	Samples	Sites	Samples	Sites	Samples
‘Mondego’ river basin														
‘Arunca’ river	1	4	2	8	2	6	-	-	-	-	-	-	3	18
‘Ega’ river	1	4	1	4	1	3	-	-	-	-	-	-	1	11
‘Foja’ river	1	4	2	8	1	6	-	-	-	-	-	-	2	18
‘Mondego’ river	4	13	3	12	3	9	-	-	-	-	-	-	5	34
‘Pranto’ river	2	8	2	8	2	6	-	-	-	-	-	-	3	22
Total	9	33	10	40	9	30	-	-	-	-	-	-	14	103
‘Sado’ river basin														
‘Sado’ river	3	18	3	15	-	-	-	-	-	-	-	-	3	33
‘Santa Catarina de Sítimos’ stream	1	6	1	5	-	-	-	-	-	-	-	-	1	11
Dam			1	2	-	-	-	-	-	-	-	-	1	2
Drainage channels	1	5	1	5	-	-	-	-	-	-	-	-	1	10
Total	5	29	6	27	-	-	-	-	-	-	-	-	6	56
‘Tejo’river basin														
‘Almonda’ river	-	-	-	-	4	7	2	6	2	6	2	8	4	27
‘Alviela’ river	-	-	-	-	2	2	2	6	2	6	-	-	3	14
‘Tejo’ river	-	-	-	-	5	6	5	14	5	15	-	-	6	35
‘Zézere’ river	-	-	-	-	1	1	1	3	1	3	-	-	1	7
‘Alverca do Campo’ embankment	-	-	-	-	2	5	3	9	2	6	2	6	4	26
Drainage channels	-	-	-	-	5	9	1	3	1	1	0	0	5	13
Total	-	-	-	-	19	30	15	41	13	37	4	14	23	122

- No data.

In the Sado river basin, a total of 56 surface water samples were collected in the 'Sado' river and the 'Santa Catarina de Sítimos' stream in 2002 and 2003, during the main period of agricultural practices (Table 1), and analysed for chlorfenvinphos, cycloxydim, endosulfan, MCPA, molinate, oxadiazon, profoxydim, propanil, triclopyr, and the metabolite 3,4-DCA.

Regarding the Tejo river basin, a total of 122 surface water samples were collected in four rivers ('Almonda', 'Alviela', 'Tejo' and 'Zézere' rivers), at the 'Alverca do Campo' embankment, a body of inland water, and drainage channels from 2004 to 2006 and 2008, during the main period of agricultural practices (Table 1). These samples were analysed for the following pesticide compounds: alachlor, atrazine, chlorfenvinphos, chlorpyrifos, cyanazine, dichlobenil, endosulfan, ethofumesate, lindane, metolachlor, metribuzin, pendimethalin, pirimicarb, prometryn, propanil, propazine, simazine, terbuthylazine, terbutryn, trifluralin, and the metabolites 3,4-DCA, DEA and DIA.

2.3.3 Analytical methods

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-DCA, DEA and DIA were extracted by solid-phase microextraction (SPME) followed by qualitative and quantitative analysis by gas chromatography with mass spectrometric detection (GC-MS) (Silva *et al.*, 2012a,b), while the pesticides cycloxydim, MCPA, profoxydim and triclopyr by solid-phase extraction (SPE) followed by liquid chromatography with mass spectrometric detection (LC-MS) (Silva *et al.*, 2006).

The above laboratory methods of pesticide analysis used for the purpose of surface water monitoring were validated in accordance with EN ISO/IEC-17025 standard (ISO, 2005).

2.4 Method for water quality standard setting

For the water column, quality standards based on direct ecotoxicity, on secondary poisoning of predators, on human consumption of fishery products and for human consumption of drinking water have to be derived. The lowest of these values is set as the overall EQS, although the drinking water standard is only adopted as an overall standard for waters intended for drinking water abstraction. It is acknowledged by the authors that the procedure adopted here for the calculation of water quality standards for the pesticide compounds under study is a simplification of the method as described in EC (2011). For the present study, only quality standards based on direct ecotoxicity were taken into consideration to set the EQS.

In line with this, two standards were set to cover both long-term and short-term exposure to a chemical: an annual average concentration (EQS-AA) to protect against the occurrence of prolonged exposure, and a maximum acceptable concentration (MAC-EQS) to protect against possible effects from short-term concentration peaks. In accordance with EC (2011), where the derivation of the MAC-EQS led to a lower value than the EQS-AA, the MAC-EQS was set equal to the EQS-AA for direct ecotoxicity.

A tiered approach was adopted to set these EQSs as outlined below:

The EQSs as set in EC (2012) for inland surface waters were adopted for nine pesticides for which these were available (Table 3).

For pesticide compounds for which no EQSs have currently been set in EC (2012), the availability of model ecosystem data in draft assessment reports ((EFSA, 2014)) was explored and, if no DAR was available, model ecosystem experiments evaluating insecticides (Brock and Van Wijngaarden, 2012; Van Wijngaarden *et al.*, 2005) and herbicides (Van den Brink *et al.*, 2006) were checked. An assessment factor (AF) of 5 and 3 was used by default to the NOEC (No Observed Concentration Effect) values of a single model ecosystem experiment to set the EQS-AA (using a study with chronic exposure) and MAC-EQS (using a study with single-peak exposure), respectively.

If no model ecosystem data was available, the probabilistic approach using species sensitivity distributions (SSDs) was considered to set the EQSs. EC (2011) requires SSDs to be constructed for both the entire dataset (i.e. all taxa, so that the relative sensitivities of taxa can be determined), and only those taxa that are expected to be particularly sensitive in case of substances with a specific mode of action. Based on previous review studies, the latter taxa used in the present study were arthropods for insecticides (Van Wijngaarden *et al.*, 2005), and primary producers (algae and macrophytes) for herbicides (Van den Brink *et al.*, 2006). The minimum number of datapoints that need to be available to construct any of the two SSDs as required in EC (2011) is ten, though a lower number (at least five datapoints) was also accepted in the present study due to low data availability. SSDs were constructed as outlined below to calculate the median estimate of the HC_5 (Hazardous Concentration for 5% of the species). An AF of 5 and 10 was used by default to HC_5 values derived from NOEC and $L(E)C_{50}$ (Median Lethal/Effective Concentration) data for setting of the EQS-AA and MAC-EQS, respectively (EC, 2011). In those cases where enough data was available for both types of SSDs, preference was given to the SSD based on the most sensitive taxonomic groups since it may evidently be anticipated that this would lead to the most conservative EQSs.

Table 3 Environmental quality standards (EQS) for nine of the pesticide compounds selected in the present study that are identified as priority substances under Directive 2013/39/EU (EC, 2013), expressed as the annual average value (AA-EQS) and the maximum allowable concentration (MAC-EQS) for inland surface waters

Pesticide	AA-EQS[a]	MAC-EQS[b]
	Inland surface waters ($\mu g\ L^{-1}$)	
Herbicide		
Alachlor	0.3	0.7
Atrazine	0.6	2.0
Simazine	1	4
Terbutryn	0.065	0.34
Trifluralin	0.03	not applicable
Insecticide		
Chlorfenvinphos	0.1	0.3
Chlorpyrifos	0.03	0.1
Endosulfan[c]	0.005	0.01
Lindane (γ-hexachloro-cyclohexane)[c]	0.02	0.04

[a] Unless otherwise specified, it applies to the total concentration of all isomers.

[b] Where the MAC-EQS are marked as "not applicable," the AA-EQS values are considered protective against short-term pollution peaks in continuous discharges since they are significantly lower than the values derived on the basis of acute toxicity.

[c] Also identified as priority hazardous substances.

In case not enough data could be encountered to allow constructing an SSD, the deterministic approach was used by applying an AF to the lowest credible toxicity value. In accordance with EC (2011), this AF ranged between 10 and 1000 depending on data availability and expert judgement. Preference was given to data as provided in DAR reports where available, followed by (in order of preference) the US-EPA database, Tomlin (2006) and the FOOTPRINT database (see next subsection for more details).

2.4.1 Laboratory toxicity data mining

As indicated above, a variety of resources was used in different levels of priority to obtain toxicity data for the deterministic and probabilistic approaches: draft assessment reports (EFSA, 2014), the ECOTOX (US-EPA, 2014) and the FOOTPRINT pesticide database (FOOTPRINT, 2014), and Tomlin (2006). 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; *vide* EC, 2011). In accordance with EC (2011), the following toxicity thresholds were used as indicated for calculation of:

MAC-EQS: L(E)C_{50}

EQS-AA: NOEC, MATC (Maximum Acceptable Toxicant Concentration)/√2, L(E)$C_{5\text{-}10}$, $EC_{10\text{-}20}/2$

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 for the EQS setting.

2.4.2 Species sensitivity distributions

SSDs were constructed as described in 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 lognormality 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 (potentially affected fraction) 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.

3. Results and discussion

3.1 Residues of pesticide compounds

During the period of the monitoring campaigns in 2002-2008, fifteen herbicides (alachlor, atrazine, ethofumesate, MCPA, metolachlor, metribuzin, molinate, oxadiazon, pendimethalin, profoxydim, propanil, simazine, terbuthylazine, terbutryn and triclopyr), three insecticides (chlorfenvinphos, chlorpyrifos and endosulfan) and two metabolites (3,4-DCA and DEA) were detected in surface waters of the ‘Mondego’, ‘Sado’ and ‘Tejo’ river basins.

Figure 1a shows the distribution of median, quartile, non-outlier range, outlier and extreme concentrations of pesticide compounds found in surface waters of the ‘Mondego’ river basin. Highest median concentrations were found for molinate (0.06 μg L^{-1}), whereas for atrazine, metolachlor and 3,4-DCA were below the quantification limit (<0.05 μg L^{-1}). Concentration variation statistics (quartiles, non-outlier range, outliers) were greater for molinate and 3,4-DCA compared to the other pesticides. Maximum non-outlier concentrations were highest for molinate (1.63 μg L^{-1}), 3,4-DCA (0.42 μg L^{-1}), atrazine (0.21 μg L^{-1}), metolachlor and simazine (0.13 μg L^{-1}), respectively.

As previously referred the crop pattern in the ‘Baixo Mondego’ area consists mainly of maize, rice and potato. Among the pesticides detected in surface waters of the ‘Mondego’ river basin, alachlor, atrazine, chlorfenvinphos, chlorpyrifos, molinate, propanil and simazine were extensively used as plant protection products in Portugal during the monitoring period (2002-2004) (DGAV, 2014). Highest outlier and extreme values were mainly detected for molinate during its application period. This may be attributed to its application conditions under rice-flooded conditions (Pereira, 2003) and very high water solubility (1100 mg L^{-1}, McBean, 2012). Atrazine was at that time the main maize applied herbicide in Portugal (officially allowed use until 31-12-2007). The chloroacetamides alachlor and metolachlor were applied in maize mainly as mixtures with atrazine. This last compound was the third most important herbicide, in terms of sales, in the ‘Montemor-o-Velho’ county, in 1999 (information provided by a representative pesticide point of sale), preceded by propanil and atrazine (Batista, 2003). In soil, rapid microbial degradation to the aniline derivative (3,4-DCA) occurs which explains the second highest detection frequency (Fig. 1a). The other detected pesticides, such as the herbicides simazine, propanil, oxadiazon, and the insecticides chlorfenvinphos and endosulfan, were also registered for use in maize and rice in the period of the monitoring campaigns. Consequently, a relation between surface water residues in terms of agricultural use pattern for 'Mondego' river basin can be suggested. In chemical monitoring programmes carried out by the Portuguese environmental authorities (SNIRH, 2014), all the pesticide compounds selected for study in this work, with the exception of oxadiazon, were detected in some surface water points of the 'Mondego' river basin, except for chlorfenvinphos and propanil. Molinate and 3,4-DCA also reached the highest concentrations (2.75 and 1.77 μg L^{-1}, respectively).

A box plot for pesticide compounds found in surface waters of the 'Sado' river basin is shown in Fig. 1b. Compounds with highest median concentrations were molinate (0.14 μg L^{-1}), oxadiazon and 3,4-DCA (<0.05 μg L^{-1}). Concentration range within quartiles and non-outlier for molinate is the greatest as compared to the other compounds.

A similar contamination pattern as discussed for the 'Mondego' river basin was observed in surface waters of the 'Sado' river basin. Six compounds (chlorfenvinphos, endosulfan, molinate, oxadiazon, propanil and 3,4-DCA) detected in surface waters of the 'Mondego' river basin were also detected in surface waters of the 'Sado' river basin since the agricultural area is mainly occupied by paddy rice, where these pesticides were used during the study. Other pesticides, not analysed in surface waters of the 'Mondego' river basin, were detected with minor frequency, such as MCPA, triclopyr and profoxydim. In relation to triclopyr, this was experimentally applied to control *Heteranthera* spp. on a more advanced phenological stage of rice and to complement the initial action of oxadiazon, while profoxydim is a recent alternative to molinate against *Echinochloa* spp. Molinate was the pesticide most often used and introduced in larger quantities by rice farmers in the 'Baixo Sado' region (Silva *et al.*, 2006). This frequent use is probably the reason for the relatively high extreme concentrations of this compound determined in this study and in other works (Pereira, 2003; SNIRH, 2014), reaching maximum values of 1380 and greater than 10 μg L^{-1}, respectively. Cycloxydim was the only pesticide compound not detected in surface waters of the 'Sado' river basin. It is registered for use in rice being only limited to control *Oryza sativa* L. after a false sowing practice.

In case of ‘Tejo’ river basin, atrazine and alachlor present the highest third quartile (0.24 and 0.1 μg L^{-1}, respectively) and maximum non-outlier concentrations (0.6 and 0.24 μg L^{-1}, respectively) in the surface waters sampled (Fig. 1c).

The wide range of pesticide compounds detected in surface waters of the ‘Tejo’ river basin can be explained through the multicrop nature of the local agriculture. Relative to them, alachlor, atrazine, ethofumesate, metolachlor, metribuzin, simazine, and 3,4-DCA present an average or a (very) high affinity for the water compartment, but others, such as chlorfenvinphos, chlorpyrifos, endosulfan, pendimethalin, propanil, terbuthylazine and terbutryn, did not (see Table 2). Atrazine, the pesticide compound most often detected, was expected due to its high affinity for the water compartment (see Table 2). All detected pesticides were approved for use in the main irrigated crops of the ‘Médio Tejo’ and ‘Lezíria do Tejo’ region. The metabolites 3,4-DCA and DEA are relevant and major metabolites of the herbicides propanil and atrazine, herbicides registered for maize and rice, crops with importance in the study area. Some pesticide compounds detected in this study (alachlor, atrazine, chlorfenvinphos, endosulfan, lindane, metolachlor, simazine, terbuthylazine, and the metabolite 3,4-DCA) were also detected in surface water samples collected in the ‘Tejo’ river basin in the scope of chemical monitoring programmes carried out by the Portuguese environmental authorities (SNIRH, 2014).

Besides ranges or distribution characteristics of concentration values of each pesticide compound detected, their frequency of occurrence should also be counted for the risk assessment process. 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% detections, respectively (Fig. 1a). 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% detections, respectively (Fig. 1b). Atrazine and alachlor were the most frequently detected pesticides in surface waters of the ‘Tejo’ river basin with 44% and 33% detections, respectively (Fig. 1c).

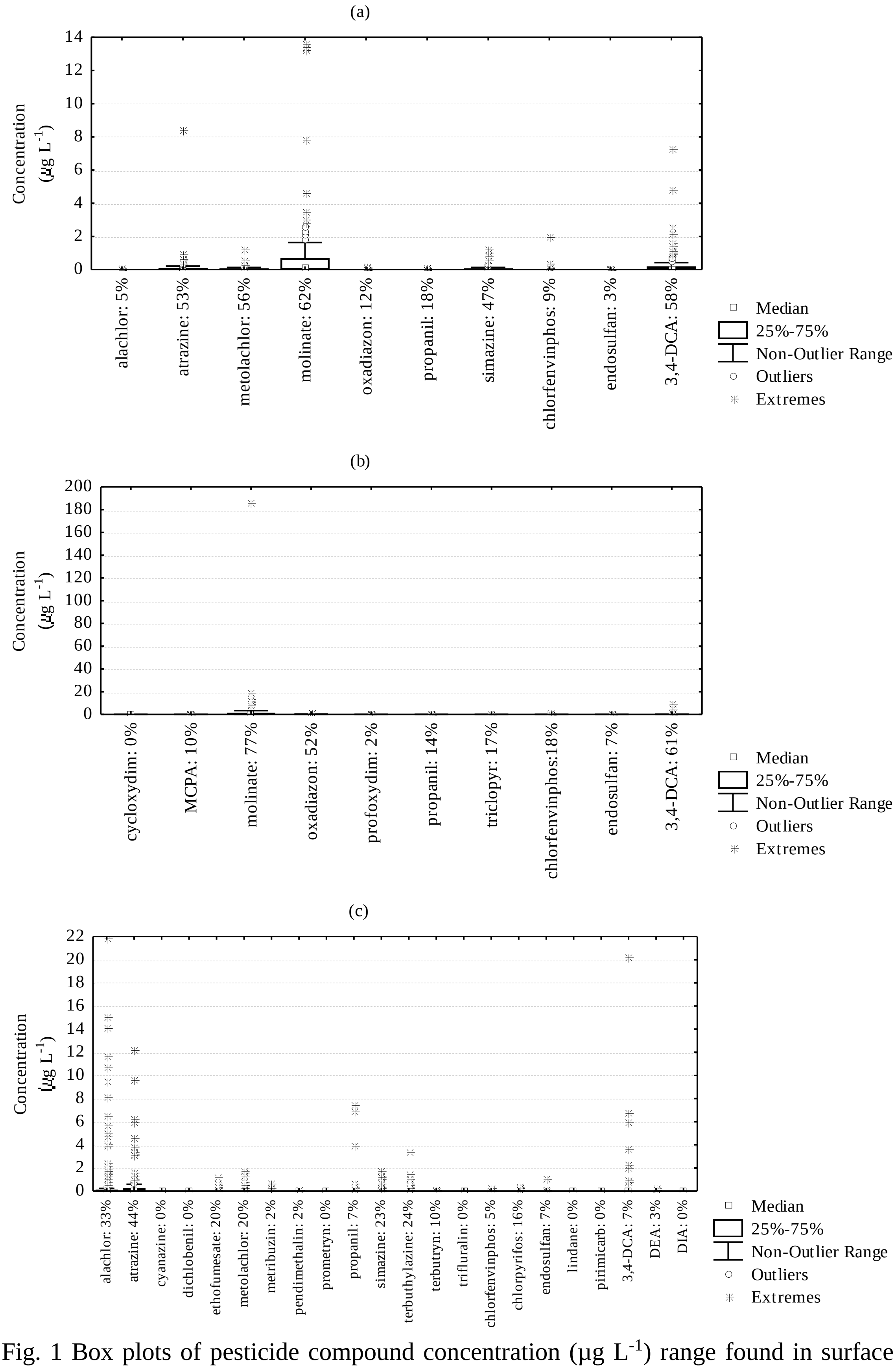

Fig. 1 Box plots of pesticide compound concentration (µg L^{-1}) range found in surface waters of 'Mondego' (a), 'Sado' (b) and 'Tejo' (c) river basin. Number in front of each pesticide compound name indicates the detection frequency of samples in percentage (%)

3.2 Aquatic risk assessment

Aquatic risks of all pesticide compounds detected in surface waters of the ‘Mondego’, ‘Sado’ and ‘Tejo’ river basins were evaluated based on two indicators using measured concentrations per sample and annual average concentrations at each site. The first indicator considers the frequency of samples with measured concentrations of a pesticide compound above the MAC-EQS as set in this study (Table 4) or established by the revised (second) list of priority substances (EC, 2013) (see Table 3) to protect against short-term exposure. Subsequently, the number of samples where the threshold was exceeded was divided by the total number of samples where the respective pesticide compound was monitored.

The second indicator considers the frequency of sites with arithmetic mean of the measured concentrations of a pesticide compound at different times during the year above the AA-EQS as set in this study (Table 4) or established by the revised (second) list of priority substances (EC, 2013) (see Table 3) to protect against long-term exposure. Subsequently, the number of sites where the threshold was exceeded was divided by the total number of sites where the respective pesticide compound was monitored.

Chlorfenvinphos and endosulfan were the pesticides that most frequently exceeded the AA-EQS (both in 14% of the sites in the ‘Mondego’ river basin), while atrazine and oxadiazon in one site (7%). The measured concentrations/MAC-EQS ratios were above 1 for oxadiazon, chlorfenvinphos, endosulfan and atrazine with values between 1-4% of the samples (Table 5). The AA-EQS were exceeded in 83% of the sites in the ‘Sado’ river basin for oxadiazon, 33% for chlorfenvinphos and endosulfan, and 17% for molinate, while the same order of pesticides exceeded the MAC-EQS in 39%, 7% (for the insecticides) and 2% of the samples (Table 5). The insecticides chlorfenvinphos and chlorpyrifos, and the herbicide oxadiazon showed the highest frequency of exceedance in surface waters of the ‘Mondego’ and ‘Sado’ river basins mainly due to their relatively low AA-EQS and MAC-EQS values (Tables 3 and 4). On the other hand, non-acceptable risks for atrazine and molinate were mainly due to their relatively high measured concentration values (see Fig. 1a,b).

Table 4 Annual average environmental quality standard (AA-EQS) and maximum allowable concentration EQS (MAC-EQS) as set in this study for 20 river basin specific pesticide compounds

Pesticide compound	AA-EQS	MAC-EQS
	(μg L^{-1})	
Herbicide		
Cyanazine	0.18	0.65
Cycloxydim	464	3820
Dichlobenil	0.98	7.5
Ethofumesate	76	390
MCPA	17	19
Metolachlor	2.1	5.6
Metribuzin	1.9	2
Molinate	18	34
Oxadiazon	0.042	0.042
Pendimethalin	0.077	0.077
Profoxydim	68	235
Prometryn	0.076	0.15
Propanil	0.37	0.67
Propazine	0.78	1.2
Terbuthylazine	0.95	0.99
Triclopyr	1.3	6.6
Metabolite		
3,4-DCA	32	71
DEA	0.1	1
DIA	0.006	0.02
Insecticide		
Pirimicarb	0.09	0.17

The ubiquitous compounds that exceeded the AA-EQS in the ‘Tejo’ river basin were atrazine (35% of the total sites) > alachlor (26%) > chlorpyrifos (17%) > endosulfan = propanil (9%) > chlorfenvinphos = DEA = simazine = terbuthylazine = terbutryn (4%). The frequency of exceedance MAC-EQS was alachlor (19% of the samples) > chlorpyrifos (10%) > atrazine = endosulfan (7%) > terbuthylazine (3%) > pendimethalin = propanil (2%) (Table 5). Alachlor and atrazine showed the highest frequencies of exceedance in surface waters of the ‘Tejo’ river basin mainly due to a combination of relatively high measured concentration values (maximum 75th percentil and non-outlier range, see Fig. 1c) and low AA-EQS and MAC-EQS values (Tables 3 and 4). The same reasons may be applied for the herbicides propanil, simazine and terbuthylazine, although these were detected in lower concentrations than alachlor and atrazine (see Fig. 1c). Aquatic risks for the insecticides chlorfenvinphos, chlorpyrifos, endosulfan, the herbicides pendimethalin and terbutryn, and the metabolite DEA were mainly due to their relatively low AA-EQS and MAC-EQS values (Tables 3 and 4).

Table 5 Frequency of exceedance of water quality standards, expressed as the annual average value (AA-EQS) and the maximum allowable concentration (MAC-EQS), of the pesticides compounds detected in surface waters of the ‘Mondego’, ‘Sado’ and ‘Tejo’ river basins

Pesticide compound	Freq. AA-EQS[a] (%)			Freq. MAC-EQS[b] (%)		
	Mondego	Sado	Tejo	Mondego	Sado	Tejo
Herbicide						
Alachlor	0	-	26	0	-	19
Atrazine	7	-	35	1	-	7
Ethofumesate	-	-	0	-	-	0
MCPA	-	0	-	-	0	-
Metolachlor	0	-	0	0	-	0
Metribuzin	-	-	0	-	-	0
Molinate	0	17	-	0	2	-
Oxadiazon	7	83	-	4	39	-
Pendimethalin	-	-	0	-	-	2
Profoxydim	-	0	-	-	0	-
Propanil	0	0	9	0	0	2
Simazine	0	-	4	0	-	0
Terbuthylazine	-	-	4	-	-	3
Terbutryn	-	-	4	-	-	0
Triclopyr	-	0	-	-	0	-
Metabolite	-	-	-	-	-	-
3,4-DCA	0	0	0	0	0	0
DEA	-	-	4	-	-	0
Insecticide	-	-	-	-	-	-
Chlorfenvinphos	14	33	4	3	7	0
Chlorpyrifos	-	-	17	-	-	10
Endosulfan	14	33	9	3	7	7

[a] Frequency of exceedance of AA-EQS = Σn/N×100, where n is the number of sites with arithmetic mean at each site/AA-EQS ratios above 1 and N is the total number of sites where analytical measurements were carried out for the respective pesticide compound.

[b] Frequency of exceedance of MAC-EQS = Σn/N×100, where n is the number of samples with measured concentration per sample/MAC-EQS ratios above 1 and N is the total number of samples where analytical measurements were carried out for the respective pesticide compound.

- No data.

Portugal has identified some of the pesticide compounds analysed in this study (3,4-DCA, DEA, MCPA, metolachlor, molinate, pendimethalin, propanil and terbuthylazine) as specific pollutants but has not derived EQS values for the herbicides pendimethalin and terbuthylazine. However, for those specific pollutants with normative values for different water categories, the EQSs were derived from Portuguese by law which may have been enacted before the WFD methodology was developed. In this study, the derivation of EQSs for 20 pesticide compounds by training largely based on the WFD technical guidance (EC, 2011) was an advance in the classification criteria of European surface water bodies, thus contributing to decline the variability of these values between MS. The results from this study also pointed out that the list of specific pollutants should be reviewed and chemical monitoring programmes adapted to the specific reality of each river basin conditions. For example, the herbicide oxadiazon should be included as river basin specific pollutant, particularly at national level where rice ecosystems are present.

4. Conclusions

From the 29 pesticides and metabolites selected for the study, the priority substances alachlor, atrazine, chlorfenvinphos, chlorpyrifos, endosulfan, simazine and terbutryn were detected in surface waters of the ‘Mondego’, ‘Sado’ and ‘Tejo’ river basins. All these pesticides exceeded at least one of the environmental quality standards set in the revised (second) list of priority substances.

On the basis of different extrapolation techniques (i.e. deterministic or probabilistic), the authors derived water quality standards expressed as an annual average value and maximum allowable concentrations for all other pesticides and metabolites selected for the study. The herbicides molinate, oxadiazon, pendimethalin, propanil, terbuthylazine, and the metabolite DEA exceeded one of these values.

Although Portugal has already set classification criteria of the status of surface water bodies, there were difficulties using them in the first generation of river basin management plans, namely due to the lack of standardized and systematized data related to the biological elements and the absence of simultaneous monitoring of biological elements and physicochemical parameters in surface waters. Moreover, the EQS values applied for the specific pollutants identified by Portugal were derived for previous legislation which may have been enacted before the WFD methodology was developed.

This study provided a classification criterion more robust within the WFD challenge level, i.e., it used similar procedures to those specified in Annex V 1.2.6 of the WFD and defined in the technical guidance for deriving EQSs, thus contributing to decline the variability of these values between MSs. Furthermore, it enabled to increase the information and knowledge on the potential aquatic risks of the most important pesticide compounds to search in surface waters of certain river basins, and it concluded that the list of specific pollutants should be adjusted, namely the inclusion of the herbicide oxadiazon in river basins with rice ecosystems. In order to reduce the aquatic risks of pesticides that are still in use, namely in vulnerable river basins, specific measures for their sustainable use should be adopted to protect the aquatic environment.

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Chapter 4 Concentration addition-based approach for aquatic risk assessment of realistic pesticide mixtures in Portuguese river basins

Based on the following manuscript:
Silva, E., Cerejeira, M.J. Concentration addition-based approach for aquatic risk assessment of realistic pesticide mixtures in Portuguese river basins. *Environmental Science and Pollution Research* (accepted, in publication)

Abstract

A two-tiered outline for the predictive environmental risk assessment of chemical mixtures with effect assessments based on concentration addition (CA) approaches as first tier and consideration of independent action (IA) as the second tier was applied based on realistic pesticide mixtures measured in surface waters from 2002 to 2008 within three important Portuguese river basins ('Mondego', 'Sado' and 'Tejo'). The CA-based risk quotients, based on acute data and an assessment factor of 100, exceeded 1 in more than 39% of the 281 samples, indicating a potential risk for the aquatic environment, namely to algae. Seven herbicide compounds and three insecticides were the most toxic compounds in the pesticide mixtures and provided at least 50% of the mixture's toxicity in almost 100% of the samples with risk quotients based on the sum of toxic units (RQ_{STU}) above 1. In eight samples, the maximum cumulative ratio (MCR) and the Junghan's ratio values indicated that a chemical-by-chemical approach underestimated the toxicity of the pesticide mixtures and CA predicted higher mixture toxicity than IA. From a risk management perspective, the results pointed out that, by deriving appropriate programmes of measures to a limited number of pesticides with the highest contribution to the total mixture toxicity, relevant benefits also on mixture impact could be produced.

Key-Words: Pesticides; mixtures; risk assessment; concentration addition; surface waters; Portugal

1. Introduction

Different agricultural practices can cause the presence of diverse types of pesticide mixtures in the environment varying in terms of their complexity, particularly with regard to the possibility to predict the composition of the mixture, i.e., the identity and concentrations of components (Altenburger et al., 2014). Consequently, with little quantitative data on mixture toxicity for relevant assessment endpoints and numerous mixture possibilities, regulators have to make decisions based on single substance data in combination with mixture extrapolation techniques (Posthuma et al., 2008). The reference models concentration addition (CA) and independent action (IA) allow the calculation of expected combined effects purely based on concentration-effect information for the components of a mixture of concern and their concentrations in the mixture. As CA model predicts the observed toxicity of mixtures with reasonable accuracy (Kortenkamp et al., 2009), a two-tiered outline was suggested by Backhaus and Faust (2012) for predictive environmental risk assessment of chemical mixtures with effect assessments based on CA approaches as the first tier and consideration of IA as the second tier. This makes optimum use of available exposure and toxicity data from the individual chemicals to calculate risk quotients by summing up EC/PNEC (=environmental concentration/predicted no effect concentration) ratios ($RQ_{EC/PNEC}$) or as sums of toxic units (RQ_{STU}) in a stepwise application, since the former provides a more conservative approach. Additional IA studies should be made if the RQ_{STU} is above threshold and only if error estimations indicate the possibility for substantial differences between CA- and IA-based assessments.

In this context, and in an attempt to provide a general screening level risk assessment of pesticide mixtures measured in surface waters of three important Portuguese river basins (‘Mondego’, ‘Sado’ and ‘Tejo’) from 2002 to 2008, the outlined approach was used adopting the procedure under the Water Framework Directive (WFD) for individual pesticides, in order (i) to determine the potential threat that pesticide mixtures pose to the aquatic biota (warranting further studies and/or risk management measures); (ii) to select the most sensitive taxonomic group; and (iii) to identify the most important compounds in the pesticide mixtures in terms of TUs.

2. Materials and methods

2.1 Study area

In terms of water resources, ‘Tejo’, ‘Sado’ and ‘Mondego’ belong to the largest hydrological basins of continental Portugal occupying 25666, 12149 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 like maize, tomato for industry, rice, sugar beet, open-air horticultural crops and potato, as well as wheat and vine (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).

2.2 Pesticide compounds selected for the study

Twenty one herbicides, five insecticides and three metabolites were selected in this study due to their indication in the list of priority substances in the field of water policy (EC, 2013), their amount sold in Portugal (DGAV, 2014) since 2002, their approval for use in the main crops of the studied agricultural areas (see section 2.1), 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,b), 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).

2.3 Risk analysis

2.3.1 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 with mass spectrometric detection (GC-MS) (Silva et al., 2012a,b), while the pesticides cycloxydim, MCPA, profoxydim and triclopyr by solid-phase extraction (SPE) followed by liquid chromatography with mass spectrometric detection (LC-MS) (Silva et al., 2006).

Table 1 presents the mean, median concentration and detection frequency values for the pesticides compounds analysed 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% detections, 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% detections, respectively. Atrazine and alachlor were the most frequently detected pesticides in surface waters of the ‘Tejo’ river basin with 44% and 33% detections, respectively. For these pesticides also match the highest mean and median concentrations.

Table 1 Mean, median concentration and detection frequency values for the pesticide compounds analysed 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^{-1})			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-<DL	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

< DL -Below the detection limit

- No data

2.3.2 Characterisation of effects

At least one short-term datum from each of the three taxonomic groups of the basic dossier data that has to be provided within the context of Regulation (EC) No 1107/2009 (EC, 2009) was used. Median effective concentration (EC_{50}) values for algae and crustaceans, and median lethal concentration (LC_{50}) values for fish of the pesticide compounds selected in the present study were obtained from McBean (2012). In those cases where data was not available, the FOOTPRINT pesticide database (FOOTPRINT, 2014) was consulted (Table 2). Limited toxicity data were available for the herbicide metabolites DEA and DIA. This last one was not detected in surface water samples; while DEA was only detected in four samples in the ‘Tejo’ river basins (see Table 1). In order to derive the first tier (standard test species approach) PNEC values for pesticides, Lepper (2005) proposed to use an assessment factor (AF) of 100 to the lowest acute toxicity value. This methodology was adopted in the technical guidance for deriving maximum acceptable concentration-quality standards (MAC-QS) to protect against possible effects from short-term concentration peaks (EC, 2011).

Table 2 Median lethal (effective) concentration (L(E)C_{50}; in mg L^{-1}) for algae, crustaceans and fish, and predicted no effect concentration (PNEC) values (in μg L^{-1}), for the pesticide compounds selected in the present study

Pesticide compound	Algae EC_{50} (72 h)	Crustaceans (*Daphnia* sp.) EC_{50} (48 h)	Fish LC_{50} (96 h)	PNEC
		(mg L^{-1})		(μg L^{-1})
Herbicide				
Alachlor	0.012	13	2.1	0.12
Atrazine	0.043	29	4.3	0.43
Cyanazine	0.2	42	16	2
Cycloxydim	44.9	71	100	449
Dichlobenil	111	6.2	5	50
Ethofumesate	3.9	13.52	10.92	39
MCPA	79.8	190	50	500
Metolachlor	0.1	25	3.9	1
Metribuzin	0.021	49.6	74.6	0.21
Molinate	0.5	14.9	16	5
Profoxydim	33	18.1	13	130
Prometryn	0.002	12.66	5.5	0.02
Propanil	0.11	4.8	5.4	1.1
Propazine	0.18	17.7[a]	17.5	1.8
Oxadiazon	0.004	2.4	1.2	0.04
Pendimethalin	0.018	0.4	0.707	0.18
Simazine	0.042	100	49	0.42
Terbuthylazine	0.016	69.3	2.2	0.16
Terbutryn	0.0017	2.66	1.1	0.017
Triclopyr	75.8	133	117	758
Trifluralin	0.0122	0.245	0.088	0.122
Metabolite				
3,4-DCA	1.65	0.12	1.94	1.2
DEA	0.1	-	-	1
DIA	-	-	-	-
Insecticide				
Chlorfenvinphos	1.6	0.0003	0.04	0.003
Chlorpyrifos	0.48	0.0017	0.002	0.017
Endosulfan	0.56	0.075	0.002	0.02
Lindane	2.5	1.6	0.022	0.22
Pirimicarb	140	0.017	55	0.17

[a] Unknown species; - No data

2.4 CA-based risk quotients for pesticide mixtures

The first tier of the approach suggested by Backhaus and Faust (2012) used exclusively CA as a basis for the preliminary risk assessment of the pesticide mixture(s) of concern. This tier itself contains two consecutive steps, based on $RQ_{MEC/PNEC}$ and RQ_{STU}. $RQ_{MEC/PNEC}$ (Eq. 1) denotes a final risk quotient for the pesticide mixture that was calculated by simply summing up MEC (measured environmental concentration)/PNEC ratios of all individual pesticide compounds. The PNEC was calculated individually for each pesticide compound selected for the study. This was derived by the standard test species (deterministic) approach according to the technical guidance for deriving environmental quality standards (EC, 2011): an AF of 100 was applied to the lowest $L(E)C_{50}$ of the acute toxicity data (Table 2). RQ_{STU} (Eq. 2) in contrast denotes a procedure where sums of toxic units (STU) were first calculated for each trophic level separately. Afterwards, that organism group that was most sensitive to the pesticide mixture (i.e. for which the highest STU was calculated) was selected and by applying the corresponding AF (100) a risk quotient for the pesticide mixture RQ_{STU} was then calculated.

$$RQ_{PEC/PNEC} = \sum_{i=1}^{n} \frac{PEC_i}{PNEC_i} = \sum_{i=1}^{n} \frac{PEC_i}{\min(EC50_{algae}, EC50_{Daphnid}, EC50_{fish})i \times \frac{1}{AF_i}} \qquad (1)$$

$$RQ_{STU} = \max(STU_{algae}, STU_{Daphnid}, STU_{fish}) \times AF = \max\left(\sum_{i=1}^{n} \frac{PEC_i}{EC50_{i,algae}}, \sum_{i=1}^{n} \frac{PEC_i}{EC50_{i.Daphnid}} \sum_{i=1}^{n} \frac{PEC_i}{EC50_{i,fish}}\right) \times AF \qquad (2)$$

The former risk quotient provides the more conservative approach, but might violate the assumption of a common biological end point that is inherent to the concept of CA. Hence, if $RQ_{MEC/PNEC}$ is above 1, i.e. indicated potential for reason for concern, RQ_{STU} can be calculated in a next step.

Only if there are still indications for a potential risk (i.e., when $RQ_{STU} > 1$) tier II commences. Tier II takes IA into consideration, either as a single model, or in the form of so-called mixed models which have been suggested in the literature as combinations of both CA and IA. This tier will, due to the data demands of IA, often require additional experimental studies.

The critical decision on whether to proceed to a tier 2 hazard assessment was driven by the possible ratio of the CA- and IA-expected mixture toxicities. A tier II assessment only makes sense when the number of pesticide compounds present in the scenario and their specific toxic unit distribution indicate that the sole application of CA might lead to a substantial risk overestimation. An appropriate approach to predict the maximum possible ratio between the mixture EC_{50}s predicted by both concepts has been put forward by Junghans et al. (2006) for mixtures of pesticides and is equal to or smaller than the sum of all toxic units divided by the highest individual toxic unit of the components that make up the mixture (Eq. 3).

$$\frac{EC50^{IA}}{EC50^{CA}} \leq \frac{\sum_{i=1}^{n} \frac{c_i}{EC50_i}}{max i \in (1, \ldots n) (\frac{c_i}{EC50_i})} \tag{3}$$

The maximal factor by which CA may predict a lower EC_{50} value and thus a higher toxicity than IA equals the number of mixture components, irrespective of the other determining factors (the concentration ratio of mixture components, the slope of the individual concentration response curves, and the effect level under consideration). However, if any mixture component has a TU that differs from the TUs of the remaining components, the maximal factor will always be smaller than n (Junghans et al., 2006).

3. Results and discussion

3.1 Aquatic risk of pesticide mixtures

The two-tiered approach for predictive environmental risk assessment of chemical mixtures proposed by Backhaus and Faust (2012) was applied to pesticide monitoring data from surface waters in the 'Mondego', 'Sado' and 'Tejo' river basins. A database considering the concentration as given of fourteen herbicides (alachlor, atrazine, ethofumesate, MCPA, metolachlor, 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 provided the input for the study (see Table 1).

The frequency of samples with summation of MEC/PNEC ratios above 1 was calculated for surface waters of the ‘Mondego’, ‘Sado’ and ‘Tejo’ river basins using measured concentrations per sample. Subsequently, the number of samples where the threshold was exceeded was divided by the total number of samples where the pesticide compounds were monitored. Table 3 shows that the $RQ_{MEC/PNEC}$ ranges from 31.1% (‘Mondego’ river basin) to 46.4% (‘Sado’ river basin). For those samples that indicate potential for reason for concern ($RQ_{MEC/PNEC} > 1$), RQ_{STU} was calculated in a next step. The frequency of samples with STU ratios above 1 was calculated for surface waters of the ‘Mondego’, ‘Sado’ and ‘Tejo’ river basins using measured concentrations per sample. Subsequently, the number of samples where the threshold was exceeded was divided by the total number of samples where the frequency of samples with summation of MEC/PNEC ratios was above 1. Table 4 shows that RQ_{STU} was above 1 in almost all samples where the frequency of samples with summation of MEC/PNEC ratios was above 1. The ratio between those two mixture risk quotients was smaller than the number of considered trophic levels, as proven by Backaus and Faust (2012), reaching 2.055 in a surface water sample from the ‘Mondego’ river basin due to differences in the sensitivities of the mixture compounds for the three organism groups.

Table 3 Frequency of exceedance of $RQ_{MEC/PNEC}$ for the pesticides compounds detected in surface waters of the ‘Mondego’, ‘Sado’ and ‘Tejo’ river basins

River basin	No. of samples	Freq. $RQ_{MEC/PNEC}$[a] (%)
Mondego	103	31.1
Sado	56	46.4
Tejo	122	42.6

[a] Frequency of exceedance of $RQ_{MEC/PNEC} = \Sigma n/N$, where n is the number of samples with $RQ_{MEC/PNEC}$ ratios above 1, and N is the total number of samples with analytical measurements for the pesticide compounds selected for the study

The taxonomic group with the highest predicted sensitivity to the pesticide mixtures (maximum STU of all analysed trophic levels) measured in surface waters of the ‘Mondego’, ‘Sado’ and ‘Tejo’ river basins was algae, followed by crustaceans and fish (Fig. 1, Table 4). This pattern is consistent with the ecotoxicity profiles of the individual pesticide compounds, mainly of herbicides to which algae are more sensitive than aquatic animals, and with higher detection frequency and measured values. Long-term trends of pesticide exposure in Swedish agricultural streams normalised to their potential effects for algae (chronic), invertebrates, and fish (both acute) were also assessed using the toxic unit approach. Although in this study, the monitoring data showed a continuous but rather low toxic potential of pesticides for all three trophic levels throughout the year (Bundschuh et al., 2014).

Table 4 Frequency of exceedance of RQ_{STU} and frequency of maxSTU for algae, crustaceans and fish for the pesticides compounds detected in surface waters of the 'Mondego', 'Sado' and 'Tejo' river basins

River basin	No. of samples	Freq. RQ_{STU}[a] (%)	Freq. max[b] STUalgae (%)	STUcrustaceans	STUfish
Mondego	32	94	56.7	40	3.3
Sado	26	100	69.2	30.8	-
Tejo	52	100	82.7	13.5	3.8

[a] Frequency of exceedance of RQ_{STU} = Σn/N, where n is the number of samples with RQ_{STU} ratios above 1, and N is the total number of samples with $RQ_{MEC/PNEC}$ ratios above 1

[b] Frequency of maxSTU = Σn/N, where n is the number of samples with maxSTU for algae, crustaceans and fish and N is the total number of samples with $RQ_{MEC/PNEC}$ ratios above 1

- No data

The ratio cumulative toxicity/maximum toxicity from one chemical defined as maximum cumulative ratio (MCR) provides a quantitative measure of the magnitude of the toxicity that is underestimated by not performing a cumulative risk assessment (Price and Han, 2011). Fig. 1 presents the MCR-RQ_{STU} plots for algae, crustaceans and fish for the pesticides compounds detected in surface waters of the 'Mondego', 'Sado' and 'Tejo' river basins. In these plots, RQ_{STU} is plotted on a log scale and MCR is plotted on a linear scale with a minimum value of 1. As the Fig.1 indicates, in almost all samples with RQ_{STU} above 1, the MCR values were smaller than 2 indicating that one pesticide compound provides at least 50% of the mixture's toxicity. For four samples in the 'Mondego' river basin (Fig. 1a) and four in the 'Tejo' river basin with maxSTU for algae (Fig. 1b), the MCR values averaged 2.528 and ranged from 2.073 to 3.127. These values indicate that the fraction of toxicity that comes from the most toxic pesticide compound averages 40% and ranges from 32% to 48%.

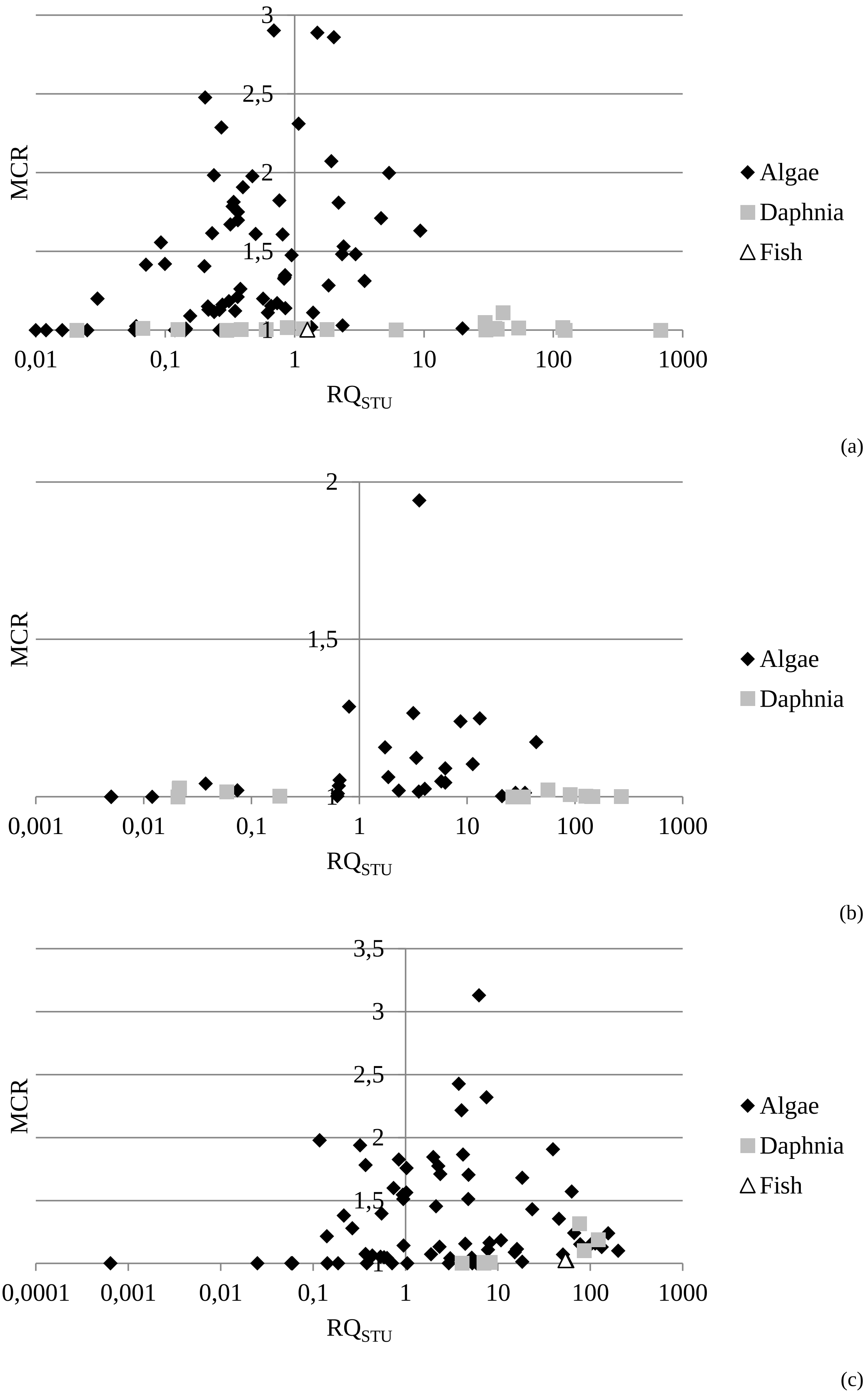

Fig. 1 Log-linear plot of MCR *versus* maxRQ$_{STU}$ for algae, crustaceans and fish for the pesticides compounds detected in surface waters of the 'Mondego' (a), 'Sado' (b) and 'Tejo' (c) river basins

Seven herbicide compounds and two organophosphate insecticides were accountable for the highest risk of toxicity for algae and crustaceans, respectively, in samples with RQ_{STU} ratios above 1 in the ‘Mondego’, ‘Sado’ and ‘Tejo’ river basins. Despite crustaceans are not be the primary ecological receptors of the herbicide metabolite 3,4-DCA, it also contributed with the greatest expected mixture toxicity to the sumTU in surface waters of the ‘Mondego’ and ‘Sado’ river basins. For fish, the insecticide organochloride endosulfan was responsible for the highest mTUs in three samples of the ‘Mondego’ and ‘Tejo’ river basins (Table 5). The mTU values were predominantly associated with herbicide compounds for which detection frequency, measured concentration and/or acute toxicity values for algae were relatively high. It has to be noted that the risk of more polar, thermo-labile and less volatile pesticide compounds was not included in the analytical survey of the present study. This can lead to a considerable underestimation of the overall toxicity of the surface water samples. In a study carried out in medium-sized Swiss rivers it was possible to evaluate how a complete pesticide screening changes the assessment of surface water quality (Moschet et al., 2014).

Table 5 Pesticide compounds and number of times accountable for mTU for algae, crustaceans and fish in samples with RQ_{STU} ratios above 1 in the ‘Mondego’, ‘Sado’ and ‘Tejo’ river basins

Pesticide compound	‘Mondego’ river basin (n=30)			‘Sado’ river basin (n=26)			‘Tejo’ river basin (n=52)		
	No. mTU for algae	No. mTU for crustaceans	No. mTU for fish	No. mTU for algae	No. mTU for crustaceans	No. mTU for fish	No. mTU for algae	No. mTU for crustaceans	No. mTU for fish
Herbicide									
Alachlor	-	-	-	-	-	-	24	-	-
Atrazine	5	-	-	-	-	-	10	-	-
Metolachlor	2	-	-	-	-	-	1	-	-
Molinate	6	-	-	4	-	-	-	-	-
Oxadiazon	2	-	-	14	-	-	-	-	-
Simazine	2	-	-	-	-	-	1	-	-
Terbuthylazine	-	-	-	-	-	-	-	-	-
Terbutryn	-	-	-	-	-	-	7	-	-
Metabolite									
3,4-DCA	-	3	-	-	-	-	-	-	-
Insecticide									
Chlorfenvinphos	-	9	-	-	8	-	-	3	-
Chlorpyrifos	-	-	-	-	-	-	-	4	-
Endosulfan	-	-	1	-	-	-	-	-	2

- No data.

Junghans et al. (2006) observed that the ratio STU/mTU (MCR) could also be used to predict when dose additive and independent action models of a mixture's toxicity produce similar or divergent estimates of toxicity. Table 6 presented the maximal possible ratio between the EC_{50} values of the pesticide mixtures predicted by IA and by CA taking into account the number of mixture components and the taxonomic group with maxSTU in surface waters of the 'Mondego', 'Sado' and 'Tejo' river basins. For each of these taxon-exposure scenarios the maximal value by which CA may predict a higher toxicity than IA was lower than the value given by the number of mixture components (between one and seven) and never exceeded a factor of 3.127. This indicates that the toxicity of all pesticide mixtures are dominated by a very small fraction of the compounds present.

Table 6 Maximal value by which CA may predict a higher toxicity than IA taking into account the number of mixture components and the taxonomic group with maxSTU in surface waters of the 'Mondego', 'Sado' and 'Tejo' river basins

No. of mixture components	Taxon	$\sum_{i=1}^{n} TU_i/\max\{TU_i\} i \in (1,\ldots,n)$[a]		
		'Mondego' river basin	'Sado' river basin	'Tejo' river basin
1	Algae	-	-	1
2	Algae	1.031	1.942	1.757
3	Algae	1.810	1.174	2.425
4	Algae	2.073	1.250	2.318
5	Algae	2.312	1.013	3.127
6	Algae	2.890	1.104	1.905
7	Algae	2.861	-	-
1	Crustaceans	-	-	-
2	Crustaceans	-	1.000	-
3	Crustaceans	1.004	-	-
4	Crustaceans	1.002	1.001	1.001
5	Crustaceans	1.012	1.022	1.000
6	Crustaceans	1.110	1.001	1.315
7	Crustaceans	1.048	-	1.007
1	Fish	-	-	-
2	Fish	-	-	-
3	Fish	-	-	-
4	Fish	-	-	1.025
5	Fish	1.003	-	1.025
6	Fish	-	-	-
7	Fish	-	-	-

[a] Calculated according to Eq. (3): the ratio between EC_x^{IA} and EC_x^{CA} can not exceed the given value
- No data

3.2 Uncertainties

The suggested approach for a predictive assessment of the mixture toxicity from a given agricultural exposure scenario is intended to give a reasonable, protective and pragmatically useful scheme for further use within risk assessment procedures. Nevertheless, one circumstance can challenge the precautionary character of the approach. Potential synergistic interactions that results in more-than-concentration-additive effects were not covered. There are a number of cases in the literature of synergistic interactions between pesticides. In some cases, the interactions were designed to be synergistic in nature (e.g., where one chemical inhibited the detoxification pathway of another) and thus were predictable. In other cases, a greater degree of synergism was observed only at higher exposure concentrations that may have affected secondary, non-target sites of action. Studies that were designed to determine mixture toxicity at environmentally relevant concentrations did not produce effects beyond those predicted by assuming concentration-addition (Rodney et al., 2013).

It is also important to highlight that for the derivation of the PNEC for the pesticide compounds selected in the present study the deterministic approach was the preferred method. However, the EQSs for some of them were already laid down in the law as a value (EC, 2013), but in that case their derivation followed a weight-of-evidence approach according to the WFD. This implicitly means that the resulting QS, whether it is derived using the AF method, the species sensitivity distribution (SSD) method, or using model ecosystem studies, are all considered reliable.

4. Conclusions

The proposed approach provides a general ‘screening level’ risk assessment of pesticide mixtures in surface waters of the ‘Mondego’, ‘Sado’ and ‘Tejo’ river basins and support the view that CA provides a pragmatic precautious but not overprotective approach, irrespective of their modes/mechanisms of action. The data generated are of importance for identifying sites of noncompliance and for obtaining a priority list of pesticide mixtures with potential aquatic risk. This list could be very useful for the evaluation of the current control measures that are mostly based on assessments of chemical risks focused on single pure toxicants.

For a given number of pesticide compounds between one and seven and a given response level of 50%, the maximal factor by which IA may predict a higher EC_{50mix} value than CA was lower than 2 in almost 100% of the samples with RQ_{STU} above 1, i.e. it indicates that only one pesticide compound provides at least 50% of the mixture's toxicity.

With respect to non-target group of organisms, they often were the primary ecological receptors of the most toxic compounds of the pesticide mixtures (e.g., herbicides on algae, and insecticides on crustaceans and fish). Despite having a different mode of action (e.g., the herbicide metabolite 3,4-DCA on crustaceans) this also contribute with the highest toxic unit in three pesticide mixtures.

Algae are most at frequent risk because of higher herbicide exposure within this analytical survey to pesticides in surface waters of the 'Mondego', 'Sado' and 'Tejo' river basins and their sensibility to these compounds.

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Chapter 5 Predicting the aquatic risk of realistic pesticide mixtures to species assemblages in Portuguese river basins

Based on the following manuscript:
Silva, E., Daam, M.A., Cerejeira, M.J. Predicting the aquatic risk of realistic pesticide mixtures to species assemblages in Portuguese river basins. *Journal of Environmental Sciences* (accepted, in publication)

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.

Key-Words: Pesticides; mixtures; risk assessment; multi-substance potentially affected fraction; surface waters

1. 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 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).

2. Experimental

2.1 Study area

In terms of water resources, ‘Tejo’, ‘Sado’ and ‘Mondego’ belong to the largest hydrological basins of continental Portugal occupying 25666, 12149 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).

2.2 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,b), 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).

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.

2.3 Risk analysis

2.3.1 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 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.

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 ($\mu g\ L^{-1}$)			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-< DL	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.

2.3.2 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.

2.3.3 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.

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.

2.4 Toxic pressure calculation for pesticide mixtures

The combined toxic risk (msPAF) per taxonomic group of all 24 pesticide compounds was evaluated by sequentially applying 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($\Sigma\text{HU}_{\text{TMoA}}$), 0, Average($\sigma_{\text{TMoA}}$), 1), where Average($\sigma_{\text{TMoA}}$) is the average of the standard deviation (σ) for pesticide compounds within the same TMoA, and $\Sigma\text{HU}_{\text{TMoA}}$ is the summation of their hazard units calculated by the following equation:

$$\sum_{i=1}^{n} \text{HU}_{\text{TMoA}} = \sum_{i=1}^{n} \frac{C_{\text{TMoA},i}}{10^{\mu i}} \tag{1}$$

where, $C_{\text{TMoA},i}$ 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-Π(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., $r = 0$.

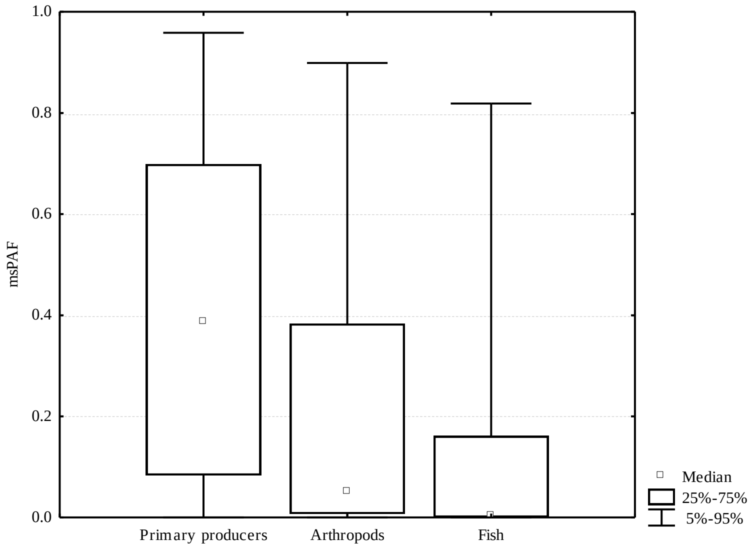
3. Results and discussion

3.1 Toxic pressure for pesticide mixtures

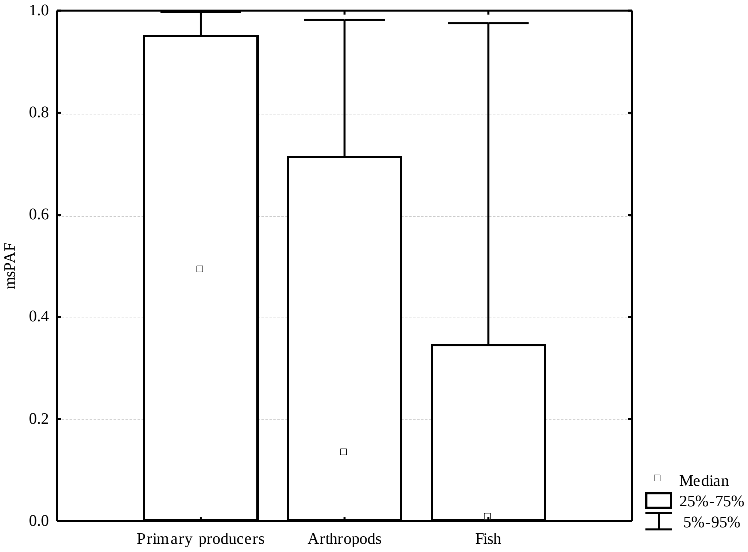
The toxic pressure, quantified as msPAF, was calculated in 281 samples considering the given concentration of fourteen herbicides (alachlor, atrazine, ethofumesate, MCPA, metolachlor, metribuzin, molinate, oxadiazon, pendimethalin, propanil, simazine, terbuthylazine, terbutryn and triclopyr), three insecticides (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.

Figure 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). 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.

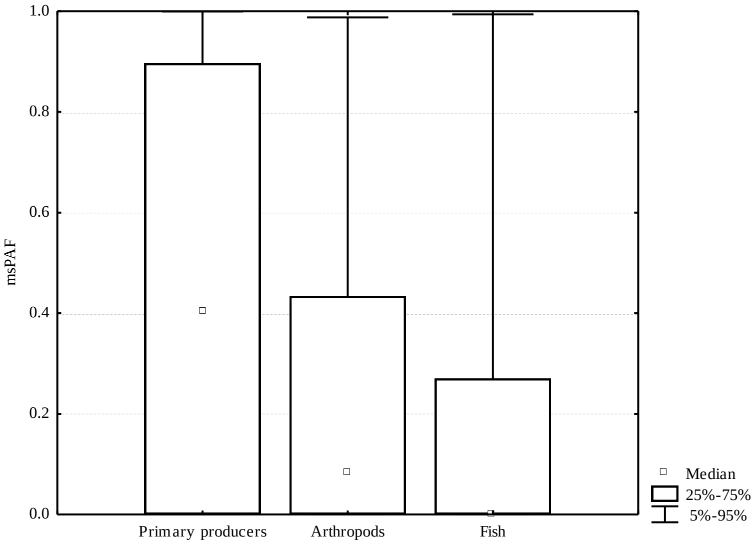
When high msPAFs for direct toxic effects are obtained for a certain taxonomic group, some possible indirect effects may be anticipated. For example, a reduction of the primary producers can lead to a decrease in the herbivore populations due to food limitation and/or habitat loss (Schäfer et al., 2011).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



Mondego



Sado



Tejo

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

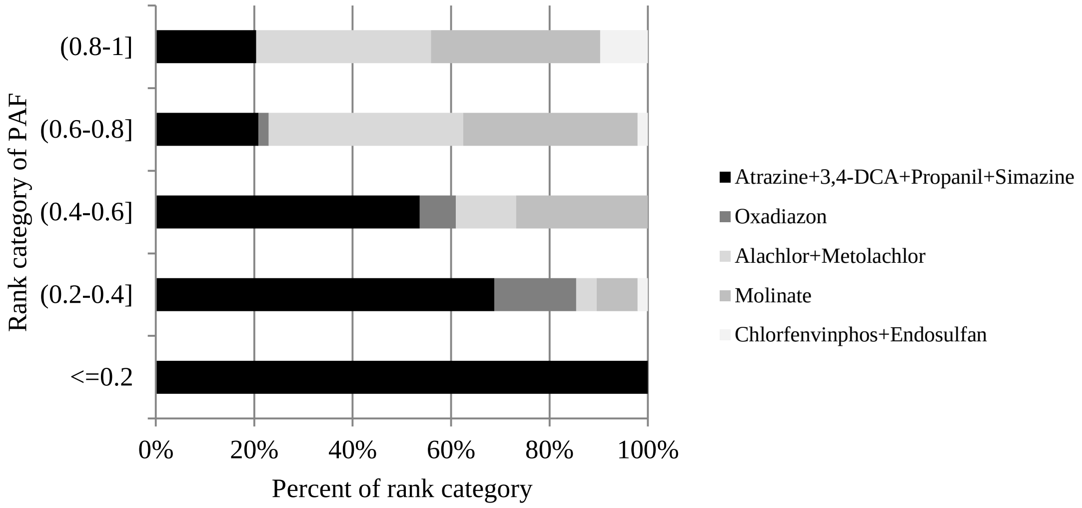
Four other case studies, which also used msPAFs posed by the pesticide mixtures as a measure of ecological risk at the local/river basin scale, were analyzed. However, caution is required when comparing the results with those of our study, because they differ in terms of methodological issues. First, 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.

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

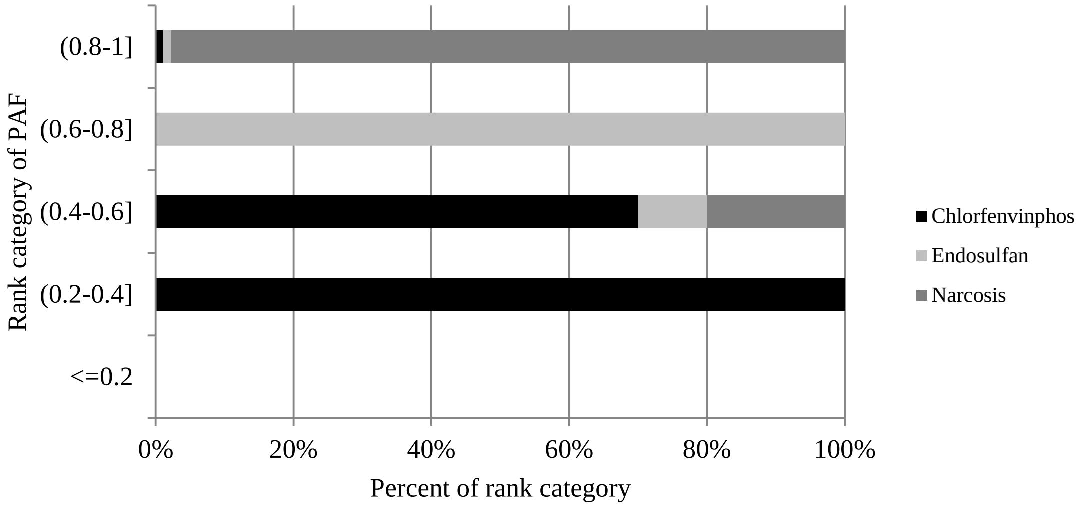
The influence of individual pesticide compounds (or groups of pesticides with the same TMoA) on the overall msPAF per taxonomic group was investigated. The approach followed by Jesenska et al. (2013) was adopted in this study. For each sample, the PAFs of individual pesticides (or class with the same 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).

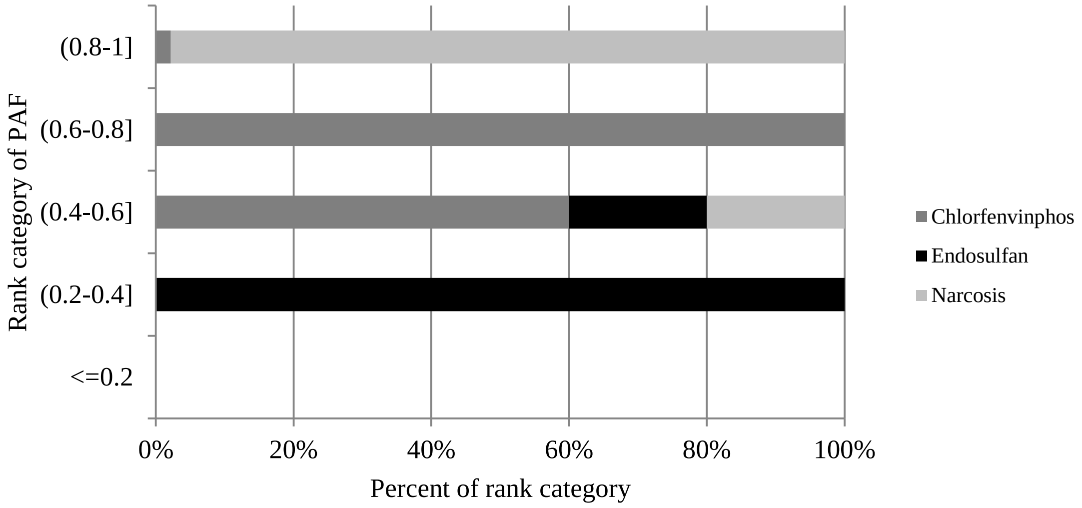
(a) Mondego



Primary producers

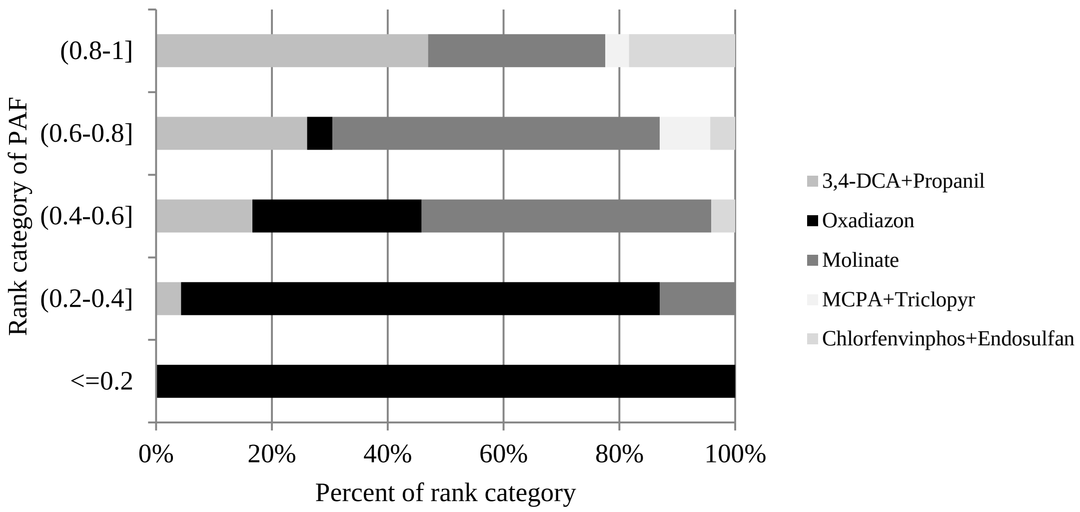


Arthropods

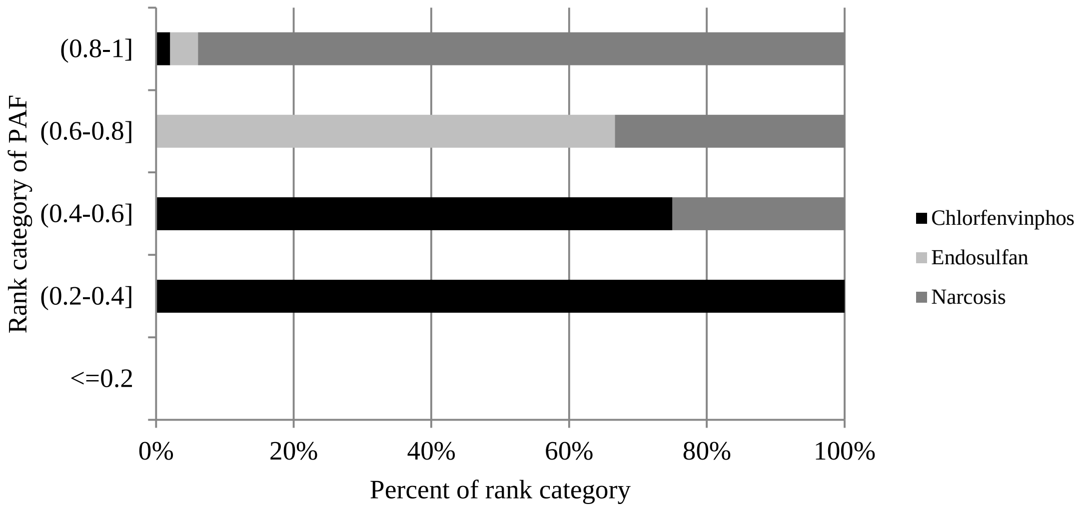


Fish

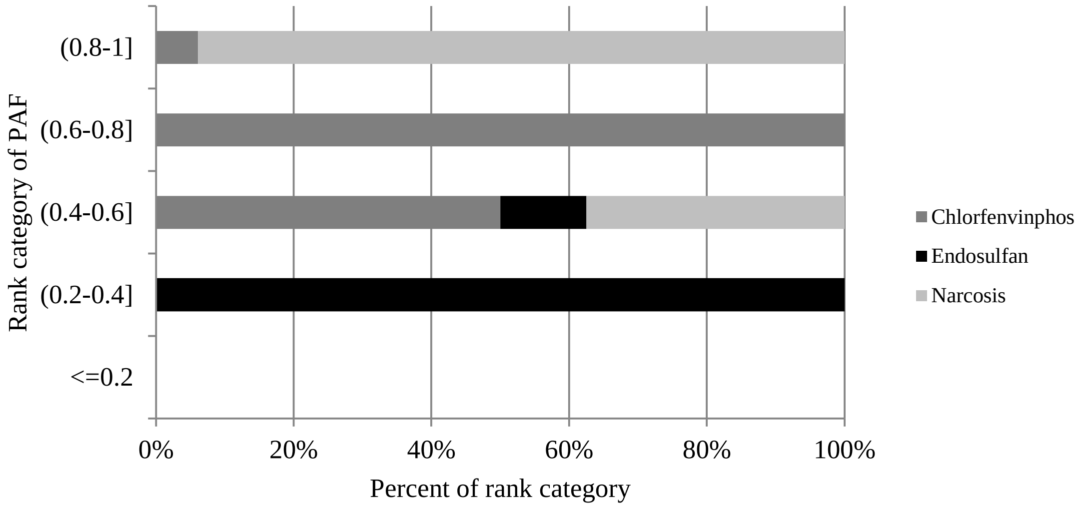
(b) Sado



Primary producers

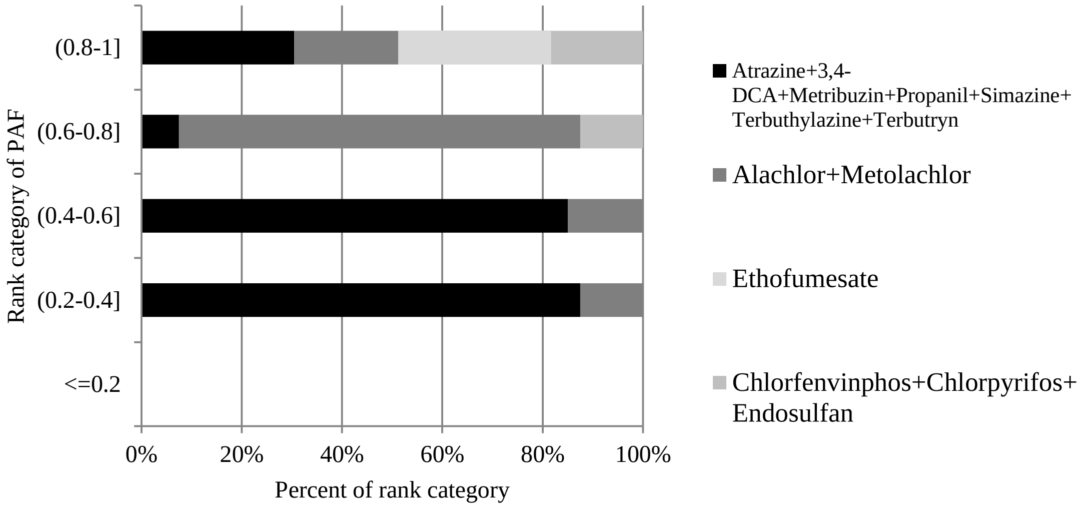


Arthropods

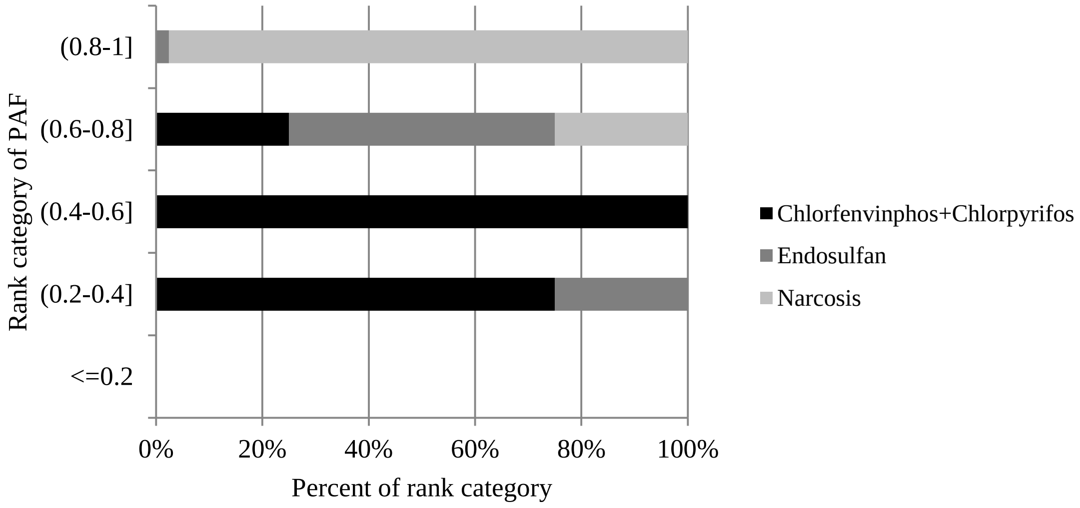


Fish

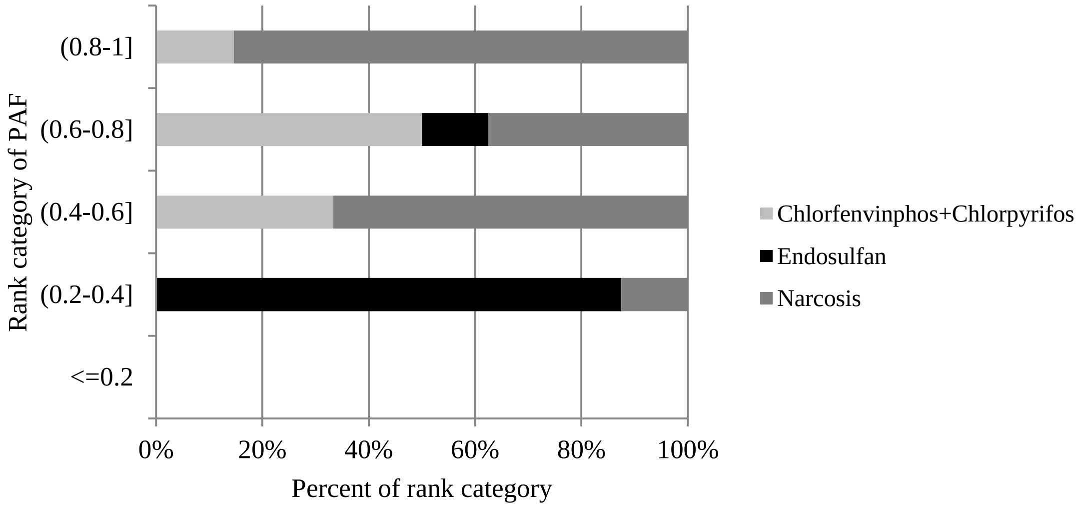
(c) Tejo



Primary producers



Arthropods



Fish

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

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).

4. 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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Chapter 6 Assessing the quality of freshwaters in a protected area within the Tagus River Basin District (Central Portugal)

Based on the following manuscript:
Silva, E., Pereira, A.C., Estalagem, S.P., Moreira-Santos, M., Ribeiro, R., Cerejeira, M.J. (2012) Assessing the quality of freshwaters in a protected area within the Tagus river basin district (central Portugal). *Journal of Environmental Quality* 41(5), pp.1413-1426

Abstract

Water-sediment quality was assessed in an agricultural zone of a protected area within the Tagus River basin district (central Portugal) combining chemical analysis to 12 pesticide compounds and whole toxicity testing using the bacterium *Vibrio fischeri*, the algae *Pseudokirchneriella subcapitata*, the crustacean *Daphnia magna,* and the midge *Chironomus riparius*. The herbicides alachlor, atrazine ethofumesate, metolachlor, terbuthylazine, the insecticides chlorfenvinphos and chlorpyrifos, and the metabolite 3,4-dichloroaniline were detected in surface water samples at four sites and in groundwater samples from six wells, during four sampling occasions. Measured concentrations were compared with parametric values for human consumption, groundwater quality standards, and environmental quality standards applicable to surface water established in European Union legislation. Most severe adverse effects were noted on the growth of *P. subcapitata* and lethality of *D. magna* in nondiluted water samples. Taking into account the values calculated by the method of toxic unit summation for pesticide mixtures, it was not possible to link the pesticides found to the toxicity detected in the water samples. Conducting this study with chemical analyses and biotests provided a more comprehensive quality assessment and realistic picture of the environmental samples analyzed, although additional studies are needed to evaluate the performance of mixture models for predicting mixture toxicity. This study underlines the importance of chemical analysis and whole toxicity testing as tools for assessing the impact of human activity on the status of water, mainly in protected zones.

1. Introduction

The Water Framework Directive (WFD) calls for the protection and restoration of clean water across Europe (EC, 2000). While prior European legislation considered chemical contamination in water, this directive provides a major innovation by also addressing the health of aquatic ecosystems. The use of whole-organism bioassays to assess lethal and sublethal toxicity, through the measurement of various biological responses, provides an additional complementary approach for the evaluation of the quality of a water body. The observed toxic impact is generally the result of the bioavailability of the complex mixture of pollutants that may be present in the sample but is also dependent on the sample physicochemical parameters (e.g., DOC content, pH). Moreover, the use of multiple test species and trophic levels may be crucial for obtaining meaningful results or for fingerprinting since many intercomparisons of biological assays have shown differences in sensitivity to different chemicals or classes of compounds (Allan *et al.*, 2006). The combination of bioassays with the chemical analysis of the contaminated samples to identify the potential toxic compounds offers a more integrated risk assessment because it encompasses biological effects and contaminant identity. When both approaches are not integrated, knowledge on the environment situation would be only partial (Blasco and Picó, 2009). However, this approach is meaningless if key contaminants are not known a priori. Numerous studies combining chemical and biological approaches for hazard assessment of complex environmental mixtures indicate that priority pollutant concentrations are a poor indicator of toxicity. Nontarget analysis of complex mixtures allows the detection and identification of a broader range of compounds; however, this approach is time consuming and not routinely applicable. The results are often difficult to evaluate since toxicological data of the compounds identified are missing (Brack, 2003). Several studies have demonstrated that the use of ecotoxicological tests concurrently with appropriate chemical analyses allows for more comprehensive estimates of water quality (Köck *et al.*, 2010; Ocampo-Duque *et al.*, 2008; Pereira *et al.*, 2000; Silva *et al.*, 2011).

For the quantification of contributions from identified toxicants, the summation of toxic units as derived from the individual components has been used (Grote *et al.*, 2005). This procedure implicitly assumes a concentration additive behavior of the components in the mixture and allows a comparison of expected and observed response for the selected effect level only.

In the present study, the above-mentioned integrated strategy was applied for the first time for a screening site-ecological risk assessment of a freshwaters protected area (Tagus vulnerable zone [TVZ], central Portugal) from the Tagus River basin district water. The area of the TVZ was selected as a case study because it is characterized by the cumulative presence of several anthropogenic pressures, the main one being agriculture, and drainage conditions favorable to contamination. Among the substances from agricultural activities liable to cause pollution, the vast majority are pesticides. The incorrect use of these products, not only through their direct field application on crops but also in agricultural related operations, such as storage, handling, dilution, and mixing, may have unacceptable effects on the environment.

In this context, and in an attempt to identify major contaminants linked to the agricultural practices in the TVZ, the objectives of the present study were (i) to assess the individual risk of pesticide compounds to surface water and groundwater ecosystems as well as human health by comparing the measured concentrations with their respective quality standards under the WFD; (ii) to quantify the toxicity of the pesticide mixtures found in water to bacteria, algae, and *Daphnia* sp. through the method of toxic unit summation (TUS); (iii) to evaluate the toxicity of water and sediment samples collected in the study area to aquatic organisms belonging to different taxonomic groups and having different functions at the ecosystem level; and (iv) to search for the potential relationships between the presence of pesticides and toxic responses in the frame of a screening ecological risk assessment.

The bacterium *Vibrio fischeri*, the algae *Pseudokirchneriella subcapitata*, and the crustacean *Daphnia magna* were selected to assess water toxicity; the midge *Chironomus riparius* was used to evaluate sediment toxicity. All the latter species are standard test species that have been widely used in ecotoxicological studies and for which standard protocols are internationally available for performing toxicity tests (e.g., OECD, 1984, 1998b, 2004).

2. Materials and methods

2.1 Study area

The study area in the TVZ is inserted in the Tagus alluvial aquifer system, whose main formations are alluvia (Holocene) and fluvial terraces (Pleistocene), and which belongs to one of the most productive hydrogeological units of the continental part of Portugal: the Tagus-Sado miopliocenic multilayer aquifer system. Groundwater flow is strongly influenced by the Tagus River. The Almonda and Alviela tributaries can recharge or discharge the alluvial aquifer system depending on the hydraulic potential. Precipitation constitutes the main source of recharge (Almeida *et al.*, 2000), primarily in the period between autumn and spring, as occurred in the hydrological year of 2007-2008 (SNIRH, 2011). Previous studies (Lobo Ferreira and Oliveira, 1995; Paralta *et al.*, 2001) concluded that the study area is highly vulnerable to diffuse groundwater contamination. Taking these hydrogeological characteristics and the intensive agricultural occupation into account, this area was designated as a vulnerable zone - TVZ (Fig. 1) - in accordance with the Council Directive 91/676/EEC of 12 Dec. 1991 concerning the protection of waters against pollution caused by nitrates from agricultural sources (EEC, 1991).

In particular, the present study was performed in an agricultural area located in Golegã (Ribatejo region, in the central part of continental Portugal). Maize (*Zea mays* L.), sugar beet (*Beta vulgaris* L.), horticultural (e.g., pepper [*Capsicum annuum* L.], onion [*Allium cepa* L.], cabbage [*Brassica oleracea* L.], and broccoli [*Brassica oleracea* L. var. *italica* Plenck]), industrial (e.g., tomato [*Solanum lycopersicum* L.]), and potato (*Solanum tuberosum* L.) crops occupy most of the agricultural area in this council (RGA, 2011), which is irrigated with groundwater extracted from wells by flooding, sprinkler systems, and drip and micro-irrigation.

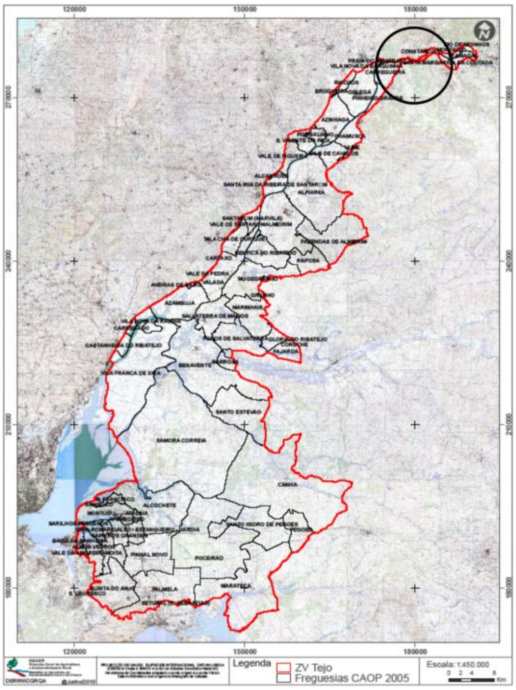

Fig. 1 Map of the Tagus vulnerable zone and the study area delimited by a circle

2.2 Water and sediment sampling

Groundwater and surface water samples were collected for analysis of pesticides and toxicity testing on June (5 and 27), 22 July, and 13 August 2008, during the main period of agricultural practices, both in terms of pesticide application and irrigation. A total of six and eight surface water samples were collected, respectively, at the Alverca do Campo embankment (from two points, EMB1 and EMB2; on the first and last sampling time no water was sampled at EMB2), a body of inland water, and the Almonda River (from two points, upstream [ALRU] and downstream [ALRD]). In summer, the water level of the embankment is maintained by pumping water from the Tagus River, while the Almonda River is fed by a perennial karstic spring. A total of 24 groundwater samples were collected from six drilled wells (GW1-GW6) for irrigation use with depths varying between 20 and 50 m. When possible, water was pumped for approximately 5 min from the well to purge the pipes to obtain a representative groundwater sample. All water samples were collected into 1-L single-use amber glass bottles and kept on dry ice until arrival at the laboratory. Sediment samples were collected only once (13 August) at the Alverca do Campo embankment (EMB1).

Surface sediment samples (0-5 cm) were collected with a spoon into 500-mL amber glass bottles.

2.3 Pesticides selected for the study

The herbicides alachlor (2-chloro-2′,6′-diethyl-*N*-methoxymethylacetanilide), atrazine (6-chloro-*N*2-ethyl-*N*4-isopropyl-1,3,5-triazine-2,4-diamine), simazine (6-chloro-*N*2,*N*4-diethyl-1,3,5-triazine-2,4-diamine), and trifluralin (α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-p-toluidine) and the insecticides chlorfenvinphos [(*EZ*)-2-chloro-1-(2,4-dichlorophenyl)vinyl diethyl phosphate], chlorpyrifos (*O*,*O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate), endosulfan (1,4,5,6,7,7-hexachloro-8,9,10-trinorborn-5-en-2,3-ylenebismethylene sulfite), and lindane (1α,2α,3β,4α,5α,6β-hexachlorocyclohexane) were selected since they are indicated in the list of priority substances in the field of water policy (EC, 2008). The herbicides ethofumesate [(*RS*)-2-ethoxy-2,3-dihydro-3,3-dimethylbenzofuran-5-yl methanesulfonate], metolachlor [2-chloro-*N*-(6-ethyl-o-tolyl)-*N*-[(1*RS*)-2-methoxy-1-methylethyl]acetamide], and terbuthylazine (*N*2-tert-butyl-6-chloro-*N*4-ethyl-1,3,5-triazine-2,4-diamine) and the metabolite 3,4-dichloroaniline (3,4-DCA) were also included in the study due to the amount sold in Portugal (DGADR, 2011) in recent years and/or to their detection in studies performed previously in Portugal (Batista, 2003; Batista *et al.*, 2001, 2002; Cerejeira *et al.*, 2000, 2003; Silva *et al.*, 2006, 2011, 2012).

2.3.1 Exposure prediction using screening models

On the basis of a set of physicochemical and partition coefficient properties selected from different databases (FOOTPRINT, 2011; Hornsby *et al.*, 1996; Tomlin, 2006), the environmental exposure and, in particular, the possibility of the occurrence in groundwater were evaluated a priori through the fugacity model level I (Mackay, 2001) and the leaching indexes developed by Bacci and Gaggi (1993) and Gustafson (1989). Based on these calculations, Table 1 presents the predicted environmental distribution (PED) for water of each pesticide compound selected for the present study and its leachability considering the criteria set through the Bacci and Gaggi index (Bacci, 1994) and the groundwater ubiquity score (GUS) index (Gustafson, 1989).

2.3.2 Characterization of effects

For an a priori ecotoxicological potential evaluation of the pesticide compounds selected for the study, their effective concentrations inducing lethal and sublethal effects on three standard test species belonging to three major taxonomic groups and throphic levels in food webs were considered. Median effective concentration (EC_{50}) values for algae and *Daphnia* sp. and no observed effect concentration (NOEC) values for *Daphnia* sp. were derived from Tomlin (2006) and FOOTPRINT (2011), while EC_{50} measures on *Vibrio fischeri* were obtained from several studies (Bonnet *et al.*, 2007; Osano *et al.*, 2002; Palma *et al.*, 2008) (Table 2).

Table 1 Predicted environmental distribution (PED) for water and leaching potential according to the Bacci & Gaggi and groundwater ubiquity score (GUS) indexes, for the pesticide compounds selected in the present study

Pesticide	PED for water (%)†	Bacci & Gaggi index‡	GUS index§
Herbicide			
Alachlor	47.3 (AA)	0.16 (L)	2.08 (T)
Atrazine	77.7 (HA)	0.62 (L)	3.56 (L)
Ethofumesate	68.8 (HA)	0.20 (L)	2.17 (T)
Metolachlor	58.1 (AA)	0.56 (L)	3.32 (L)
Simazine	89.8 (VHA)	0.56 (L)	3.35 (L)
Terbuthylazine	40.5 (AA)	0.44 (L)	3.18 (L)
Trifluralin	1.56 (VLA)	0.02 (T)	0.17 (IL)
Metabolite			
3,4-DCA	67 (HA)	0.64 (L)	3.77 (L)
Insecticide			
Z-chlorfenvinphos	13.5 (VLA)	0.11 (L)	1.87 (T)
E-chlorfenvinphos	6.23 (VLA)	0.11 (L)	1.87 (T)
Chlorpyrifos	2.15 (VLA)	1.08E-2 (T)	0.32 (IL)
α-endosulfan	1.96 (VLA)	8.79E-3 (IL)	-0.16 (IL)
β-endosulfan	1.75 (VLA)	8.79E-3 (IL)	-0.16 (IL)
Lindane	25.7 (LA)	0.3 (L)	2.49 (T)

† Mackay fugacity model (Level I, version 3.00, 2004, Trentu University, Canada) if $PED_W < 20\%$: Very Low Affinity (VLA); if $20\% \leq PED_W < 40\%$: Low Affinity (LA); if $40\% \leq PED_W < 60\%$: Average Affinity (AA); if $60\% \leq PED_W < 80\%$: High Affinity (HA); if $PED_W \geq 80\%$: Very High Affinity (VHA).

‡ If index ≥ 1E-1: Leacher (L); if 1E-2 ≤ index ≤ 9E-2: Transition (T); if 1E-4 ≤ index ≤ 9E-3: Improbable Leacher (IL) (Bacci, 1994).

§ If GUS > 2.8: Leacher (L); if 1.8 < GUS < 2.8: Transition (T); if GUS < 1.8: Improbable Leacher (IL) (Gustafson, 1989).

2.4 Analysis of pesticides in water

2.4.1. Chemicals

Pesticide standards of 99% purity (BASF Aktiengesellschaft, Limburgerhof, Germany) or higher (Dr. Ehrenstorfer GmbH, Augsburg, Germany), analytical-grade reagents (Merck, Darmstadt, Germany), and sodium chloride (Panreac Quimica, Barcelona, Spain) were used throughout the analysis.

2.4.2 Multiresidue method

A commercial autosampler (Combi PAL from CTC Analytics, AG, Switzerland) using a solid-phase microextraction (SPME) module, mounted on a gas chromatography-mass spectrometry (GC-MS) system was used; SPME fibers of 65-μm carbowax divinylbenzene (CW/DVB) were supplied by Supelco (Bellefonte, PA, USA). The SPME step was performed at the following conditions: water samples (10 mL) were placed in vials and extracted by immersion of the coated fiber for 60 min at room temperature; sample agitation was used at 250 rpm after the addition of 10% NaCl (w/v); no pH adjustment was needed. After extraction, the fiber was directly exposed to the hot injector of the GC system for analysis. Thermal desorption was performed for 5 min.

The GC-MS analyses were performed with a Varian ChromPack CP-3800 gas chromatograph coupled to a Saturn 2000 GC/MS (Varian, Walnut Creek, CA, USA) equipped with an ion-trap detector. A J&W DB-5MS 30-m × 0.25-mm Low Bleed/MS column was used (J&W Scientific, Folsom, CA, USA). Helium was used as carrier gas at 83 kPa (12 psi). Injector, interface, and mass-spectrometric detector temperatures were 270, 260, and 190°C, respectively. Column temperature was programmed from 50 to 170°C at 10°C min^{-1}, from 170 to 180°C at 1°C min^{-1}, from 180 to 200°C at 5°C min^{-1}, maintained 6 min at this temperature, and then from 220 to 240°C at 15°C min^{-1}, with final time of 4 min. The ionization mode was electronic impact (EI). Ions used for identification and quantification were as follows (in parentheses): alachlor (160+188), atrazine (200+215), E-chlorfenvinphos (267+323), Z-chlorfenvinphos (267+323), chlorpyrifos (197+314+316), 3,4-DCA (126+161+163), α-endosulfan (195+241+339), β-endosulfan (195+241+339), ethofumesate (161+207), lindane (181+183+219), metolachlor (162+238), simazine (173+186+201), terbuthylazine (173+214+229), and trifluralin (264+306). Detection limits varied between 0.1 ng L^{-1} (trifluralin) and 17 ng L^{-1} (E-chlorfenvinphos) following the recommendations of the ISO/IEC 17025 norm (ISO, 2005).

Table 2 Median effective concentration (EC$_{50}$; in mg L^{-1}), with 95% confidence limits and coefficient of variation (CV; in %) inside parentheses, and no observed effect concentration (NOEC; in mg L^{-1}) values for algae, *Daphnia* sp. and *Vibrio fischeri*, for the pesticide compounds selected in the present study

Pesticide	Algae†	*Vibrio fischeri*‡	*Daphnia* sp.§	
	EC$_{50}$ (72 h)	EC$_{50}$ (30 min)	EC$_{50}$ (48 h)	NOEC (21 d)
	mg L^{-1}			
Herbicide				
Alachlor	0.012	159 (114-210)	13	0.22
Atrazine	0.043	69.4 (68.8-70.0)	6.9	0.25
Ethofumesate	3.9	-¶	13.52	0.32
Metolachlor	0.1	215 (200-220)	25	0.707
Simazine	0.042	-	>100	2.5
Terbuthylazine	0.016	-	21	0.019
Trifluralin	0.0122	-	0.245	0.051
Metabolite				
3,4-DCA	1.65	0.61 ± 0.08 CV (13.11%)	0.12	0.005
Insecticide				
Chlorfenvinphos	1.6	-	0.0003	0.0001
Chlorpyrifos	0.48	2.84 (2.52-3.16)	0.0017	0.0046
Endosulfan	>0.56	-	0.075	-
Lindane	0.78 (120 h)/2.5	-	1.6	0.0002

† Tomlin (2006), FOOTPRINT (2011).
‡ Bonnet *et al.* (2007); Osano *et al.* (2002); Palma *et al.* (2008).
§ FOOTPRINT (2011).
¶ -, no data.

2.5 Risk assessment of pesticides under the Water Framework Directive (2000/60/EC)

The Directive 2008/105/EC of the European Parliament and of the Council lays down environmental quality standards (EQS) for priority substances and certain other pollutants, which include some pesticides, with the aim of achieving good surface water chemical status (EC, 2008). Table 3 presents the EQS parameters for eight of the pesticides selected in the present study that are included among the priority substances, expressed as an annual average value (AA-EQS) and as a maximum allowable concentration (MAC-EQS) established at a level providing aquatic environment and human health protection against long-term exposure and short-term exposure, respectively. For any given surface water body, applying the AA-EQS means that for each representative monitoring point within the water body, the arithmetic mean of the concentrations measured at different times during the year does not exceed the standard. For any given surface water body, applying the MAC-EQS means that the measured concentration at any representative monitoring point within the water body does not exceed the standard.

Table 3 Environmental quality standards (EQS) for eight of the pesticide compounds selected in the present study that are identified as priority substances under Directive 2008/105/EC (EC, 2008), expressed as the annual average value (AA-EQS; in μg L^{-1}) and the maximum allowable concentration (MAC-EQS; in μg L^{-1})

Pesticide	AA-EQS†		MAC-EQS‡	
	Inland surface waters	Other surface waters	Inland surface waters	Other surface waters
	μg L^{-1}			
Herbicide				
Alachlor	0.3	0.3	0.7	0.7
Atrazine	0.6	0.6	2.0	2.0
Simazine	1	1	4	4
Trifluralin	0.03	0.03	not applicable	not applicable
Insecticide				
Chlorfenvinphos	0.1	0.1	0.3	0.3
Chlorpyrifos	0.03	0.03	0.1	0.1
Endosulfan§	0.005	0.0005	0.01	0.004
Lindane (γ-hexachloro-cyclohexane)§	0.02	0.002	0.04	0.02

† Unless otherwise specified, it applies to the total concentration of all isomers.

‡ Where the MAC-EQS are marked as “not applicable,” the AA-EQS values are considered protective against short-term pollution peaks in continuous discharges since they are significantly lower than the values derived on the basis of acute toxicity (EC, 2008).

§ Also identified as priority hazardous substances.

The Directive 2006/118/EC of the European Parliament and of the Council sets out quality standards for plant protection products as community criteria for the assessment of the chemical status of bodies of groundwater (EC, 2006), including those for use as water supply for human consumption (EC, 1998). The concentrations of 0.1 μg L^{-1} of an active substance in plant protection products (including their relevant metabolites, degradation and reaction products) and of 0.5 μg L^{-1} for the sum of all such products should not be exceeded, to protect human health and the environment (EC, 2006).

To assess the risk for pesticides within the context of the WFD, the measured concentrations of the compounds selected for the present study in water were compared with the EQS indicated above.

2.6 Mixture Risk Assessment Method: Toxic Unit Summation

The method of toxic unit summation (TUS) is a direct application of the concentration addition concept and is defined by the following formula:

$$\text{TUS} = \sum_{i=1}^{n} TU_i = \sum_{i=1}^{n} \frac{c_i}{ECx_i}$$

where c_i is the actual concentration (or doses) of the individual substances in a mixture and ECx_i denotes equi-effective concentrations (or doses) of these substances if present singly (e.g., EC_{50i}). The quotients c_i/ECx_i are termed toxic units (TU); TU rescales absolute concentrations (or doses) of substances to their different individual toxic potencies. It expresses the concentrations (or doses) of mixture components as fractions of equi-effective individual concentrations (or doses) ECx_i. Typically, x = 50% (EC_{50i}) is used as the reference level, but TUS can also be calculated for any other effect level x. If TUS = 1, the mixture is expected to elicit the total effect x. If the sum of TU is smaller or larger than 1, the mixture is expected to elicit effects smaller or larger than x, respectively (Kortenkamp *et al.*, 2009).

The TUS mixture risk assessment method used in the present study was based on the measured concentrations of the pesticide compounds in the water samples and their respectively effect measures for algae, *Daphnia* sp., and *Vibrio fischeri* (see Table 2).

2.7 Bioassays for Water and Sediment Toxicity Testing Assessment

The ecotoxicological evaluation of the water samples was performed based on the 5-min luminescence of the bacterium *V. fischeri* Lehmann & Neumann, 72-h growth of the green algae *P. subcapitata* Koršhikov, 48-h lethality and 21-d reproduction of the cladoceran *D. magna* Straus, whereas that of the sediments was based on the 10-d growth of the midge *C. riparius* Meigen. All selected bioassays utilize standard test organisms widely used in ecotoxicological studies and have established standardized protocols that allow their easy and reliable execution. The 72-h algae growth test and the 48-h cladoceran lethal test were performed on all undiluted water samples (i.e., 100%) according to the Algaltoxkit F (SOP, 2004) and Daphtoxkit F magna (SOP, 2003) standard operation procedures, respectively. In addition, because on the last sampling time the Algaltoxkit F and Daphtoxkit F magna tests revealed toxic effects equal to or higher than those observed on the previous sampling times (see "Results and Discussion" section), the toxicity of a dilution gradient of samples EMB1 and ALRD was explored with more detail by performing the following conventional bioassays: the 72-h *P. subcapitata* growth (OECD, 1984), 21-d *D. magna* reproduction (OECD, 1998b) and 5-min *V. fischeri* luminescence (Azur Environmental, Carlsbad, CA, USA). Toxicity assessment of a dilution gradient of sediment sample EMB1 was also performed using the 10-d *C. riparius* growth (OECD, 2004).

The Algaltoxkit F and Daphtoxkit F magna toxkits both adhere to the International Organization for Standardization guidelines for the correspondent conventional assays (ISO 1996, 2004). The Algaltoxkit F test estimates the 72-h growth of *P. subcapitata* in each test solution and all materials used are purchased with the kit. As the correspondent conventional assay (e.g., OECD, 1984), the algae concentration at the start of the test was approximately 10^4 cells mL^{-1} replicate^{-1} culture, and all cultures were incubated at 25°C under continuous cool-white fluorescent illumination (100 μE m^{-2} s^{-1}). Algal growth rate was determined by optical density measurements in a Hitachi U-2000 spectrophotometer UV-Vis (Hitachi, Ltd., Tokyo, Japan) and expressed as the percentage of inhibition relatively to the control. The Daphtoxkit F magna test estimates the 48-h lethality/immobility of *D. magna* neonates (less than 24-h old) exposed to the test solutions. Each replicate consisted of five organisms per 10 mL of medium and was incubated in darkness at 20°C. The percentage of mortality was determined at the end of the 48-h exposure by quantifying the number of immobile organisms.

The luminescence test with *V. fischeri* was conducted according to the 81.9% basic test protocol (Azur Environmental, Carlsbad, CA, USA). The light emission of the bacteria was measured using the microtox toxicity analyzer model 500 (Strategic Diagnosis, Newark, DE, USA) within a short exposure time of 5 min, since preliminary tests did not reveal any toxicity change for periods up to 30 min. A series of nine dilutions (factor of 2) was tested.

The 72-h *P. subcapitata* growth test was performed following OECD (1984) and Environment Canada (1992) guidelines. The *P. subcapitata* (strain Nr. WW 15-2521) was acquired from the Carolina Biological Supply Company (Burlington, NC, USA) and maintained in 100-mL nonaxenic batch cultures, with Woods Hole MBL growth medium (Stein, 1973), at 19 to 21°C under continuous cool-white fluorescent illumination (100 μE m^{-2} s^{-1}). Samples EMB1 and ALRD were subjected to a dilution gradient of 6.25, 12.5, 25, 50, and 100%. The MBL medium was diluted 2.5 times to be used as the control and dilution medium according to the required N/P ratios. All waters and respective dilutions were tested without and with the addition of nutrients (in the same proportion as the diluted MBL medium) as an approach to discriminate potential toxic effects from those due to natural poor nutrient levels (Environment Canada, 1992). Three 1-mL replicate cultures of each test dilution and six of the control were set up and inoculated with a 100-μL algal inoculum, so that the initial cell concentration was 10^4 cells mL^{-1}. The tests were performed under the same conditions used for culturing. At the end of the 72-h exposure, the mean specific growth rate per day was estimated. Initial and final cell densities were counted on well-mixed aliquots of each replicate under a microscope (×400 magnification), using a Neubauer chamber (American Optical, Buffalo, NY, USA).

The 21-d *D. magna* reproduction test was performed according to OECD (1998b) guidelines. Organisms used for testing were third-brood 6- to 24-h-old neonates, obtained from cultures maintained at 20 to 22°C under a 14-h/10-h light/dark photoperiod. Culture medium was a reconstituted hard water (ASTM, 2002) supplemented with vitamins (7.5 μg L^{-1} of B_1, 1 μg L^{-1} of B_{12}, and 0.75 μg L^{-1} of biotin) and Marinure extract (Glenside, Stirling, UK; 7.5 mL L^{-1} of a suspension with an absorbance of 620 units at 400 nm). Cultures (25 and 15 daphnids L^{-1} up to the first brood and from there onward, respectively) were fed daily with *P. subcapitata* (3×10^5 cells mL^{-1}), and the medium was renewed every other day. Samples EMB1 and ALRD were subjected to a dilution gradient of 12.5, 25, 50, and 100%. The culture medium was used as the control and dilution medium. Ten replicates were set up for each treatment, with 50 mL of the test solution and one organism each. During testing, the feeding regime, medium renewal frequency, and incubation conditions were similar to those used for culturing. After the 21-d exposure, the percentage survival of the parent animals was estimated and reproduction was determined as the total number of living neonates released per female.

The 10-d *C. riparius* growth test was performed following the OECD (2004) and Environment Canada (1997) guidelines. For testing, first- to second-instar larvae were used and obtained from cultures maintained in a transparent plastic cage (40 by 60 by 120 cm) sufficiently large to allow swarming and copulation of adults (OECD, 2004), at 20 to 22°C, under a 14-h/10-h light/dark photoperiod with 90-min dawn and dusk periods. Cultures consisted of crystallizing dishes containing 185 g of quartz sea sand (0.1-0.4 mm particle size; Merck, Darmstadt, Germany) and 300 mL of reconstituted hard water (ASTM, 2002) were continuously aerated and fed a suspension of ground Tetramin (Tetrawerk, Melle, Germany) every other day (0.1 g dish^{-1}, with 30 and 15 larvae dish^{-1} up to Day 7 and from there onward, respectively; for further details see Castro *et al.* (2003). The samples dilution gradient of EMB1 was 12.5, 25, 50, and 100%. The culture medium was used as the control and dilution medium. Four replicates were set up for each test dilution and control, each with 50 g of sediment and 120 mL of medium. Vials were prepared 12 h before the beginning of the test and left with continuous aeration. Three larvae were added per replicate, and after 30 min, aeration was restarted. During testing, the organisms were fed daily with 1 mg of Tetramin per larvae. The tests were incubated under the same temperature and light conditions used for culturing. After a 9-d exposure (instead of 10 d because larvae started to pupate), growth was estimated as the final dry weight (g), and the percentage of survival was also determined.

2.8 Data analysis

For all four conventional tests (bacterium, algae, cladoceran, and midge), except for the *V. fischeri* test, the organism responses were examined for significant differences between the control and tested water/sediment dilutions using one-way ANOVA. Although a nested ANOVA design was used in the *C. riparius* test, a one-way ANOVA was applied to the growth data due to the occurrence of pupation. When significant differences were found, the Dunnett’s test was performed to determine the no observed effect dilution (NOEC) and the lowest observed effect dilution (LOEC). The median effective dilution (EC_{50}) and respective 95% confidence limits (CL) were obtained by fitting organism responses to a logistic model using the least squares method (OECD, 1998a). For the *V. fischeri* test, effective dilutions resulting in the inhibition of light emission were determined using the microtox Omni Software 1.18 (Azur Environmental). As the microtox software did not provide replicate data to perform ANOVA, the water dilutions causing a luminescence inhibition lower and higher than 10% were considered as the NOEC and LOEC, respectively.

3. Results and discussion

3.1 Risk assessment of pesticides in groundwater and surface water

The herbicides atrazine, ethofumesate, metolachlor, and terbuthylazine were detected in all surface water samples from the Alverca do Campo embankment. The maximum concentration was determined for metolachlor (1.83 μg L^{-1}) on 27 June, when the total pesticides (2.69 μg L^{-1}) also reached its highest concentration (Fig. 2a, Table 4). However, the sum of the TU of all active substances based on the effect measures for algae and *Daphnia* sp. (see Table 2) was less than 1 (Fig. 3a).

The herbicides alachlor and terbuthylazine, the insecticides chlorpyrifos and chlorfenvinphos (E+Z), and the metabolite 3,4-DCA were detected in surface water samples from the Almonda River. Total concentration of pesticides was higher than that quantified in surface waters from the Alverca do Campo embankment in all sampling dates, reaching the maximum value (32.63 μg L^{-1}) at the furthest downstream sampling point of the Almonda River on 22 July due mainly to the presence of 3,4-DCA and alachlor, with 20.19 and 10.75 μg L^{-1}, respectively (Fig. 2b, Table 4). This may be related to the lesser dilution of the compounds in the surface water of the Almonda River, only maintained in summer by the perennial nature of the groundwater source, while a larger quantity and dynamic of water flow occur at the Alverca do Campo embankment due to the pumping from the Tagus River. On the other hand, the former is more susceptible to the impact of agriculture activities, owing to the proximity of the streambanks to cropped areas along its run. In fact, in the two surface water samples with the major total concentration of pesticides, collected at downstream (22 July) and upstream (27 June) sampling points of the Almonda River, the sum of the TU of all active ingredients was greater than 1 for algae and *Daphnia* sp., respectively, due mainly to the presence of alachlor and Z-chlorfenvinphos. The sum of the TU of all active substances based on $NOEC_{Daphnia}$ (see Table 2) was also greater than 1 for all surface water samples, with the exception of those collected on the last sampling date (13 August) (Fig. 3b). The metabolite 3,4-DCA and the insecticide Z-chlorfenvinphos contributed to the total chronic toxicity with the highest TU. The arithmetic mean of the concentrations of alachlor, atrazine, chlorfenvinphos, and chlorpyrifos measured in surface water samples collected at the four monitoring points during 2008 exceeded their AA-EQS (0.3, 0.6, 0.1 and 0.03 μg L^{-1}, respectively) (Table 4; see Table 3). Concentrations of alachlor and chlorpyrifos were above their MAC-EQS (0.7 and 0.1 μg L^{-1}, respectively) for priority substances in the field of water policy (EC, 2008) (Fig. 2b; see Table 3) in eight (all) and five surface water samples, respectively, from the Almonda River.

The herbicides alachlor, atrazine, and metolachlor were detected in groundwater samples collected in six wells. The parametric value for human consumption and the groundwater quality standard for individual pesticides (0.1 μg L^{-1}) (EC, 1998, 2006) was exceeded for atrazine and metolachlor in four groundwater samples, whereas metolachlor and total pesticides reached the maximum concentration (0.13 and 0.19 μg L^{-1}, respectively) on 22 July (Fig. 2c, Table 5). Although the sum of the TU of all active substances based on the effect measures for algae and *Daphnia* sp. (see Table 2) was less than 1 (Fig. 3c), groundwater should be protected from deterioration and chemical pollution. This is particularly important for the use of groundwater in water supply for human consumption and for groundwater-dependent ecosystems, as in the interaction between the Tagus alluvial aquifer system and watercourses. The Almonda River is fed by a spring, which causes flow throughout the year, even when no precipitation occurs. This may, in turn, at some point of its run recharge the aquifer. Thus, the contamination that exists in surface water can be transmitted to groundwater and conversely. Although pesticides occur to a lesser degree than in surface water, they may be more persistent due to the reduced microbial activity and the absence of light in groundwater conditions.

The herbicides simazine and trifluralin and the insecticides endosulfan and lindane were not detected in the water samples. Relative to the pesticide compounds that were detected, alachlor, atrazine, ethofumesate, metolachlor, terbuthylazine, and 3,4-DCA present an average or a high affinity for the water compartment, but others, such as chlorfenvinphos (E+Z) and chlorpyrifos, did not (see Table 1). Some of the pesticides that were not detected have a low or a very low affinity for water, such as trifluralin, endosulfan (α+β), and lindane. However, simazine has a very high affinity for this environmental compartment and is classified as leacher (see Table 1), but it was not registered for use at the time of the study. Moreover, it was not used in the main crops of the study area. Atrazine, the pesticide most often detected in the groundwater samples, was expected since it shows high affinity for the water compartment and leaching potential (see Table 1). All detected pesticides except ethofumesate and chlorfenvinphos were approved for use in maize, the main crop of Golegã council. Ethofumesate was authorized for use in sugar beet, while chlorfenvinphos was registered for cabbage until 2007, crops also grown in the study area. The compound 3,4-DCA can be a metabolite of the herbicides diuron, linuron, and propanil. However, it is more likely that the parent compound was linuron, considering that it was registered for crops with importance in the study area (e.g., onion and potato), while the herbicides diuron and propanil were generally applied in orchards and rice.

Previous studies already concluded that the study area was vulnerable to pesticide contamination. From a total of 21 wells selected in agricultural areas of the Golegã council from 1996 to 2000, the detection of at least one of the pesticides in analysis was observed in 81% (Batista, 2003; Batista *et al.*, 2002). Lindane and α-endosulfan were also detected between 1983 and 1993 in surface water samples collected in the Almonda River (Cerejeira *et al.*, 2003). The values of some pesticides detected in this study (alachlor, atrazine, chlorpyrifos, metolachlor, simazine, and terbuthylazine) in surface waters from the Alentejo region of Portugal were reported by other authors (Palma *et al.*, 2008, 2009, 2010; Pérez *et al.*, 2010).

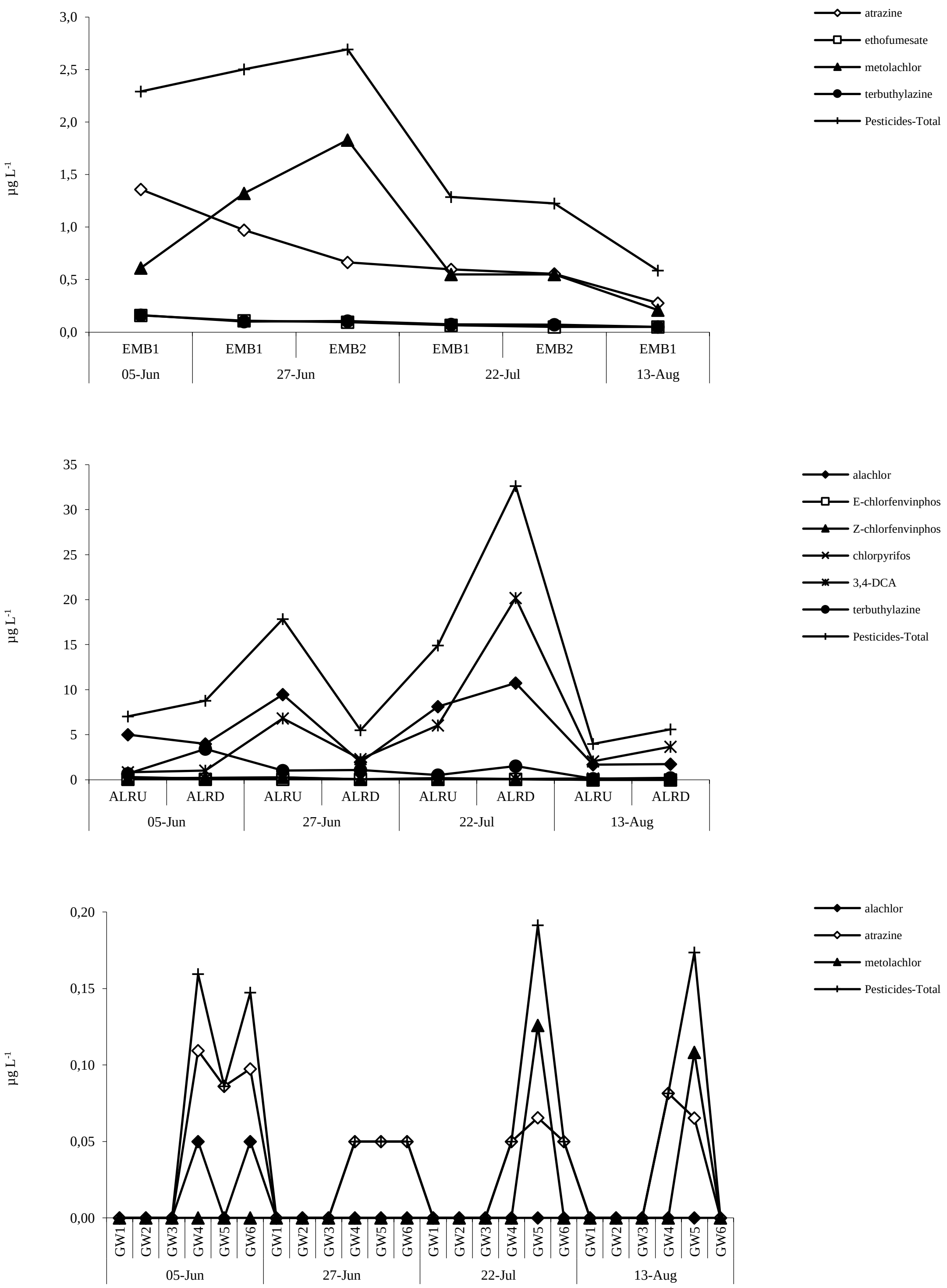

Fig. 2 Time variation of the concentration of the pesticides detected in a) surface water samples collected at the two points of the Alverca do Campo embankment (EMB1, EMB2); b) surface water samples collected at the Almonda River (upstream-ALRU and downstream-ALRD); and c) groundwater samples collected in six wells (GW1-GW6)

Table 4 Mean, median and maximum concentration (in μg L^{-1}) of the pesticides detected in surface water samples collected at the two points of the Alverca do Campo embankment (EMB1, EMB2) and at the Almonda River (upstream-ALRU and downstream-ALRD) from June to August 2008

Pesticide		EMB1	EMB2	ALRU	ALRD
		μg L^{-1}			
Herbicide					
Alachlor	Mean	<LD†	<LD	6.07	4.61
	Median	<LD	<LD	6.56	2.98
	Maximum	<LD	<LD	9.46	10.75
Atrazine	Mean	0.80	0.61	<LD	<LD
	Median	0.78	0.61	<LD	<LD
	Maximum	1.36	0.66	<LD	<LD
Ethofumesate	Mean	0.1	0.07	<LD	<LD
	Median	0.09	0.07	<LD	<LD
	Maximum	0.16	0.1	<LD	<LD
Metolachlor	Mean	0.67	1.19	<LD	<LD
	Median	0.58	1.19	<LD	<LD
	Maximum	1.32	1.83	<LD	<LD
Terbuthylazine	Mean	0.1	0.09	0.58	1.55
	Median	0.09	0.09	0.6	1.29
	Maximum	0.16	0.11	1.03	3.41
Metabolite					
3,4-DCA	Mean	<LD	<LD	3.93	6.79
	Median	<LD	<LD	4.04	2.98
	Maximum	<LD	<LD	6.82	20.19
Insecticide					
Chlorfenvinphos	Mean	<LD	<LD	0.15	0.11
	Median	<LD	<LD	0.15	0.1
	Maximum	<LD	<LD	0.31	0.26
Chlorpyrifos	Mean	<LD	<LD	0.21	0.06
	Median	<LD	<LD	0.2	0.07
	Maximum	<LD	<LD	0.3	0.12

† LD, limit of detection.

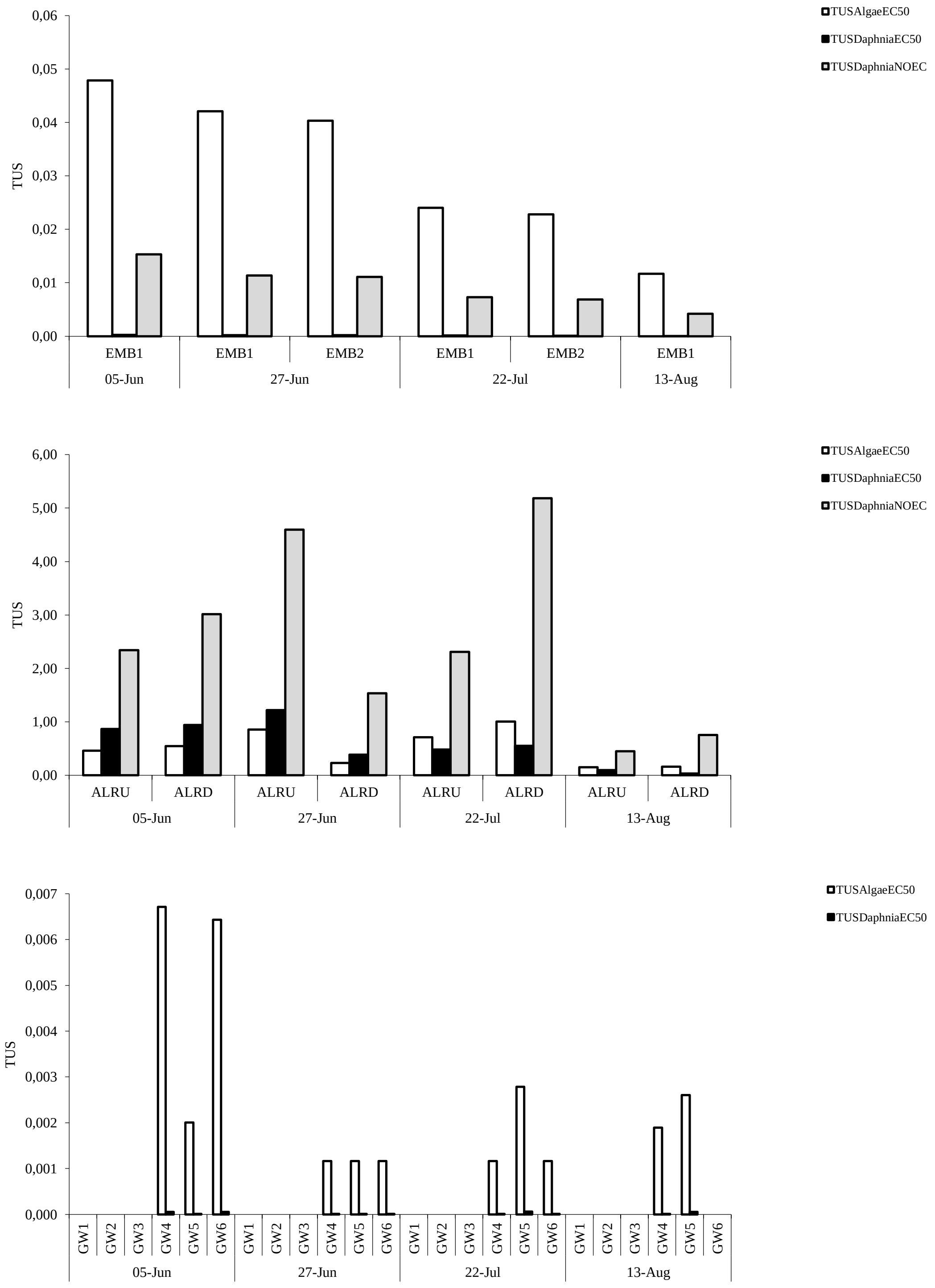

Fig. 3 Theoretical toxic unit summation (TUS) for algae and Daphnia in a) surface water samples collected at the two points of the Alverca do Campo embankment (EMB1, EMB2); b) surface water samples collected at the Almonda River (upstream-ALRU and downstream-ALRD); and c) groundwater samples collected in six wells (GW1-GW6)

Table 5 Mean, median and maximum concentration (in μg L^{-1}) of the pesticides detected in groundwater samples collected in six wells (GW1-GW6) from June to August 2008

Pesticide		GW1	GW2	GW3	GW4	GW5	GW6
		μg L^{-1}					
Herbicide							
Alachlor	Mean	<LD†	<LD	<LD	0.01	<LD	0.01
	Median	<LD	<LD	<LD	<LD	<LD	<LD
	Maximum	<LD	<LD	<LD	<0.05	<LD	<0.05
Atrazine	Mean	<LD	<LD	<LD	0.07	0.07	0.05
	Median	<LD	<LD	<LD	0.07	0.07	0.05
	Maximum	<LD	<LD	<LD	0.11	0.09	0.10
Metolachlor	Mean	<LD	<LD	<LD	<LD	0.06	<LD
	Median	<LD	<LD	<LD	<LD	0.05	<LD
	Maximum	<LD	<LD	<LD	<LD	0.13	<LD

† LD, limit of detection.

3.2 Comparison with effects on aquatic organisms in water and sediments

All toxicity tests fulfilled the validity criteria for control performance required in the respective guidelines. Concerning the toxicity of the nondiluted samples EMB1 and EMB2 using the algal toxkit, the growth inhibition of *P. subcapitata* was below 50% at all times, with the exception of an inhibition of 89% on 13 August. Moreover, on the first two sampling dates (5 and 27 June), a negative inhibition of 2 and 15%, respectively, was noted (Fig. 4a). Effects on algal growth with samples ALRU and ALRD were in general higher than those observed in both the previous samples, varying between 73 and 107% (Fig. 4b).

The present results on the algal toxkit are in agreement with the sum of the TU calculated for the pesticides measured in the two surface sampling points, which are also higher for samples ALRU and ALRD than for samples EMB1 and EMB2. However, according to that method, pesticide mixtures at ALRD are only expected to elicit effects (TUS > 1) once (on 22 July), most likely due to the contribution of the herbicide alachlor to the total risk (see Fig. 3a,b). The value 1 was not exceeded in any of the groundwater samples (see Fig. 3c), even though the observed inhibition effects on the growth of *P. subcapitata* were above 50% in 25% of the total (Fig. 4c). Synergistic toxic interactions among individual pesticides on this alga also could explain differences between observed and predicted toxicities (Pérez *et al.*, 2011). Previous studies with mixtures of pesticides demonstrated that the mixtures either behave in a toxicologically additive or antagonistic manner (Cedergreen *et al.*, 2006, 2007, 2008; Munkegaard *et al.*, 2008).

The results of the lethal toxicity of all waters toward the crustacean *D. magna* using the respective toxkit microbiotests were also generally higher for the surface water samples collected at the Almonda River (ALRU and ALRD), with mortality values above 50%, than for the water samples collected at EMB1 and EMB2 (Fig. 4a,b). These effects on mortality/immobility were consistent with those on the sum of the TU for the same water samples. However, in some groundwater and surface water samples, namely, in those collected at the Almonda River, the lethal effects were greater than expected, with the exception of one sample ALRU collected on 27 June, due to the contribution of the insecticide Z-chlorfenvinphos to the total acute risk (see Fig. 3a,b,c). As for the algae, other studies show interaction of pesticide mixtures on this crustacean (Cedergreen *et al.*, 2006; Loureiro *et al.*, 2010; Syberg *et al.*, 2008), so the toxicity observed can be caused by a number of stressors that may or may not interact.

Regarding the toxicity tests performed with samples collected on the last sampling time at points EMB1 and ALRD, no toxic effects were detected on the bacterium *V. fischeri* with either of the water samples; the highest percentage of luminescence inhibition was 5%, and thus the NOEC values were higher than the highest water concentration tested (100%) (Table 6). The effect measures for *V. fischeri* (30-min EC_{50}) (see Table 2) and the measured concentrations of the herbicides alachlor, atrazine, and metolachlor and the metabolite 3,4-DCA in the sampling points EMB1 and ALRD (see Fig. 2a,b, Table 4) indicated that acute effects above 50% were not expected. Taking into account the specific mode of action of the herbicides ethofumesate and terbuthylazine, for which no data are available, a relatively low sensitivity of *V. fischeri* may be anticipated. This test did not reveal toxicity to the surface water tested, probably due to the absence or lower sensitivity of stressors to the *V. fischeri*. On the other hand, interactions between pesticides and different components of the sample can affect the availability of organic chemicals and also their potential toxicity to the bacteria (Ruiz *et al.*, 1997).

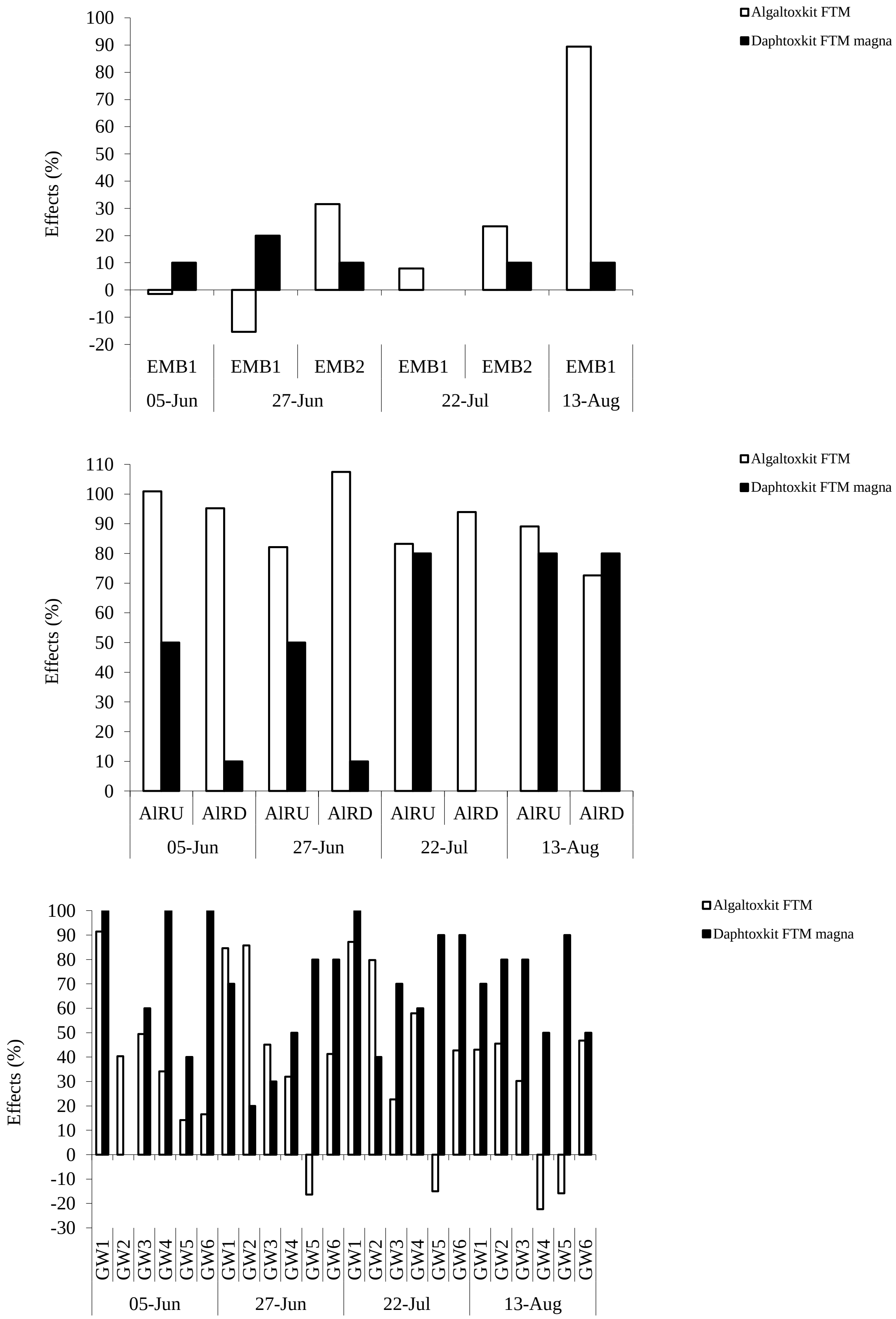

Fig. 4 Effects on the growth inhibition of *Pseudokirchneriella subcapitata* and on the mortality and/or immobility of *Daphnia magna* in the nondiluted a) surface water samples collected at the two points of the Alverca do Campo embankment (EMB1, EMB2); b) surface water samples collected at the Almonda River (upstream-ALRU and downstream-ALRD); and c) groundwater samples collected in six wells (GW1-GW6), based on the respective toxkit

Differences in *P. subcapitata* growth between the control and all tested water dilutions were observed for both samples EMB1 and ALRD either in the test performed without (EMB1: $F_{5,15} = 211$, $P < 0.001$; ALRD: $F_{5,15} = 116$, $P < 0.001$) or with (EMB1: $F_{5,15} = 684$, $P < 0.001$; ALRD: $F_{5,15} = 204$, $P < 0.001$) the addition of nutrients. Values of percentage inhibition in growth higher than 10% were only observed for 100% ALRD and up to the 12.5% dilution for EMB1, regardless of the nutrient levels. Thus, NOEC and LOEC values were 6.25 and 12.5%, respectively, for EMB1 and 50 and 100% for ALRD (Table 6). Values of EC_{50} and associated CL were only estimated for samples with nutrient additions as only these allow discriminating toxicity form differences in nutrient levels; toxicity to *P. subcapitata* of sample EMB1 was higher than that of sample ALRD (Table 6).

Table 6 Range of mean responses of *Vibrio fischeri* 5-min luminescence (in lt), *Pseudokirchneriella subcapitata* 72 h growth rate (in d^{-1}), *Daphnia magna* 21 d reproduction (in number of neonates/female), and *Chironomus riparius* 10 d growth (mg), and values of no observed effect concentration (NOEC; in %), lowest observed effect concentration (LOEC; in %), and median effective concentration (EC_{50}), with 95% confidence limits inside brackets, for all tests performed with water (bacterium, microalgae and cladoceran) and sediment (chironomid) dilutions (12.5, 25, 50, and 100%) prepared form samples collected at points EMB1 and ALRD on the last sampling time

Sample	Estimate	*V. fischeri*	*P. subcapitata*†	*D. magna*	*C. riparius*
EMB1	Response	72.9-104	0.610-1.72	101-155	0.727-1.34
	NOEC	100	6.25	<12.5	100
	LOEC	ne‡	12.5	12.5	ne
	EC_{50}	ne	50 (44-56)	> 100	ne
ALRD	Response	77.9-108	0.810-1.72	145-165	nt‡
	NOEC	100	50	25	nt
	LOEC	ne	100	25 to 50	nt
	EC_{50}	ne	99 (94-104)	25 to 50	nt

† To discriminate effects due to low nutrient levels and/or toxicity, results are for waters with a supplement of nutrients.

‡ ne, no effect; nt, not tested.

In the reproduction test with *D. magna*, effects on survival were also observed for both EMB1 and ALRD samples. Whereas survival was 70, 40, 60, and 50% in the 12.5, 25, 50, and 100% dilutions of EMB1, respectively, it was greater than 90% up to the 25% dilution and 0% in the 50 and 100% dilutions of sample ALRD. For EMB1 dilutions, differences in reproduction were also detected ($F_{4,26} = 14$, $P < 0.001$). The Dunnett´s test revealed differences between all dilutions relatively to the control, which corresponded to inhibition percentages ranging between 11 and 35%, and thus the EC_{50} value could not be estimated (Table 6). Differences in reproduction were found among the three ALRD dilutions where there were parent survivors ($F_{2,26} = 11$, $P < 0.001$), even though differences were below 5%, and thus the 25% dilution was considered the NOEC (Table 6).

The sum of the TU calculated with $NOEC_{Daphnia}$ as the reference level for the pesticides measured at the two surface sampling points on the last sampling date (13 August) was less than 1 (see Fig. 3a,b), which suggests that there were no alterations expected on the reproduction of *D. magna*. The results indicate that most likely, interaction effects among the pesticides and/or other abiotic and biotic factors may be responsible for the toxicity observed.

In the growth test with *C. riparius*, 100% survival was registered at all tested sediment dilution of sample EMB1. Although growth differences were observed among the tested sediments ($F_{4,41} = 5.90$, $P < 0.001$), the Dunnett´s test revealed that larval growth was not inhibited relatively to the control. Instead, the observed significant differences were due to a higher growth at the 12.5% dilution than in the control, most probably related to the presence of nutrients in the sediment sample which can stimulate the growth of larval *C. riparius*. In fact, this organism is considered opportunistic, tolerant to contamination and with a high adaptive capacity (de Haas *et al.*, 2002; Groenendijk *et al.*, 1998). Contrary to our findings, numerous studies have found interactions among organophosphate insecticides and triazine herbicides using *Chironomus tentans* (reviewed by Belden *et al.*, 2007).

These quality stations belong to the monitoring network quality of surface water resources (SNIRH, 2011) in Portugal where some parameters are or were monitored. During the study period, only the biochemical oxygen demand, phosphates, and phosphorus parameters were measured at the ALRD. The biochemical oxygen demand reached a value above 20 mg L^{-1} O_2 in June but decreased in July and August, while based on other parameters, this surface water was classified as bad status for multiple uses.

Taking into account the pressures to which these chemical quality elements and others measured in the previous years respond, it can be concluded that the study area was influenced by agricultural, domestic, and industrial discharges. This may lead to eutrophication and organic pollution, also detected through the observed effects on the indicator organisms. The stress from low oxygen, resulting from organic matter degradation or related to eutrophication phenomena, could combine with that from chemicals, as indicated by a review on the interactions between effects of environmental chemicals and natural stressors (Holmstrup *et al.*, 2010). Moreover, the hydrochemical facies of the Tagus alluvial aquifer system was classified as calcium-bicarbonate, sodium-chloride or mixed (Almeida *et al.*, 2000), i.e., groundwaters are characterized by chemicals for which algae and *Daphnia* may have sensitivity.

4. Conclusions

The TU sum for pesticide mixtures predicted the toxicity found with the 72-h algae growth and the 48-h cladoceran lethal tests only in two Almonda River samples due mainly to the presence of alachlor and Z-chlorfenvinphos. However, most adverse effects detected by the biotests could not be explained by the toxic measures. This allows for the conclusion that there are other stressors with variable nature together with pesticides that can act independently or jointly or that can interact. Available information suggests that other anthropogenic activities may be causing ecological risk in the water bodies of the study area. Additional studies are needed to evaluate the performance of mixture models for predicting mixture toxicity based on our current knowledge of the composition of pesticide mixture that occur in the aquatic environment.

This interesting and promising approach still has many restrictions, as there is limited information available on effect-based key toxicants for ecosystems due to chemical contamination and with respect to toxicological endpoints to be considered at specific sites. More studies should be performed to provide evidence of the main stressors on a broader scale and possible mitigation measures to improve the ecological status of different ecosystems.

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Chapter 7 Conclusions

Based on the results and discussion of the approaches developed and applied to improve the ecological risk assessment of pesticides in freshwaters, several conclusions can be drawn. These conclusions are listed below and related to the specific aims of this thesis, outlined in Chapter 1.

Provide a preliminary ecotoxicological evaluation of whether the current standard of 0.1 µg/L overprotects or underprotects the risks of PPPs to groundwater life

- Although the trigger value of 0.1 µg/L appears to be sufficiently protective for the majority of the PPPs presently included in Annex I, effects may ensue at this concentration for a number of PPPs, especially insecticides. Leachability to groundwater and ways that groundwater organisms perceive pollutants to their environment will have to be evaluated for these compounds as to assess whether they may exert environmental side-effects to groundwater ecosystems.
- Ecotoxicological studies evaluating the sensitivity of true groundwater organisms are needed to validate whether and to what extent surface water toxicity data may be used for the risk assessment of groundwater life.
- The bacterium *V. fisheri* was more sensitive than the crustacean *D. magna* for two PPPs with specific modes of action (i.e., microtubule assembly inhibitor and protein synthesis inhibitor). Given the importance of microorganisms for groundwater ecosystems, the sole evaluation of the sensitivity of a crustacean may not be sufficient in such cases.

Define candidate compounds for monitoring and prioritising as RBSPs in surface waters of three important Portuguese river basins

- An integrated approach, based on modelling, field and laboratory studies, was used to selected priority and other substances of concern in relation to pesticide compounds to be analysed in surface waters of three Portuguese river basins (‘Mondego’, ‘Sado’ and ‘Tejo’, Portugal) from 2002 to 2008. Pesticide use in dominant crops of several agricultural areas located within the catchment of those river basins, and having affinity for the water compartment were selected, being considered, also, other pesticides included in priority lists, defined in Europe.

- EQSs expressed as an AA and MAC for potential RBSPs were derived using different extrapolation techniques (i.e. deterministic or probabilistic) based on the method described in view of the WFD.
- From the 29 pesticides and metabolites selected for the study, 20 were detected in surface waters of the ‘Mondego’, ‘Sado’ and ‘Tejo’ river basins, seven of which were priority substances: alachlor, atrazine, chlorfenvinphos, chlorpyrifos, endosulfan, simazine and terbutryn. All the priority substances exceeded at least one of the environmental quality standards set in the revised (second) list of priority substances. Non-acceptable aquatic risks were revealed for molinate, oxadiazon, pendimethalin, propanil, terbuthylazine, and the metabolite DEA.
- The derivation of EQSs for 20 pesticide compounds by training based on the WFD technical guidance contributed to decline the variability of these values between MSs and to improve the classification of ecological status of Portuguese surface waters.
- Therefore, it was suggested 1) to replace the EQSs of specific pollutants established in Portugal by update EQS values based on the WFD technical guidance, 2) to include the herbicide oxadiazon in the list of RBSPs, and 3) in general to use an integrated approach such as the one outlined in this thesis, based on modelling, field and laboratory studies, to identify candidates compounds for monitoring and prioritisation as RBSPs in other river basins.

Prioritise pesticide mixtures that might pose ecological risks in surface waters of three important Portuguese river basins, but also site-specific exposures and single-species/species assemblages, by applying component-based approaches

Taking into account this third main objective, to apply new integrated approaches for the ecological risk assessment of pesticide mixtures in surface waters, two exposure metrics (risk quotients and msPAF) were calculated.

- Because toxicity data for the basic standard test species usually are available for pesticides and CA seems to be a broadly applicable model for pesticides, CA-based approaches ($RQ_{MEC/PNEC}$ and RQ_{STU}) were applied as a first tier - irrespective of the modes/mechanisms of action of the mixture components to provide a general ‘screening level’ risk assessment of mixtures of measured pesticides in surface waters of three Portuguese river basins (‘Mondego’, ‘Sado’ and ‘Tejo’) from 2002 to 2008.

- Overall, our study highlights that the CA model can be assumed as a reliable approach for assessing risk for ecologically relevant pesticide mixtures. There are a number of further considerations that support the suitability of this model for the described application.
- The results generated in this study demonstrate that even in mixtures with a high number of components (up to 7 in the 'Mondego' and 'Tejo' river basins), one pesticide compound was responsible for >50% of the toxicity. This observation renders differences between CA and IA predictions very small.
- Seven herbicide compounds and two organophosphate insecticides were accountable for the highest risk of toxicity for algae and crustaceans, respectively, in samples with RQ_{STU} ratios above 1 in the 'Mondego', 'Sado' and 'Tejo' river basins. Consequently when implementing restoration programmes, it seems cost-effective to focus on these pesticides in first instance. It is important to construct exposure and effect databases for frequently occurring pesticide combinations that likely may dominate the potential for risk in water bodies of agricultural landscapes.
- Algae were most at frequent risk because of higher herbicide exposure within this analytical survey to pesticides in surface waters of the 'Mondego', 'Sado' and 'Tejo' river basins and their sensibility to these compounds.
- The other exposure metric proposes the PAF of species, related to the 'forward' use of SSDs, as a quantitative measure of ecological risk to aquatic biota communities. By using dimensionless PAF values instead of risk quotients, the ecological risk of a mixture of compounds can be calculated with aggregation protocols based on fundamental theory on mixture toxicity and the TMoA of compounds in the mixture. The multi-substance PAF (msPAF) was proposed as a parameter to quantify the overall ecological risk of mixtures of pesticides measured in surface waters of three important Portuguese river basins ('Mondego', 'Sado' and 'Tejo') from 2002 to 2008 for different groups of species of the aquatic community.
- The median msPAF was higher for primary producers and arthropods than for fish, exceeding 5%, the threshold PAF used in the prospective SSD approach for deriving individual EQSs. The inhibitors of photosystem II (atrazine, 3,4-DCA, metribuzin, propanil, simazine, terbuthylazine and terbutryn) and oxadiazon were identified having the relatively largest toxic effects on primary producers, while the organophosphorus chlorfenvinphos and chlorpyriphos, and the organochlorine endosulfan on arthropods

and fish, respectively. This has been explained by the number of detected pesticides with the same TMoA, mainly herbicides, their measured concentrations and toxicity data.

- In this approach, the risk characterisation, instead of a yes-or-no responses, provided the quantification of the effects that occurred on a management-relevant endpoint (i.e with relevance for protection of ecosystem services) as a function of a likely exposure.
- As a general approach, the risk quotients applied in the Chapter 4 could be assumed as a first tier risk assessment procedure for pesticide mixtures, while the methodology for assemblage-level mixture risk assessment (Chapter 5) represents a higher tier ecological risk assessment, with complete management-relevant information.
- For the time being, a more ecologically-based procedure for ecological risk assessment could appear difficult to apply. Deeper knowledge (often largely incomplete) is needed on structure and function of natural communities of water bodies. Moreover, for developing reliable SSD approaches, toxicity data on a large number of organisms are needed, and at present this information is available only for a very low number of chemicals.
- The outcome of PAF calculations should be confirmed and validated to improve ecological interpretation of the output.

Assess the role of pesticide stress for Portuguese freshwater ecosystems in relation to the impact of other stressors by integrating modelling approaches, chemical analysis, and effect-based tools

- Taking into account that retrospective mixture risk assessments may also be founded on a WOE approach, chemical analyses and toxicity testing were combined to assess the impact of chemical pollution on benthic species. Such methods were partly oriented on whole mixtures and partly on risk of identified pesticide mixtures from a subset of the exposure data of the case study, ‘Tejo’ river basin. Whole toxicity testing in water and sediment were conducted with four organisms and test types: survival and growth were determined for (i) midge larvae (*Chironomus riparius*), survival and reproduction for (ii) crustacean (*Daphnia magna*), growth for (iii) algae (*Pseudokirchneriella subcapitata*), and light production was tested with (iv) a luminescent bacterium (*Vibrio fischeri*). Furthermore, water toxicity was estimated on the basis of mixtures of measured pesticides using the sum of TUs. Finally, it was studied whether the effects that were measured in toxicity tests could be explained by the water mixture-toxicity estimate TU.

- The sum of TUs for pesticide mixtures predicted the toxicity found with the 72-h algae growth and the 48-h cladoceran lethal tests only in two Almonda River samples due mainly to the presence of alachlor and Z-chlorfenvinphos.
- However, most adverse effects detected by the biotests could not be explained by the toxic measures. This allows for the conclusion that there are other stressors with variable nature together with pesticides that can act independently or jointly or that can interact. Available information suggests that other anthropogenic activities may be causing ecological risk in the water bodies of the study area.
- Additional studies are needed to evaluate the performance of mixture models for predicting mixture toxicity based on our current knowledge of the composition of pesticide mixture that occur in the aquatic environment.
- Linking chemical pollution in ecosystems to measurable ecotoxic effects is an interesting and promising approach. However, it still has many restrictions, as there is limited information available on effect-based key toxicants for ecosystems due to chemical contamination and with respect to toxicological endpoints to be considered at specific sites. More studies should be performed to provide evidence of the main stressors on a broader scale and possible mitigation measures to improve the ecological status of different ecosystems.

Concluding, this research addresses some of the new challenges for risk assessment, particularly for an integrated assessment of pesticide stress in aquatic ecosystems, in order to achieve good ecological and chemical status in European water bodies. Better criteria for the assessment of the ecological and chemical status of water bodies was provided by calculating groundwater TVs for pesticides taking into account ecotoxicology knowledge for the biota of that ecosystem, and by focusing on RBSPs in relation to their identification and derivation of EQS values. Furthermore, the importance of chemical monitoring studies for constructing exposure and effect databases of frequently occurring pesticide mixtures in surface waters is highlighted. Two exposure metrics were calculated, CA-based and msPAF approaches. Take into account that the aim of ecological risk assessment is to produce information in support of risk management and of political and regulatory choices, the msPAF methodology represents a more ecologically-based approach for ecological risk assessment and more relevant for risk management. However, more toxicity data for aquatic species is needed.

As retrospective mixture risk assessments can use multiple lines of evidence, possible multiple stress caused by pesticides (and other toxicants) not included in chemical monitoring programs was identified by conducting *in vivo* bioassays. Thus, combined biological and chemical-analytical approaches provided an important progress towards an identification of those toxicants that are relevant for site-specific risks and towards an estimation of the portion of an effect that can be explained by the analysed chemicals.

The thesis outcomes is of importance for reducing the risks of pesticide exposure in surface waters and obtaining a reality check of the prospective registration procedure for them, helping to implement the feedback mechanisms between the three complementary European Regulations/Directives with regard water protection and management, as wells as marketing and sustainable use of pesticides. However, it may be concluded that not all new science is ready for use, and an effort is needed, by both the scientific and regulatory communities, for making the new scientific achievements realistically applicable.

Chapter 8 Areas for further research

To further improve ecological risk assessment of pesticides in freshwaters, the following recommendations are suggested:

- Conduct ecotoxicological tests for evaluating the sensitivity of true groundwater in order to validate whether and to what extent surface water toxicity data may be used for the risk assessment of groundwater life.
- Perform more ecologically-based procedures for ecological risk assessment, using more extensive information on ecologically relevant endpoints and ecologically realistic exposure assessment.
- Improve the reliability of experimental and predictive approaches for exposure assessment taking into account chemical emissions and spatial-temporal patterns.
- Understand and assess the role of pesticides in relation to the impact of other stressors. These approaches should include the development and application of 1) aquatic effect-base monitoring tools (bioassays, biomarkers, ecological indicators, such as PICT-pollution-induced community tolerance and SPEAR-SPEcies At Risk) to support the assessment of water quality and provide a link between chemical and ecological assessments, 2) multivariate methodologies to identify those stressors which are major drivers of biological variability in an aquatic ecosystem, and 3) eco-epidemiological methods to quantify the intensity of stressors and to relate this to the biodiversity of river basins (msPAF output validation).
- Understand the processes in the toxicokinetic and toxicodynamic phase involved in sequential exposure against mixtures where exposure pulses overlap or recovery does not take place, but also on those involved in time-varying pulse exposures with intervening recovery periods.
- In-depth knowledge on the toxicological mechanisms by non-biased and discovery-driven approaches such as that provided by toxicogenomics (OMICS), including to mechanistically understand the potential interactions of stressors.
- Evaluate the cumulative risks of simulated regimes realistically reflecting those of the co-occurring pesticides in the specific surface waters of concern by means of experimental studies with model ecosystem approaches through the analysis of food web interactions.