LINKING EXPOSURE OF MEDITERRANEAN FRESHWATER ECOSYSTEMS TO PESTICIDES MIXTURES WITH THEIR ENVIRONMENTAL SIDE-EFFECTS

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TESE ELABORADA PARA OBTENÇÃO DO GRAU DE DOUTOR EM ENGENHARIA DO AMBIENTE

2017

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“Ao meu amado PAI”

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List of Abbreviations and Acronyms

AChE Acetylcholinesterase

ACR acute-to-chronic ratio

AF Application Factor, Adjustment Factor, Assessment Factor

ANOVA analysis of variance

BBCH Biologische Bundesanstalt, Bundessortenamt and Chemical Industry

BD bulk density

CA Concentration (Dose) Addition

CCME Canadian Council of Ministers of the Environment

Cw water concentration

DARs draft assessment reports

DCA Detrended correspondence analysis

DO Dissolved oxygen

DT50 detection time 50%

EC electrical conductivity

EC50 Median Effective Concentration

EFSA European Food Safety Authority

EPT Ephemeroptera, Plecoptera and Trichoptera

EQS Environmental Quality Standard

ERA environmental risk assessment

ERO ecological recovery option

ETO ecological threshold option

EU European Union

FOCUS Forum for the Co-ordination of Pesticide Fate Models and Their Use

FQPA Food Quality Protection Act

GC-MS Gas Chromatography-Mass Spectrometry

GWD Groundwater Directive

HCp Hazardous Concentration to p% of the species

HI Hazard Index

HU Hazard unit

IA Independent Action

ICN Instituto da Conservação da Natureza

ISO International Organisation for Standardisation

Kd water-sediment partitioning coefficient

Koc organic carbon partitioning coefficient

LC50 Median Lethal Concentration

LC-ESI-MS Liquid Chromatography–Electrospray Ionization–Mass Spectrometry

LC-MS Liquid-Cromatography-Mass Spectrometry

LOD limit of detection

LOEL Lowest Observed Effect Level

LOQ limit of quantification

MAC-EQS Maximum Allowable Concentration-Environmental Quality Standard

MCR Maximum Cumulative Ratio

MDR Model Deviation Ratio

MEC Measured Environmental Concentration

MED-Rice Mediterranean Rice group

MRL maximum residue limit

MS Member State

msPAF Multi-Substance Potentially Affected Fraction

nAChR acetylcholine receptor

NOAEL No Observed (Adverse) Effect Level

NOEC No Observed Effect Concentration

OC organic carbon

p significance level

PAF Potentially Affected Fraction

PAT Pesticide Application Tool

PDMS/DVB Polydimethylsiloxane/Divinylbenzene

PEARL Pesticide Emission Assessment at Regional and Local scales

PEC Predicted Environmental Concentration

PED Predicted Environmental Distribution

PELMO Pesticide Leaching Model

PNEC Predicted No Effect Concentration

PPP Plant Protection Product

PRC principal response curve

pRDA partial redundancy analysis

PRZM Pesticide Root Zone Model

RA Risk Assessment

RAC Regulatory Acceptable Concentration

RDA Redundancy Analysis

RICEWQ Rice Water Quality Model

RQ Risk Quotient

SDB-XC Styrene Divinyl Benzene eXtra Clean

SOP Standard Operational Procedure

SPE Solid-Phase Extraction

SPME Solid-Phase Microextraction

SSD Species Sensitivity Distribution

SWASH Surface Water Scenarios Help.

SWB Small Water Bodies

TER Toxicity Exposure Ratio

TGD Technical Guidance Document

TK/TD Toxicokinetics/Toxicodynamics

TMoA Toxic Mode of Action

TU Toxic Unit

TUS Toxic Unit Summation

TOXSWA Toxic Substances in Surface Waters

Trel Average Relative Tolerance

TV Threshold Value

TWA Time-Weighted Average

USEPA United States Environmental Protection Agency

WFD Water Framework Directive

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Resumo

Em ecossistemas de água doce associados a zonas agrícolas, os organismos encontram-se expostos a uma multiplicidade de pesticidas, toxicologicamente e estruturalmente distintos, e em concentrações variáveis ao longo do tempo. No entanto, os efeitos ambientais dos químicos são tradicionalmente avaliados e regulados substância a substância. Compreender e melhorar a ligação entre a avaliação de efeitos e da exposição constituem um passo importante nos atuais desafios da avaliação de risco por forma a aumentar a sua relevância ecológica. Com este objetivo, foram desenvolvidas e aplicadas abordagens integradas de diferentes níveis hierárquicos de complexidade e realismo ecológico, que incluem: modelação da exposição, testes laboratoriais com organismos individuais, distribuição da sensibilidade de espécies, modelos de ecossistemas e avaliação das interações das comunidades aquáticas, por forma a avaliar os efeitos de combinações realistas de pesticidas em corpos de água associados aos agroecossistemas do arroz, tomate e milho típicos das condições Mediterrânicas. Contribuiu-se para o conhecimento global da adequabilidade da avaliação de risco prospetiva e demonstrou-se que o risco dos pesticidas pode ser subestimado durante o atual procedimento de registo. Os resultados do estudo constituem também um contributo para a elaboração de procedimentos otimizados de medidas no âmbito da legislação europeia; a identificação de locais com os maiores impactos esperados de misturas de pesticidas; a avaliação dos principais compostos de pesticidas que mais contribuíram para os riscos aquáticos identificados. Além disso, aprofundaram-se os conhecimentos sobre os efeitos revelados de coocorrência de fatores químicos, ambientais e biológicos em ecossistemas aquáticos considerando os efeitos das interações bióticas e abióticas a nível da comunidade e do ecossistema. Os resultados contribuem ainda para a redução dos riscos de pesticidas em águas doces.

Palavras-chave: Avaliação de Risco Ecológico; Pesticidas; Misturas; Modelação; Ecossistemas de Água doces

Abstract

In freshwater ecosystems associated with agricultural areas, organisms are exposed to a multitude of toxicologically and structurally distinct pesticides in concentrations that may fluctuate over time. However, the environmental risks of chemicals are traditionally evaluated and regulated on the basis of single substance. Understanding and improving the link between effects and exposure assessment is an important step in the current challenges of risk assessment in order to increase its ecological relevance. To this end, integrated approaches of different hierarchical levels of complexity and ecological realism have been developed and applied, including: exposure modelling, laboratory testing with individual organisms, species sensitivity distribution, ecosystem models and assessment of aquatic community interactions to evaluate the effects of realistic pesticide combinations on water bodies associated with rice, tomato and maize typical agroecosystems of Mediterranean conditions. Contributing to the overall knowledge of the adequacy of the prospective risk assessment and demonstrating that pesticide risk may be underestimated during the actual registration procedure. The data generated in the present study contributed to the derivation of optimized programs of measures under the scope of European legislation; the identification of sites with the highest expected impacts of pesticide mixtures; the evaluation of the major pesticide compounds that contributed mostly to the identified aquatic risks. Furthermore contribute to a deeper knowledge and unravel the effects of co-occurring chemicals, environmental and biological stressors in aquatic ecosystems considering the effects of biotic and abiotic interactions at community and ecosystem levels. The results contribute to reducing the risks of pesticides in freshwater.

Keywords: Ecological Risk Assessment; Pesticides; Mixtures; Modelling; Freshwater Ecosystem

Resumo alargado

Em ecossistemas de água doce associados a zonas agrícolas, os organismos encontram-se expostos a misturas de pesticidas, toxicologicamente e estruturalmente distintos e em concentrações variáveis ao longo do tempo. Os esquemas legislativos para a autorização dos pesticidas (regulamento EU nº 1107/2009) e o estabelecimento de normas de qualidade ambiental para águas de superfície e subterrâneas (2000/60/CE e 2006/118/CE respetivamente) são atualmente baseados em avaliações dos compostos individuais. No entanto, é frequente serem aplicados diferentes pesticidas na proteção fitossanitária durante a época cultural, com especial importância nos países do Sul da Europa, dada a dimensão globalmente inferior das explorações agrícolas comparativamente com as do Norte da Europa, existindo uma maior multiplicidade de pesticidas envolvidos a nível regional, pois cada agricultor toma a sua própria decisão. Para além disso, os cenários ambientais utilizados na avaliação da exposição dos pesticidas são maioritariamente focados nas condições do Norte e Centro da Europa, pelo que é necessário avaliar a sua adequabilidade as condições do Sul e especialmente as distintas condições da área Mediterrânica.

Por forma a avaliar o risco, apoiar a legislação e a gestão do risco das misturas, são necessários dados quantitativos da toxicidade de misturas para parâmetros relevantes na avaliação dos efeitos. Contudo, com poucos dados e numerosas possibilidades de misturas, têm que ser tomadas decisões com base na toxicidade das substâncias individuais em combinação com técnicas de extrapolação para as misturas. Tem sido demonstrado que os conceitos Concentração da Adição (CA) e Ação Independente (IA) são ferramentas importantes na previsão da toxicidade de misturas, quer estas sejam compostas completamente por substâncias com modos de ação tóxicos similares ou dissimilares. Assim, compreender e melhorar a ligação entre a avaliação de efeitos e da exposição de misturas reais de pesticidas constitui um passo importante nos atuais desafios da avaliação de risco por forma a aumentar a sua relevância ecológica. Com este objetivo foram desenvolvidas e aplicadas abordagens integradas de diferentes níveis hierárquicos de complexidade e realismo ecológico associadas aos agroecossistemas do arroz, tomate e milho típicos das condições Mediterrânicas.

Avaliaram-se a precisão e adequabilidade dos modelos de exposição utilizados para o cálculo da concentração ambiental prevista de pesticidas em águas subterrâneas (FOCUS PELMO) e superficiais (FOCUS Step 3) em diferentes níveis da avaliação prospetiva. Verificaram-se diferenças significativas entre os valores previstos para as águas subterrâneas e superficiais com os modelos, comparativamente a concentrações medidas em ambos os compartimentos. Relativamente à adequabilidade dos cenários ambientais do Sul da Europa, verificou-se também uma elevada percentagem de subestimação das previsões dos modelos de água superficial.

A produção de arroz apresenta um cenário ambiental único relativamente ao potencial transporte de pesticidas devido às condições contínuas de inundação, pelo que os modelos utilizados na avaliação de risco de pesticidas não são adequados. Neste sentido foram propostas por um grupo de peritos, um conjunto de orientações e modelos para avaliação da exposição de pesticidas nas águas dos canteiros de arroz, ressaltando a necessidade da criação de cenários nacionais e da calibração e parametrização dos modelos. Avaliou-se a precisão e adequabilidade de dois dos modelos propostos (MED-Rice e RICEWQ). Apesar dos modelos de primeiro nível como o MED-Rice, serem por definição mais simplicistas e conservadores, verificou-se que existe uma subestimação das concentrações do inseticida analisado para diferentes cenários aplicados. Pelo contrário o RICEWQ, modelo menos conservador e mais complexo, foi parametrizado e calibrado revelando uma boa precisão e adequabilidade.

Obtiveram-se níveis de exposição a misturas de pesticidas em canais de água envolventes às culturas de tomate e milho com potenciais riscos aquáticos, através do cálculo de quocientes de risco (QR), com base nos modelos de adição da concentração como primeira etapa da avaliação do risco de misturas de pesticidas. Evidenciou-se que, mesmo em misturas com elevado número de componentes, uma única substância ativa foi responsável por mais de 50% da toxicidade da mistura. Os inseticidas na generalidade, principalmente os piretróides e organofosforados, foram os principais responsáveis pela toxicidade em amostras com QR mistura> 1 na Lezíria do Tejo.

Atendendo aos pesticidas com maior coocorrência ao longo das diferentes amostragens de campo e da potencial possibilidade de efeitos sinérgicos, a terbutilazina e o clorpirifos foram selecionados para estudo pormenorizado. Foi estudada a aplicabilidade dos modelos de referência CA e IA e a exatidão da sua previsão, utilizando o modelo MixTox

que avalia os possíveis desvios (devido a interações entre os pesticidas. Foram examinados os efeitos destes pesticidas sobre a imobilidade de *Daphnia magna* e sobre a taxa de crescimento da *Raphiodocelis subcapitata,* observando-se um ajuste ao modelo IA e CA respetivamente. Porém, relativamente à *D. magna* foi observado um padrão específico; antagonismo, em doses baixas e sinergismo, em doses elevadas. Posteriormente, os potenciais efeitos foram analisados para duas combinações relevantes da mistura ao nível da comunidade do zooplâncton, utilizando microcosmos interiores de água doce. Observou-se toxicidade direta da terbutilazina sobre o fitoplâncton, o que poderá ter indiretamente potenciado os efeitos observados sobre os daphnideos por diminuição dos níveis de alimento e oxigênio dissolvido, em combinação com a diminuição verificada das taxas de alimentação, hipoteticamente resultado da ingestão de partículas contendo terbutilazina. A terbutilazina potenciou o efeito do clorpirifos sobre as taxas de alimentação, desencadeando a transformação deste em análogos de oxon, que têm uma maior toxicidade que o clorpirifos. Foram verificadas alterações ao longo da cadeia alimentar causadas por efeitos diretos e indiretos dos compostos testados, tendo ocorrido recuperação das populações. No entanto, se a opção de recuperação ecológica for a adotada, com o objetivo de proteção do meio aquático, torna-se necessário avaliar as possíveis interações entre os *stressors* químicos (e outros) que podem estar presentes nos corpos de água adjacentes às culturas.

Avaliou-se ainda a relação entre os efeitos das aplicações de pesticidas em campos de tomate e milho e a sua previsão através da estimativa da fração de espécies potencialmente afetada por multi-substâncias (msPAF), método proposto em ecotoxicologia para a avaliação do risco de misturas a um nível mais elevado de integração biológica, utilizando o conceito de distribuição de sensibilidade das espécies e os modelos de toxicidade das misturas. Foi utilizado o procedimento de partição de variância com base na análise de redundância (pRDA) para avaliar os efeitos previstos dos pesticidas, conjuntamente com a influência dos fatores ambientais e das interações entre biota nas comunidades aquáticas. Por isso, a variância total na composição da comunidade biológica foi dividida em: variância explicada pelo msPAF produtores primários e msPAF artrópodes, fatores ambientais, interações bióticas, variância compartilhada e variância inexplicada.

A análise dos dados revelou que as comunidades de plâncton e invertebrados apresentaram respostas semelhantes aos *stressors*, com diminuição da biodiversidade e uma simplificação da estrutura biológica associada à presença de maior risco previsto para os artrópodes e para os produtores primários. A generalidade dos locais e datas de amostragem com um mais alto msPAF foi associada a uma diminuição dos táxons conhecidos por serem vulneráveis a pesticidas, indicando uma elevada relação entre os efeitos dos perfis de exposição reais e a previsão através do msPAF. A análise de pRDA indicou ainda que os fatores ambientais e as interações bióticas influenciaram consideravelmente as comunidades de água doce. A abordagem msPAF explicou uma parte significativa da variância na abundância de espécies (24%). Não obstante, incluindo as interações bióticas na análise, a biota explicou a maior percentagem de variância nos diferentes grupos (56%), seguido pelo msPAF (8%). Os resultados demonstraram assim a importância das interações bióticas e das condições ambientais específicas do local na estruturação da composição das comunidades aquáticas.

O trabalho desenvolvido contribui de forma relevante para o aumento do conhecimento global acerca da adequabilidade da metodologia da avaliação da exposição ambiental, demonstrando que a exposição e o risco real de pesticidas poderá ser subestimado no procedimento de avaliação prospetiva, reforçando a necessidade de desenvolver estudos acerca da adequabilidade e precisão do modelos e cenários utilizados. Destaca-se ainda a importância dos estudos de monitorização química para a construção de bases de dados de exposição e efeitos dos pesticidas que ocorrem frequentemente em águas doces. A abordagem hierarquizada melhorada e desenvolvida nestes estudos, entre exposição e efeitos, contribui para poder melhor abordar a complexidade da avaliação de risco ambiental.

Os dados gerados pelo presente estudo contribuíram também para a elaboração de programas otimizados de medidas no âmbito da legislação europeia; a identificação de locais com os maiores impactos esperados de misturas de pesticidas; a avaliação dos compostos de pesticidas que maioritariamente contribuíram para os riscos aquáticos identificados. Além disso, este trabalho constitui um importante contributo para melhor entender os efeitos das interações bióticas e abióticas ao nível da comunidade e do ecossistema, realçando a necessidade de aumentar o conhecimento acerca das mesmas.

A ligação global entre a avaliação do risco regulamentar e a situação no cenário agrícola real deverá ser consideravelmente reforçada, devendo as conclusões do presente estudo e de outros estudos de campo sobre a exposição e efeitos dos pesticidas serem integradas na avaliação prospetiva de risco. Os dados de exposição e os riscos ecológicos em campo devem também ser considerados na futura identificação e priorização das substâncias no âmbito da Diretiva Quadro da Água.








CHAPTER 1

GENERAL INTRODUCTION

1. Background

Agricultural areas cover 40% (174.1 million hectares) of the total land area of the EU-28, and approximately two thirds (65.8 %) of these farmlands are used for the cultivation of arable and permanent crops (Eurostat 2016). In the Mediterranean countries the proportion of utilized agricultural area occupied by permanent crops was relatively high (a little over 19%) (Eurostat 2016). To prevent losses of harvestable crop products due to pests, diseases, and weeds, it is common practice to use insecticides, fungicides, and herbicides as crop protection products. In 2013, pesticides with an approximate input value of 11 billion Euros were applied to European arable lands (EC 2014). In 2014, it was recorded in Portugal about 1.084 million hectares of crops potentially treated with pesticides, from that: herbicides and fungicides account for 39% and 37% of hectares treated, respectively, followed by the areas treated with insecticides (26%) (INE 2015). The widespread and intentional release of these highly biologically active substances may pose threats to non-target aquatic and terrestrial ecosystems across the EU.

Many studies have reported **pesticide pollution of groundwaters, marine systems, rivers and lakes** (e.g. Gilliom 2007; Malaj et al. 2014; Hull et al. 2015, Silva et al. 2006, 2011, 2012, 2015a). Surface waters are especially at risk as systems that are likely to receive agricultural nonpoint source inputs due to their often close proximities to arable lands (Von der Ohe et al. 2011; Knäbel et al. 2012; Stehle and Schulz 2015). Pesticides may enter water systems through different exposure pathways. The primary transport routes for pesticides, particularly to small surface water bodies in non-irrigation agriculture, are surface run-off and tile drainage induced by heavy precipitation events (Leu et al. 2004; Rabiet et al. 2010; Taghavi et al. 2010; Bereswill et al. 2012; Stehle and Schulz 2015). Whereas spray drift is assumed to be the main route of edge-of-field surface waters in North/Central Europe, runoff and soil erosion can be the largest contributors to pesticide surface water contamination in South European countries, especially in Mediterranean countries were short and intensively periods of rain are frequent (Tarazona 2005; Ramos and Martinez-Casasnovas 2006). Soil particles and associated pesticides that enter freshwater ecosystems may result in longer-term exposure regimes, whereas spray drift will generally result in short-term pulsed

exposure regimes (Tarazona 2005; Daam et al. 2011a). The magnitude of pesticide transport is determined by various climatic and geological factors such as the amount and intensity of rainfall, hydrology, slope of the agricultural area and soil moisture (Schulz 2004).

Given the fact that pest organisms and weeds are taxonomically related to many non-target freshwater organisms, there is a potential for adverse ecological effects in surface waters (McKnight et al. 2012; Schäfer et al. 2012; Brock 2013). A recent study (Malaj et al. 2014) using governmental monitoring data and standard toxicity data derived from single species laboratory tests showed that, out of various **organic pollutants, insecticides particularly jeopardize the integrity of EU freshwater ecosystems**. In addition, several additional small-scale field studies conducted in the EU reported that pesticide exposure exerted adverse effects on the aquatic ecosystem structure and function (e.g., Schulz 2004; Schäfer et al. 2012; Beketov et al. 2013; Bereswill et al. 2013). Although many studies addressing organic toxicant effects on freshwater organisms at the individual, population and community level have been published in ecotoxicological journals, **very few were conducted under field conditions,** for instance, only 0.6% of the studies on pesticide effects (Beketov and Liess 2012).

Small water bodies (SWB) constitute an important component of freshwater ecosystems as they support higher proportions of biodiversity compared to larger freshwater systems (e.g. Biggs et al. 2014) and represent an important inland water–carbon flux (Holgerson and Raymond 2016). In current literature, SWB refer to both small lentic (ponds, small lakes) and lotic waters (headwater streams and ditches, but also springs and flushes; Biggs et al. 2014). Lotic SWB represent 80–90% of the European river network, with catchments comprising 58% of the total EU area. In total, 2589 studies (1,466 lotic and 822 lentic) addressed pesticides in freshwaters, and 13.2% (8% lotic and 5.2% lentic) of these focused on SWB (Lorenz et al. 2016). The low amount of pesticide studies conducted in SWB contrasts with their spatial dominance among the water bodies receiving pesticide inputs. Anthropogenic impacts on SWB may strongly constrain the chemical and ecological qualities of downstream water bodies (e.g. Dodds and Oakes 2008). Therefore, the EU requests member states to implement national action plans on the sustainable use of plant protection products, with the specific aim to protect water bodies in the agricultural landscape (EC 2009a).

Groundwater has long been considered as an extreme environment inhabited by only a few specialized species. In the past decades, however, research into groundwater has increased considerably and several studies have shown that groundwater environments harbor relatively diverse communities of animals (e.g., Gibert et al. 1994; Rouch and Danielopol 1997; Galassi et al. 2009; Gibert et al. 2009). Many authors subsequently started to dispute groundwater legislation for only considering groundwater as a source of drinking water and not as an ecosystem (e.g., Notenboom 2001; Daam et al. 2010). In the EU, this was acknowledged with the implementation of a new Groundwater Directive (GWD) in 2006.

The **importance of water quality protection in agricultural areas is highlighted in different environmental policies at the European and national levels**. In acknowledgement of pesticide effects on the environment, the Directive **2009/128/EC** (EC 2009a) and the Regulation no. **1107/2009** (EC 2009b) of the EU on plant protection products demand a sustainable use of pesticides. Both also include the conservation of biodiversity and the prevention of unacceptable effects on non-target organisms.

However, to reach that aim, a profound understanding of **pesticide effects under realistic conditions** and effect propagation from individual to ecosystem level is needed (Knillmann 2013). The importance of retrospective impact assessment [e.g., as undertaken under the **Water Framework Directive** – WFD (EC 2000)] for informing prospective ERA is widely recognized (Ragas 2011; Boxall et al. 2012; EC 2012a).

In 2012, the European Commission published a communication on the **combined effects of chemicals** (EC 2012b), expressing concerns about the current limitations of assessing compounds individually and proposing a path forward to ensure that risks associated with chemical mixtures are properly understood and assessed. It states that EU laws set strict limits for the amounts of particular chemicals allowed in food, water, air and manufactured products, but that the **potential risks of these chemicals in combination are rarely examined** (EC 2012b). The hazard and/or risk assessment (RA) requirements for (components of) products on the European market are laid down in specific EU legislations primarily depending on the intended use of the product. As the composition of these products (e.g. pesticides) is generally known, and the relevant compounds are relatively well assessed individually, the RA is performed prospectively, based on the properties of the individual constituents and the same is also carried out on the

formulated products. However, when several formulated products are used in combination, i.e. for the application of plant protection products (PPPs) in the field or for the use of personal care products at home, the combined resulting risk is generally not assessed.

1.1.Prospective and Retrospective Environmental Risk Assessment

Prospective environmental risk assessment (ERA) concerns the evaluation of the probability of adverse effects of pesticide exposure in ecosystems before the marketing, release, or agricultural use of the pesticide (Solomon et al. 2008). Consequently, a prospective risk assessment procedure always follows a more or less reductionist, **bottom-up approach** by making use of scenarios and models to estimate a tiered environmental exposure and by adopting a tiered effect assessment procedure based on more or less standardized ecotoxicity tests and extrapolation techniques. In addition, assessments for regulatory reasons pesticide registration purposes are usually conducted for one chemical at the time. If the marketed plant protection product (formulated pesticide) contains more active substances, however, mixture toxicity of these active substances is considered (EFSA 2013).

Currently the **prospective environmental risk assessment** (ERA) of pesticides in Europe aims to assess the probability that an adverse effect occurs in the environment before a pesticide has been placed on the market and used in agricultural fields under the umbrella of **Regulation 1107/2009/EC**. The approach traditionally entails two different phases, the exposure and the effect assessment, which are combined in a risk characterization.

In the **exposure assessment**, pesticide concentration dynamics are calculated for different environmental compartments (e.g., soil, water, sediments) using mathematical models and scenarios that represent the environmental compartments that are potentially exposed to the pesticide. In Europe, the aquatic exposure assessment is performed based on a series of pesticide exposure scenarios for several climatic regions, crops, and water bodies (e.g., ditches, streams, ponds) that were developed by the Forum for the Coordination of Pesticide Fate Models and Their Use (FOCUS) Surface

Water Group (FOCUS 2001, 2007) and from the Groundwater Group (FOCUS 2000, 2009). For the different types of edge-of-field surface waters, the pesticide exposure simulations provide peak exposure concentrations (90th percentile), time-weighted average concentrations, and annual exposure profiles within a standardized water body length (i.e., 100 m for streams and ditches, 30 m for ponds) (FOCUS 2001). The spatial–temporal frame used by those simulations was chosen with the intention to represent a realistic worst-case exposure situation, but it may be disputed whether it also represents a realistic worst-case situation from an ecological perspective (Rico et al. 2016).

Recently several studies have demonstrated that concentrations in the field are frequently greater than those predicted in both groundwater and surface water using FOCUS pesticide fate models, as commonly used in the EU for this purpose (e.g. Knäbel et al. 2012, 2014; Stehle and Schulz 2015). The protection goal of the FOCUS approach was not achieved for fungicides and insecticides field concentrations. In detail, the authors found that 23% and 15% of the measured insecticides and fungicides field concentrations, respectively, were underpredicted by the step-3, PECs calculated with FOCUS when applied exactly as it is done within the regulatory risk assessment for pesticides, which questions the protectiveness of the FOCUS exposure assessment (Knäbel et al. 2012, 2014).

The **pesticide risk assessment** for edge-of-field surface waters in the European Union (EU) follows a tiered approach. Each tier is characterized by an exposure assessment, which results in a **predicted environmental concentration (PEC), and an effect assessment, which results in a regulatory acceptable concentration (RAC)**. In each tier, the calculated PEC for edge-of-field surface waters should be smaller than the corresponding RAC. The principle behind the tiered risk assessment approach is to start with a simple conservative assessment (Tier-1) and to do more complex and environmentally realistic evaluations only when the lower tiers indicate a clear risk so as to focus resources on more complicated substances. According to the European Food Safety Authority (EFSA) guidance document on the aquatic effect assessment of pesticides (EFSA 2013), the RACs derived in Tier-1 should be based on results from laboratory toxicity tests performed with standard test species and the application of an assessment factor (AF). Tier-2 also includes results of laboratory toxicity tests with

additional test species, allowing the geometric mean (geomean) approach or the species sensitivity distribution (SSD) approach. The SSD approach is applied if toxicity data are available for eight or more species of the sensitive taxonomic groups, and the geomean approach can be used if more toxicity data are available than under Tier-1 but less than required for the SSD-approach. The highest experimental tier (Tier-3) described in the EFSA Aquatic Guidance Document is based on the evaluation of pesticide effects in model ecosystems (i.e., micro- and mesocosms). In this Tier-3 procedure, the RACs can be derived on the basis of 2 options: 1) the ecological threshold option (ETO-RAC), accepting negligible population level effects only, and 2) the ecological recovery option (ERO-RAC), accepting some population-level effects under the condition that recovery takes place within a given time frame (EFSA 2013).

The **ecological realism** of the effect assessment might be substantially improved by the use of **higher tier** testing methods, such as **model ecosystems**, **field and semi-field studies** (Vighi and Villa 2013).

Higher tier studies have been performed mainly in Atlantic Central Europe and North America and results of such studies **have been extrapolated** to other climatic regions including the **Mediterranean** (Ramos et al. 2000; López-Mancisidor et al. 2008). However, the climatic and ecological conditions of those regions are quite different (e.g. temperature, light intensity, community structure) so it may be expected that fate, bioavailability and effects of pesticides are also different (Ramos et al. 2000; Daam et al. 2011b; López-Mancisidor et al. 2008). That is especially important in the case of rice agroecosystems. Rice is commonly cultivated at river basins in southern Europe, where paddy fields, artificial and natural surface-water bodies create a unique ecosystem which should be realistically considered as a whole (Capri and Karpouzas 2008). Pesticide risk assessment in rice paddies in Europe has been focused on the development of lower tier tools and techniques such the Med-Rice guidelines overlooking the need for higher tier analysis (Karpouzas et al. 2006).

Recent field studies (Schäfer et al. 2012; Beketov et al. 2013; Peters et al. 2013) reported **pesticide-induced adverse effects at concentrations well below (i.e., 1/10 to 1/100) conservative tier-1 RAC_{SW} (RAC surface water)**. In addition, based on statistical analyses, Luttik et al. (2011) argued that the AFs of 100 used for (acute) tier-1 RAC_{SW} derivation may not adequately cover interspecies sensitivity variation. These findings

provide evidence that even the conservative RAC_{SW} are potentially not protective in the field. An even worse protection level may thus be expected for the even less conservative higher-tier ERO-RAC_{SW}, although they have been established under conditions that are considered more realistic.

A **retrospective risk assessment** often follows a more holistic, top-down approach with a focus on the ecological status of the stressed ecosystem or watershed of concern. Such an approach may also consider the cumulative effects of multiple stressors by applying eco-epidemiological approaches. **Ideally**, retrospective risk assessments use **multiple lines of evidence** by considering both holistic (e.g., ecological indicators for different types of stressors) and reductionist (e.g., evaluating the chemical status of surface water by means of water quality guidelines) approaches (Solomon et al. 2008; Suter et al. 2010; Artigas et al. 2012; Beketov and Liess 2012; Burton et al. 2012).

The **WFD follows a retrospective approach** and aims to improve the ecological and chemical status of water bodies in Europe. The ecological status of the usually larger water bodies that fall under the domain of the WFD is, among others, assessed by monitoring of biological quality elements (e.g., fish, macro-invertebrates, macrophytes, benthic diatoms and phytoplankton). These quality elements monitored in a specific water body are compared with those of more or less pristine reference ecosystems. Besides evaluating the ecological status, the chemical status of WFD water bodies is assessed by comparing chemical monitoring data with EQSs (environmental quality standards) for EU-wide priority substances and other relevant, river basin, or Member State-specific substances. Note that these EQSs usually are derived for individual chemicals and seldom for chemical mixtures.

With regards to groundwater, a new **Groundwater Directive (GWD)** was implemented in 2006, which states in recital 20: “Research should be conducted in order to provide better criteria for ensuring groundwater ecosystem quality and protection” (EC 2006). The GWD maintained the EU-wide groundwater quality standards of 0.1 µg/L for any individual compound and 0.5 µg/L for the sum of all individual pesticides as was laid down in the "old" Groundwater Directive (80/68/EEC). These trigger values relate to the contemporary detection limits for pesticides, and hence lack any ecotoxicological base. What is new is that if these groundwater quality standards are considered not to be adequate for achieving the environmental objectives as set out in the WFD, more

stringent threshold values (TV) have to be established by Member States (MS), in which local or regional conditions should also be taken into account (EC 2006).

In March 2010, the Commission published a report presenting these TVs as set by the MS (EC 2010). Regarding pesticides, six MS established TVs for 36 different active substances which are below the quality standard of 0.1 µg/L and ranged from 0.0001 µg/L to 0.1 µg/L. The number of TVs established by each MS varied between zero (Portugal) and 62 (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). This may at least be considered surprising, since several studies conducted over the past three decades have demonstrated pesticide contamination at concentrations indicating environmental risks in various Portuguese groundwater bodies (e.g., Cerejeira 1993; Cerejeira et al. 1995a,b, 2000, 2003; Batista 2003; Batista et al. 2001, 2002; Silva et al. 2006, 2011, 2012).

Daam et al. (2010) set groundwater TVs based on ecotoxicological data for all PPPs allowed for use at that time in the EU. In the almost complete lack of data for groundwater organisms, they used data for surface water taxa known to be well represented in groundwater as surrogates. TVs lower than 0.1 µg/L were calculated for 16 PPPs, most of which have an insecticidal mode of action. This thus reveals that the effect assessment of these PPPs may not be fully adequate, but would still only indicate risk if the (expected) concentrations of these PPPs are greater than their calculated TVs.

From the above, it appears that European legislation allows a retrospective reality check of the prospective registration procedure for pesticides under Regulation 1107/2009/EC. To make such a **reality check effective, however, requires strengthening the links between the four complementary Regulations/Directives** (Brock 2013). Because the management of total pesticide use in EU Member States does not fall under the scope of Regulation 1107/2009/EC, this regulation does not provide options and tools for this purpose. To address this apparent gap and to provide management tools for EU Member State authorities, another Directive with a focus on the sustainable use of pesticides was adopted (2009/128/EC). This Sustainable Use Directive requires the Member States of the European Union to introduce National Action Plans while setting quantitative objectives, measures, and timelines to reduce pesticide risks for human health and the environment. In principle, the adequacy of the

prospective environmental risk assessment approaches for safeguarding the ecosystem's integrity in freshwaters must be evaluated. Within this context, also pesticide risk indicators are developed that allow the evaluation of annual trends in potential toxic stress of pesticides in surface waters (e.g., the HAIR indicator; www.hair.pesticidemodels.eu; Kruijne et al. 2011). Directive 2009/128/EC thus forms an important risk management link between the prospective ERA under Regulation 1107/2009/EC and the retrospective ERA under the WFD.

1.2. Addressing Pesticides Mixtures In Freshwater Ecosystems

It is relatively easy to understand and predict effects of a single toxic substance. However, in the real world organisms may be exposed to various mixtures of different compounds and to assess their impact on the individuals and ecosystems is one the biggest challenge in ecotoxicology for the next few decades (Schwarzenbach et al. 2006; Von der Ohe et al. 2011; EC 2012b; Altenburger et al. 2013).

Under the umbrella of Regulation 1107/2009/EC, RAC derivation is primarily based on a single substance toxicity assessment approach, except in the registration procedure for pesticide formulations that contain several active substances. In this case, the concentration addition (CA) concept is used as a default when setting RACs for mixtures (EFSA 2013). 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., PCBs, dioxins), again by applying the CA concept (EC 2011). Although compliance with good chemical status is primarily based on EQSs for individual substances, cumulative stress (including mixtures) of toxicants may be identified as a main pressure affecting ecological status. In that case the **cumulative risks have to be evaluated and reduced**. An important question at stake is whether compliance to the relevant set of RACs (and EQSs) is sufficient to also prevent cumulative risks from different pesticides (Brock 2013).

As mentioned above the European **ERA of pesticides is currently based on assessments of individual compounds** while it is common practice in agriculture to use several pesticides simultaneously. Due to the overall smaller size of farms in **South Europe** as

compared to countries in North Europe, it is more likely that **different pesticides could be involved at the regional level** because each farmer will take their own decision (Ramos et al. 2000). The need for studies into **environmental side-effects of pesticide mixtures may thus be especially important for Southern European** countries (Ramos et al. 2000).

One way of addressing combined environmental risks from pesticide co-exposure could be to base the selection of co-occurring pesticides on their use patterns in specific crops or based on common tank mixes. Data collections on use patterns have been performed throughout Europe that could serve as a basis (Garthwaite et al. 2015). However, most experimental research on aquatic risks due to multi-stress by pesticides is based on laboratory single species tests whereas community-level experiments are relatively scarce (Brock 2013). Nevertheless, from model ecosystems experiments that addressed exposure to realistic packages of pesticides used in potato (Arts et al. 2006), flower bulb (Van Wijngaarden et al. 2004), and wheat (Auber et al. 2011), it appeared that the largest proportion of the risk was caused by one or a few active ingredients only.

1.2.1. Prediction of mixture toxicity

The **two basic principles of mixture effects** were defined already around the middle of the twentieth century. These are the concepts of additivity and interaction (Bliss 1939; Plackett and Hewlett 1952). The concept of additivity is based on the assumption of no interaction between substances in mixture (Greco et al. 1995).

Nowadays, two main mathematical models exist to assess the combined toxicological effect of chemicals, either assuming that individual compounds act via a dissimilar mode of action (**independent action, IA**, or Response Addition) or by the same mode of action (dose or **concentration addition, CA**)

In **CA based models**, the total response corresponds to the sum of all the individual concentrations multiplied with their respective potencies and **generally provide reliable estimates of combined effects**. They can more easily be used with existing toxicity data and are considered to be slightly more conservative than IA models. However, the results obtained by both models are usually very similar and the difference between the predictions rarely exceed a factor of five (Backhaus et al. 2004; Kortenkamp et al. 2009;

Backhaus and Faust 2012). Therefore, CA and IA models are generally used in overall assessment of toxic mixtures as a first choice and possible interactions should be taken into account when dealing with uncertainties of these approaches (Altenburger et al. 2013; Hernández et al. 2013).

Multiple stress by pesticides in aquatic ecosystems can not be ignored in ERA, however 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)** (Schäfer et al. 2007; Verro et al. 2009; Gregorio and Chévre 2014, Silva and Cerejeira 2015). Consequently, when addressing cumulative stress of pesticides in ERA, it seems cost-effective to focus on those pesticides that dominate the exposure in terms of TUs (>90%). **Read-across information from similar mixtures can be used to identify mixtures where interactions** could play a role and which should hence be further investigated (Bopp et al. 2015).

The **pesticide toxicity index** methodology has also been used as a **screening tool** to assess potential **aquatic toxicity** of complex pesticide mixtures. That approach combines measures of pesticide exposure and acute toxicity in an additive toxic-unit model (Nowell et al. 2014). In that way it is possible to construct exposure and effect databases for frequently occurring pesticide combinations (in water and sediment) that likely may dominate the potential for risk in water bodies of agricultural landscapes. Nonetheless this methodology is a relative ranking system that indicates that one sample is likely to be more or less toxic than another sample, without indicating that toxicity will necessarily occur.

Moreover, the above described methodologies are limited because they do not consider synergistic effects, which are known to be possible with pesticides (Cedergreen 2014). For example, the combination of pyrethroid insecticides and azoles fungicides such as deltamethrin and prochloraz is known to be much more toxic to bees than the chemicals individually with a ratio ranging from 366 to 1786 fold (Colin and Belzunces 1992; Yoder 2011). The proposed mechanism is that these fungicides, by inhibiting ergosterol biosynthesis via the inhibition of cytochromes P450 also involved in detoxification, decrease the capacity of the organisms to detoxify other chemicals, such as the pyrethroid insecticides. Similar interactions have been found between miticides and pyrethroids, or between miticides (Yoder 2011). Synergism has also been shown to occur

between organophosphates and triazines pesticides the main mechanism responsible for the cases of synergy is that compounds that can induce the production of P450 monooxygenases, will increase the rate of oxon formation and hence increase the toxicity of the organophosphates (Cedergreen 2014). Synergism has also been shown to occur between organophosphates and carbamates pesticides in salmon (Cedergreen 2014). Most studies analysing synergistic interactions of pesticides mixtures in aquatic organisms applied standardized exposure conditions (Kretschmann et al. 2015).

Other methodologies have also been developed, such as a **two-step model approach mixing CA for modelling mixture toxicity** of compounds with the same MoA, **and IA to combine the toxicity of compounds with different MoA** (de Zwart and Posthuma 2005). CA and IA can also be evaluated with species sensitivity distributions (SSD), which can be much more robust, but require a large quantity of ecotoxicity data, which are often not available (Gregorio et al. 2013). This allows the **prediction of the fraction of species in the species assemblage** which is likely to be affected at a certain mixture concentration (**multiple substance potentially affected fraction; msPAF**) (Traas et al. 2002; de Zwart and Posthuma 2005). The msPAF results were applied to comparative risk analyses, and addressed comparisons in space, in time and between compounds (Gregorio et al. 2012; Jesenska et al. 2013; Silva et al. 2015b). Only a limited number of studies are available that validate the SSD model by through comparison of SSD predictions with real effects in ecosystems. A range of model ecosystem (i.e. micro- and mesocosms) studies demonstrated a sufficient match of their thresholds with SSD derived thresholds (e.g. HC5 values) for particular individual substances (Hose and Van den Brink 2004; Schmitt-Jansen and Altenburger 2005; Kefford et al. 2006; Maltby et al. 2009; Mebane 2010). **However only few studies have compared mixture effect predictions (msPAFs) from SSDs with the real-world ecosystem situations** (Posthuma and de Zwart 2006, 2012; Carafa et al. 2011; Smetanová et al. 2014). Posthuma and de Zwart (2006) found no statistically significant correlation between msPAF values of mixtures (metals, ammonia, household chemicals) and fish species richness or abundance in rivers in Ohio, USA. This was attributed to the influence of additional stressors. A significant correlation and good agreement (in terms of values) were observed between msPAF and the estimated “fraction of species likely lost due to toxicant mixture. In a study by Carafa et al. (2011), a significant correlation between

msPAF for mixtures of 60 different substances and two biotic indices for macroinvertebrates and diatoms is reported.

Most of the case studies on pesticide mixture toxicity are carried out retrospectively, based on monitoring data. Such types of risk assessments, however, could also be carried out prospectively, prior to placing a product on the market, and based on calculated Predicted Environmental Concentration (PEC) data, in order to screen and detect the combinations that could be of concern, using the above described methodologies. Furthermore the model ecosystem approach (Arts et al. 2006; Ippolito et al. 2012), or in silico approaches such as TK/TD (toxicokinetics/toxicodynamics) models (Ashauer et al. 2007) and food web models may be used if the exposure regimes simulated realistically, reflect those of the co-occurring pesticides in the specific surface waters of concern.

Interactions between stressors may exacerbate effects of individual stressors and result in **unanticipated ecological effects** (Townsend et al. 2008; Shears and Ross 2010). There is now ample evidence that the ecological effects of organic toxicants on populations and communities are moderated in the presence of additional stressors such as habitat degradation (Rasmussen et al. 2012), nutrients (Alexander et al. 2016) and a wide range of other environmental factors (Laskowski et al. 2010). Clearly, adopting a multiple stressor context including chemical, environmental and biological stressors is imperative for an integrated ecological and ecotoxicological assessment of freshwaters systems.

1.3. The need for more ecologically-based approaches

Ecological studies on water pollution have mainly focused on excessive nutrient loading, acidification and organic pollution (i.e. excessive organic matter loading). Organic toxicants (defined as organic chemicals above natural levels with biochemical or physiological modes of action that adversely affect organisms) such as pesticides were rarely considered. Studies published in five major freshwater ecological journals on stressors focused predominantly on nutrients, followed by climate change, invasive species and habitat degradation, whereas organic toxicants were relatively poorly

covered despite their widespread occurrence and potential ecological effects (Schwarzenbach et al. 2006; Beketov et al. 2013; Malaj et al. 2014). Although many studies addressing organic toxicant effects on freshwater organisms at the individual, population and community level have been published in ecotoxicological journals, very few were conducted under field conditions, for instance, only 0.6% of the studies were previously reported to deal with pesticide effects (Beketov and Liess 2012). A result of this "division of labour" between ecologists and ecotoxicologists is a paucity of studies on the field effects of organic toxicants (Thompson et al. 2016).

Efforts to **understand how a complex ecosystem may respond to mixtures of chemicals with different modes of action on the different taxonomic groups of living organisms are still scarce.** Moreover, the interactions between the combined effects of toxic chemicals and other stress factors such as variable environmental parameters (e.g., temperature, pH, oxygen depletion in water and water shortage in soil) or, more in general, their dependence upon environmental factors is largely unknown (Meek et al. 2011).

Clements et al. (2012) introduced a theoretical framework of the context-dependency approach in ecotoxicology, which introduces abiotic and biotic factors into the assessment of toxicant effects on communities. In the past, a number of studies have included ecological factors in the assessment of pesticide effects on aquatic biota in the field. For instance, in the study of Berenzen et al. (2005) the effects of pesticides on aquatic invertebrates in freshwater streams were analysed in combination with environmental factors. Martin et al. (2011) studied the responses of aquatic invertebrates to pesticide runoff accounting for physical-chemical and hydrological parameters as well as vegetation coverage. Bollmohr et al. (2011) studied the effects of pesticides along with environmental factors on benthic communities in an estuary ecosystem. Species interactions, such as predation or competition, were shown to be important factors affecting the responses of aquatic invertebrates to pesticides in several studies, for instance, in Trekels et al. (2011) and in Foit et al. (2012).

The sensitivity of organisms to pesticides and their potential to recover from toxic stress is largely determined by the species functional characteristics (species traits) (Poff 1997). Trait-based approaches represent a promising tool capable of complementing taxonomically-based assessments with functionally-based assessment (Baird et al.

2011). At present, they represent a tool for the analysis of population vulnerability and for many other approaches relevant for ERA (Van den Brink et al. 2013). One of the bottlenecks for the development and application of the approach is the lack of data for the precise characterization of suitable traits, particularly for traits describing detailed anatomical characteristics, as well as physiological or metabolically traits.

2. Research needs and aims of the thesis

In freshwater systems located in agricultural areas, organisms are exposed to a multitude of structurally and hence toxicologically different pesticides in concentrations that may fluctuate over time. The environmental effects of chemicals are traditionally evaluated and regulated on the basis of single substances with single-peak or chronic treatment regimes.

From the previous section, it is clear that there is an increasing need for approaches capable of answering more complex questions than dose/concentration-response relationships, based on single species and toxicants, allow. To do this, it is essential to **improve the capability to extrapolate from specific test conditions to the variability of characteristics in natural ecosystems**. However, very little knowledge is currently available regarding the prediction and assessment of mixture toxicity in higher-tier settings. Efforts to understand how a complex ecosystem may respond to chemical mixtures with different modes of action on the different taxonomic groups of living organisms are still scarce. Moreover, the interactions between the combined effects of toxic chemicals and other stress factors or variable environmental parameters (e.g., temperature, pH, oxygen depletion in water, water shortage in soil or, more in general, their dependence upon environmental factors) is largely unknown (Vighi and Villa 2013). The **link** between results obtained by **microcosms with the real situation in the field require** to be s**trengthened**. Recent studies have indicated several research needs that have to be addressed in these regards:

I. Scarcity of **monitoring data**, especially to evaluate model prediction adequacy, generally called "**verification/calibration/benchmarking/validation**" data sets (Knäbel et al. 2012,2014)

II. Whereas spray drift is assumed to be the main route of edge-of-field surface waters in North/Central Europe, runoff and soil erosion can be the largest contributors to pesticide surface water contamination in Mediterranean countries, particularly after heavy rainfall following a period of drought (Tarazona 2005; Daam et al. 2011a). The **generic FOCUS scenarios are mostly based on North/Central European** conditions and therefore **need further experimental and monitoring work** to underpin the validity of exposure profiles for **Mediterranean areas** (Brock et al. 2010; Daam et al. 2011b).

III. The current European ERA of pesticides is based on **assessments of individual** compounds while it is **common practice** in agriculture and horticulture to use several **pesticides simultaneously**. Due to the overall smaller size of farms in **South Europe** as compared to countries in North Europe, it is **more likely that different pesticides could be involved at the regional level** because each farmer will take their own decision (Ramos et al. 2000). The **need for studies** into environmental side-effects of pesticide mixtures may thus be especially important for **Southern European countries** (Ramos et al. 2000).

IV. Due to the large number of potential chemical contaminants and the great complexity of natural systems it is not feasible to perform ecotoxicity tests for each potential mixture. In addition, non-chemical factors may also act as stressors and add to the complexity of multiple stressor situations. Therefore, **a simplified and robust approach to assess the ecotoxicity of chemical mixtures is needed for use in environmental risk assessment** (ERA) and in regulatory toxicology (Smetanová et al. 2014).

V. Only a **few studies have compared SSD** (Species Sensitivity Distribution) predictions to the effects **in real-world ecosystems** other than micro- or mesocosms. Overall, there is thus a **need for the validation of SSD** predictions regarding the effects of toxicant mixtures on **biological communities in the field** (Smetanová et al. 2014).

VI. Model ecosystem experiments have almost exclusively been conducted in Central/North Europe. Since **indirect effects** of pesticides **may be more**

pronounced and (hence) recovery may take longer **under South European** conditions, **there is an urgent need for model ecosystem studies in South Europe (Daam et al. 2011a).**

Following the above described problems of pesticide effects in the environmental context, the aim of the thesis is to contribute to a better understanding of the linkage between pesticide mixture exposure and effects under relevant South European conditions by tackling the research needs indicated. Hence, the following specific objectives (A to C) may be distinguished:

A. **Evaluate the accurateness** of **models used in** EU in **ERA of pesticides**, namely by the FOCUS group and analyse how well existing FOCUS surface water scenarios predict measured environmental concentrations under specific **Mediterranean conditions**;
B. **Increase our understanding** of the risk evaluation **of pesticides in Mediterranean freshwater ecosystems;**
C. Assess **how well effects of the obtained real-world exposure profiles** may be **predicted** by comparing **mixture effect predictions** with those observed in the field.

3. Outline of the thesis

In this Chapter 1, a synthesis is presented of a number of important issues and pointed out the need for research to improve the linkage between pesticide mixture exposure and effects, namely the need for a more 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 work is detailed in a series of seven manuscripts that contributed and provided data to meet the specific aims proposed in this study:

- Chapter 2 - the predictiveness of FOCUS groundwater predictions was evaluated and a preliminary risk evaluation of predicted pesticides was provided (attending to pesticides with trigger values lower than 0.1 µg/L) to increase the knowledge concerning their potential underprotection of the risks to groundwater life (Objective A and B).

- Chapter 3 - the accurateness of the proposed tiered approach to predict the exposure of pesticides in rice crop was evaluated and the ecological risk assessment of the neocotinoid imidacloprid is performed (Objective A and B).
- Chapter 4 - a maize and tomato crop based approach is used to analyse the predictiveness and accurateness of FOCUS surface water models for South European scenarios and a tiered approach was developed to increase our understanding on the risk evaluation of pesticides in Mediterraean freshwaters ecosystems (Objective A and B).
- Chapter 5 - The pesticides with frequent co-occurrence and high potential for synergistic effects, the triazine terbuthylazine and organophosphate chlorpyrifos, were also evaluated concerning deviations from the reference models, i.e. concentration addition (CA) and independent action (IA), potential side-effects on single-species (*D. magna* and *R. subcapitata*) and on zooplankton community (microcosm-) level at environmental-realistic concentrations (Objective B)
- Chapter 6 – The effects of pesticide mixtures in edge-of-field tomato and maize agroecosystems were predicted by the multi-substance PAF approach (msPAF) quantifying the overall ecological risk of mixtures of pesticides measured in surface waters of 'Lezíria do Tejo' for different groups of species of the aquatic community. A variance partitioning procedure based on redundancy analysis (pRDA) was used to evaluate the predicted effects of pesticides along with environmental factors and biota interactions on macroinvertebrate, zooplankton and phytoplankton community compositions in ditches adjacent to Portuguese maize and tomato crop areas (Objective C).

Chapter 7 aims to state the draw of several conclusions related to the specific aims of this thesis, outlined in this Chapter 1, and providing insights in areas for further research to improve overall linkage between fate and effects in freshwaters.

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CHAPTER 2

COMPARING ECOTOXICOLOGICAL STANDARDS OF PLANT PROTECTION PRODUCTS POTENTIALLY TOXIC TO GROUNDWATER LIFE WITH THEIR MEASURED AND MODELLED CONCENTRATIONS

Based on the following manuscript:

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Abstract

Trigger Values (TVs) for groundwater ecosystems in the European Union (EU), as elsewhere, are not based on toxicity data for the biota of that ecosystem. At present, very few toxicity tests have been conducted with groundwater organisms so the true sensitivity of groundwater ecosystems is largely unknown. Daam et al. (2010) set groundwater TVs for all plant protection products (PPPs) allowed for use at the time of the study based on toxicity data for surface water organisms as surrogates for groundwater organisms and calculated TVs lower than the current EU standard of 0.1 µg/L for 16 PPPs. This thus reveals that the effect assessment of these PPPs may not be fully adequate, but would still only indicate risk if the (expected) concentrations of these PPPs are greater than their calculated TVs. The present study was therefore initiated to evaluate whether predicted and measured concentrations of these PPPs are higher than the previously calculated TVs lower than 0.1 µg/L. To this end, predicted environmental concentrations (PECs) were calculated using the PELMO and SCI-GROW models that are currently used for this purpose in the EU and USA, respectively, and measured concentrations (MECs) were obtained from the open literature. In addition, the empirical PERPEST model was used to assess the severity and probability of effects that may be expected at these concentrations on taxonomic groups known to be well represented in groundwater ecosystems. In addition, only for dimethoate a PEC greater than 0.1 µg/L was calculated. However, when considering concentrations actually measured in the field, 99.7% showed risk quotients (RQ as MEC/TV) values higher than 1 and 36.7% even higher than 100. Future field monitoring studies are needed to validate and eventually calibrate the way PEC values are currently calculated with the different models and scenarios currently in use. Such studies would also aid in the question to what extent the high MEC values may be attributed to diffuse or point-source pollution.

Keywords: Groundwater, environmental risk assessment, plant protection products, predicted environmental concentrations, exposure models

1. Introduction

Groundwater has long been considered as an extreme environment inhabited by only a few specialized species. Up to the 1980s, the subsurface was even generally considered to be sterile (Gibert et al., 2001). In the past decades, however, research into groundwater biodiversity has revealed that groundwater environments harbour diverse communities of animals (e.g., Gibert et al., 1994; Galassi et al., 2009). Many authors subsequently started to dispute groundwater legislation for only considering groundwater as a source of drinking water and not as an ecosystem (e.g., Notenboom, 2001; Daam et al., 2010). In the EU, this was acknowledged with the implementation of a new Groundwater Directive (GWD) in 2006, which states in recital 20: “Research should be conducted in order to provide better criteria for ensuring groundwater ecosystem quality and protection” (EC, 2006). The GWD maintained the EU-wide groundwater quality standards of 0.1 µg/L for any individual compound and 0.5 µg/L for the sum of all individual pesticides as was laid down in the "old" Groundwater Directive (80/68/EEC). These trigger values relate to the contemporary detection limits for pesticides, and hence lack any ecotoxicological base. What is new is that if these groundwater quality standards are considered not to be adequate for achieving the environmental objectives as set out in the Water Framework Directive (2000/60/EC), more stringent threshold values (TV) have to be established by Member States (MS), in which local or regional conditions should also be taken into account (EC, 2006).

In March 2010, the Commission published a report with an accompanying working document, presenting these TVs as set by the MS (EC, 2010). Regarding pesticides, six MS established TVs for 36 different active substances which are below the quality standard of 0.1 µg/L and ranged from 0.0001 µg/L to 0.1 µg/L. The number of TVs established by each MS varied between zero (Portugal) and 62 (UK). 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). This may at least be considered surprising, since several studies conducted over the past two decades have demonstrated pesticide

contamination at concentrations indicating environmental risks in various Portuguese groundwater bodies (e.g., Cerejeira et al., 2003; Silva et al., 2006; Daam et al., 2011).

The TVs as set by the other MS were generally based on i) background levels for naturally occurring substances (not applicable to pesticides); ii) water quality standards (EQS) set for associated surface water and dependent terrestrial ecosystems (e.g., surface water EQS for priority substances in Directive 2008/105/EC); iii) actual and potential legitimate uses of functions of groundwater (mostly based on drinking water standards); and/or iv) saltwater intrusion (e.g., for sulphate and chloride TV settings; not applicable to pesticides) (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. Given the differences in taxonomic composition between groundwater ecosystems and their (terrestrial and) surface water counterparts, as well as the traits (physiological, morphological and ecological attributes) of the species they are composed of, it may be questionable whether sensitivity of groundwater life may be based on toxicity data from other environmental compartments (Sket, 1999; Daam et al., 2010). At present, however, very few toxicity tests have been conducted with true groundwater organisms (stygobionts) so the true sensitivity of groundwater ecosystems is largely unknown (Daam et al., 2010; Korbel and Hose, 2011).

Daam et al. (2010) set groundwater TVs based on ecotoxicological data for all PPPs allowed for use at that time in the EU. In the almost complete lack of data for groundwater organisms, they used data for surface water taxa known to be well represented in groundwater as surrogates. Three different approaches were used: i) a "first-tier" approach, using toxicity data for the crustacean *Daphnia magna* and the bacterium *Vibrio fischeri* since crustaceans and bacteria have been reported to be the most diversified, dominant and fundamental components of groundwater ecosystems, respectively (e.g., Notenboom, 2001); ii) species sensitivity distributions (SSDs), constructed with toxicity data of surrogate surface water organisms for the truncated groundwater diversity in accordance with Hose (2005); iii) the case-base model PERPEST (Van den Brink et al., 2002). Although the trigger value of 0.1 µg/L appeared to be sufficiently protective for the majority of pesticides, Daam et al. (2010) calculated TVs lower than 0.1 µg/L for 16 PPPs, most of which have an insecticidal mode of action. This

thus reveals that the effect assessment of these PPPs may not be fully adequate, but would still only indicate risk if the (expected) concentrations of these PPPs are greater than their calculated TVs.

In the present study, the TVs for the PPPs for which Daam et al. (2010) calculated a TV lower than 0.1 µg/L were compared with their expected and measured concentrations in groundwater. To this end, predicted environmental concentrations (PECs) were calculated using the models PELMO, one of the FOCUS (FOrum for Co-ordination of pesticide fate models and their Use) models as currently used in the PPP registration procedure in the EU, and SCI-GROW (Screening Concentration In GROundWater), a screening model frequently used in the USA for this purpose. In addition, measured environmental concentrations (MECs) of these PPPs were obtained from the open literature. Subsequently, the PECs and MECs were compared with the TVs as calculated in Daam et al. (2010) to evaluate whether actual risks are likely to occur for these PPPs. Where possible, species sensitivity distributions (SSDs) and the empirical PERPEST model were used to assess the severity and probability of effects that may be expected at the calculated and measured concentrations. Ultimately, this was aimed at evaluating whether the previously calculated TVs lower than 0.1 µg/L may potentially lead to risks for groundwater life under the current EU legislation.

2. Materials and Methods

2.1. PELMO and SCI-GROW simulated PPP concentrations

In the lower risk assessment of PPPs before registration in the EU (Regulation (EC) No 1107/2009; EC, 2009) a number of mathematical models are used to assess the fate of pesticides in the different environmental compartments (FOCUS, 2000, 2009). For groundwater, four different models are currently used for this end: i) the pesticide leaching model (PELMO), ii) the pesticide emission assessment at regional and local scales model (PEARL), iii) the pesticide root zone model (PRZM), and iv) the macropore flow model (MACRO). In the present study, PELMO was chosen since this model was used for three out of five compounds for which previous PEC calculations were available

for groundwater from published draft assessment reports (DARs; http://dar.efsa.europa.eu/dar-web/provision), enabling a comparison of our simulations with those made in the DARs. Furthermore, these three PPPs included dimethoate, the only PPP for which a PEC greater than 0.1 µg/L was reported in these five DARs.

The simulation model FOCUS PELMO 4.4.3 was used to estimate the PECs for the nine realistic worst-case scenarios as set by FOCUS (2000) as a realistic worst-case Tier-1 exposure assessment to represent agriculture across Europe. Using these scenarios, PECs were calculated for all representative uses of the PPPs in South and North Europe, as indicated in the DAR reports, EU review reports, and/or reasoned opinions on MRL modifications as published by EFSA (European Food Safety Authority; Table 1). For the simulations, worst-case values were used, i.e. highest application rate and shortest interval between applications. In order to calculate the amount of the PPP that actually reaches the soil surface after application, the dose rates were corrected for the amount of crop interception. Interception values for the different crops and growth stages were used according to FOCUS (2000, 2009).

The simulation model PELMO 4.4.3 contains a number of defined crop scenarios. However, no respective crop scenarios exist for olives and orchards within the FOCUS models. In these cases, a crop scenario that was considered most suitable for the missing crop was chosen based on similarity in cultivable area (location), root depth, leaf area index (LAI), and time between planting and harvest. In this way, citrus was considered to be the most suitable crop scenario for olives and the apple scenario for orchards. To calculate the application dates for each crop scenario, the harvest date as provided in FOCUS (2000, 2009) and the shortest interval between the applications and security interval as described in the representative uses were used. Values for the other input parameters were also selected from DAR and EU review reports (Table 1). For a number of input parameters (e.g. diffusion coefficients), substance specific data were not available. In these cases, default values as recommended by the FOCUS group (FOCUS, 2000, 2009) were used. The simulation set-up and the output processing followed EU procedures (FOCUS, 2000), i.e. a simulation period of 26 years, in which the first 6 years are used as a warming-up period in order to minimize the influence of the initial conditions, and the last 20 years are used as output. The yearly average pesticide flux

concentration in leachate at 1m depth was calculated and the 80th-percentile concentration (i.e. the year with the fourth largest average leachate concentration) was identified as the target output to be predicted by the meta-model.

SCI-GROW (Screening Concentration In GROundWater) is the model used by the US-EPA (United States Environmental Protection Agency) in the initial tier screening of pesticides in groundwater. This model provides an estimate of likely groundwater concentrations at the maximum allowable use rate for areas with groundwater systems that are exceptionally vulnerable to contamination. In most cases, a large majority of the use areas will have groundwater that is less vulnerable to contamination than the areas used to derive the SCI-GROW estimate. The model estimation procedure can't currently be adjusted (e.g., divided by a factor) to estimate a more realistic exposure level for groundwater that is not especially vulnerable to contamination (US-EPA, 2007). Version 2.3 of the SCI-GROW of the model was used to estimate the concentrations of the PPPs under study. This enabled a comparison between PECs calculated through the initial tiers of the registration procedures of the EU and USA, the more as the input data of the SCI-GROW simulations (application rate, number of applications, Koc, and soil DT50) were also selected from the DAR and EU review reports (Table 1).

Table 1. Input values used for the PEC (predicted environmental concentration) calculations with PELMO (version 4.4.3) and SCI-GROW (version 2.3) in accordance with FOCUS (2009) and US-EPA (2007), respectively.

Parameters	CHLO	CHLOR	C-MET	CYF	CYP	DELT	DIFLU	DIME	ESFE	FIPRO	FENA	ʎ -CYH	PHOS	THIR	Source
Molecular weight (gmol-1)	265.9	350.6	350.6	434.3	416.3	505.2	310.7	229.3	419.9	437.2	303.4	449.9	317.3	240.4	A,B,C
Solubility in water (mgL-1)	0.81	1.05	2.74E-03	2.66E-03	9.00E-03	2.00E-04	0.08	39.8	0.001	3.78	353.25	5.00E-03	20	16.5	A,B,C
Vapour pressure (Pa)	7.62E-05	3.35E-03	1.95E-03	2.70E-07	2.30E-07	1.24E-08	1.20E-07	2.64E-04	1.17E-09	2.00E-06	1.20E-04	2.70E-07	6.50E-05	2.00E-06	A,B,C
pKa-value [-]	-	--	--	--	--	--	--	--	--	--	--	--	--	--	A,B,C
Henry coefficient (Pa m^3 mol-1)	2.50E-02	4.78E-01	2.35E-01	1.90E-01	2.40E-02	3.10E-02	0	1.42E-06	4.92E-04	2.31E-04	9.10E-05	2.00E-02	1.03E-03	3.30E-02	A,B,C
Diffusion coefficient air (cm^2s-1)	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	F
Volatilisation depth (cm)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	F
DT50 (d)	30.9	36.1	65.8	28.8	50.7	27.4	3.4	4.1	73.6	76	32.1	37.5	5	7.2	A,B,C
Reference temperature (°C)	20	20	20	20	20	20	20	20	20	20	20	20	20	20	F
Reference soil moisture (kPa)	10	10	10	10	10	10	10	10	10	10	10	10	10	10	F
Q10-factor [-]	2.58	2.58	2.58	2.58	2.58	2.58	2.58	2.58	2.58	2.58	2.58	2.58	2.58	2.58	F
Koc-value (mLg-1)	850	8151	4645	112004	85572	1.02E+07	9148	28.3	6.31E+05	727	754	1.57E+05	716	9629	A,B,C
Exponent Freundlich isotherm [-]	0.9	0.9	0.9	0.9	0.9	0.9	1.2	0.9	0.9	0.9	0.9	0.9	0.9	0.9	F
Crops [-]	Winter cereals	Grap vines	Citrus	Maize, Potatoes	Olive	Olive	Apples	Olives , tomatoes, wheat, sugar beet	Pome Fruit	Maize	tomatoes	Orchads	Olives , Pome fruit , Potatoes	Apples, wine grapes	A,B,C
Number of applications [-]	2	1	2	2 -- 3	2	4	2	1 -- 3	1 --3	1	2	2	1 --4	3--4	A,B,C
Dosages (kgha-1)	1	0.245	2.25	0.02 - 0.312	0.05	0.134	0.18	0.4 -0.60	0.015 - 0.025	0.05	10.08	2.3	0.5 – 0.75	2.4 – 3.2	A,B,C
Dates of application and intervals [-]	1st BBCH31 Last BBCH69	BBCH71	1st at BBCH89 110 days	1st at BBCH50 14 days	Whole season	Last at BBCH59 for Olives	1st at BBCH11 14 days	Last at BBCH59 for Olives	1st at BBCH11 14 days	at BBCH00	1st at BBCH05 14 days	1st at BBCH05 14 days	28 days before harvest for citrus, 7 days for potatoes and Apples	1st at BBCH10 to apples (7 days);1st at BBCH60 to wine grapes	A,B,C
Incorporation depth (cm)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	A,B,C
Interception by crops (%)	50 - 90	70	70	75	70	20- 80	50	20-80	50	0	0-50	0-50	15-70%	50-70%	A,B,C

CHLOT - chlorothalonil ; CHLOR - chlorpyrifos; C – MET - chlorpyrifos-methyl; CYF - cyfluthrin; CYP – cypermethrin; DELT – deltamethrin; DIFLU –diflubenzuron; DIME – dimethoate; ESFE –esfenvalerate; FENA-fenamiphos; FIP –fipronil; ʎ -CYH - lambda-Cyhalothrin; PHOS –phosmet; THI -thira

A - Draft Assessment report for PPPS, http://dar.efsa.europa.eu/dar-web/provisio; B - EU Review reports, http://ec.europa.eu/sanco_pesticides/public/?event=activesubstance.selection; C - reasoned opinions on MRL modifications as published by EFSA , http://www.efsa.europa.eu/en/publications/efsajournal.htm; F - FOCUS defaults in PELMO - FOCUS, 2009

2.2. Trigger value calculations using the first-tier and SSD approach

The trigger values (TVs) below which no effects on groundwater life is expected that were used in the present study were those as calculated by Daam et al. (2010). These authors calculated four different TV values: a short-term and a long-term value using both a first-tier and species sensitivity distribution (SSD) approach. For a detailed description of these TV calculations and rationale, the reader is referred to Daam et al. (2010). In brief, the short-term and long-term TVs were calculated using EC50 and NOEC toxicity values, respectively, obtained from the US Environmental Protection Agency (US-EPA) ECOTOX database (http://cfpub.epa.gov/ecotox/). In the almost complete absence of toxicity data for true groundwater organisms, toxicity vales for surface water invertebrates (and the marine bacterium *Vibrio fischeri*) from taxonomic groups known to dominate groundwater ecosystems were used as surrogates. Only data that fulfilled defined selection criteria related with test parameter and test duration were used for further analysis. In this way, the first-tier TV was calculated by applying an uncertainty factor (value differing per organism and toxicity value type, i.e. EC50 or NOEC) to selected toxicity data for the crustacean *Daphnia* spp. and the bacterium *V. fischeri*. For those pesticides for which five or more toxicity data were available, SSDs were constructed using the ETX computer program, version 2.0 (Van Vlaardingen et al., 2004). Since this program assumes a log-normal distribution of the data, log-normality was tested with the Anderson–Darling Test included in the ETX software package. If log-normality 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. Subsequently, the lower HC5 estimate from an SSD based on acute EC50 values was set as the TV short-term. Since few NOEC values were available, the TV long-term for the SSD approach was calculated by dividing the lower HC5 estimate with an acute-to-chronic ratio (ACR) of ten (Daam et al., 2010).

The SSD curve for dimethoate constructed with the ETX program did not pass the lognormality test, and the reciprocal Pareto distribution curve that was subsequently constructed with the BurrliOZ program showed clear (visual) misfits with the data points. These included misfits in the lower tail, which is evidently most crucial for the HC5 and hence TV estimation (*vide* Figure 4B in Daam et al., 2010). Therefore, a new SSD curve

was constructed in the present study to calculate the TV of dimethoate. Toxicity data for saltwater invertebrates belonging to the same taxonomic groups as accepted for the freshwater invertebrates were also included in this SSD since recent studies have shown that freshwater and saltwater toxicity datasets may in principle be pooled for organic compounds (EC, 2011; Klok et al., 2012). The fit of resulting SSD to the data points indeed clearly improved (Figure 1B) when compared to the SSD without inclusion on the saltwater toxicity data (Figure 4B in Daam et al., 2010).

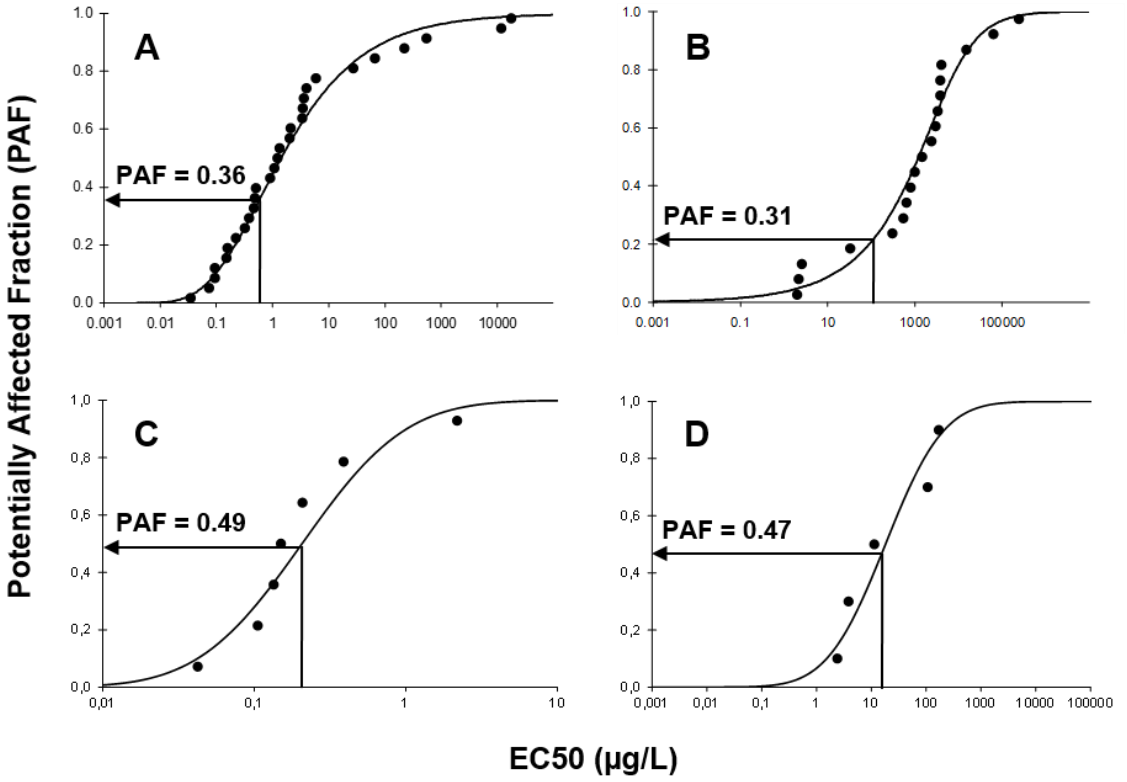

Fig. 1 Species sensitivity distributions (SSD) as constructed based on published EC50 (effect concentration 50%) values of chlorpyrifos (A), dimethoate (B), esfenvalerate (C) and phosmet (D). The potentially affected fraction (PAF), i.e. the number of taxa for which their toxicity values are expected to be exceeded based on the SSD curve, is indicated at the maximum MECs encountered in the open literature for these substances

2.3. Ecological risk assessment

The environmental risk of the PPPs under study was evaluated by calculating risk quotients (RQ), i.e. by dividing the predicted (PEC) and measured (MEC) environmental

concentrations with the TV values. Hence, an RQ value lower than 1 indicates that the compound involved is less likely to pose a significant risk since the exposure (PEC or MEC) is lower than the concentration estimated to be safe for groundwater life. On the other hand, an RQ greater than one implies potential risks, where especially compounds with an RQ value exceeding 100 have been indicated to be of very high concern referring to concentration levels of 1/10 of the acute LC50. (James et al., 2009).

The PECs were estimated using the PELMO and SCI-GROW models as described in section 2.1, whereas MEC values were obtained from studies published in the open literature (Table 2). As outlined in section 2.2, TVs were calculated using the first-tier and SSD methods as short-term and long-term trigger values. PELMO and SCI-GROW, however, only calculate one PEC value for a give scenario. Subsequently, both short-term and long-term trigger values were compared with this single PEC. In line with this, in the prospective risk assessment of PPPs for surface waters, a single PEC value is compared with both the acute EC50 and chronic NOEC value of the most sensitive species. Similarly, the maximum MEC as encountered in the literature was compared with both short-term (and long-term) TVs. This may further be justified with the reported long residence times of PPPs in groundwater due to e.g. low temperatures and oxygen levels and the relatively slow dynamics of groundwater (e.g., Müller et al., 2006).

Table 2. Measured environmental concentrations (MEC) obtained from studies published in the open literature.

	Mean (ug/L)	Median (ug/L)	Minimum (ug/L)	Maximum (ug/L)	Frequency of detection (% of samples)	Sources
chlorothalonil				1.95		E-Papadopoulu-Mourkidou et al., 2004
			0.00	0.15	58.3%	Ahad et al., 2000
				0.09	16.6%	Tariq et al., 2007
dimethoate		0.033		0.9	20.3%(n=64)	Postigo et al., 2010
	81	81	48	110	2.2%(n=181)	Gonçalves et al., 2007
				2.3	--	Jurado et al., 2012
	0.2681 ±1.201			10.90	17.08% (n=15)	Loewy et al., 2003
				0.03084		Mansilha et al., 2011
	0.096	0.021	0.003	0.58	22.8%(n=114)	Gonçalves et al., 2007
chlorpyrifos	0.06			0.3	---	Murray et al, 2010
			0.00	0.03	58.3%	Ahad et al., 2000
				0.002	--	Estevez et al., 2012
	0.019			0.52	16.3%(n=15)	Loewy et al., 2003
esfenvalerate			0.01	0.2	100%	Ahad et al., 2001
				0.08	--	PPSGDP, 2002
fenamiphos	0.25	0.25		0.25	0.6%(n=181)	Gonçalves et al., 2007
lambda - cyhalothrin	0.059	0.059		0.059	0.6%(n=181)	Gonçalves et al., 2007
phosmet	0.498			15.5	18.7% (n=15)	Loewy et al., 2003

2.4. PERPEST

The empirical PERPEST model was run for the PPPs under study. PERPEST predicts the effects of a particular concentration of a pesticide on various (community) endpoints simultaneously based on a large database of aquatic (surface water) model ecosystem experiments. This results in a prediction showing the probability of classes of effects (no, slight, or clear effects) on the various endpoints. The model was run with the maximum PEC and MEC concentrations obtained as described previously at default settings (total of 7 compounds in 16). For a detailed description of the model, the reader is referred to Van den Brink et al. (2002).

3. Results and Discussion

3.1. Modelled pesticide concentrations

PEC calculations with the FOCUS PELMO model were performed for 14 out of the 16 compounds for which Daam et al. (2010) calculated TVs below 0.1 µg/L. The PEC simulations were not made for dinocap since this compound is no longer allowed to be used in the EU. Simulations were not also made for pirimiphos-methyl because it may be anticipated that the specific authorised use of this compound (direct post-harvest treatment of crops and for structural treatment of storage rooms or equipment in contact with food) in the EU should not result in significant direct nor indirect exposure of the water compartment either during or after application (Table 1).

For these fourteen compounds, only the PEC as calculated for dimethoate using the Piacenza (0.109 µg/L) scenario slightly exceeded 0.1 µg/L (Table 3).

The respective values as reported in the draft assessment report for dimethoate (EC, 2005), which were simulated using an older version of FOCUS PELMO (version 3.3.2), are slightly higher for the Porto scenario than those calculated in the present study. In a comparison test of output values between the old and new version of the model for dummy substances as provided with the model, the new version also generally simulated lower PECs as compared to the older version.

It should be noted that the model characteristics of the latest version of FOCUS PELMO have also been reported to potentially lead to a underestimation of the PEC in groundwater. For example, a small top-layer of soil (small soil compartment of 1 mm on top of the soil where all of the applied mass is assumed to be deposited after pesticide application) from which all volatilization is assumed to occur was implemented in the latest version of FOCUS PELMO some previous field testing has demonstrated that this approach may lead to an overestimation of volatilization from the soil surface (Van den Berg et al., 2003) and hence an underestimation of the PEC values.

Table 3. Maximum predicted environmental concentrations (PECs) in groundwater calculated with PELMO and SCI-GROW and environmental risk of the PPPs under study evaluated by calculating risk quotients (RQ), i.e. by dividing the PEC values calculated with PELMO with the TV values from Daam et al. (2010).

Substance	PEC max		First tier				SSD			
	SCI GROW[1]	PELMO [2]	TV ST	TV LT	PEC[2]/TV ST	PEC [2]/TV LT	TV ST	TV LT	PEC[2]/TV ST	PEC[2]/TV LT
chlorothalonil	0.0852	< 0.001	2.8	-	-	-	0.22	0.0215566	-	-
chlorpyrifos	0.00151	0.000	0.00035	0.0055	-	-	0.031	0.0031	-	-
chlorpyrifos-methyl	0.053	0.000	0.0094	-	-	-	-	-	-	-
cyfluthrin	0.005	0.000	0.00084	-	-	-	-	-	-	-
cypermethrin	0.00054	0.000	0.17	0.03	-	-	0.0038	0.00038	-	-
deltamethrin	0.00288	< 0.001	0.0045	-	-	-	-	-	-	-
diflubenzuron	0.000469	< 0.001	0.056	-	-	-	-	-	-	-
dimethoate	0.00572	0.109	0.026	7.3	4.1923	0.0149	0.00097	0.000097	112.37113	1123.711340
esfenvalerate	0.000234	0.000	0.0011	-	-	-	0.018	0.0018	-	-
fenamiphos	0.983	0.000	0.023	-	-	-	0.092	0.0092	-	-
fipronil	0.00981	0.001	0.156	-	-	-	0.0046	0.00046	-	-
lambda-cyhalothrin	0.0246	0.000	0.0029	-	-	-	-	-	-	-
phosmet	0.00559	0.000	0.11	-	-	-	0.0054	0.00054	-	-
pirimiphos-methyl	NP	NP	0.0018	-	-	-	-	-	-	-
thiram	0.0496	0.000	2.1	-	-	-	0.033	0.0033	-	-

TV - trigger value, ST -short-term, LT -long-term

NP - not performed

1 - simulations with SCI GROW version 2.3

2- simulations with FOCUS PELMO version 4.4.3.

SCI-GROW calculated a PEC greater than 0.1 µg/L for only fenamiphos (0.99 µg/L; Table 3). Values were generally greater than those simulated using PELMO, with the exception of dimethoate (Table 3). This is not surprising since the SCI-GROW is a conservative first-tier screening tool, whereas PELMO may be considered a robust higher-tier models with regional agricultural scenarios at the outset of its analysis. In the SCI-GROW model the DT50 plays a larger influence in the results, so for active substances like dimethoate that are mobile in soil (Koc= 28.3 mg/L) but with a DT50 < 6 days (in that case the first step of the result is given by D = log(Koc + 5.0)and C= log10(DT50/6), where PEC = C * D) the PEC value modelled decrease largely when compared with substances with a DT50 > 6 days (then C = log(DT50 - 5.0). In FOCUS PELMO, none of the input parameters has such a great influence on the sensitivity and output results, so the DT50 value does not have such a significant impact on the final PEC result as in the SCI-GROW model (Dubus et al., 2003). The US-EPA does not currently conduct higher-tier modelling when groundwater concerns are identified, but instead requests monitoring studies (US-EPA, 2007). Hence, values simulated using SCI-GROW should be interpreted with caution.

The sole use of pesticide fate models for the exposure assessment of groundwaters to pesticides has indeed been disputed by many authors. For example, Trevisan et al. (2003) and Kubiak et al. (2003) discussed that, despite the large experience that has been gained with some pesticide fate models, there is still uncertainty about the validation status of the models used to calculate PECs and that the different approaches used in the European pesticide registration procedure may result in varying output values (Trevisan et al., 2003, Kubiak et al., 2003). Calculations with groundwater pesticide fate models could also become more realistic if not only one application date is used per country, which is the current practice in the model scenarios used, but the actual range of application dates in different countries and years. When calculating concentrations for application dates varying by ± two weeks, concentrations in groundwater usually varied very little. The highest variation was found for application at BBCH 30 in maize (6.6% variation over all scenarios) although variations of up to 36.6 % from the average were observed for single scenarios as in the case for the Piacenza scenarios (Gericke et al., 2010).

3.2. Measured pesticide concentrations

One of the major drawbacks of the deterministic risk assessment as currently conducted are false negatives: a (potentially) non-leachable compound could percolate because of particular local agricultural practices (i.e. basin irrigation; Balderacchi & Trevisan, 2010). In line with this, significant differences between PECs simulated with the two models and the MECs reported in the literature were observed (*vide* Table 2 and 3). MEC values could be encountered for six insecticides (chlorpyriphos, dimethoate, esfenvarelate, fenamiphos, λ-cyalothrin and phosmet) and one fungicide (chlorothalonil) of the pesticides under study. However, it should be taken into account that water quality objectives for PPPs are frequently far below analytical detection limits and that the lack of any positive experimental finding does not necessarily mean absence of risk. In chemical databases, the LOQ and LOD (limit of detection) are often not reported, leading to uncertainties whether a concentration is just below LOQ or even below LOD (James etal., 2009)

It may be argued whether the high MEC values represent diffuse pollution since high pesticide concentrations in freshwaters have often been attributed to specific and punctual pollution episodes, local treatments and/or accidental spills (e.g., Lacorte et al., 2001; Nabais et al., 2007). For example, the highest MEC value reported for dimethoate (110 µg/L) is approximately three order of magnitude higher than the highest simulated PEC of 0.11 µg/L (Tables 2 and 3). MECs reported in other studies in different sampling periods and localities were comparable to this PEC value (0.15 µg/L and 0.09 µg/L), although also slightly (0.9 µg/L) to clearly higher (2.3 µg/L and 10.9 µg/L) values were encountered. This could thus partially indicate that the scenarios adopted to calculate PECs do not fully cover particular local agricultural practices in case of diffuse pollution and/or a high occurrence of specific and punctual pollution episodes with this insecticide.

3.3. Risk assessment

The potential risks of the PPPs under study to groundwater life as indicated by the RQ (RQ = PEC/TVs) values are provided in Table 2. Based on this approach, no risk is expected. Data of 14 compounds modelled with FOCUS PELMO in groundwater system showed that for all the compounds, scenarios and crops simulated with exception of dimethoate, the 80th percentile is < 0.01 µg/L. Subsequently, only dimethoate indicated risk with high RQ values for both short-term (RQ=112) and long-term (RQ=1123) calculated using TVs obtained through the SSD approach. According to James et al. (2009), compounds exceeding a risk ratio of 100 are of very high concern, referring to concentration levels of 1/10 of the acute LC50.

The potential risks of the PPPs as indicated by the ratio of maximum MEC and TVs are visualized in Figure 2. All the pesticides for a MEC/TV ratio could be calculated indicate risk, although for fenamiphos, esfenvalerate and chlorothalonil also RQ values lower than one were obtained for certain MECs, depending on the approach used. Interestingly when compared the PEC_PELMO/TV and PEC_SCI Grow/TV risk quotient, the second fit remarkable well to the results of measured concentrations. Dimethoate was the pesticide with the greatest RQ for short-term (RQ =1170) calculated using the SSD approach. Chlorpyrifos showed very high RQ values mainly due to their relatively high toxicity to aquatic invertebrates, hence producing low TV values. In a similar study for Australian groundwaters, Hose (2005) also identified chlorpyrifos as an insecticide with potentially significant risks to groundwater ecosystems. On the other hand, non-acceptable risks for dimethoate and phosmet may be attributed not only to a low TV value, but to a combination of relatively high MEC values and low TV values. Hose (2005) also reported MEC values for dimethoate above the trigger values that were calculated in that study. Loewy et al. (2003) conducted a chronic risk evaluation of PPPs for groundwater ecosystems by comparing the ratio of medium values from all positive pesticide detections encountered in the open literature with reference NOEC values. In that study, the dimethoate medium concentration did not exceed the chronic risk value, but chlorpyrifos and phosmet exceeded the aquatic quality criteria by factors of up to 16 (Loewy et al., 2003).

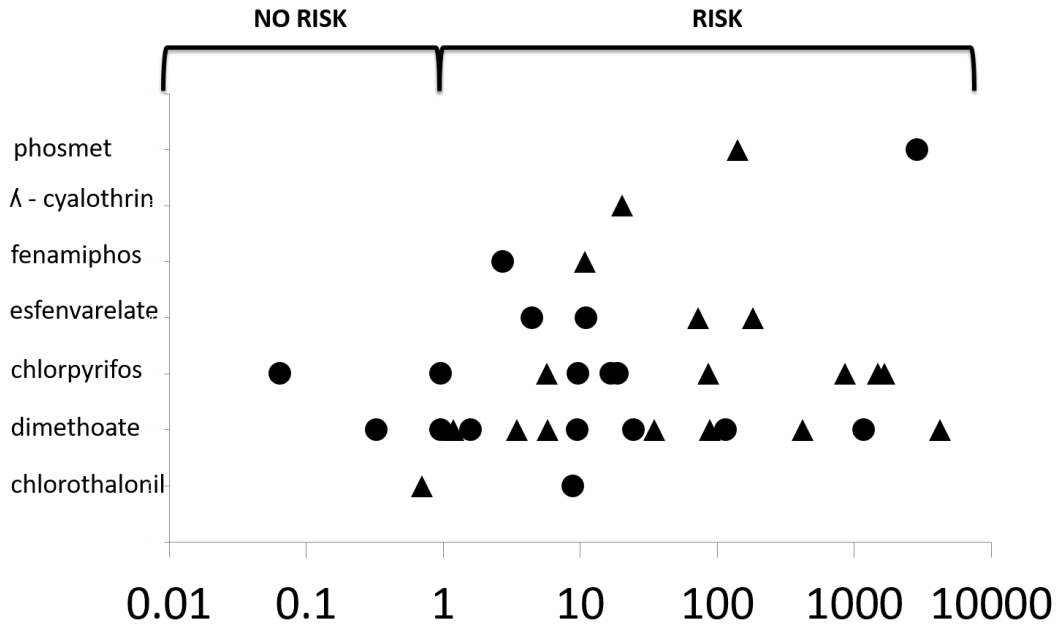

Fig. 2 Visualization of the potential risk of the PPPs to groundwater life as indicated by the ratio of encountered maximum concentrations in the open literature and TVs lower than 0.1 µg/L as established by Daam et al. (2010). Each black dot represents a datapoint using TV values based on the SSD approach, whereas black triangles refer to datapoints using TV values based on the first-tier approach (for details, see text).

The SSDs of the four PPPs for which the highest MECs values were found in the open literature (i.e. chlorpyriphos, esfenvalerate, dimethoate and phosmet) are visualized in Fig. 2. Based on the SSD curves and the maximum MECs for these four substances, the potentially affected fraction of the species assemblage was always greater than 20%, varying between 21% for dimethoate up to as high as 49% for esfenvalerate (Figure 1). Also the three (out of the seven compounds for which at least one MEC was encountered) pesticides included in the PERPEST model indicate a large probability of clear effects on taxonomic groups likely to be encountered in groundwater ecosystems (Figure 3).

Available information on unacceptable effects of pesticides in the field indicates that effects are not covered by the current prospective PPPs environmental risk assessment (first tier or higher tier) in practice the risk assessment based on existing methodology is, in some cases, not protective enough for aquatic non-target (EFSA, 2013).

Vrysas et al., 2011 concluded that the combination of pesticide concentration from monitoring studies as well as chronic toxicity to aquatic organisms is crucial for the

assessment of likelihood of aquatic environmental threat, the EQS assessment system alone is not enough to assess environmental risks.

Both WFD and pesticide regulations consider single substance risks separately. However, monitoring results show that in practice, aquatic ecosystems are exposed to a mixtures of pesticides simultaneously. These concentrations usually do not exceed the individual EQS or probable no-effect concentrations (PNECs). However, the combined effect of these substances is not accounted (Babut et al., 2013). Also other factors as environmental stress may alter effects of toxicants on populations and communities by a factor of more than 10 (Knillmann et al. 2012).

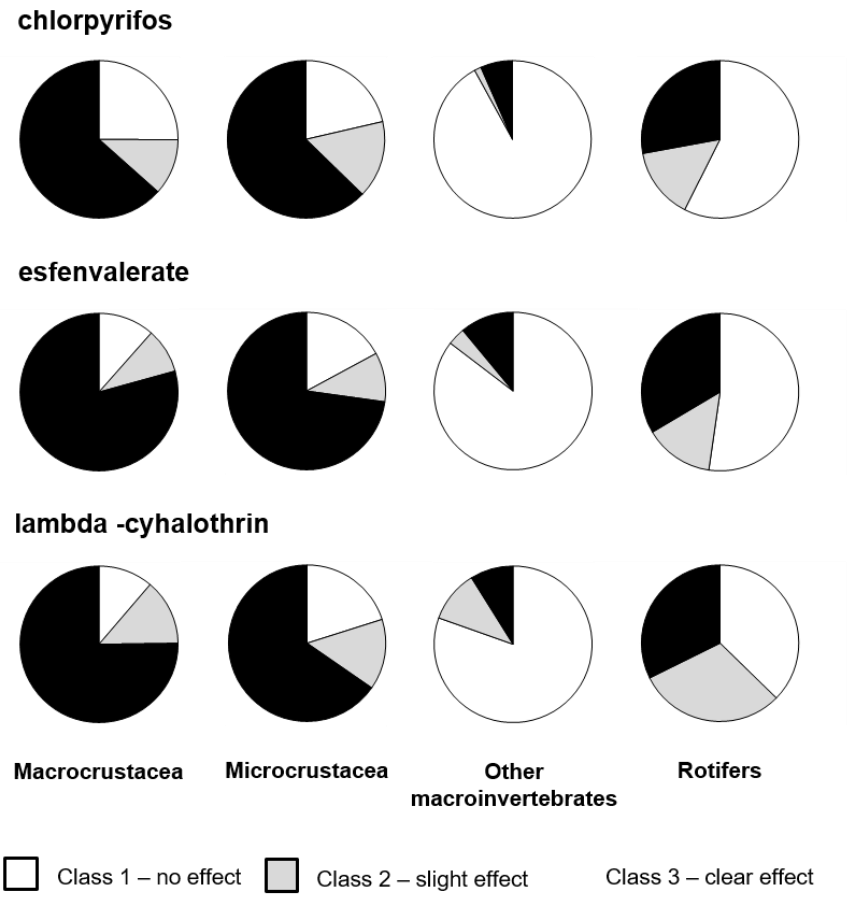

Fig. 3 Potential effects of the maximum MEC concentrations as obtained from the open literature for chlorpyrifos, esfenvarelate and lambda-cyhalothrin on different taxonomic groups as predicted by PERPEST.

4. Conclusions and way forward

Based on the simulated PEC values, no risks are expected and the TVs calculated for groundwater thus appears to be sufficiently protective for those PPPs. However, when considering concentrations actually measured in the field, 99.7% showed RQ values higher than 1 and 36.7% even higher than 100. Future field monitoring studies are needed to validate and eventually calibrate the way PEC values are currently calculated with the different models and scenarios currently in use. Such studies would also aid in the question to what extent the high MEC values may be attributed to diffuse or point-source pollution. Similarly Vrysas et al. (2011) concluded that the combination of pesticide concentration from monitoring studies as well as chronic toxicity to aquatic organisms is crucial for the assessment of likelihood of aquatic environmental threat, and that the EQS assessment system alone is not enough to fully assess environmental risks. On the effect side, the use of toxicity data generated with surface water taxa for the sensitivity assessment of groundwater organisms should be evaluated by developing toxicity testing with true groundwater taxa and subsequently comparing results obtained with their surface water counterparts.

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CHAPTER 3

IMIDACLOPRID ECOLOGICAL RISK ASSESSMENT IN RICE PADDIES

Based on the following two manuscripts:

Daam MA, Pereira ACS, Silva E, Caetano L, Cerejeira MJ (2013) Preliminary aquatic risk assessment of imidacloprid after application in an experimental rice plot. *Ecotoxicology and environmental safety*, 97, 78-85.

Pereira AS, Cerejeira MJ, Daam MA (2017) Ecological risk assessment of imidacloprid applied to experimental rice fields: Accurateness of the RICEWQ model and effects on ecosystem structure. *Ecotoxicology and Environmental Safety*, 142, 431–440.

Preliminary aquatic risk assessment of imidacloprid after application in an experimental rice plot.

Abstract

The potential aquatic risk of application of the neonicotinoid insecticide imidacloprid for aphid control in rice was assessed. To this end, imidacloprid was applied as Confidor ® 200 SC the recommended field dose of 100 g a.i./ha to a Portuguese rice plot. Subsequently, fate of the test compound in water and potential effects of water samples on a battery of test species were determined. As compared to the first-tier predicted environmental concentrations (PECs) calculated using MED-Rice (around 30 µg/L depending on the scenario used) and US-EPA (78 µg/L) simulations, the actual peak concentration measured in the paddy water (52 µg/L) was higher and lower, respectively. As was anticipated based on 50% effect concentrations (EC50 values) for *Daphnia magna* published in the open literature and that calculated in the present study (48h-EC50 immobility = 84 mg/L),no effects were observed of field water samples on daphnids. The sediment-dwelling ostracod *Heterocypris incongruens*, however, appeared relatively sensitive towards imidacloprid (6d-EC50 growth inhibition = 0.01–0.015 µg/L) and as light effect was indeed noted in field samples taken the first week after application. Species sensitivity distributions based on published EC50 and NOEC values also revealed that other species are likely to be affected at the peak and time-weighted average imidacloprid concentrations, respectively. By applying the relative tolerance approach (i.e. by dividing the EC50 value of a certain species with that of *Daphnia magna*), ostracods appear to contain the most sensitive taxa to imidacloprid, followed by EPT (Ephemeroptera, Plecoptera and Trichoptera) taxa. Future field studies into (higher-tier)fate modelling of pesticides in rice paddies and effect assessment on field communities are required to ensure protection of aquatic life and wildlife (e.g. birds)from pesticide stress.

Keywords: Imidacloprid; Experimental rice field; Risk assessment; Fate modelling

1. Introduction

Rice culture is one of the most important irrigated crops in Portugal and involves a large consumption of pesticides (Leitão et al., 2007). Previous studies performed in Portugal on this crop pointed out the need to use new active ingredients that are less toxic and less persistent in the environment and also more effective against some particular threats to this crop, like the weeds *Heteranthera* spp. and aphid insects. For example, since no insecticide was authorized in Portugal for use in rice fields to control aphids, the neonicotinoid imidacloprid was proposed for this end ([EFSA] European Food Safety Authority, 2010a). Since this new intended use implied that the existing maximum residue limit (MRL) for rice of 0.05 mg/kg (set at the limit of quantification) would have to be raised to 2 mg/kg, a consumer risk assessment was conducted by the European Food Safety Authority (EFSA). It was concluded that this MRL would not raise any consumer health concerns so [EFSA] European Food Safety Authority (2010a) concluded that the proposed temporary MRL for imidacloprid in rice was acceptable. The potential environmental risk related with the application of imidacloprid in rice plots at the authorized field dose of 100 g/ha is evaluated in the present paper.

Since their introduction in the early 1990s, neonicotinoids like imidacloprid have shown the fastest growth in the market share and has become the most widely used class of insecticides worldwide (Elbert et al., 2008; Miranda et al., 2011). Imidacloprid acts by selectively disrupting nicotinic acetylcholine receptors in the insect central nervous system (Tomizawa and Casida, 2005). Given this mode of action, it has been used to control sucking insects, such as aphids, leafhoppers, psyllids, thrips, whiteflies and beetles in various agricultural crops, to control white grubs in lawns and turfgrass, as well as to control domestic pests such as fleas and cockroaches ([CCME] Canadian Council of Ministers of the Environment, 2007).

Neonicotinoids have often been reported to contaminate surface waters (e.g. Kreuger et al., 2010; Lamers et al., 2011; Starner and Goh, 2012), although data on the environmental fate of neonicotinoids in other environmental compartments may be rather inconsistent (Fossen, 2006; Tišler et al., 2009; Miranda et al., 2011; Thuyet et al., 2011a). The need to increase our understanding of the fate of imidacloprid may especially be true for rice fields, since mathematical methods as developed by FOCUS

(Forum for the Co-ordination of Pesticide Fate Models and Their Use) are not applicable to rice cultivation. In order to address this problem, a small group of experts (the Mediterranean Rice or MED-Rice group) was formed to produce general guidelines for how risk assessment should be performed in rice paddies (MED-Rice, 2003). The Med-Rice group developed a simple tier-1 spreadsheet which could be used for calculating predicted environmental concentrations (PECs) in groundwater (GW) and surface water (SW) bodies. Similarly, the Tier I Rice Model v1.0 was developed by the United States Environmental Protection Agency (US-EPA) to estimate pesticide concentrations in rice paddies following their application ([US-EPA] United States Environmental Protection Agency, 2007). In terms of environmental effect assessment of imidacloprid, most studies have focused on honey bee (*Apis mellifera* L.) colonies. Relatively few toxicity studies have been performed on the potential effects on aquatic non-target organisms despite its increasing use (Jemec et al., 2007; Tišler et al., 2009), although research efforts have increased over the past years (e.g. Stoughton et al., 2008; Hayasaka et al., 2012c; LeBlanc et al., 2012; Roessink et al., 2013).

The aim of this paper was to evaluate the fate of imidacloprid after application to an experimental rice plot in Portugal. The accurateness of the first-tier scenarios as developed by MED-Rice and US-EPA in predicting measured imidacloprid concentrations was also assessed. Environmental side-effects on aquatic organisms were evaluated by testing field samples with a battery of single species tests. The potential of measured concentrations to exert risks to aquatic life was also evaluated using species sensitivity distributions (SSDs) and by comparing them with toxicity values reported in previously published (semi) field studies. Implications for the risk assessment of pesticides in rice paddies and indications for future research needs are discussed.

2. Materials and methods

2.1. Experimental rice plot

The experimental rice field was located in city council Alcácer do Sal in the Baixo Sado region (Portugal). This rice field area receives water from the water catchments Vale do Gaio and Pego do Altar, which is distributed through an irrigation canal over the different rice plots through passive irrigation (i.e. the water entering the first rice plot from the irrigation canal passes through to the following field; Fig. 1). To avoid contamination from pesticide applications made in other rice plots, the selected rice plot was one that received water directly from the water catchments. This rice plot had a surface area of 0.62 ha with an average water layer of approximately 10 cm throughout the experiment. The soil was sandy-loam with pH 6.1 and an organic matter content of 1.55%. These characteristics are representative for Portuguese rice fields (MED-Rice, 2003). To evaluate possible contamination of water entering the rice field plot from the water catchments, water from the nearest water catchment (Pego do Altar; Fig. 1) was analysed for the main pesticides used in the area. In addition, the water in the irrigation canal was analysed for imidacloprid residues.

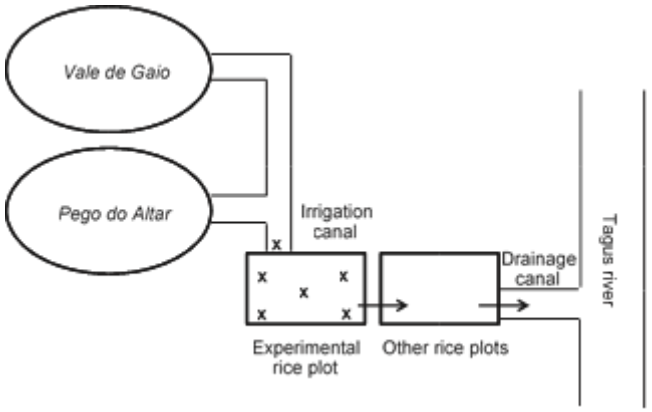

Fig. 1 Schematic overview of waterways in the study area. A “x” indicates a sampling point in the paddy and irrigation canal.

2.2. Imidacloprid application and water sampling

Imidacloprid was applied as the commercial product Confidor ® 200 SC at the recommended field dose of 100 g a.i./ha with a spray volume rate of 300 L/ha ([EFSA] European Food Safety Authority, 2010a). Water samples for chemical analysis and toxicity evaluations were taken from the rice plots a day before imidacloprid application, 8 h after treatment, as well as 2, 5, 7, 14 and 28 days after application. In the irrigation canal, water was collected 5, 7, and 28 days after treatment. These water samples were taken as a 5 L sample composed of various subsamples of 1 L taken at 5 points distributed over the plot (Fig. 1). Sediment samples were taken a day before imidacloprid application, 8 h and 5, 14 and 28 days after treatment by collecting the top 5 cm layer and transferring it to a 500-mL bottle. Water and sediment samples were transported to the laboratory under refrigeration conditions, where they were stored at 4°C without light until analysis.

2.3. Chemical analysis

Water samples of 500 mL obtained as described in the previous section were extracted within 48 h using solid phase extraction with styrene divinyl benzene extra clean (SDB-XC) Empore disks. The extraction disks were conditioned with 5 mL dichloromethane, 5 mL methanol, and 5 mL distilled water. After extraction, imidacloprid was eluted from the disks with two successive portions of 5 mL acetonitrile and concentrated by evaporating to 0.5 mL by applying compressed nitrogen gas. Within 7 days after extraction, imidacloprid was analysed via Liquid Chromatography–Electrospray Ionization–Mass Spectrometry (LC-ESI-MS), mounted with a Zorbax SB-C18 column (length 150 mm, width 4.6 mm) at quantification and identification fragments 254 nm and 256 nm, respectively. The retention time for the imidacloprid peak was 6 min with a detection limit in water of < 25 ng/L. Imidacloprid recovery from the water was 85 ±4% (mean± S.D., n=6).

2.4. First-tier PEC calculations

The first-tier PECs in rice paddy water were calculated using the methodology as developed by MED-Rice (2003) and [US-EPA] United States Environmental Protection Agency (2007). The tier-1 spreadsheet developed by MED-Rice (2003) also allows for calculating PECs in paddy soil, groundwater, and water and sediment of adjacent surface water bodies at different days after application. In addition, the time-weighted average (TWA) concentrations over a time period (T) in these environmental compartments were calculated based on the initial PEC (i.e. predicted peak-concentration after application) and the half-life (DT50; detection time 50%) in these compartments by applying the following equation (after MEDRice, 2003):

$$\text{TWA} = = \frac{\text{PEC initial x } (1-\exp\left(\frac{-\text{T x ln}(2)}{\text{Dt50}}\right))}{T\ x \ln(2)/Dt50}$$

Two standard European scenarios, corresponding to two different but representative situations, were developed by MED-Rice: a sandy soil with a high infiltration rate, representing a situation vulnerable to groundwater contamination, and a clay soil with poor infiltration, representing a situation vulnerable to surface water contamination. Besides these two scenarios, a site-specific scenario was run based on parameters measured at the experimental rice plot (Table 1). Simulations allowed for the estimation of both the actual PEC values at various days post application and the time-weighted average (TWA) concentrations over these time periods.

The Tier I Rice Model v1.0 as developed by the United States Environmental Protection Agency (US-EPA) calculates a single screening-level PEC that represents both short and long term surface water exposure in the paddy ([US-EPA] United States Environmental Protection Agency, 2007). This water concentration (Cw; in µg/L) is calculated from the application dose (in kg/ha) and the water-sediment partitioning coefficient (Kd; in L/kg), eventually based on the organic carbon partitioning coefficient (Koc; in L/kg) if a Kd value is not available. The following formulas are applied for this purpose (after [US-EPA] United States Environmental Protection Agency (2007)):

$$Cw = \frac{\text{Application dose}}{(0.00105 + 0.00013\ Kd)}$$

And; if appropriate: Kd = 0.01 Koc

Input values for pesticide properties for both model simulations were obtained from the draft assessment report of imidacloprid ([EC] European Commission, 2006; Table 1). In addition, a scenario was run with input parameters set at levels measured at the field site.

2.5. Single species toxicity testing and chemical analysis of imidacloprid

Laboratory single species tests were conducted to determine the toxicity of the water and sediment samples collected as described in Section 2.2 and to establish EC50 values for a battery of test organisms. These tests were conducted with the cladoceran *Daphnia magna*, the sediment-dwelling ostracod *Heterocypris incongruens*, the green algae *Pseudokirchneriella subcapitata*, and the macrophyte *Lemna minor*. The *L. minor* test was conducted to determine effects on frond number and area in accordance with [ISO]

International Organisation for Standardisation (2005). For the other organisms, Toxkit microbiotests (MicroBioTests, Gent, Belgium) were used in accordance with their corresponding standard operational procedures (SOPs) as developed by MicroBioTests to determine relevant endpoints for the different species (Table 2). These SOPs adhere to the ISO standard methods for *D. magna* ([ISO] International Organisation for Standardisation, 2012a), *P. subcapitata* ([ISO] International Organisation for Standardisation, 2012b), and *H. incongruens* ([ISO] International Organisation for Standardisation, 2012c).

Table 1. Input values used for the first-tier PEC (predicted environmental concentration) calculations in accordance with MEDRice (2003) and US-EPA (2007).

	MED-RICE (2003)		US-EPA (2007)	This study	Source
	Scenario 1 clayey	Scenario 2 sandy			
INPUT: Scenario data I					
OC soil (%)	1.8	0.9	1	1.55	Default / measured in this study
Depth water (m) (water level in field)	0.1	0.1	0.1	0.1	Default
Water velocity field (L/sec/ha)	3	3	–	As MED-RICE (2003)	Default
Water velocity outflow (L/sec/ha)	0.5	0.5	–	0.5	Default
Leakage (mm/d) (infiltration rate)	1	10	–	As MED-RICE (2003)	Default
t close (d) (time of closure of field)	5	5	–	As MED-RICE (2003)	Default
t flood (d) (time of flooding)	120	120	–	As MED-RICE (2003)	Default
Depth canal (m) (deepness of outflow)	1	1	–	As MED-RICE (2003)	Default
INPUT: Scenario data II					
Area (m^2) (area of rice field)	10,000	10,000	–	6,200	Default/measured in this study
Volume of water in field (L)	1000,000	1000,000	–	620,000	Calculated
Depth soil (m)	0.05	0.05	0.01	As MED-RICE (2003) and US-EPA (2007)	Default
BD soil (kg/dm^3) (soil bulk density)	1.5	1.5	1.3	As MED-RICE (2003) and US-EPA (2007)	Default
Grain density (kg/m^3)	–	–	2650	As US-EPA (2007)	Default
Sediment porosity ()	–	–	0.509	As US-EPA (2007)	Default
Outflow rate (1/d)	0.0432	0.0432	–	As MED-RICE (2003)	Default
Dilution factor	10	10	–	As MED-RICE (2003)	Default
Depth sediment (m) (active sediment depth)	0.05	0.05	–	As MED-RICE (2003)	Default
OC (%) of sediment	1.6	1.6	–	As MED-RICE (2003)	Default
BD sediment (kg/dm^3) (sediment bulk density)	1.5	1.5	–	As MED-RICE (2003)	Default
INPUT: Product					
Dose (g/ha) (application rate of product)	100	100	100	100	This study
F dep (fraction of dose deposited to paddy field)	1	1	1	1	Default
F drift (fraction drift to adjacent water body)	0.0277	0.0277	–	0.0277	In accordance with FOCUS (2001)
Koc (dm^3/kg)	212	411	175	175	Studies in EC (2006) with comparable soil
Kd (soil) (dm^3/kg)	3.8	3.7	1.75	2.7	Calculated
Kd (sediment) (dm^3/kg)	3.4	6.6	–	2.8	Calculated
F sorbed (soil) (fraction partitioning to soil)	0.74	0.74	–	0.671	Calculated
F sorbed (sediment) (fraction partitioning to sediment)	0.20	0.33	–	0.174	Calculated
DT50 total, pw (d) in flooded soil system	14	14	–	14	Study in EC (2006) with comparable sediment
DT50 pw (d) in water phase	1.4	1.4	–	1.4	Study in EC (2006) with comparable sediment
DT50 soil (d) in solid phase	14	14	–	14	Study in EC (2006) with comparable sediment
DT50 total, sw (d) in sediment/water system	14	14	–	14	Study in EC (2006) with comparable sediment
DT50 sw (d) in water phase	1.4	1.4	–	1.4	Study in EC (2006) with comparable sediment
DT50 sed (d) in solid phase	14	14	–	14	Study in EC (2006) with comparable sediment

Table 2. EC50 (effect concentration 50%) and LC50 (lethal concentration 50%) values of imidacloprid as calculated for the organisms tested in the laboratory bioassays conducted in this study.

Organism	Endpoint	Value (mg a.i./L)
Daphnia magna	48 h-EC50, immobility	84
Heterocypris incongruens	6 d-EC50, growth inhibition	0.01–0.015
	6 d-LC50, mortality	40.015
Pseudokirchneriella subcapitata	72 h-EC50, growth inhibition (light inhibition)	4600
Lemna minor	7 d-EC50, growth inhibition (frond number and area)	740

2.6. Species sensitivity distributions (SSDs)

Given the insecticidal type of action of imidacloprid, arthropods may be expected to be the most sensitive taxonomic group (Maltby et al., 2005; Sánchez-Bayo, 2012). Subsequently, toxicity data for arthropods (crustaceans and insects) were obtained from various reports (draft assessment report: [EC] European Commission, 2006; RIVM, 2008; Junghans et al., 2011), a study by Becker et al. (2011), and the US Environmental Protection Agency (US-EPA) ECOTOX database (available via: http://cfpub.epa.gov/ecotox/).

Selected endpoints for acute toxicity were the median effect concentrations for immobility or mortality observed in toxicity tests (EC50 or LC50) with a test duration of 1–4 days. For chronic NOECs, data with a test duration of 21 and 28 days evaluating mortality, development, reproduction and growth (as well as swimming behaviour and emergence for insects) were considered valid. Since recent studies have demonstrated that toxicity data for freshwater and saltwater organisms may in principle be pooled for pesticides ([EC] European Commission, 2011; Klok et al., 2012), obtained EC50 values for the saltwater crustaceans *Americamysis bahia* and *Artemia* sp, and the insect *Aedes taeniorhynchus* were included in the SSDs. Geometric means were calculated when more than one toxicity value was reported for the same endpoint of a species. Subsequently, the geometric mean of the most sensitive endpoint (e.g. either that calculated for mortality or immobility in case of EC50) was selected for that species.

Log-normal distributions of threshold values 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 log-normal distribution of the data, log-normality was tested with the Anderson–Darling Test included in the ETX software package, which was evaluated at the 5% significance level.

3. Results and discussion

3.1. Dissipation of imidacloprid in rice paddy water

Half-lives of imidacloprid in the paddy water as determined based on concentrations measured in the present study were between 1 and 3 days, depending on the time-period over which they were calculated (DT50-7d=0.9d; DT50-14d=1.8d; DT5028d=3.0d). These values are in line with those reported in previous studies conducted in rice plots. For example, DT50 values of imidacloprid reported in Japanese rice field water ranged from approximately 2 days (DT50-7d: Thuyet et al., 2011a; DT50-14d: Phong et al., 2009) to 4 days (DT50-1m: Sanchez-Bayo and Goka, 2006a). In Indian paddies, Kanrar et al. (2006) reported aquatic half-lives between 1.6 and 2.8 days for imidacloprid applied as granular formulation. Wu et al. (2004) also found comparable halflife values of 2.6–2.7 days in water from a rice paddy in China.

3.2. Measured versus modelled imidacloprid concentrations

In the EU risk assessment of imidacloprid, PEC estimates for surface waters were made using FOCUS scenarios for applications of Confidor SL 200 in apple and tomato (both as spray application) and Gaucho FS 600 in sugar beet (as seed treatment; [EC] European Commission, 2006). The highest modelled PEC surface water was 7.962 mg a.i./L for application of Confidor SL 200 on apple trees in the FOCUS scenario R3 stream (Bologna, Italy) and was subsequently used to calculate toxicity exposure ratios (TERs; [EC]

European Commission, 2006). In addition, a PEC up to 36 µg/L was calculated using the FQPA (Food Quality Protection Act) Index Reservoir Screening Tool assuming a worst-case 100% crop treatment in the USA (Fossen, 2006). The PECs calculated in the present study for rice pond water using MED-Rice and EPA model scenarios are all substantially higher (between 33 µg/L and 71 µg/L depending on the scenario used; Table 3) than these previous PEC calculations. This is not surprising given that, unlike in these previous simulations for spray drift on water, imidacloprid application in our study was made by direct overspray.

Table 3. Predicted environmental concentrations (PECs) in paddy water and soil, groundwater, and adjacent water body water and sediment for the scenarios provided in Table 1. The paddy water concentration as measured in the present study was 52 µg/L.

PEC/scenario	MED-RICE —clayey	MED-RICE —sandy	MED-RICE — this study	US-EPA
Paddy water (µg/L)	26	26	33	78
Paddy soil (µg/kg)	99	98	89	–
Groundwater (µg/L)	0	0	0	–
Water of receiving waterbody (µg/L)	0.21	0.22	0.27	–
Sediment of receiving waterbody (µg/kg)	1.2	1.9	1.1	–

As compared to the first-tier PECs simulated using the MED-Rice (around 30 µg/L depending on the scenario used) and US-EPA (78 µg/L) models, the actual peak concentration measured in the paddy water (52 µg/L) was higher and lower, respectively (Table 3; Fig. 2). This difference between the two simulations is probably due to differences in how the PEC is calculated and assumptions that are made in this, e.g. the sediment depth used by MED-Rice and EPA are five and one cm, respectively (Table 1). Given the similar DT50 values (Table 1; Section 3.1) and the lower peak-concentrations modelled by the MED-Rice method, TWA concentrations calculated over 28 days post application from MED-Rice simulations (between 2 µg/L and 2.5 µg/L) were logically also lower than those obtained from actual field measurements (8.0 µg/L).

The environmental fate of imidacloprid is rather inconsistent depending on the application method, formulation of the pesticide and field conditions (Fossen, 2006; Tišler et al., 2009; Miranda et al., 2011; Thuyet et al., 2011a). For example, Tišler et al.

(2009) reported that, although some authors consider imidacloprid as relatively immobile and do not expect it to leach to groundwater, other studies concluded the opposite. Simulated PEC groundwater values in our study were negligible (Table 3), whereas concentrations varying from less than 0.1 µg/L up to 6.7 µg/L were detected in a well over a five month sampling period (Fossen, 2006). In a paddy rice cultivation area in Vietnam where imidacloprid was applied at a field dose similar to that used in the present study (on average 0.12 kg/ha), Lamers et al. (2011) detected imidacloprid in five of the eight wells surveyed. In these five wells, detected concentrations varied in time between non-detectable up to 1.53 µg/L (Lamers et al., 2011). Future studies into the monitoring of imidacloprid in groundwater systems of our study area are needed to evaluate the actual contamination occurring in the field and should adopt a sampling strategy that considers this potential spatial–temporal variation in pesticide concentrations.

Despite the great variety of application methods, formulation and field conditions, the peak-concentration in paddy water as measured in the present study is comparable to those previously detected in rice fields following imidacloprid application. For example, Thuyet et al. (2011b) set their test concentrations at 58 µg/L based on the concentration range that was previously reported in paddy fields. The paddy water concentration of 52 µg/L measured in the present study is also in the range of 40 µg/L to 90 µg/L reported by Kanrar et al. (2006), even though they used broadcast application after mixing with sand.

The lack of data on the environmental fate of imidacloprid in aquatic ecosystems has often been discussed (e.g. Jemec et al., 2007; Tišler et al., 2009; Kreuger et al., 2010; Lamers et al., 2011; Starner and Goh, 2012). This may be especially true for rice paddies, and further testing and improvement of model scenarios in order to predict pesticide exposure in rice paddies more realistically have previously been recommended (e.g. MED-Rice, 2003; Karpouzas et al., 2006; Phong et al., 2009). For example, although the RICEWQ model is generally concluded to be the most appropriate model for higher-tier pesticide fate predictions in European rice fields, further validation studies have also been recommended to evaluate its predictive value for national (site specific) conditions (MED-Rice, 2003, Karpouzas et al., 2006). In this regard, although the first-tier assessment as conducted in the present study may be expected to lead to worst-case

predictions, the measured surface water concentration in the paddy water was greater than simulated PEC values. Future studies also evaluating the RICEWQ model for predicting pesticide concentrations are therefore needed to validate the accurateness of the current exposure assessment of pesticides in European rice paddies.

3.3. Toxicity thresholds and toxicity of field samples in bioassays

As was anticipated based on the insecticidal type of action of imidacloprid, the green algae *P. subcapitata* and the macrophyte *L. minor* were relatively tolerant to imidacloprid, with calculated EC50 values of 4600 mg/L and 740 mg/L, respectively (Table 2). The EC50-48 h for immobility as calculated in the present study for *D. magna* (84 mg/L) was the same as that reported in the draft assessment report of imidacloprid (85 mg/L; [EC] European Commission, 2006). No toxicity values for *H. incongruens* were encountered in the literature for comparison with the value obtained in the present study (Table 2).

Effects as observed from the field samples were generally in line with the toxicity values calculated in the laboratory tests: based on the calculated EC50 values, no clear effects would indeed be expected on *D. magna* and *P. subcapitata* at a peak-concentration of 52 µg/L (Table 2; Fig. 2). Similarly, no effects were anticipated on the frond number and area of L. minor at this imidacloprid concentration given the calculated EC50 of 740 mg/L (Table 2). The greatest growth inhibition that was recorded for this species (71% after 7 days; Fig. 2).

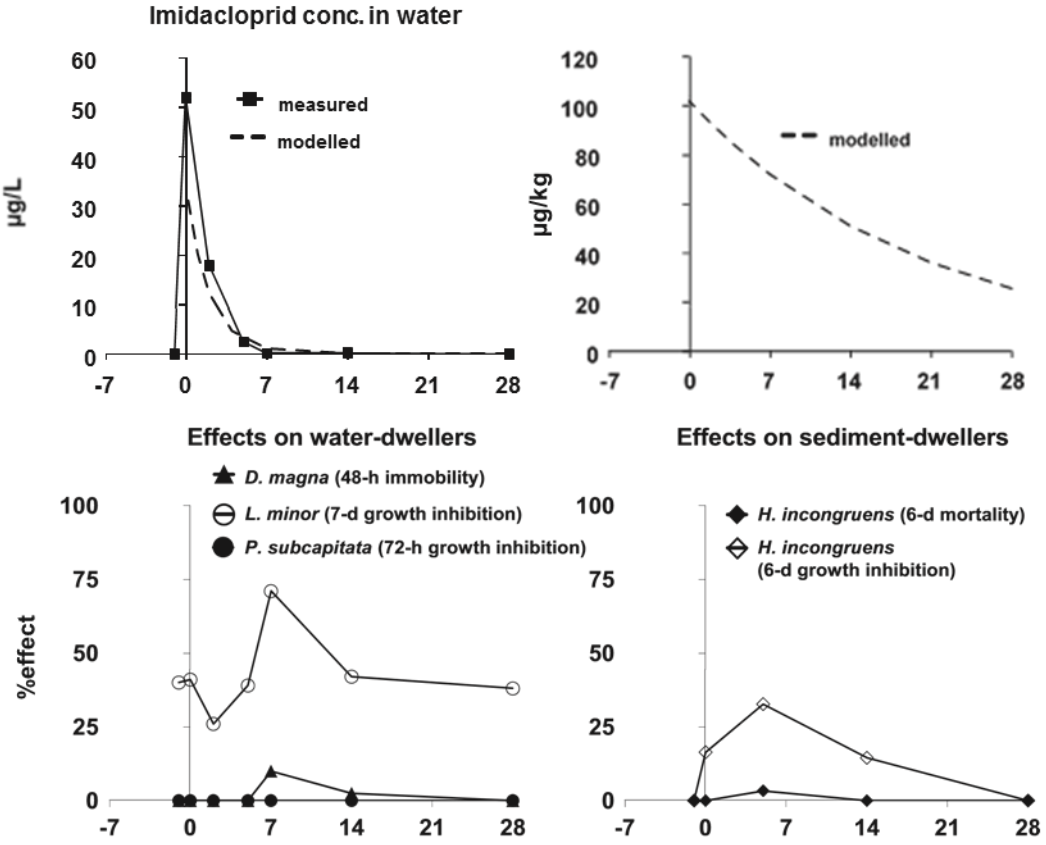

Fig. 2. Imidacloprid concentrations as measured and modelled in the paddy water (in µg/L) and sediment (in µg/kg), and effects as recorded from the field water samples on the test organisms during the course of the experiment.

Similarly, no effects were anticipated on the frond number and area of *L. minor* at this imidacloprid concentration given the calculated EC50 of 740 mg/L (Table 2). The greatest growth inhibition that was recorded for this species (71% after 7 days; Fig. 2) was also noted in the water collected from the irrigation canal that was devoid of imidacloprid (growth inhibition 72%; data not shown). The greatest total pesticide concentration measured in the irrigation canal was 0.08 µg/L and corresponded to the herbicide propanil. Since at this low propanil concentration no effects are expected on *L. minor* (Mitsou et al., 2006), the observed effects are likely to be due to (mixture) toxicity of unknown compounds or other water quality factors (conductivity, pH, etc.). Although no effects on survival rates of *H. incongruens* were observed from sediment samples taken in the rice field, sublethal effects were noted on growth (Fig. 2). Since no effects on either parameter were noted in samples taken from the irrigation canal, the effect on growth rate is likely to have resulted from the imidacloprid application. Interestingly, growth rate inhibition was greater in sediment samples taken 5 days post application than in those taken 8 h after application (Fig. 2). This is probably due to the fact that the

translocation of imidacloprid from the water to the sediment was not instantaneously, but took some time to occur. In line with this, the relative amount of imidacloprid in sediment increased from approximately 8% immediately after application to 24% after one week in a water-sediment study ([EC] European Commission, 2006).

3.4. Species sensitivity distributions

On several occasions it has been discussed that the widely employed test species *Daphnia magna* is unsuitable for predictive risk assessment of neonicotinoids due to its low sensitivity towards this class of insecticides (e.g. Kreutzweiser et al., 2007; Beketov and Liess, 2008; Lukančič et al., 2010; Miranda et al., 2011). *Daphnia magna* even appeared to be the least sensitive arthropod species in SSDs constructed for the neonicotinoid thiacloprid (Beketov and Liess, 2008) and imidacloprid (Miranda et al., 2011; Hayasaka et al., 2012c).

Therefore, the potential of the measured imidacloprid concentrations to exert effects on arthropods was also evaluated by constructing SSDs based on previously published acute (EC50) and chronic (NOEC) toxicity data (see Section 2.6). Since the SSD constructed using the entire arthropod EC50 dataset did not pass lognormality ($p>0.05$), separate curves were constructed for crustaceans and insects (Fig. 3).

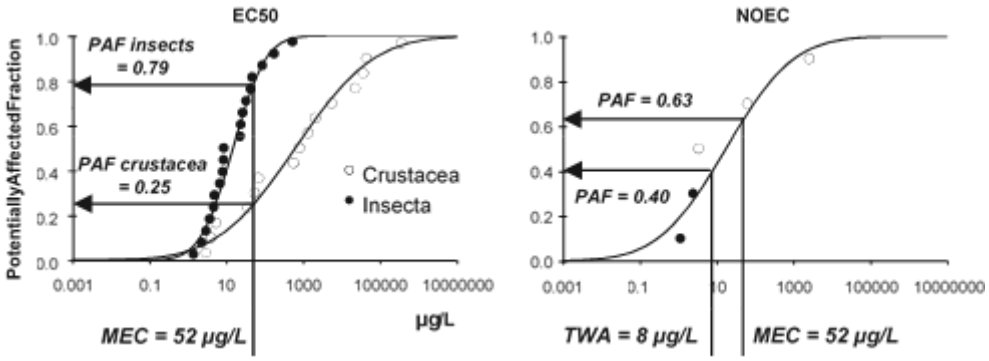

Fig. 3. Species sensitivity distributions (SSD) as constructed based on published EC50 (effect concentration 50%; left) and NOEC (no observed effect concentration; right) values. The potentially affected fraction (PAF), i.e. the number of taxa for which their toxicity values are expected to be exceeded based on the SSD curve, is indicated at the measured peak-concentration (MEC) of 52 µg imidacloprid/L in both cases. In addition, the PAF at the time-weighted average (TWA) concentration as calculated over 28 days is also indicated in the SSD based on NOEC values.

Since neonicotinoids were specifically designed to act as agonists at the nicotinic acetylcholine receptor (nAChR) of insects, the overall greater sensitivity of insects relative to crustaceans as observed in the SSD curves was indeed anticipated (Fig. 3). To evaluate which arthropod groups are especially vulnerable to imidacloprid, the Trel approach as developed by Wogram and Liess (2001) was applied to the EC50 data. To this end, the (geometric mean of the) EC50 value of a certain species was divided by that of *D. magna* (Fig. 4). Subsequently, a Trel of one indicates a relative tolerance equal to that of *D. magna*. For species more sensitive than *D. magna*, Trel is less than one and for less sensitive species it is greater than one. Interestingly, despite the overall greater sensitivity of insects as compared to crustaceans as observed from the SSD curves (Fig. 3), taxa belonging to the crustacean class Ostracoda appeared to be the most sensitive taxonomic group towards imidacloprid (Fig. 4). In line with this, the EC50 values as calculated in the present study for the ostracod *H. incongruens* yield a Trel between 0.00044 and 0.00066. Sánchez-Bayo and Goka (2006b) also demonstrated that the sensitivity of three field-collected typical rice paddy ostracods to imidacloprid was two to three orders of magnitude greater than that of *D. magna*.

The midge *Chironomus riparius* and the mysid *A. bahia* have recently been indicated as additional standard test species in the prospective acute risk assessment of pesticides to aquatic invertebrates in Europe ([EFSA] European Food Safety Authority, 2013). With calculated Trel values of 0.0012 and 0.0016, respectively, they are indeed three orders of magnitude more sensitive than *D. magna* to imidacloprid. *H. incongruens* appears even slightly more sensitive, and has proven great potential as a reliable and sensitive low cost alternative for traditional whole sediment assays to a variety of compounds (e.g. Belgis et al., 2003; Kudlak et al., 2011). In general, the role of toxicity testing using benthic species in the prospective risk assessment of pesticides needs further evaluation as to ensure protection of sediment communities from chemical stress (see Diepens et al., 2013 for a recent review). Following ostracods, the insect orders Ephemeroptera (mayflies), Plecoptera (stoneflies) and Trichoptera (caddisflies) were found to be the most sensitive taxonomic groups to imidacloprid (Fig. 4). These three orders, jointly commonly referred to as EPT taxa, have indeed often been reported to be especially prone to chemical stress, including pesticides (e.g. Wogram and Liess, 2001). Recent studies have further indicated that the most sensitive taxa and taxonomic group may

depend on the exposure regime, i.e. whether a single-peak, repeated pulse or chronic exposure is evaluated (Roessink et al., 2013; Van Dijk et al., 2013).

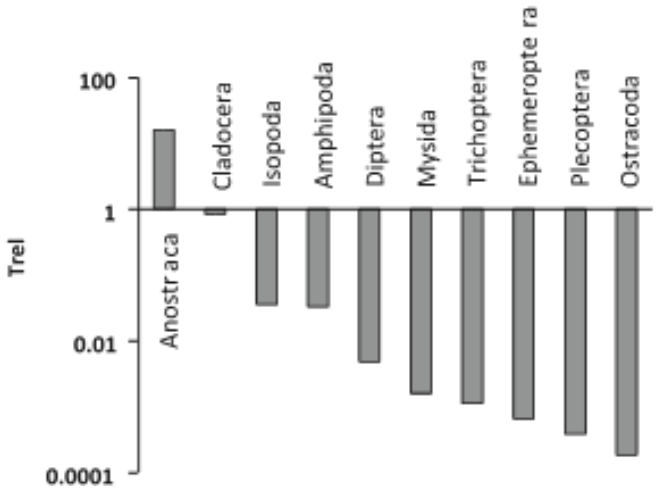

Fig. 4. Average relative tolerance (Trel) values based on EC50 values for different taxonomic arthropod groups. In accordance with Wogram and Liess (2001), a Trel was calculated by dividing the (geometric mean of the) EC50 value of a certain species with that of *D. magna*. Subsequently, a Trel of one indicates a relative tolerance equal to that of *D. magna*. For species more sensitive than *D. magna*, Trel is less than one and for less sensitive species it is greater than one.

Based on the SSD constructed with EC50 data, as much as 25% and 79% of the crustacean and insect taxa, respectively, are expected to be affected by at least 50% at the measured peak concentration of 52 µg/L (Fig. 3). For the SSD curve based on NOEC data, 63% of the arthropods are potentially affected at this peak concentration. Although NOECs are often compared with PECmax in the prospective risk assessment, the use of the time-weighted average (TWA) concentration has in recent years been proposed for use in chronic risk assessment (e.g. Schäfer et al., 2011; Zafar et al., 2012). The 28 d-TWA of 8 µg/L, calculated by applying the formulas in MED-Rice (2003) to measured field concentrations, still indicates effects on 40% of the arthropod species assemblage based on 28 d-NOECs (besides the NOEC for *D. magna*, which was based on a 21 d test).

3.5. Implications for risk assessment

Based on the discussed above, there is little doubt that the application of imidacloprid at the recommended dose rate will affect various species in the rice plot. Model

ecosystem studies evaluating imidacloprid indicate clear long-lasting effects at concentrations as measured in the present study (e.g. [EC] European Commission, 2006; Hayasaka et al., 2012a, b). For example, Hayasaka et al., 2012a, b reported significant treatment effects on zooplankton, benthic and neuston communities in paddy cosms at initial concentrations of 40 µg/L to 50 µg/L, and also reported reduced growth of medaka fish (Oryzias latipes) at these concentrations. In the EU draft assessment report, a NOEC model ecosystem of as low as 0.6 µg/L was set based on available studies, to which still a safety factor of two was applied ([EC] European Commission, 2006).

It has been questioned whether ecosystem structure should be the protection goal in rice fields or whether maintaining ecosystem function should be the main goal. For example, [EC] European Commission (2002) states that if "the use pattern of the compound includes direct application of the plant protection product into aquatic systems (e.g. in-crop areas like rice paddies or aquatic weed control uses)[…], unacceptable impacts on ecological function instead of biodiversity parameters should be the main consideration when effects on aquatic systems are assessed". Although this was later disputed ([EC] European Commission, 2003) and was not included in the final version of the MED-Rice (2003) guidance document, protecting ecosystem structure in rice paddies will provide constraints in practical terms. For example, since chironomid larvae have been recorded as pests of rice growing in many temperate countries, various insecticides have in the past been applied against these organisms (e.g. MED-Rice, 2003; Leitão et al., 2007). Since chironomids are amongst the most sensitive test organisms to imidacloprid used in aquatic risk assessment, protecting ecosystem structure maybe an impossible objective when target organisms are also the most sensitive test species. On the other hand, chironomids are important macroinvertebrates in the ecology of the aquatic ecosystems. They play a key role in recycling organic matter and are important prey items for birds and fish (Faria et al., 2007; Poulin, 2012). In this regard, rice paddies are often located in or in the vicinity of Natural Reserves with great importance as habitats for waterfowl and migratory bird species (MED-Rice, 2003), as is the case for our study area ([ICN] Instituto da Conservação da Natureza, 2007; Lourenço and Piersma, 2009). In addition, the ecological effect chain following pesticide stress may evidently also affect ecosystem function and even crop productivity. This may be illustrated with a study by Sánchez-Bayo and Goka (2006a) who evaluated imidacloprid

in experimental rice fields. These authors concluded that the absence of *Chironomus yoshimatsui* and typical paddy ostracods from fields with imidacloprid concentrations as low as 1 µg/L led to green algae (*Spirogyra* sp.) blooms, thus increasing the pH of the water. The reduced abundance of predators was not fully attributed to a direct toxic effect but also by a lesser availability of prey, because aphids were completely absent. This lower abundance of predators could in theory mean that the rice crop would in turn be more liable to attack by late-season pests, such as rice bugs (*Leptorisa* sp.) and planthoppers (*Delphacidae*) (Sánchez-Bayo and Goka, 2006a). Filamentous and unicellular green algae have also been reported as a problem in Portuguese rice fields since they compete with the rice crop for nutrients (Med-Rice, 2003). Future field studies are needed to set light on these issues ensuring adequate pesticide risk management in rice ecosystems. These studies should include (i) assessment of pesticide fate to evaluate and validate pesticide fate modelling scenarios; (ii) monitoring of aquatic ecosystem structure and functioning following pesticide application in paddy water and surrounding waterways, as well as evaluating possible impact on wildlife (e.g. birds); (iii) evaluation of possible positive and negative effects of pesticide application on crop productivity and pest occurrence. Ultimately, such studies should aid in setting in crop and off-crop protection goals ([EFSA] European Food Safety Authority, 2010b) in rice paddies and ensuring sustainable rice production.

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Ecological risk assessment of imidacloprid applied to experimental rice fields: accurateness of the RICEWQ model and effects on ecosystem structure

Abstract

The fate of imidacloprid and its potential side-effects on biological communities and ecosystem functioning was studied in experimental rice plots. In addition, the influence of applying a withholding period of zero days (actual practices) and seven days (recommended in EU) on this was evaluated. Predicted environmental concentrations (PEC) of imidacloprid calculated with the higher-tier model RICEWQ agreed well with concentrations measured in the field. Methodologies generally used in the EU and USA for lower-tier PEC calculation, however, severely underestimated actual field concentrations and hence appear to need further evaluation and eventually amendments. Effects on several biological communities (especially ostracods, dipterans and coleopterans) were noted following imidacloprid application, with greatest effects in the paddy were as applied a withholding period of 7 days. An increase in the density of snails (*Physa acuta*), however, may have prevented effects on ecosystem functioning through functional redundancy. Implications of study findings for the ecological risk assessment of imidacloprid and potential mitigation measures are discussed.

Keywords: neonicotinoid; fate modelling; ecological risk assessment; rice

1. Introduction

Pesticide exposure assessment conducted in prospective risk evaluations for regulatory purposes depends on the use of pesticide fate simulation models to determine predicted environment concentrations (PEC) of pesticides. Rice agroecosystems present a unique environmental scenario with respect to pesticide fate so that traditional mathematical methods as developed, for example, by FOCUS (Forum for the Co-ordination of Pesticide Fate Models and Their Use) are not applicable to rice cultivation (Capri and Karpouzas, 2008; Luo et al., 2012; Daam et al., 2013). In order to address this problem, a small group of experts (the MEDiterranean Rice or MED-Rice group) was formed to produce general guidelines for how risk assessment should be performed in rice paddies. The MED-Rice group developed a simple tier-1 spreadsheet that can be used for calculating PECs in groundwater, paddy water and adjacent surface water bodies (MED-Rice, 2003). Similarly, the Tier I Rice Model was developed by the United States Environmental Protection Agency (US-EPA) to estimate pesticide concentrations in rice paddies following their application (US-EPA, 2007).

A preliminary risk assessment previously conducted by our research team indicated that the first-tier models described in the above paragraph underestimated actual paddy field concentrations of the neonicotinoid insecticide imidacloprid following its application (Daam et al., 2013). In line with this, further testing and improvement of (first-tier) model scenarios in order to more realistically predict pesticide exposure in rice paddies have previously been recommended (e.g. MED-Rice, 2003; Karpouzas et al., 2006; Phong et al., 2009). In addition, although the higher-tier RICEWQ model is generally considered to be the most appropriate model for higher-tier pesticide fate predictions in European rice fields, further validation studies are needed to evaluate its predictive value (MED-Rice, 2003, Karpouzas et al., 2006; Daam et al., 2013).

Based on laboratory toxicity testing of paddy field water and available single species toxicity data, the measured (peak) imidacloprid concentration in the preliminary risk assessment study is likely to have toxic effects on non-target aquatic organisms (Daam et al., 2013). Especially, effects on ostracods and EPT (Ephemeroptera, Plecoptera and Trichoptera) taxa were found likely to occur. Subsequently, this preliminary risk

assessment study recommended to conduct field studies to assess the effects of imidacloprid on rice field communities and to evaluate the predictiveness of the RICEWQ model (Daam et al., 2013).

The aim of the present study was to increase our understanding on the fate and potential side-effects of imidacloprid following a single application according to its authorized use. The possible influence of actual (no withholding period) and recommended (7 days withholding period) water management practices on this was also assessed. By comparing measured field concentrations with calculated PECs, the accurateness of the RICEWQ model in predicting imidacloprid concentrations could be evaluated. In addition, field communities were monitored to assess whether realistic imidacloprid exposure exerts toxic effects on aquatic rice paddy life.

2. Material and methods

2.1. Experimental design and pesticide application

The study was performed in three experimental rice plots of the "Lezíria Grande de Vila Franca de Xira" area, situated in the vicinity of the River Tagus Estuary Natural Reserve (Portugal). This rice field area receives water from the Conchoso water catchment of the Tagus River, which is distributed through an irrigation canal by water adduction over the different rice plots. The three experimental rice plots used for the present study had a median surface area of 2.56 ha and contained a silty-clay soil with pH 5.9 ± 0.2 and an organic matter content of 3.8 ± 0.01 %. The climate is Mediterranean and the average annual rainfall is 700 mm, most of which falls between October and March.

Rice plots were set up according to standard agricultural practices in April 2013, which included one application of the preemergence herbicide oxadiazon. No insecticide applications had been made during the last three years prior to the study since no insecticide had been allowed for use in rice during that period. The rice was at the tillering (vegetative) stage and had been seeded 30 days prior to the start of the experiment. To avoid contamination from pesticide applications made in surrounding

rice plots, the selected rice plots were disconnected from the adjacent plots and only received water directly from the water catchment. Imidacloprid was applied to two of the three selected rice plots (T1 and T2) in July 2013 as the commercial product Confidor® 200 SC (200 g imidacloprid/L) at the recommended field dose of 100 g a.i./ha (EFSA, 2010) through direct aerial overspray with a spray volume rate of 300 L/ha. After application, the water outflow was closed in one of the applied plots (T1), whereas it was left open during the first seven days following application in the other rice plot (T2). Typical rice cropping strategies in Europe, including Portugal, have been reported to maintain rice fields closed (i.e., no water inlet or outlet) for two to seven days following pesticide application (MED-RICE, 2003). In actual agricultural practices in the study area, however, rice fields are not closed during or after pesticide applications (personal communication with the regional Portuguese Association of Rice Producers, AOP). Subsequently, the T1 and T2 treatments were chosen to include realistic recommended and actual pesticide application scenarios, respectively. The third rice plot (C) did not receive any pesticide application to serve as control and its water inflow and outflow were also left open.

2.2. PEC calculations in rice paddy fields

The first-tier PECs in rice paddy water were calculated in accordance with MED-Rice (2003) and US-EPA (2007). Input pesticide properties data for both model simulations were obtained from the draft assessment report of imidacloprid (EC, 2006; Table 1).

Besides using the two standard scenarios developed by MED-Rice (sandy soil and clay soil), the first-tier MED-Rice PEC was also calculated using input parameters measured at the field site (Table 1). For a detailed description of these methods and basic input parameters for the two standard scenarios, the reader is referred to Daam et al. (2013).

Higher-tier PECs in the paddy water and soil were calculated using the RICEWQ model. This corresponds to a step 3 PEC estimation, in which a site-specific calculation is performed taking all the required information of the local situation into account (MedRice, 2003). The RICEWQ model simulates the water and chemical mass balance associated with the unique flooding conditions, overflows, and controlled water releases

that are typical for a rice cropping system. The model applies the principle of mass balance to simulate water volume changes in the paddy and chemical residues in three media of the rice paddy (rice foliage, water column and benthic sediments) from the point of chemical application. To this end, water balance (e.g. precipitation), pesticide application (e.g. foliage interception), crop (e.g. plant growth) and water (e.g. partitioning between water and paddy soil) algorithms are applied (for a detailed description of the model see Williams et al., 2011). Values for crop practice and water management parameters used as input parameters were based on those estimated at the field site (Table 2). To this end, water depth was measured manually by using rulers mounted at both ends and the middle of the rice paddies. Hydrological properties of the soil like field capacity, wilting point, suspended sediment concentration and bulk density were measured at the beginning of the experiment. In case of the absence of measured data for certain parameters, parameterization was done according to expert judgement (Table 2).

The model calibration was perfomed using the two-step process (water balance calibration followed by pesticide balance calibration) as described in Christen et al. (2006). The dates of irrigation and rainfall and the amount of rainfall were known from field observations. The calibration of the irrigation of the rice plots was done both using the “fixed-volume” mode, which allows for the input of specified amounts to make the water balance as accurate as possible, and the automatic mode (Table 2). Evapotranspiration was assumed to be equal to pan evaporation, which is considered a valid assumption for an aquatic rice environment (Christen et al., 2006).

After the water balance was adequately calibrated, the pesticide balance was calibrated using field data where possible, supplemented with data from literature and general rice cultivation practices (Table 2). First, calibration was undertaken to match the initial pesticide concentration predicted by the model with that measured in the paddies by varying the application efficiency value. Initial water content of the rice pady soil was assumed to equal the field capacity. The value used in the model for the dissipation of imidacloprid (DT50) from the water layer was determined by regression analysis of measured pesticide concentrations throughout the experimental period.

Table 1. Input values used for the first-tier PEC (predicted environmental concentration) calculations in accordance with MEDRice (2003).

	This study	Source
INPUT: Scenario data I		
OC soil (%)	1.8	Measured in this study
Depth water (m) (water level in field)	0.1	Default
Water velocity field (L/sec/ha)	As MED-RICE (2003)	Default
Water velocity outflow (L/sec/ha)	0.5	Default
Leakage (mm/d) (infiltration rate)	As MED-RICE (2003)	Default
t close (d) (time of closure of field)	As MED-RICE (2003)	Default
t flood (d) (time of flooding)	As MED-RICE (2003)	Default
Depth canal (m) (deepness of outflow)	As MED-RICE (2003)	Default
INPUT: Scenario data II		
Area (m2) (area of rice field)	25600	Measured in this study
Volume of water in field (L)	2560000	Calculated
Depth soil (m)	As MED-RICE (2003))	Default
BD soil (kg/dm3) (soil bulk density)	As MED-RICE (2003))	Default
Grain density (kg/m3)	As US-EPA (2007)	Default
Sediment porosity (-)	As US-EPA (2007)	Default
Outflow rate (1/d)	0.017	calculated
Dilution factor	As MED-RICE (2003)	Default
Depth sediment (m) (active sediment depth)	As MED-RICE (2003)	Default
OC (%) of sediment	As MED-RICE (2003)	Default
BD sediment (kg/dm3) (sediment bulk density)	As MED-RICE (2003)	Default
INPUT: Product		
Dose (g/ha) (application rate of product)	100	This study
f dep (fraction of dose deposited to paddy field)	1	Default
f drift (fraction drift to adjacent water body)	0.0277	In accordance with FOCUS (2001)
Koc (dm3/kg)	178	US-EPA (2007)
Kd (soil) (dm3/kg)	3.2	Calculated
Kd (sediment) (dm3/kg)	2.8	Calculated
F sorbed (soil) (fraction partitioning to soil)	0.706	Calculated
F sorbed (sediment) (fraction partitioning to sediment)	0.176	Calculated
DT50 total,pw (d) in flooded soil system	14	Study in EC (2006) with comparable sediment
DT50 pw (d) in water phase	1.4	Study in EC (2006) with comparable sediment
DT50 soil (d) in solid phase	14	Study in EC (2006) with comparable sediment
DT50 total,sw (d) in sediment/water system	14	Study in EC (2006) with comparable sediment
DT50 sw (d) in water phase	1.4	Study in EC (2006) with comparable sediment
DT50 sed (d) in solid phase	14	Study in EC (2006) with comparable sediment

Table 2. Input values used for the Higher-tier PEC (predicted environmental concentration) simulations with RICEWQ in accordance with MEDRice (2003).

Data	Parameters	Units	Value (Paddy T1/T2)	Comments
Simulation management	Date simulation		June 16 2013	
	Date simulation ends		August 14 2013	
	Number steps per		24	
Crop practices	Seeding date		May 17 2013	Field data
	Emergence Date		May 22 2013	Field data
	Maturation Data			Not relevant simulation stops before
	Maximum crop		0.75/ 0.80	Crop interception at maturation time
	Deposition of crop		-1	-1=left alone; -2=crop residues removed
	Surface area of paddy	ha	2.39/2.56	Field data
	Number of pesticide		1	Field data
	Pesticide app. rate	$g.ha^{-1}$	100	Field data
	Pesticide app. date		July 16 2013	Field data
	Closure of paddy field	day^{-1}	C1 July 16 to July 25	Field data
	Application efficiency		1	Field data
Hydrology	Paddy water depth at	cm	11.5/10	Field data
	Depth of paddy	cm	17.5/ 18	Field data
	Irrigation rate	$cm.day^{-1}$	1.3	Varies
	Maximum drainage	$cm.day^{-1}$	5	Varies
	Starting date of	day^{-1}	June 19 2013	Field data
	Type of irrigation—			Both used
	Evaporation—read			Data from local meteorological station
Soil	Field capacity of	$cm^3\ cm^{-3}$	0.49	Field data
	Wilting point of	$cm^3\ cm^{-3}$	0.35	Field data
	Initial soil moisture of	$cm^3\ cm^{-3}$	0.49	Low relevance as the paddy is ponded
	Bulk density of	$g\ cm^{-3}$	0.99	Field data
	Suspended sediment	$mg\ L^{-1}$	62	Field data
	Depth of active	cm	5	A-horizon (Hornbuckle et al., 1999)

Table 2 (continued)

Data	Parameters	Units	Value (Paddy T1/T2)	Comments
Chemical	Initial concentration	ppm	0.056/0	Field data
	Aqueous metabolism	day^{-1}	0.02567	EPA (2007)
	Aqueous hydrolysis	day^{-1}	0	EPA (2007)
	Aqueous photolysis	day^{-1}	0.0866	EPA (2007)
	Saturated sediment	day^{-1}	0.02567	EPA (2007)
	Foliar decay rate	day^{-1}	0.017	From Inao et al. (1999)
	Wash off coefficient	cm day^{-1}	0.2	Assumed—model default
	Water–sediment	$cm^3\ g^{-1}$	7.52	EPA (2007)
	Solubility in water	ppm	580	EPA (2007)
	Volatilisation	m dia^{-1}	0	EC (2006)
	Settling velocity	m dia^{-1}	2	Calibrated, begins with model default
	Mixing depth for	cm	0.1	Calibrated, mixing depth is linked to mixing velocity
	Slow release		0	Liquid application

2.3. Chemical analyses

To enable verifying how well predicted imidacloprid concentrations (see previous section) relate to those actually occurring in the field, imidacloprid concentrations were determined in water from the rice paddies and drainage canals. In addition, a possible presence of pesticides in water from the water catchment entering the rice field plots was also evaluated. To this end, inlet water as well as water from the drainage canal were analyzed at the beginning of the experiment for the main pesticides used in the study area: the herbicides oxadiazon and profoxydim; the insecticides chlorpyrifos, imidacloprid and indoxacarb; and the fungicide azoxystrobin (AOP, personal communication). To evaluate exposure concentration dynamics of imidacloprid throughout the course of the 28 d-experimental period, three replicate water samples were taken at each sampling location one day before application, 3h after application, as well as 1, 3, 6, 14, and 28 days post application in all rice plots and the surrounding watershed. To this end, five 1-L samples were collected in amber glass bottles from different spots at each sample location and sent to the laboratory on ice for chemical analysis. Azoxystrobin, chlorpyrifos, imidacloprid, indoxacarb, oxadiazon and profoxydim were analyzed by liquid chromatography/mass spectrometry/mass spectrometry (LCMS/MS) according to DIN EN ISO/IEC 17025:2005. Limits of quantification (LOQ) were 0.05 µg/L for all coumponds analyzed, with an analytical recovery as determined for imidacloprid of 84 ± 6 % (mean ± SD; n = 4).

2.4. Field communities and water quality

On several moments throughout the experiment, nine replicate macroinvertebrate samples were taken in each rice plot by passing a 500-µm mesh-size Surber Sampler (Hydro-Bios, Kiel, Germany) through the entire water column of an approximately 50 x 25 cm surface area. Samples were preserved with formalin (4% v/v) and transported to the laboratory for identification. Macroinvertebrates were identified to the lowest possible taxonomic level, which was at the genus level for most taxa.

On sampling days, a 15-L bulk water sample was collected in a bucket by taking several depth-integrated water samples using a perspex tube. After taking subsamples for analysis of chlorophyll-a and nutrients as described below, the bucket was emptied until a final volume of 5 L. This was passed through a zooplankton net (mesh size 55 µm; Hydrobios Kiel, Germany) and the concentrated zooplankton sample was fixed with formalin to a final concentration of 4% (v/v). Three replicate samples were taken at each rice plot. Rotifers, cladocerans and ostracods were identified in subsamples to the lowest possible taxonomic level (genus level for most taxa) with an inverted microscope (Olympus CH-2). Copepods were separated into nauplii (immature stages), calanoids, and cyclopoids (mature stages). Numbers were recalculated to numbers per liter rice paddy water.

Phytoplanktonic chlorophyll-a measurements were made by filtering a known water sample volume over a Whatman GF/C glass fibre filter (mesh size 1.2 µm). Pigment extraction was performed with 90% acetone and quantified by spectrophotometry (Parsons et al., 1984). Subsequently, chlorophyll-a concentrations were calculated as described in Lorenzen (1967).

Dissolved oxygen (DO), water temperature, electrical conductivity (EC) and pH were measured directly in the paddy water using a WTW Multiline F/set-3 multiprobe. Water samples for nutrient analysis were collected in triplicate, filtered immediately after collection (0.2-mm nylon membrane filters, Whatman) and frozen until analysis. The nutrients analyses for ammonium, nitrate, and orthophosphate were determined by molecular absorption spectrophotometry using a Skalar segmented flow analyser (Houba et al., 1988).

2.5. Data analysis

The accuracy of the predicted pesticide concentrations was assessed by comparing them with the measured imidacloprid concentrations. Modelling accuracy was determined through the goodness of fit as indicated by the root mean square error (RMSE; Loague and Green, 1991).

The effects of the insecticide treatment on the zooplankton and macroinvertebrate communities were analyzed by the principal response curve (PRC) method (van den Brink and ter Braak, 1999) performed using the CANOCO software package (Ter Braak, 2009). The canonical coefficients calculated by PRC express the part of the variance in community structure, which can be attributed to treatment. By plotting the community-level multivariate response against time (x-axis), treatment effects are separated from temporal changes in community structure and therefore easy to interpret. Treatment effects are expressed as deviations from the control so that control becomes a straight line over time, to which treatments may contrast. With the PRC, calculated species weights can be interpreted as the affinity of the taxon to the principal response curve. Species with a high positive weight are indicated to show a response similar to that indicated by the PRC, whereas those with a negative weight show a response opposite to that indicated by the PRC. Species with a near-zero weight are indicated to show either a response very dissimilar to that indicated by the PRC or no response at all. To assess significant differences between the biological communities of the two treated paddies (T1, T2) and the control paddy (C) for each sampling date, a one-way analysis of variance (ANOVA) was performed using the sample scores on axis 1 from each RDA analysis, followed by post hoc multiple comparasion post hoc testing using Dunnett´s and Newman-Keuls tests. Before running the ANOVAs, homogeneity of the variance was tested and could be confirmed for each sampling date using the Levene's test.

The responses of individual taxa and taxonomic groups were analysed using RM-ANOVA followed by a Tukey's test to assess whether there was a significant response to treatments exhibited by these taxa/taxonomic groups over time. One-way ANOVA was also used to compare the aquatic abiotic factors (including chlorophyll a, pH, temperature, dissolved oxygen, water depth, ammonium, nitrate, orthophosphate) among the three treatments. These analyses were performed using STATISTICA 7.0 (Stat Soft Inc., 2004). Statistical significance was accepted at $p < 0.05$.

3. Results and discussion

3.1. Pesticide concentrations measured in the field

Besides oxadiazon and imidacloprid, concentrations of all other pesticides in the field samples were below their detection limit (DL). The maximum concentrations of the herbicide oxadiazon determined over the course of the experiment were DL (irrigation canal), 1.3-1.5 µg/L (three paddy plots) and 0.81 µg/L in the drainage canal. Given its absence in the irrigation water and the fact that this herbicide is often used at preemergence (EU, 2007), it is most likely that the oxadiazon detections in paddy and drainage water originated from residues that remained in the paddy from previous applications on the paddy soil.

Imidacloprid concentrations in the control paddy plot and irrigation water remained 0.1 µg/L throughout the experimental period. Peak imidacloprid concentrations following its application to the open and closed paddy plots were 56 µg/L and 60 µg/L, respectively. In the irrigation canal, the highest imidacloprid concentration (8.8 µg/L) was also measured on the first sampling moment following the imidacloprid applications.

Half-lives (DT50) of imidacloprid in the paddy water as determined based on measured concentrations were 2.7 days for the closed paddy plot and 1.3 days for the open paddy plot. In line with this, Daam et al. (2013) reported DT50 values of one to three days for imidacloprid in another Portuguese rice field area. Interestingly, these DT50 values also correspond to those reported in Japonese, Indian and Chinese rice fields following imidacloprid application (DT50 = 1.6 – 4 days; Daam et al., 2013 and references therein). The peak imidacloprid concentrations of 56 - 60 µg/L discussed above are also similar to those reported in these previous studies (e.g., 52 µg/L: Portugal, Daam et al., 2013; 49 µg/L: Japan, Hayasaka et al., 2012; 53 µg/L: Vietnam, La et al., 2015). It hence appears that despite differences in application methods, pesticide commercial formulation tested and field conditions, peak loading and fate of compounds like imidacloprid are very consistent in spatial-temporal distinct rice agroecosystems.

3.2. Accurateness of simulated exposure profiles in paddy and drainage water

The first-tier PEC in paddy water calculated with the MED-Rice method using a site specific scenario was 30 µg/L. Although first-tier assessments may be expected to lead to worst-case predictions, the peak concentrations actually measured in the imidacloprid-treated rice plots (56-60 µg/L) were approximately two times higher than the simulated first-tier concentration. Daam et al. (2013), using the two standard first tier MED-Rice scenarios, also reported that the measured field concentration was about twice as high as the PECs calculated with these scenarios. Similarly, the modelled peak imidacloprid concentration in the drainage canal water (0.43 µg/L) was more than an order of magnitude lower than that actually measured in the field (8.8 µg/L). Future studies are therefore needed to validate the accurateness of the current first-tier exposure assessment of pesticides in European rice paddies.

The water balance calibration conducted for the higher-tier RICEWQ fate model showed a good match between simulated and observed water depths (RMSE = 0.060 cm for T1 and RMSE = 0.080 for T2). The initial peak concentration in paddy water as well as the overall exposure profile over the course of the experiment also closely matched with those resulting from measurements in the field (Figure 1; RMSE= 5 µg/L for T1 and RMSE= 6.8 µg/L for T2). Previous validation studies of the model under European conditions also showed a high agreement between observed and predicted pesticide concentrations (e.g. Capri and Miao, 2002; Karpouzas et al., 2005; Karpouzas and Capri, 2006; Christen et al., 2006; Infantino et al., 2008). Subsequently, RICEWQ is currently considered to be the most reliable model for higher-tier exposure assessment in European rice paddies.

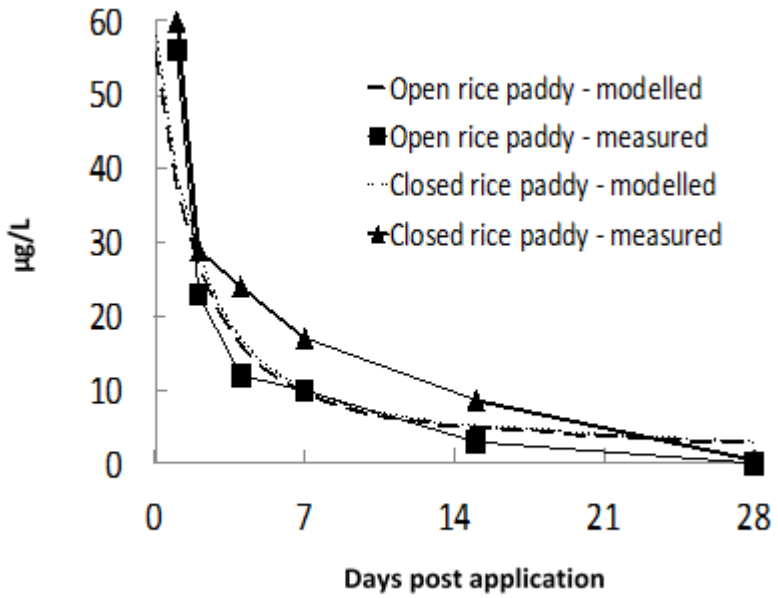

Fig. 1. Measured and modelled (using the RICEWQ model) imidacloprid concentrations in the paddy water (in µg/L).

3.3. Effects of imidacloprid on ecosystem structure

Both in terms of number of species and their abundances, the zooplankton communities in the rice plots were dominated by rotifers, followed by cladocerans, copepods and ostracods. The PRC diagram indicates clear deviations of the communities in both treated rice plots from the control (Figure 2). For example, the most discriminating species from the PRC analysis, the rotifer *Polyarthra euryptera*, completely disappeared from the open paddy field, whereas only a relatively small effect on this species was noted in the closed field (Figures 2 and 3A). The clearest toxic effect of the imidaclopid application on zooplankton was denoted for ostracods (Figure 3B). Laboratory as well as (semi) field studies have indeed shown that ostracods are sensitive to imidaclopid, especially when compared to other zooplankton groups (Sánchez-Bayo and Goka, 2006a,b; Hayasaka et al., 2012; Daam et al. 2013).

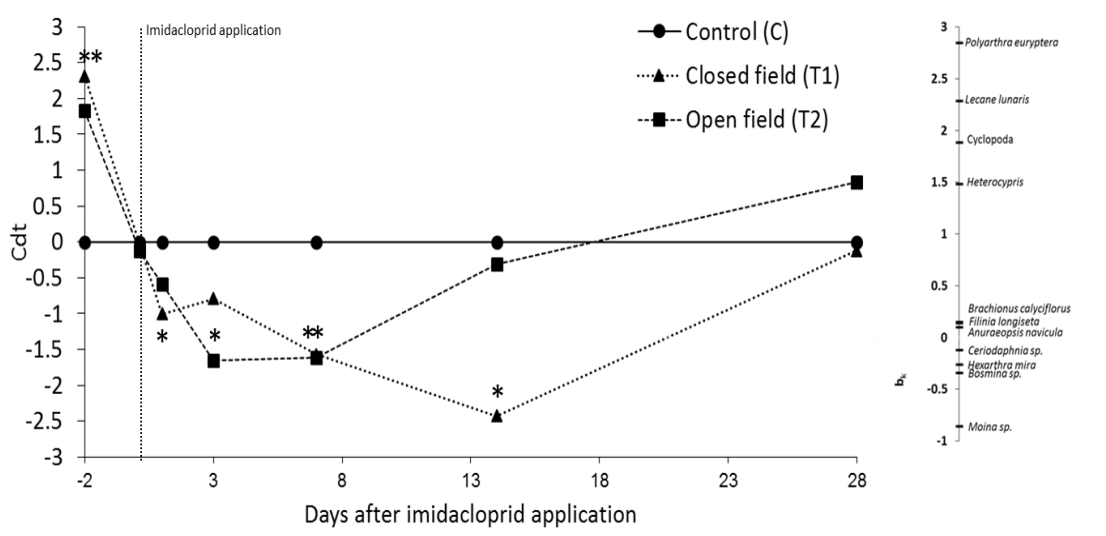

Fig. 2. Principal response curves (PRCs) resulting from analysis of the zooplankton data set, indicating the treatment effects of imidacloprid on the zooplankton community. Of all variance, 25% could be attributed to sampling date; this is displayed on the horizontal axis. Of the total variance, 38.2 was allocated to the treatment regime by the PRC analysis. Of this variance, 41% is displayed on the vertical axis. The lines represent the course of the treatment levels over time. The species weight (bk) can be interpreted as the affinity of the taxon with the PRCs. The PRC diagram displays a significant amount of the treatment variance ($p = 0.0020$). Asterisks indicate significant differences between treatments and the control.

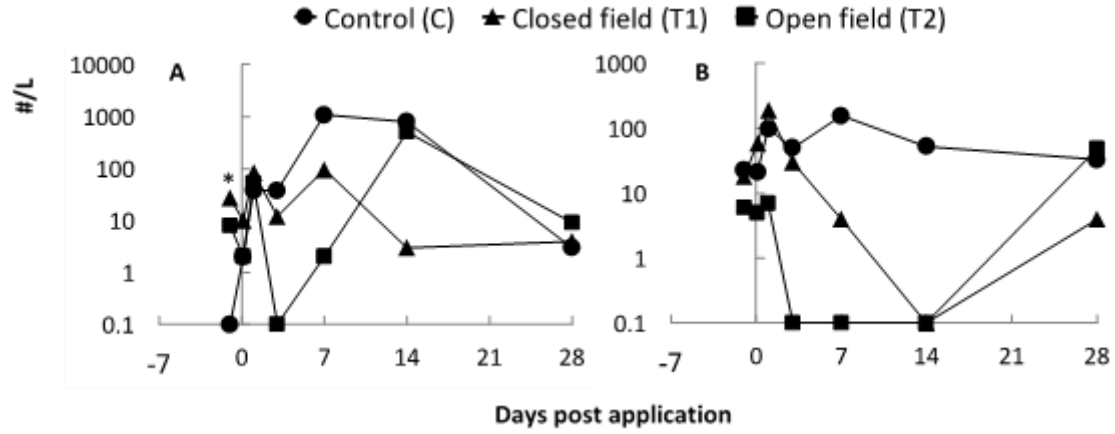

Fig. 3. Change over time in densities (#/L) of the two most discriminative zooplankton taxa: Polyarthra euryptera (A) and ostracoda (B). A value of 0.1 denotes absence of the taxon. Asterisks indicate significant differences between treatments and the control in paired comparison (Tukey's, $p \leq 0.05$).

The insect communities were dominated by Diptera (mostly Ephydridae, Culicidae and Chironomidae) and, to a lesser extent, Coleoptera (Hydrophilidae and Dytiscidae). Taxa from other taxonomic groups (Odonata, Plecoptera, Decapoda, Hemiptera and Ephemeroptera) were only encountered in low numbers and/or on individual sampling days. Previous studies in European rice paddies also showed dominance of insects with

short life cycles belonging to Diptera, Coleoptera and Hemiptera, whereas species species with longer cycle like some dragonflies were localised mostly in adjacent water bodies with longer water permanence periods (Leitão et al., 2007; Lupi et al., 2013; Simpson and Roger, 1995). Subsequently, insects belonging to Ephemeroptera, Plecoptera and Trichoptera (EPT) are generally not well represented in European rice paddies, as was the case in our study.

Based on toxicity data evaluations, EPT taxa have been demonstrated to possess the greatest sensitivity to imidacloprid, whereas dipterans and coleopterans may be expected to be only moderately sensitive (e.g. Daam et al., 2013; Rico and Van den Brink, 2015; Morrissey et al., 2015 and references therein). Despite this, significant deviations in macroinvertebrate community structure between treated and untreated rice plots were indicated by the PRC (Figure 4). In line with this, reductions in numbers of dipterans (Figure 5A to 5D) and coleopterans (Figure 5E) could also be demonstrated in paddies treated with imidacloprid with the univariate statistical analyses (Tukey's test, $p \leq 0.05$).

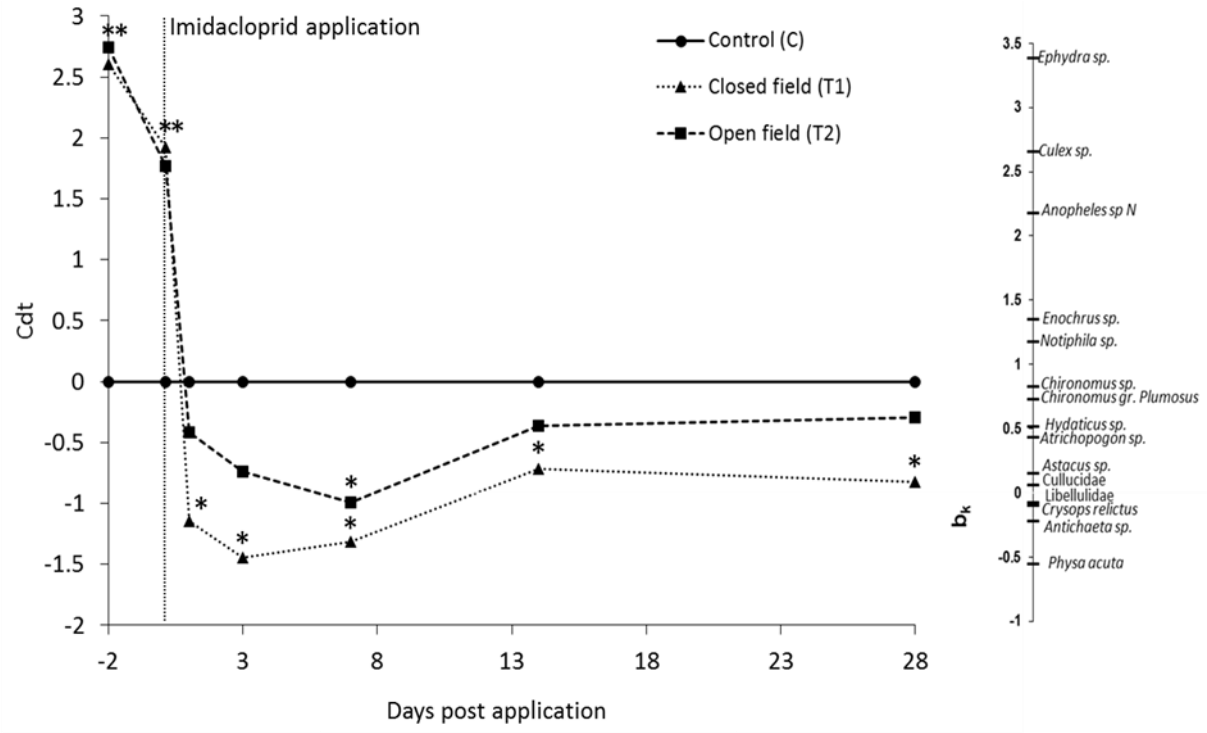

Fig. 4. Principal response curves (PRCs) resulting from analysis of the macroinvertebrates data set, indicating the treatment effects of imidacloprid on the macroinvertebrate community. Of all variance, 21% could be attributed to sampling date; this is displayed on the horizontal axis.Of the total variance, 29.2% was allocated to the treatment regime by the PRC analysis. Of this variance, 31% is displayed on the vertical axis. The lines represent the course of the treatment levels over time. The species weight (bk) can be interpreted as the affinity of the taxon with the PRCs. The PRC diagram displays a significant amount of the treatment variance ($p = 0.0020$). Asterisks indicate significant differences between treatments and the control.

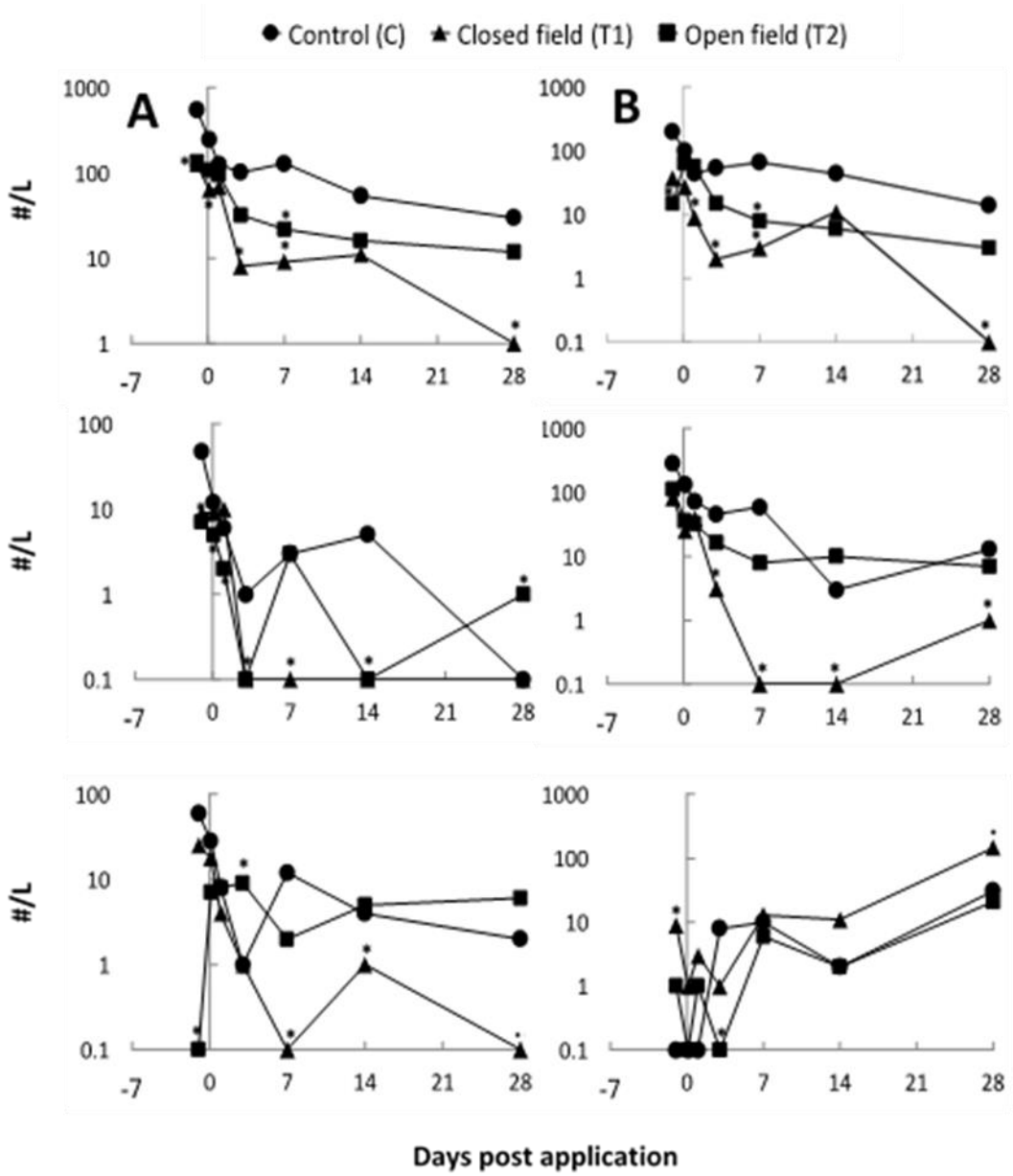

Fig. 5. Change over time in numbers of the most discriminative macroinvertebrates: Diptera (A), Ephydra sp. (Diptera; B), Chironomus plumosus (Diptera; C), Culicidae (Diptera; D), Coleoptera (E), Mollusca (F). A value of 0.1 denotes absence of the taxon. Asterisks indicate significant differences between treatments and the control in paired comparison (Tukey's test, $p \leq 0.05$).

Pestana et al. (2009) reported that outdoor stream microcosms treated with imidacloprid up to 17.6 µg imidacloprid/L did not result in significant decreases in abundances of coleopterans and dipterans. However, these authors also discussed that this tolerance of aquatic coleopterans was unexpected given that imidacloprid is used to control terrestrial coleopteran pest species (Pestana et al., 2009). The presence of

sensitive non-target coleopterans in the paddies used in our study may hence be related to this. Similarly, the effects on shore flies (Ephydridae; Figure 5B) and chironomids (*C. plumosus*; Figure 5C) in our study is not surprising given the fact that they are also known pest organisms in rice fields (Leitão et al., 2007; USAID, 2009). In field-based microcosms, decreased survival of four out of five chironomid species of the subfamilies Tanypodinae and Orthocladiinae were observed after exposure to 7.5 µg imidacloprid/L (Colombo et al., 2013). Laboratory bioassays with different chironomid species have also indicated that Chironomidae contain sensitive taxa to imidacloprid (Stoughton et al., 2008; Azevedo-Pereira et al., 2011; LeBlanc et al., 2012). A species sensitivity distribution (SSD) constructed with laboratory EC50 data for dipterans (Figure 6) indeed shows that effects on chironomids could be expected at the (peak) imidacloprid concentration measured in the treated paddy fields.

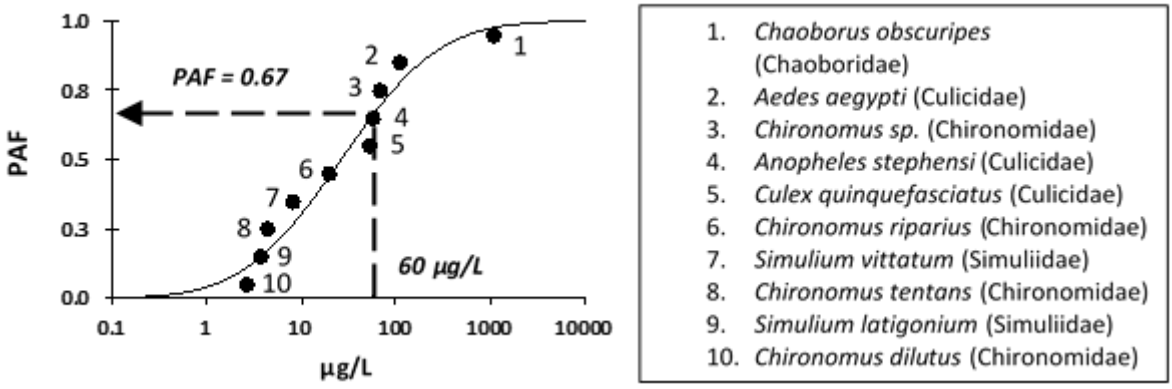

Fig. 6. Species sensitivity distributions (SSD) as constructed based on published 2d-4d EC50 (effect concentration 50%) for dipterans available in the US Environmental Protection Agency (US-EPA) ECOTOX database (available via: http://cfpub.epa.gov/ecotox/). Since immobility data were hardly available, only (geometric mean) lethal toxicity data were included in the curve that was constructed with the ETX computer program, version 2.0 (Van Vlaardingen et al., 2004). The Anderson–Darling Test included in this software indicated that lognormality could be accepted at the 5% significance level. The potentially affected fraction (PAF), i.e. the number of taxa for which their toxicity values are expected to be exceeded based on the SSD curve, is indicated at the measured peak-concentration (MEC) of 60 mg imidacloprid/L. For details on SSD construction, the reader is referred to Daam et al. (2013).

Based on the LC50 values of imidacloprid available for Culicidae, no complete elimination of Aedes sp. (Culicidae) as noted in the closed paddy field would be anticipated (Figure 5D). In this regard, Sánchez-Bayo and Goka (2006a) discussed that the mortality endpoint (LC50) is not a reliable predictor of the effects of imidacloprid

under field situations (e.g. rice paddies). These authors discussed that this is because the paralysis effect induced by this insecticide takes place at much lower concentrations than those required to cause the death of the animals: regardless of the taxa, differences as large as 100- or 600-fold were observed between the EC50 and LC50 for the same exposures (Sánchez-Bayo and Goka, 2006a). Camp and Buchwalter (2016) also highlighted the importance of evaluating sublethal endpoints and further demonstrated that an increase in temperature is a powerful modulator of sublethal toxicity within a range of environmentally relevant temperatures by impacting both uptake rates and metabolic rates. The higher temperatures in the closed treated paddy plot (Figure 7A) may hence be a possible explanation for the greater toxicity on several macroinvertebrates observed in this plot as compared to the open treated paddy plot (Figures 4 and 5) since exposure profiles were not very different between the two plots (Figure 1).

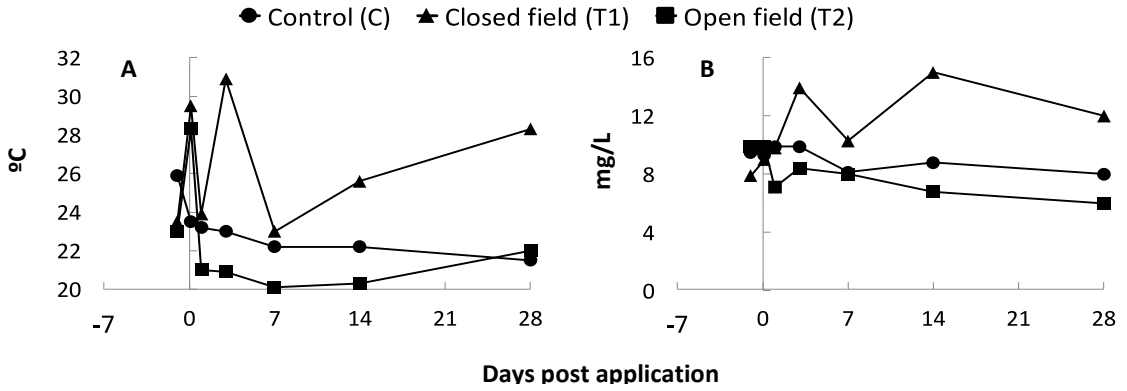

Fig. 7. Dynamics of temperature (A) and dissolved oxygen concentration (B) over the course of the experiment in the different treatments. Asterisks indicate significant differences between treatments and the control in paired comparison (Tukey's test, $p \leq 0.05$).

From two weeks post application onwards, the numbers of the snail *Physa acuta* were approximately five times higher in the closed paddy applied with imidacloprid than in the other two treatments (Figure 5F). Increased abundances of (tolerant) snails have previously been demonstrated in rice fields treated with imidacloprid (Colombo et al., 2013) and other insecticides (e.g. Leitão et al., 2007) and may be explained by the decrease in competition for food and substrate with sensitive invertebrates.

Direct and indirect effects of the imidacloprid applications could be demonstrated on several zooplankton and macroinvertebrate groups (Figures 3 and 5). The zooplanktonic populations and communities appeared to recover relatively fast, while not all the

macroinvertebrate populations recovered within the study period (Figures 2 through 5). This could indeed be anticipated based on the short generation times and high passive dispersal characteristics of zooplanktonic taxa as compared to macroinvertebrates (Rico and Van den Brink, 2015).

The analysis on community level of as visualized in the PRCs for zooplankton (Figure 2) and macroinvertebrates (Figure 4), also demonstrated differences in community structures between treated and untreated rice plots in the pre-treatment sampling (Figure 2 and 4). Invertebrate communities in rice paddies are indeed known to present large variability and temporal fluctuation due to agronomic water management leading to continuous inflow and elimination of populations with irrigation and drainage water, respectively (Capri and Karpouzas, 2008). Small differences in agricultural practices (irrigation and drainage, seeding density etc) between rice plots have been demonstrated to result in large spatial-temporal variation in invertebrate communities (e.g. Leitão et al., 2007; Capri and Karpouzas, 2008; Hayasaka et al., 2012). The number of rice plots that can be included in field studies as replicates is limited for logistic reasons. The use of several enclosures in multiple rice plots, and allocating these aleatorically within each rice plot to the different treatments, could be a way forward in future rice field studies to allow a greater replication with lower variation.

3.3. Aquatic risk assessment and mitigation options

Imidacloprid caused a decrease in several invertebrates and an increase in the snail *Physa acuta*, with clearest effects in the closed paddy plot (Figures 2 through 5). Given that pesticides may be applied by direct overspray in rice paddies, unacceptable impacts on ecological function instead of biodiversity parameters should be the main consideration when effects on aquatic rice systems are assessed (EC, 2002). In other words, structural changes as observed in our study should be acceptable within the paddy as long as the function is maintained (Capri and Karpouzas, 2008). As discussed in the previous section, two of the most sensitive organisms (chironomids and shore flies) are known target organisms underlying imidacloprid applications (Leitão et al., 2007; USAID, 2009). Paradoxically, chironomids are also often requested as standard test

species in aquatic effect assessments (e.g. EFSA, 2013). Protecting ecosystem structure in aquatic rice agroecosystems may hence indeed be considered to be an unrealistic protection goal (Daam et al., 2013).

Macroinvertebrates in rice paddies play an important role in recycling organic matter and their decomposition rates have indeed been demonstrated to be reduced even at sublethal imidacloprid concentrations (e.g. Kreutzweiser et al., 2008; Pestana et al., 2009). In addition, reduced grazing on algae resulting from the decrease in ostracods and other invertebrates after imidacloprid exposure may lead to algae blooms that may compete with the rice plants for nutrients (Sánchez-Bayo and Goka, 2006b; Daam et al., 2013). The absence of effects on the low nutrient levels (ammonia <7 mg/L; ortho-phosphate and nitrate <0.2 mg/L in all treatments) and chlorophyll-a levels (mean ± SD: 12 ± 4 µg/L) in the present study indicates that ecosystem functioning could be maintained in the plots treated with imidacloprid (one-way ANOVA; $p > 0.05$). This is most likely the result of the increase abundances of the snail *Physa acuta* that took over the role of the more sensitive grazers (i.e. functional redundancy; Figure 5F). Furthermore, no effects on microbial decomposing activity are to be expected at the imidacloprid concentrations measured in the treated plots (Pestana et al., 2006; Kreutzweiser et al., 2008). The increase in oxygen concentrations in the closed rice plot (Figure 7B) might have been due to decreased respiration rates resulting from the death of invertebrates, the more since the substituting snail grazers are air-breathers. Invertebrate death resulting from insecticide exposure have indeed previously been reported to possibly contribute to decreased oxygen levels (e.g. Brock et al., 2000). The fact that the water in the closed plot was not renewed, and hence that less oxygen may have been lost through drainage, propably also played a large role in this.

If ecosystem functioning rather than structure is chosen as the protection goal of infield rice agroecosystems, the effects noted in the treated plots discussed above would be considered acceptable. Although effects on ecosystem structure are indeed generally considered to be inevitable in in-field rice systems, specific local conditions, agricultural practices and particular aspects of environmental protection (e.g. in cases where paddies are located close to protected areas or irrigation water is feeding into protected water bodies) should also be taken into consideration (MED-Rice, 2003). South

European rice field areas are often located in or in the vicinity of Natural Reserves with great importance as habitats for waterfowl and migratory bird species

(MED-Rice, 2003; Toral et al., 2012), as is the case for our study area (e.g. Alves et al., 2010). Since the log Kow for imidacloprid is 0.57, a risk assessment for secondary poisoning for invertebrate or fish eating birds and mammals was not required in the prospective European risk assessment of imidacloprid (EC, 2006). However, invertebrates constitute a substantial part of the diet of many bird species during the breeding season and are indispensable for raising offspring, indicating that the loss of food through the decrease in invertebrates as observed in the present study could affect bird populations indirectly. Hallman et al. (2014), for example, demonstrated that at imidacloprid concentrations of more than 20 nanograms per litre, insectivorous bird populations tended to decline by 3.5 per cent on average annually.

Depending on agricultural practices and their physical-chemical parameters (e.g. solubility, Koc and DT50), pesticides applied to rice paddies may leach to groundwater and spread over waterways though drainage and exert ecological side-effects (e.g. Faria et al., 2007; Christen et al., 2008; Daam et al., 2013; Jin et al., 2016). Lamers et al. (2011), for example, reported that 16% of the imidacloprid mass applied to the paddy was lost to surrounding watersheds. The peak imidacloprid concentration of 8.8

µg/L as measured in the drainage canals of the present study is more than an order of magnitude higher than the maximum ecological quality reference value of 0.2 µg/L of imidacloprid in Europe, whereas annual-average benchmark values set for imidacloprid are even as low as 0.0083 to 0.067 µg/L (Morrissey et al., 2015 and references therein). At the end of the 28d experiment, imidacloprid concentrations in the paddy water were still 0.2 (open field) and 0.56 µg/L (closed field). Subsequently, a withholding period of at least 28 days would be needed to allow time for imidacloprid residues in the paddies to dissipate to levels that may be considered acceptable for (acute) environmental protection prior to being discharged from the field. The current withholding period of zero days (actual practices) and two to seven days (MED-RICE, 2003) hence do not suffice to avoid potentially unacceptable ecological risks from pesticide drainage to adjacent waterways. Longer withholding periods have indeed frequently been recommended (e.g. Inao et al., 2008; Jin et al., 2016) and applied (e.g. 21-28 days for molinate; Christen

et al., 2008) to reduce the concentrations and hence risks of pesticides in paddy field drainage water.

4. Conclusions

The calibration and validation exercise of the RICEWQ model indicated that this model adequately predicted imidacloprid concentrations in the rice paddy water. This thus supports the use of this model for higher-tier PEC estimatons, as was also concluded in previous studies with different pesticides. The method currently used in the EU for lower-tier PEC calculations in rice paddy water, however, underestimated peak imidacloprid concentrations and hence needs further evaluation and improvement.

Several invertebrates (ostracods, dipterans and coleopterans) decreased in numbers following imidacloprid treatment. The increase in snails (*Physa acuta*), however, seemingly contributed to the ecosystem functioning through functional redundancy, implying no unacceptable risks in the paddy if ecosystem function is set as the protection goal. In this case, however, care has to be taken to have sufficient adjacent non-agricultural wetlands to avoid secondary poisoning and food limitations to bird populations. In addition, withholding periods currently used and recommended in the EU are insufficient to avoid the spread of imidacloprid over watersheds and hence need to be increased..

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CHAPTER 4

EVALUATION OF FOCUS SURFACE WATER PESTICIDE CONCENTRATION PREDICTIONS AND RISK ASSESSMENT OF FIELD-MEASURED PESTICIDE MIXTURES – A CROP-BASED APPROACH UNDER MEDITERRANEAN CONDITIONS

Based on the following manuscript:

Pereira AS, Daam MA, Cerejeira MJ (2017). Evaluation of FOCUS surface water pesticide concentration predictions and risk assessment of field-measured pesticide mixtures – a crop-based approach under Mediterranean conditions. *Environmental Sciences and Pollution Research.* doi:10.1007/s11356-017-9393-7

Abstract

FOCUS models are used in the European regulatory risk assessment (RA) to predict individual pesticide concentrations in edge-of-field surface waters. The scenarios used in higher tier FOCUS simulations were mainly based on Central/North European conditions and work is needed to underpin the validity of simulated exposure profiles for Mediterranean agroecosystems. In addition, the RA of chemicals are traditionally evaluated on the basis of single substances although freshwater life is generally exposed to a multitude of pesticides. In the present study we monitored 19 pesticides in surface waters of five locations in the Portuguese 'Lezíria do Tejo' agricultural area. FOCUS step 3 simulations were performed for the South European scenarios to estimate predicted environmental concentrations (PECs). We verified that 44% of the PECs underestimated the measured environmental concentrations (MEC) of the pesticides, showing a non–compliance with the field data. Risk was assessed by comparing the environmental quality standards (EQS) and regulatory acceptable concentrations with their respective MECs. Risk of mixtures was demonstrated in 100% of the samples with insecticides accounting for 60% of the total risk identified. The overall link between the RA and the actual situation in the field must be considerably strengthened, and findings from field studies on pesticide exposure and effects should be carried out to assist the improvement of predictive approaches used for regulatory purposes.

Keywords: modelling, $FOCUS_{sw}$, pesticide mixtures, Risk assessment, Mediterranean surface waters

1. Introduction

The application of pesticides to agricultural areas can result in the contamination of edge-of-field surface water bodies (Schwarzenbach et al. 2006). To protect sensitive freshwater ecosystems against pesticide side-effects, a so-called tiered approach has been adopted in the European Union (EU) for the admission of pesticides on the market. These tiers are based on the comparison of a RAC (Regulatory acceptable concentration) with the environmental predicted exposure (PEC: Predicted Environmental Concentration) (EC 2009a; EFSA, 2013). The PEC is calculated using environmental fate modelling as developed by FOCUS (Forum for the Co-ordination of pesticide fate models and their Use; FOCUS 2001). The FOCUS modelling approach consists of four different tiers that increase in complexity and realism from Step 1 to Step 4. Whereas Steps 1 and 2 of the $FOCUS_{sw}$ (FOCUS surface water) package consist of lower-tier exposure assessments based on generic worst-case approaches, Step 3 also considers the diversity of soil and climate across the EU. For Step 3, the $FOCUS_{sw}$ Workgroup developed ten soil–climate scenarios at the EU level for calculating PEC in surface water bodies that receive pesticide residues through spray drift, runoff and drainage. The FOCUS surface water working group reported that the Step 3 PEC_{sw} (PEC surface water) estimated from the ten developed scenarios are likely to represent at least a 90th percentile worst-case for surface water exposures resulting from agricultural pesticide use within the EU (FOCUS 2001). Subsequently, these scenarios should hypothetically represent the so-called 'realistic worst-case' situations with respect to the heterogeneity of European soil and climate conditions. The recent revision of the legal framework for authorization of use of plant protection products (Regulation EC 1107/2009 and Directive 2009/128/EC; EC 2009a, b) imposes a need for close collaboration across country borders within the three pesticide authorization zones (designated the north, central, and south zones) in Europe. The principles of zonal evaluation and mutual recognition embedded in Regulation EC 1107/2009 are intended to reduce the approval times for pesticides. However, the three authorization zones represent a very simplified view compared to the 16 climatic zones/scenarios that have been outlined for pesticide modelling in Europe (Blenkinsop et al. 2008).

The European Food Safety Authority (EFSA) responsible for the EU peer review of active substances used in plant protection products (PPPs) and risk assessment methodologies in the EU advised to "critically evaluate and improve the surface water exposure assessment in the future" (EFSA 2013). The intention of $FOCUS_{sw}$ was not to perform a specific national exposure assessment but to cover the range of possible conditions that could occur throughout Europe. However, $FOCUS_{sw}$ is also routinely used in many Member States to assess national pesticide exposure in prospective risk assessments. The level of protectiveness of $FOCUS_{sw}$ soil-climate scenarios for specific national agro-environmental conditions, however, remains unclear and needs field validation (EFSA 2013).

The generic FOCUS scenarios are mostly based on North/Central European conditions and therefore need further experimental and monitoring work to underpin the validity of exposure profiles for Mediterranean areas (Brock et al. 2010; Daam et al. 2011a). For example, although spray drift is assumed to be the main route of edge-of-field surface waters in North/Central Europe, runoff and soil erosion can be the largest contributors to pesticide surface water contamination in Mediterranean countries, particularly after heavy rainfall following a period of drought (Ramos et al. 2000). Studies into the comparison of FOCUS predictions with field-measured pesticide exposure may therefore be especially needed in the South European countries.

A study by Knäbel et al. (2012) reported that, although FOCUS Step 1 and 2 PECs generally overpredict MECs (measured environmental concentrations) in surface water, MECs exceeded 23% of step 3 and 31% of Step 4 PECs. These authors hence concluded that the protectiveness of the higher-tier FOCUS exposure assessment may be disputed (Knäbel et al. 2012). A follow-up study also demonstrated that FOCUS model predictions are neither protective nor appropriate for predicting concentrations of fungicides in the field in the context of European pesticide risk assessment (Knäbel et al. 2014).

After a pesticide is authorized and in use, field concentrations should not exceed their RACs as not to compromise pre-authorization risk assessments and to adhere to the general and specific protection goals outlined in EU pesticide legislation (EFSA 2010, 2013; EC 2009a; Nienstedt et al. 2012). Monitoring data of pesticides in surface waters are very useful to review the authorization retrospectively, even though the routine monitoring is often restricted to larger water bodies and differs therefore from the edge-

of-field approach used in the authorization process (Knauer 2016). In larger water bodies concentrations of pesticides are expected to be lower than in the edge-of field water bodies, since they are not the first entry point (Knauer 2016).

Exceedances of RACs and/or environmental quality standards (EQS) by concentrations measured in surface waters indicate a possible need for action, e.g. to adjust the conditions of use of certain products (Crommentuijn et al. 2000; Boye et al. 2012; Bundschuh et al. 2014; Kreuger and Nilsson 2001). In addition, although the current European ERA of pesticides is based on assessments of individual compounds, different agricultural practices may cause the presence of pesticide mixtures that may vary in terms of their complexity (Altenburger et al. 2013). As cumulative stress of toxicants has been identified as one of the main pressures affecting ecological status, mixture risks have to be evaluated and reduced (Brock 2013). Due to the overall smaller size of farms in South Europe as compared to countries in North Europe, it is more likely that different pesticides could be involved at the regional level because each farmer will take their own decision (Ramos et al. 2000). The need for studies into environmental side-effects of pesticide mixtures may thus be especially important for Southern EU (Ramos et al. 2000; Daam et al. 2011a).

Predicting the risk for mixtures of all the compounds applied on a specific crop allows estimating the risk posed to aquatic ecosystems at a crop-based level rather than as a function of individual chemicals (e.g. Daam et al. 2011b).

The need for field investigations to exemplarily verify exposure and effect predictions in environmental risk assessments has frequently been stressed in recent years (e.g. Artigas et al. 2012; EFSA 2013). The present study aimed at contributing to this by a) evaluating the predictive power of FOCUS Step 3 scenarios by comparing in edge-of-field water bodies in a typical Mediterranean crop area; b) evaluating the actual aquatic risks in the field by comparing MECs with their respective maximum acceptable concentrations as determined through prospective (RACsw) and retrospective (MAC-EQSs) effect assessments; and c) evaluate the risks of environmental-realistic pesticide mixtures.

2. Materials and methods

2.1. Study field characteristics and sampling points

The research area “Lezíria Grande de Vila Franca de Xira” located on the river Tagus lowlands, is an alluvial plain with approximately 13000 ha of irrigated farmland. It is bounded by two rivers, the Tagus and the Sorraia, and located in the highest part of the estuary of the River Tagus, about 25 km upstream from Lisbon. The climate is Mediterranean and the average annual rainfall is 700 mm, most of which falls between October and March. About 20 per cent of the area is covered by light-to-medium-textured, mainly fluvial, deposits; the remaining 80 per cent is heavy-textured marine deposits, most of which is moderately to very saline. The study area is located within one of the most important areas for Portuguese horticulture and cereal crops and is mainly dominated by rice, tomato and maize crops. Part of the research area lies in the Natural Reserve of the Tagus Estuary, a portion of the Tagus estuary that became a nature reserve by the Portuguese Decree Law 565/76 and has a high biotic diversity (Caçador et al. 2000, 2013) with a vast number of migratory birds using this estuary regularly (Delany et al. 2009). The reserve has an area of almost 15000 ha and includes estuarine waters, marshes, mudflats, salt pans, islands, channels, and agricultural land. During 2014, water samples were collected at 5 sites within the area: two sites in ditches alongside maize agricultural areas (M1 and M2 Locations) and three in tomato crop areas (T1, T2 and T3 locations). The schematic map of the research area can be found in Figure 1. Sampling was performed on nine occasions during May and August 2014 in order to account for the main period of agricultural activities in the area.

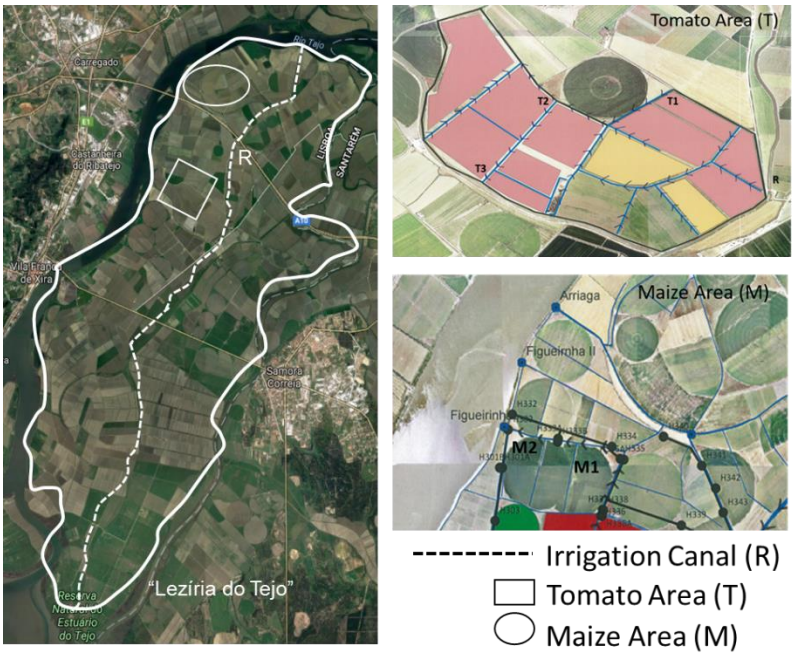

Fig. 1. Sampling sites at the "Lezíria do Tejo" agricultural area. Sampling sites M1, M2 were located in ditches on a maize area, whereas sampling sites T1, T2 and T3 were located in ditches on a tomato crop area.

2.2. Pesticide compounds selected for the study and exposure assessment

Nineteen compounds from fourteen different chemical classes (8 herbicides, 8 insecticides and 3 fungicides) were selected for inclusion in the study based on the following criteria: (A) their allowance for use on the main crops in the study area as well as their actual use as indicated by farmers and local associations (personal communication); (B) affinity for water compartment through the Level I fugacity model (Mackay 2001); (C) detections (frequent and/or in high concentrations – above their respective parametric and quality standards in surface waters) in previous monitoring studies conducted by our research team (e.g. Cerejeira et al. 2003; Silva et al. 2012, 2015); (D) high PECs obtained in preliminary (default) runs with the FOCUS STEP 1 & 2 model (FOCUS 2001) (i.e. Toxicity Exposure Ratio ≥ regulatory trigger values) and (E) compounds of particular concern with regard to mixture toxicity (e.g. known synergists; Cedergreen 2014).

Concentrations of the selected pesticides in the water samples were measured by an external Laboratory following the standard guidelines (analysis GC–MS and LC–MS/MS) according to DIN EN ISO/IEC 17025:2005.

2.3. FOCUS modelling

PECs were calculated using the software tool SWASH 5.3 (Surface Water Scenarios Help; Van den Berg et al. 2015) for the nine substances that were applied by the farmers during the monitoring period in the maize and tomato area. Within SWASH, the models PRZM 4.3.1 (Carsel et al.1998) and MACRO 5.5.4 (Jarvis and Larsbo 2012) calculate water and substance fluxes that enter the water body via runoff/erosion and drainage. The model TOXSWA 4.4.2 (Adriaanse et al. 2014; Beltman et al. 2012) simulates the fate of the pesticide in the water body following loading caused by spray drift deposition and either runoff/erosion or drainage. The software PAT (Pesticide Application Tool) is implemented in the SWASH shell to determine actual application dates. The model selects appropriate dates from an application window that is specified by the user according to the application range, number of applications and the interval between applications.

FOCUS calculations rely on several input parameters related to the pesticide, application, crop type, climate and landscape scenarios. The selection of the most appropriate scenarios was mainly based on climatic conditions relevant for the respective field study, cultivated crops (crops are associated with particular scenarios) and pesticides input pathway (as detailed in Table 1). FOCUS Step 3 simulations of each of the eight compounds were made for all crops on which this compound was applied and for all scenarios that are representative for South-European conditions. Pesticide characteristics were mainly taken from European review and draft assessment reports, whereas application parameters (application rate, frequency and interval) were set at levels indicated by farmers and local associations (personal communication; Table 1).

Table 1. Pesticide parameters and field data used in FOCUS Step 3 surface water simulations.

	Unit	CTP	CPF	CYP	IMI	IND	CYM	DET	TBT	MET	Note
Molar Mass	g/mol	483.15	350.89	416.3	255.7	527.83	198.18	201.68	229.71	214.29	[A],[B]
Saturated Vapor Pressure	mPa	6.3E-08[a]	1.43[b]	2.3E-04[b]	4E-07[a]	6E-03[a]	1.5E-04[b]	0.35[b]	0.12[a]	1.21E-04[a]	[A],[B]
Solubiliy in water	mg/L	0.88[a]	1.05[a]	0.009[a]	610[a]	0.2[a]	780[a]	327.1[a]	6.6[a]	1050[a]	[A],[B]
Molar enthalpy of dissolution	J/mol	27000	27000	27000	27000	27000	27000	27000	27000	27000	[C]
Diffusion coefficient in water	m^2/d	4.3E-05	4.3E-05	4.3E-05	4.3E-05	4.3E-05	4.3E-05	4.3E-05	4.3E-05	4.3E-05	[C]
Diffusion coefficient in air	m^2/d	0.43	0.43	0.43	0.43	0.43	0.43	0.43	0.43	0.43	[C]
Koc	L/kg	301	8151	26492	223	5125	43.6	78	231	11.5	
Freundlich exponent		0.95	0.9	1	0.8	0.9	0.86	0.86	0.93	0.9	[A],[B],[C]
Ref. Concentration in liquid phase	m^3/d	1	1	1	1	1	1	1	1	1	[C]
Factor for uptake by plants roots in soil		0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	[C]
Wash-off factor from crop	1/mm	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	[C]
Half-life time water	d	1000	5	3	138.8	1.4	0.3		6	31.1	
Half-life time soil	d	138	50	60	118	3-11	0.7	71	71	14.5	
Half-life time sediment	d	343	1000	17	1000	1000	0.3	1000	1000	1000	[C]
Half-life time crop	d	10	10	10	10	10	10	10	10	10	[C]
Activation energy	J/mol	65400	65400	65400	65400	65400	65400	65400	65400	65400	[C]
Exponent	1/K	0.0948	0.0948	0.0948	0.0948	0.0948	0.0948	0.0948	0.0948	0.0948	[C]
Q10 factor		2.58	2.58	2.58	2.58	2.58	2.58	2.58	2.58	2.58	[C]
Exponent for the effect of water content		0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	[C]
Half-life measured at pF 2		2	2	2	2	2	2	2	2	2	[C]
Exponent for the effect of water content		0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	[C]
Half-life measured air moisture content	%	100	100	100	100	100	100	100	100	100	[C]
Use bi-phase degradation	y/n	n	n	n	n	n		n	n	n	
Crop		Tomato	Maize	Maize	Tomato	Tomato	Tomato	-	Maize	Tomato	[D]
Applicattion rate	g a.s./ha	40	500	75	0.1	37.5	2000	-	900	750	[D]
number of applicattions	n	2	1	1	2	1	1	-	2	2	[D]
Minimum Interval between applications	d	7	n.a.	n.a.	18	n.a.	7	-	n.a.	13	[D]

CTP -chlorantraniliprole; CPF-chlopyrifos; CYP-cypermethrin; IMI-imidacloprid; IND-indoxacarb; CYM-cymoxanil; DET-desethyl - terbuthylazine; TBT-terbuthylazine; MET –metribuziN
[A] Data were taken from the European review report (EU, 2016); [B] Data were taken from the Draft assessment report (EFSA, 2016); [C] Focus default (FOCUS, 2001); [D] Field data
[a] measured at 20ºC
[b]measured at 25ºC
n.a. not applicabl

The MECs were compared to the calculated PECs to assess the degree of correctness in the predictions. All the pesticide concentrations measured on individual sampling moments in surface waters were assessed rather than only using the maximum measured concentrations, since it has been discussed that the latter may result in a data set biased toward worst-case conditions (Knäbel et al. 2012). To assure a more correct way to compare MECs with FOCUS PECs, the 90th percentile of measured concentrations over all sampling sites and sampling events was used, i.e. the 90th percentile in space and time was calculated (after Reichenberger 2013).

Subsequently, the FOCUS predictions were evaluated by a) comparing the highest MEC for each substance with its highest PEC_{sw} as calculated by FOCUS and b) comparing the 90th percentile MEC of a substance with its PEC max for a given substance/crop combination.

2.4. Evaluation of the prospective and retrospective risk assessment using the MECs

The protectiveness and field relevance of the prospective risk assessment for a Mediterranean typical agroecosystem was evaluated following the approach developed by Stehle and Sulchz (2015).

To this end, to assess the overall protectiveness of the prospective risk assessment procedure we compared the MEC_{max} with the respective EU-level RACs for the approval of active substances using the RAC data from Stehle and Sulchz (2015).

The EU WFD 2000/60/EEC (EC 2000) uses a retrospective risk assessment approach by comparing chemical monitoring data with environmental quality standards (EQSs) for EU-wide priority substances. Therefore, the aquatic risks of all pesticide compounds detected in surface waters of the “Leziria do Tejo” were also evaluated retrospectively by evaluating the Frequency of exceedance of their respective maximum acceptable concentration EQS values (MAC-EQSs) as an indicator. The MAC-QS were taken from the revised (second) list of priority substances (EC 2013) and, for those not listed in EC (2013), from reports by different EU Member states for specific pollutants (DEFRA 2014; Ecotoxcentre 2016; Johnson 2012; Vorkamp and Sanderson 2016; RIVM 2016). MAC-

EQS values could be encountered for all compounds measured in outfield sites with the exception of the fungicide cymoxanil. To assess the frequency of MAC-EQS exceedance, the number of samples where the threshold was exceeded was divided by the total number of samples where the respective pesticide compound was monitored (c.f. formula 1).

Frequency of exceedance $\mathrm{MAC-EQS} = {\sum n}/{N}$ x 100 (formula 1)

where n is the number of samples with MAC-QS exceedance and N is the total number of samples where analytical measurements were carried out for the respective pesticide.

2.5. Risk assessment of pesticides mixtures

Mixture toxicity was assessed using environmental quality standards (EQS values) together with the MEC values of the pesticides, following the tiered approach described by Moschet et al. (2014). Firstly, as a worst-case estimation of the mixture toxicity, no grouping of the compounds by toxic mode of action was performed in the first tier assessment, which was hence performed by summing up the MEC/EQS ratios ($RQ_{mixture}$) of all compounds. For a mixture with i pesticides, the risk characterization ratio of the mixture (RQ) is the sum of all the risk characterization ratios of the individual compounds (RQi), assuming that concentration addition model is applicable (c.f. formula 2).

$$RQ = \sum_{i=1}^{n} \frac{MEC}{EQS} \text{ (formula 2)}$$

When the value of the RQ of the mixture is larger or equal to 1 a potential environmental risk is identified (Backhaus and Faust 2012). Secondly, the risk was calculated for the two main pesticide classes encountered in the study area (herbicides and insecticides; Table

2) separately. In this way, only RQs of substances from the same pesticide type were summed ($RQ_{herbicides}$ and $RQ_{insecticides}$). This realistic worst-case approach was done in order to determine which pesticide type most affects the total risk (Moschet et al. 2014). In addition, this allowed to have a better estimation of which taxonomic group may be expected to be especially at risk, i.e. arthropods with high $RQ_{insecticides}$ values (e.g. Maltby et al. 2005) and primary producers with high $RQ_{herbicides}$ values (e.g. Van den Brink et al. 2006). The relative importance of each substances for the RQs was evaluated through the concept of maximum cumulative ratio (MCR) that provides a quantitative measure of the magnitude of the toxicity that is underestimated by not performing a cumulative risk assessment (Price and Han 2011).

3. Results and Discussion

3.1. Pesticide exposure

During the monitoring campaign, five herbicides (glyphosate, metolachlor, metribuzin, terbuthylazine and rimsulfuron), six insecticides (chlorantraniliprole, chlorpyrifos, cypermethrin, imidacloprid, indoxacarb and lambda-cyhalotrin), two fungicides (chlorothalonil and cymoxanil) and two metabolites [aminomethylphosphonic acid (AMPA) and desethyl-terbuthylazine (DET)] were detected in surface waters of “Leziria do Tejo”. Table 2 presents the maximum and average concentrations for the pesticides encountered in the surface waters of the field site “Lezíria do Tejo”. Detection frequencies varied from 1 to 45 occurrences per pesticide in a total of 45 samples. Sites showing particularly high levels of certain pesticide classes were M2 (organophosphates and triazines), M1 (pyrethroids) and T1 (anthranilic diamides and phosphonoglycines; see Fig. 1 and Table 2), the peak measured concentrations of that substances corresponding with the information from the farmers and organizations regarding pesticide applications made in these sites (personal communication). As expected from the sales and application information in the study area (personal communication), herbicides had the highest detection frequencies (88%), followed by insecticides (70%) and fungicides (24%). In line with this, Silva et al. (2015) also found herbicides as the

most frequently and generally in highest concentrations of the different pesticide types analysed in the “Tejo” river basin. In the present study, triazines were detected in 100% of the samples with a highest concentration of 8.5 µg/L for terbuthylazine. Nevertheless, the highest median concentration was found for the organophosphate herbicide glyphosate (1.6 µg/L) and its metabolite AMPA (4.1 µg/L). Herbicides detected in the five ditches are mainly applied to widespread arable crops such as maize (e.g., S-metolachlor, terbuthylazine) and tomato (e.g., metribuzine, rimsulfuron) so their detections most likely resulted due to spray drift and/or run-off from adjacent agricultural fields. From the detected herbicide compounds, terbuthylazine was indeed applied during the monitoring campaign in the maize fields (M1 and M2) and metribuzin was applied in the tomato fields (T1, T2 and T3) (Tables 1 and 2). Glyphosate is allowed for use in the channels of the study area since 2000 to control of the water hyacinth *Eichhornia crassipes* that could lead to irrigation and drainage canal obstruction due to its excess growth (Moreira et al. 2002a, b). The presence of high glyphosate (and its metabolite AMPA) in high concentrations and frequencies in both canal types (75%) could thus be associated with the use of glyphosate for aquatic hyacinth control.

From the organophosphates insecticides, chlorpyrifos was the pesticide with the highest detected concentration, with a maximum of 12 µg/L (in M2). In the case of the pyrethroids, cypermethrin had the highest concentration (up to 10 µg/L in M1). In line with this, the insecticides chlorpyrifos and cypermethrin were applied in the maize crop field during the monitoring period and the insecticides chlorantrinilaprole, imidacloprid and indoxacarb, together with the fungicide cymoxanil had been applied in the tomato fields (see Table 1 and Table 2). The presence of these insecticides can hence be associated with the respective pesticide application practices in the maize and tomato crops.

Table 2. Measured environmental concentrations (MECs) of pesticides in the 45 samples taken at the 5 ditches in tomato and maize crop areas “Lezíria do Tejo” in 2014.

Common name	**Type**[a]	**Chemical Group**	**Avg. MEC (µg/L)**	**σ**	**Max. MEC (µg/L)**
aminomethylphosphonic acid	M	organophosphate	4.01	4.51	16.0
chlorantraniliprole	I	diamide	0.83	1.03	4.50
chlorothalonil	F	aromatic	0.03	0.00	0.03
chlorpyrifos	I	pyridine organothiophosphate	0.56	2.15	12.0
cymoxanil	F	aliphatic nitrogen	0.03	0.00	0.03
cypermethrin	I	pyrethroid ester	0.57	2.10	10.0
desethyl-terbuthylazine	M	chlorotriazine	0.24	0.32	1.10
glyphosate	H	organophosphate	1.67	3.70	3.90
imidacloprid	I	neonicotinoid	0.38	0.81	3.00
indoxacarb	I	oxadiazine	0.03	0.91	1.70
lambda-cyhalothrin	I	pyrethroid ester	0.05	0.01	0.05
metolachlor	H	chloroacetanilide	0.61	0.95	2.80
metribuzin	H	triazinone	0.09	0.13	0.96
rimsulfuron	H	pyrimiddinylsulfonylurea	0.03	0.00	0.03
terbuthylazine	H	chlorotriazine	0.58	1.78	8.50

[a] F = fungicide; H = herbicide; I = insecticide; M = metabolite

The substances azoxystrobin; ethoprophos; folpet; glufosinate were always below the detection limit

The other detected pesticides, such as the herbicides rimsulfuron, S- metolachlor, the insecticides indoxacarb, lambda-cyalothrin and the fungicide chlorothalonil were also registered for use in maize and tomato in the period of the monitoring campaigns, so their detections most likely resulted due to spray drift and/or run-off from adjacent agricultural fields or from applications in fields not indicated by the farmers. Consequently, a relation between surface water residues in terms of agricultural use pattern for 'Lezíria do Tejo' ditches is clearly visible. Some of the pesticides detected in this study (metolachlor, terbuthylazine, and chlorpyrifos) have also previously been detected in surface water samples collected in the 'Tejo' river basin in the scope of chemical monitoring programmes carried out namely by the Portuguese environmental authorities (Cerejeira et al. 2003; SNIRH 2016; Silva et al. 2015).

3.2. Compliance of PECsw FOCUS Step 3 simulations

The accuracy of FOCUS Step 3 PECs (calculated using application information from the field sites) in predicting MECs under typical Mediterranean maize and tomato crop conditions was evaluated (Table 3). By comparing the PEC_{max} calculated for the four scenarios representative of South European conditions (D6, R2, R3, R4) with MEC_{max} in the field, we verified that 44% of the predictions underestimated actual concentrations measured in the field (PEC < MEC). The average underestimation was 31 times, with the highest underestimation encountered for cypermethrin (98 times). The remaining 56% PEC values overestimated their respective MEC value (PEC> MEC), with an average of 11 times and a maximum of 43 times (metribuzin). When evaluations were made using the 90^{th} percentile for each substance, the percentages of compounds with an underestimation and overestimation of PECs showed a similar pattern (Table 3). However, the extent by which MECs were underestimated was reduced, as may be illustrated by the reduction of 98 times to 29 times as noted for cypermethrin. On the other hand, compounds for which PECs showed an overestimation of the MEC showed an increase in its extent when the 90^{th} percentile was used (e.g. for metribuzin from 43 to up 160 times; Table 3). The best agreement in PEC and MEC was verified for the insecticides imidacloprid and indoxacarb with an underestimation of 0.25 and 0.22 times respectively when using the 90^{th} percentile concentrations. In spite of the low number

of compounds analysed, herbicide concentrations appear to be generally overpredicted and insecticides and fungicide concentrations tend to be underpredicted by the FOCUS model scenarios. Recent studies have shown that 23% and 15% of the measured insecticide and fungicide field concentrations, respectively, were more than ten times underpredicted by the Step 3 PECs calculated with FOCUS when applied exactly as is done within the regulatory risk assessment for pesticides, which questions the protectiveness of the FOCUS exposure assessment (Knäbel et al., 2012, 2014). In addition, Knäbel et al. (2012) also verified that 42% of all simulated FOCUS Step 3 sediment concentrations underestimated the measured fungicide concentrations in sediment. For the higher-tier FOCUS predictions (Step 4), Knäbel et al. (2012) discussed that approximately a third (i.e., 31%) of the simulated insecticide water concentrations underestimated the field concentrations and 6.5% were underestimated by more than 10 times. However no other study with respect to herbicides FOCUS Step 3 overprediction and underprediction exists to our knowledge. Overall, the results show a discrepancy between the PEC_{sw} Step 3 predictions using South European scenarios with real-world concentrations measured in a typical Mediterranean field scenario. This could hence reflect unacceptable ecological effects that are not assessed by the current regulatory risk assessment. Further research is needed to evaluate whether this is the result of incompatibility of current FOCUS scenarios with Mediterranean field conditions and/or point source pollution resulting from applications that do not comply with agreed intended uses (e.g. higher application doses and/or frequencies) by pesticide applicators.

Table 3. Predicted surface water concentrations with FOCUS Step 3 for pesticides applied in tomato and maize crop study areas during the monitoring campaign in 2014 and their respective maximum measured concentrations and 90th percentile for all the samples during the monitoring campaign in 2014

	chlorantraniliprole	chlorpyrifos	cypermetrin	imidaclopride	indoxacarbe	cymoxanil	DET	terbuthylazine	metribuzin
PEC_{sw} (D6 Ditch) (µg/L)	0.311	**2.622**	**0.102**	1.747	**6.246**	**0.003**	0.780	5.242	5.602
PEC_{sw} (R2 Stream) (µg/L)	0.377	2.410	0.070	0.917	0.205	0.001	1.684	9.124	12.470
PEC_{sw} (R3 Stream) (µg/L)	1.004	2.564	0.076	3.018	0.219	0.001	0.582	5.118	23.140
PEC_{sw} (R4 Stream) (µg/L)	**2.262**	1.812	0.054	**3.315**	0.155	0.002	**2.607**	**30.712**	**42.000**
MEC_{max} (µg/L)	4.500	12.000	10.000	3.000	1.700	0.061	1.100	8.500	0.960
90^{th} percentile MEC (µg/L)	2.510	2.940	3.040	0.832	1.430	0.050	0.900	2.800	0.261

Bold values – PECmax

3.3. Single substances aquatic risk assessment

For all of the 15 detected substances with exception of the fungicide cymoxanil (detection frequency of 6%), a MAC-EQS value could be encountered in the literature (Table 4). Firstly, the risk of individual substances was evaluated, which was considered as the least conservative risk assessment scenario. In total, ten substances contributed at least once to an exceedance of a MAC-EQS in the 45 water samples taken at the different sites and sampling moments (Table 4). These substances consisted of 3 herbicides, 6 insecticides, and 1 metabolite. The most critical substances were cypermethrin and lambda-cyalothrin that exceeded the MAC-EQS in 50% of the samples taken in the maize crop area, followed by DET (37%), terbuthylazine (34%) and chlorpyrifos (25%) (Table 4). In the tomato crop area, rimsulfuron (50%), chlorantraniliprole (28%), metolachlor (16%), imidacloprid (15%) and indoxacarb (12%) exceeded the MAC-EQS in the most frequent manner. These results are in agreement with Sthele and Schulz (2015) who verified that WFD priority substances such as chlorpyrifos and cypermethrin largely exceeded their respective MAC-EQS. Similarly, recent studies into the prioritization of pollutants in Mediterranean rivers concluded that pesticides and their derivates were the most important compounds in contributing to risk to aquatic ecosystems, with chlorpyrifos identified as one of the most important compounds (Kuzmanović et al. 2015; López-Doval et al. 2012, Silva et al. 2015).

The WFD comprises a list of substances subject to review for possible identification as priority substances or priority hazardous substances, although EQS remain undefined for these substances. This list includes the neonicotinoid insecticide imidacloprid that was also monitored in the present study. The maximum concentration detected of 3.0 µg/L is substantially higher than the maximum ecological quality reference value of 0.2 µg/L of imidacloprid in Europe, whereas annual-average benchmark values set for imidacloprid are even as low as 0.0083 to 0.067 µg/L (Morrissey et al. 2015 and references therein). Recently, Silva et al. (2015) derived EQS for some of pesticide compounds detected in the “Tejo” river basin, including the compounds metolachlor, metribuzin and terbuthylazine for which MAC-EQS of 5.6, 2 and 0.99 µg/L, respectively, were set. The maximum detected values for those substances in the present study, 2.8, 0.96 and 8.5 µg/L, respectively, all exceeded these MAC-EQSs.

With regards to the protectiveness of the prospective risk assessment, the MECs of the organophosphate insecticide chlorpyrifos and the pyrethroid insecticide cypermethrin MEC_{max} were up to 100 and 480 times higher than their respective regulatory RAC_{sw}. Also the pyrethroid lambda-cyalothrin, the neonicotinoid imidacloprid and the triazine terbuthylazine were detected in concentrations up to 23, 10 and 7 times higher than their RAC_{sw}, respectively. Stehle and Schulz (2015) conducted a comprehensive meta-analysis in which they verified that 44.7% of the 1566 measured insecticide concentrations in EU surface waters exceeded their respective RACs. Pyrethroids (n=108) appeared to be the pesticide class with the highest percentage of RAC_{SW} exceedances (70.4%); followed by organophosphorus insecticides (37.5%; n=1100) and neonicotinoids (24.2%; n=33) (Stehle and Schulz 2015).

From the above it can be deducted that especially insecticides may form an important threat to European freshwater biodiversity, as insecticide levels above their RACs may lead to severe biodiversity reductions (Stehle and Schulz 2015). Previous field studies have also reported pesticide-induced adverse effects on ecosystem function and aquatic biodiversity in small agricultural surface waters (e.g., Berenzen et al. 2005; Bereswill et al. 2013; Malaj et al. 2014; Schäfer et al. 2012). Malaj et al. (2014), for example, concluded that of all the 223 chemicals they evaluated on a continental scale, pesticides were among the major contributors to the chemical risk. No scientific knowledge on insecticide surface water exposure is available for approximately 80% of European high-intensity agricultural areas, which indicates that future monitoring studies are needed to further quantify risks across the EU, the more since climate change is expected to lead to increasing insecticide applications in European agriculture (Kattwinkel et al. 2011).

Table 4. Frequency of exceedance of quality standards to protect aquatic organisms, expressed as the maximum allowable concentration (MAC-EQS) for 15 compounds in surface waters of the 'Lezíria do Tejo'

Common name	MAC-EQS (µg/L)	RAC_{sw}[g] (µg/L)	Freq. Of MAC-EQS (%)	
			Maize crop ditches	Tomato crop ditches
aminomethylphosphonic acid	1500 [a]	-	0	0
chlorantraniliprole	0.97 [b]	-	n.d.	28
chlorothalonil	1.2 [c]	-	n.d.	0
chlorpyrifos	0.1 [d]	0.1 [Higher tier (0.001)]	25	0
cymoxanil	-	-	n.p.	n.p.
cypermethrin	0.0006 [d]	0.025 [Higher tier (0.003)]	50	n.d.
desethyl-terbuthylazine	0.5 [e]	-	37	0
glyphosate	200 [e]	-	0	0
imidacloprid	0.2 [e]	0.3 [Higher Tier (0.552)]	n.d.	15
indoxacarb	0.42 [f]	-	1	12
lambda-cyhalothrin	0.00047 [b]	0.0021[Tier I]	50	n.d.
metolachlor	2.1 [b]	-	n.d.	16
metribuzin	1.1 [b]	-	n.d.	0
rimsulfuron	0.012 [f]	-	n.d.	50
terbuthylazine	1.3 [b]	1.2 [Tier I]	34	3

[a]Ecotoxcentre (2016)
[b]RIVM (2016)
[c]DEFRA (2014)
[d]EC (2013)
[e]Vorkamp and Sanderson (2016)
[f]Jonhson (2012)
[g]Regulatory acceptable concentrations for water (RAC_{sw}) their respective tiers (higher tiers denote microcosm/ mesocosm studies) of the RAC_{sw} setting. See Stehle and Schulz (2015) for further details on RAC_{sw}
n.p. - not possible
n.d. - not detected

3.4. Risk assessment for pesticide mixtures

When applying the worst-case scenario (i.e. summation of all RQs), 100% of the surface water samples (n=45) exceeded the $RQ_{mixture}$ of 1, up to an RQ of 16721, indicating high potential environmental risks of the pesticide mixtures (Figure 2a). Insecticides were responsible for the largest part of the mixture risk, accounting for 60% of the total risk on average. As can be concluded from Figure 2a, in almost all the samples with an $RQ_{mixture}$ above 1, the MCR values were smaller than 2 indicating that one pesticide compound contributed at least 50% of the total mixture toxicity. The maize crop ditches (M1 and M2) clearly showed the highest mixture risks, with cypermethrin (36%),

chlorpyrifos (26%) and lambda-cyalothrin (21%) as the main contributors to the mixture toxicity in samples taken in these agricultural field sites.

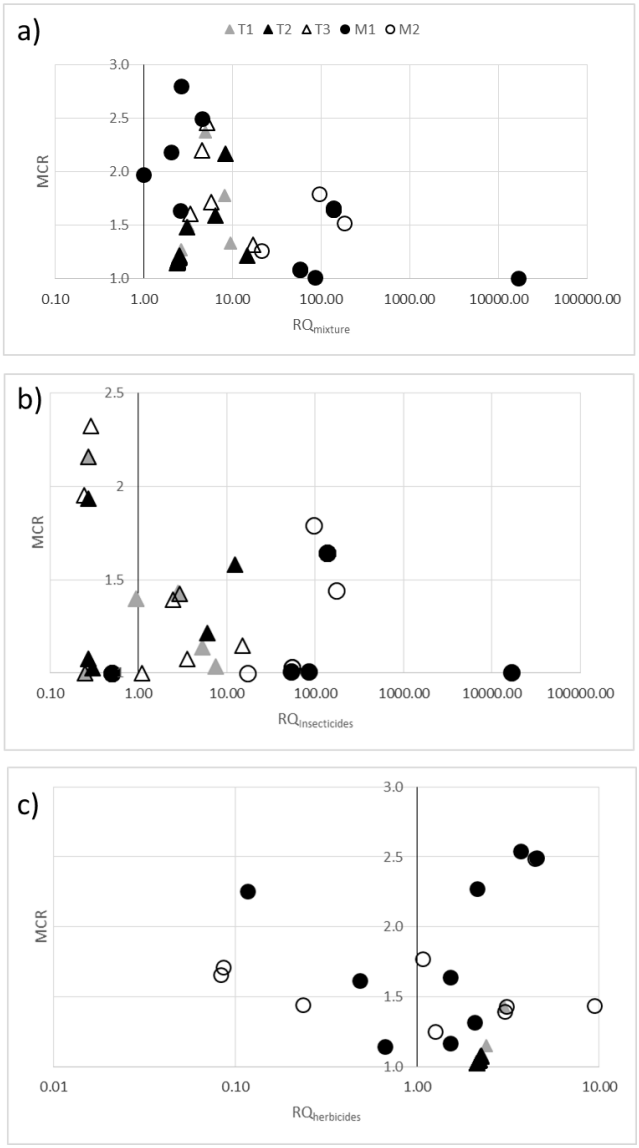

Fig. 2. Log-linear plot of MCR versus RQ (MAC-EQS/ MEC) of the (a) mixture (all pesticides), (b) insecticides and (c) herbicides detected in surface waters of the study area.

In relation to the risk of insecticides, an $RQ_{insecticides}$ greater than 1 was encountered in 53% of the samples (Figure 2b). The MCR values were always smaller than 2, indicating that one insecticide contributed at least 50% of the insecticide mixture toxicity in all samples. Chlorpyrifos and cypermethrin contributed 36% and 35% of the mixture toxicity in M1 and M2, respectively, whereas chlorpyrifos (32%), chlorantraniliprole (32%) and imidacloprid (24%) were the main contributors for the insecticide mixture toxicity in tomato ditch samples.

For herbicides, 84% of the samples showed an $RQ_{herbicides}$ greater than 1, with 9.4 as the highest RQ (Figure 2c). Similarly to the pattern discussed for insecticides, MCR values were smaller than 2 in almost all the tomato crop samples, indicating that one pesticide compound contributed at least 50% of the mixture's toxicity, with rimsulfuron as the main contributor to this pattern. For four samples in the maize crop ditches the MCR values ranged between 2.26 and 2.54 (Figure 2c). These values indicate that the fraction of toxicity exerted by the most toxic pesticide compound ranged from 34 to 35%, with the metabolite DET and the herbicides terbuthylazine and metolachlor as the main contributors.

The results of our study are in accordance with chemical monitoring data that reveal that in edge-of-field surface waters usually a limited number of pesticides dominate the mixture of contribution for the overall calculated risk (Belden et al. 2007; Moschet et al. 2014; Silva et al. 2015; Schäfer et al. 2007; Verbruggen and Van den Brink, 2010; Verro et al. 2009). Gregorio and Chèvre (2014) also used the risk quotient methodology to retrospectively assess the risk posed by mixtures of chemicals (mainly pesticides) in the Geneva Lake and the Rhone River and identified the most problematic substances demanding risk reduction. The authors showed that the risk levels associated with mixtures of compounds can rapidly exceed critical aquatic thresholds, and that the sum of substances may lead to risk situations. Consequently, the question which mixtures are present and which have associated combined risks becomes central for defining adequate monitoring and risk assessment. For this reason, the construction of exposure and effect databases for frequently occurring pesticide combinations (in water and sediment) that are likely to dominate the potential for risk in water bodies of agricultural landscapes will be an important way forward (Brock 2013).

The approach used in the present study did not consider synergistic effects, which indicates that actual risks of the pesticide mixtures may even be greater. A systematic review of environmental mixture toxicity studies by Cedergreen (2014) revealed that the difference between observed and predicted effect concentrations was rarely more than 10-fold. However, synergistic pesticide mixtures were especially noted for mixtures containing cholinesterase inhibitors or azole fungicides (Cedergreen 2014). In addition, the contamination of surface waters by herbicides and insecticides has the potential to cause 'ecological synergism' in which top down and bottom up trophic effects interact

(Relyea and Hoverman 2006). After reviewing model ecosystem studies evaluating pesticide mixtures, Verbruggen and van den Brink (2010) indeed concluded that when mixtures of pesticides that affect different biological endpoints (e.g., insecticides and herbicides) are evaluated, increased indirect effects are often observed due to food web interactions. Future studies should hence also include mixture effects in evaluating the risks associated with co-occurring pesticides in the field.

4. Conclusions and way forward

Our study revealed clear differences between MECs and PECs in a crop based approach under Mediterranean conditions, evidencing the inaccuracy of current predictive tools used in the EU regulatory framework for pesticides and highlighting the need to better tune them. Monitoring data are becoming increasingly important and part of the re-evaluation procedure of pesticides. The results of the prospective and retrospective aquatic risk assessment showed the importance of a tiered approach contributing to i) the identification of sites with the highest expected impacts of pesticide mixtures; ii) the evaluation of the major pesticide compounds that contributed mostly to the identified aquatic risks. The data generated in the approach followed can be used to the: derivation of optimized programs of measures under the scope of European legislation as well as for the identification of risk mitigation measures. The overall link between the regulatory risk assessment and the actual situation in the field should be considerably strengthened, and findings from our and other field studies on pesticide exposure and effects should should be carried out to assist the improvement of predictive approaches used for regulatory purposes. Real-world exposure data and actual ecological risks in the field should also be considered in future identification and prioritization of WFD priority substances.

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CHAPTER 5

TERBUTHYLAZINE AND CHLORPYRIFOS REALISTIC MIXTURES IN THE FIELD ARE SYNERGETIC?

Based on the two following manuscripts:

Silva E, Martins C, Pereira AS, Loureiro S, Cerejeira MJ.Toxicity assessment and prediction of an environmentally realistic pesticide mixture to *Daphnia magna* and *Raphidocelis subcapitata* (submitted to *Ecotoxicology*)

Pereira AS, Cerejeira MJ, Daam MA (2017). Toxicity of environmentally realistic concentrations of chlorpyrifos and terbuthylazine in indoor microcosms. *Chemosphere* 182, 348-355.

Toxicity assessment and prediction of an environmentally realistic pesticide mixture to *Daphnia magna* and *Raphidocelis subcapitata*

Abstract

Previous work showed the co-occurrence of the organophosphate chlorpyrifos and the s-triazine herbicide terbuthylazine in surface waters of agricultural areas in "Lezíria do Tejo", Portugal. In the present study, we examined the effects of these pesticides, singly and as a binary mixture, on the immobility of *Daphnia magna* and on the growth rate of the microalgae *Raphidocelis subcapitata*. Terbuthylazine and chlorpyrifos at single exposure caused a very toxic or toxic response in both organisms. Usually, the toxicity of mixtures is evaluated in relation to the reference models Concentration Addition (CA) and Independent Action (IA). Initially, in this study the CA and IA model were used to evaluate the joint effects of chlorpyrifos and terbuthylazine. For immobility endpoint, the data fits better to the IA model, due to different mode of action of the pesticides, however a specific pattern was showed; at low dose levels, the immobility was lower than modelled (antagonism), whereas at high dose levels the immobility was higher than modelled (synergism). On the other hand, no deviation was observed from independent action in algal tests. A Model Deviation Ratio (MDR) approach was applied to evaluate the predictability of CA and IA models to mixture toxicity of realistic pesticide concentrations. Results indicated either and additive or a synergistic interaction depending on the concentrations combination and the test species. This study represents an important step to understand the interactions among relevant pesticides in aquatic ecosystems.

Keywords: Mixture, chlorpyrifos, terbuthylazine, *Daphnia magna*, *Raphidocelis subcapitata*, toxicity

1.Introduction

Plant production has a very important place in the Community (EC, 2009). 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, PPPs can also have non-beneficial effects on plant production. Their use may involve risks and hazards for humans, animals and the environment, especially if incorrectly used. Since the use of pesticides in agriculture inevitably leads to exposure of non-target organisms (including humans), undesirable side-effects may occur on some species, communities or on ecosystems as a whole (van der Werf, 1996).

Aquatic organisms are not only exposed to single substances, but typically to a mixture of pesticides. Experimental studies have shown that exposure against pesticide mixtures as present in the aquatic environment (Junghans 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 them resulting in adverse biological outcomes.

The Regulation on PPPs (EC, 2009) requires that interaction between the active substance, safeners, synergists and co-formulants shall be taken into account in the evaluation and authorisation. This explicitly refers to marketed PPP, which are, by origin, technical mixtures containing one to several a.s., plus, typically, several co-formulants. It does not apply to the potential combined effect resulting from the concomitant use of several formulations, as applied in practice, or to the combined effects in the environmental matrix where they end up. Similarly, the potential aggregate exposure to the same AS coming from other sources is currently not addressed for PPPs (Kienzler et al., 2016).

Since two pesticides (the insecticide chlorpyrifos and the herbicide terbuthylazine) were detected at the same time and/or in sequence in surface waters under a maize field condition within “Lezíria do Tejo”, Portugal, (Silva et al., 2015, Pereira, in press), it is crucial to assess their joint toxicity to aquatic organisms.

Bottom-up approaches aim to predict the toxicity of a defined mixture, based on knowledge on the chemical composition and toxicity of the mixture components. The aims of bottom-up approaches are: to test the predictive power of Concentration Addition (CA) (Loewe and Muischnekand, 1926) and Independent Action (IA) (Bliss, 1939) for certain chemicals and biological test system; to analyze deviations from conceptual expectations (interactions), and finally to provide quality targets for chemical mixtures (Cedergreen et al., 2013; Vighi et al., 2003). Jonker et al. (2005) proposed an approach (MIXTOX) in which both CA and IA were generalized to describe synergistic or antagonistic, concentration-ratio-dependent, and concentration-level-dependent deviations from either reference model. Different likelihood functions can be incorporated, and the approach can take into account differences in individual nonlinear concentration curves (slopes and functional form) and differences in relative toxicities of individual chemicals.

Only very few environmental studies tested the effect of binary mixtures consisted of terbuthylazine and chlorpyrifos, e.g. Munkegaard et al., 2008; Pérez et al., 2013a,b, focussing on toxicity to algae *R. subcapitata*, macrophyte Lemna minor, insect larvae *Chironomus riparius* and fish *Danio rerio*. To our knowledge, there is no data on the toxicity of such compounds to the crustacean *Daphnia magna*, and with MIXTOX model for algae *R. subcapitata*.

This study aims to overcome this limitation and to achieve more environmental realism in the scientific basis for forecasting risks and associated uncertainties of agricultural exposure situations, namely of a binary mixture of pesticides (chlorpyrifos and terbuthylazine), by addressing the following questions:

- Do the reference models correctly describe the joint effects of such pesticide mixture, and if not, which deviation patterns are revealed? Are deviation patterns conserved over different taxonomic groups?

- Are CA and IA models accurate for predicting the toxicity of two realistic proportions of chlorpyrifos and terbuthylazine for *R. subcapitata* and *D. magna* using the Model Deviation Ratio (MDR) (Belden et al., 2007)?

2.Materials and methods

2.1.Test-organisms and chemical compounds

The dormant eggs (ephippia) of the crustacean *D. magna* were hatched according to the Daphtoxkit F magna standard operation procedure (SOP, 2003), in a petri dish. The ephippia were incubate for 72h, at 20-22°C under continuous illumination of min. 6000 lux (light intensity at the top of the petri dish), with a "reconstituted" natural freshwater, according to the formula recommended by the International Standardization Organization (ISO, 1996), for the acute toxicity test with *D. magna*. After that, the eggs develop into neonates can then be used immediately for the toxicity tests.

The microalgae *R. subcapitata* was de-immobilized from algal beads and transferred into an adequate culturing medium (ISO, 2004) according to the Algaltoxkit F standard operation procedure (SOP, 2004).

In order to check the correct execution of the test procedures and the sensivity of the tests, a reference test with the chemical potassium dichromate ($K^2Cr^2O^7$) was performed for both.

The use of ephippia and algal beads, in Toxkits, allows to prevent the variability associated with recruitment/maintenance of live stocks in conventional biossays, keeping an identical sensitivity. Other advantages of these tests, when compared with the conventional, is that allows obtaining uniform exposure conditions (due to the biologically inert materials), obtaining a high uniform quality of the medium and minimizing the necessary equipment and the labour time.

The organisms were exposed to chlorpyrifos (with 99.0% of purity) and terbuthylazine (with 98.5% of purity) singly and as a binary mixture. The stock solutions were prepared in acetonitrile and stored at 5ºC. In order to execute the toxicity tests, at the different concentrations tested, stock solutions were dissolved in the culture medium, to each test-organism.

2.1.1.Immobility or mortality test with *D. magna*

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. A major condition for the validity of the test is that the number of dead + immobile organisms should not exceed 10% in the controls.

2.1.2.Growth inhibition test with *R. subcapitata*

The Algaltoxkit F test estimates the 72-h growth of *R. subcapitata* in each test solution and all materials used were purchased with the kit. As the correspondent conventional assay (e.g., OECD, 2011), the algae concentration at the start of the test was approximately 1.10^6 cells mL^{-1} $replicate^{-1}$ culture, and all cultures were incubated at 24°C under continuous cool white fluorescent illumination (100 µE m^{-2} s^{-1}). Algal growth rate was determined by optical density measurements, at 670 nm in a Hitachi U-2000 spectrophotometer UV-Vis (Hitachi, Ltd., Tokyo, Japan), and expressed as the percentage of inhibition relatively to the control.

The test validation criteria, according to OECD 201 (OECD, 2011), indicates that the control growth rate must be at least 0.92 per day, which corresponds to an increase in cell density by a factor 16 in 72h.

In order to obtain the EC50s value for *D. magna* for each pesticide, five concentrations were tested for chlorpyrifos and terbuthylazine with four replicates each; in addition, a control with artificial culturing medium was also tested in quadruplicate. The same was done for the algae, but with three replicates for each concentration.

2.2. Experimental Design

The dose response surfaces for the binary pesticide mixtures were performed by using a ray design. This design consisted on exposure to a number of binary mixture doses at predefined mixture ratios (Figure 1).

The number mixture ratios were selected according to the methodology presented in Pérez et al. (2011). The aim of this choice was to obtain a reliable coverage of effect of the two pesticides. In this article the nominal concentrations of the mixtures were calculated based on expected toxic strengths (TU) of: 0.375 (0.125 + 0.25; 0.25 + 0.125), 0.5 (0.125 + 0.375; 0.25 + 0.25; 0.375 + 0.125), 0.75 (0.125 + 0.625; 0.25 + 0.5; 0.375 + 0.375; 0.5 + 0.25; 0.625 + 0.125), 1 (0.125 + 0.875; 0.25 + 0.75; 0.375 + 0.625; 0.5 + 0.5; 0.625 + 0.375; 0.75 + 0.25; 0.875 + 0.125), 1.5 (0.75 + 0.75; 1 + 0.50; 0.50 + 1), 1.75 (1 + 0.75; 0.75 + 1) and 2 (1 + 1). With the EC_{50s} values for single exposures and these ratios, is possible to convert the TUs into the concentrations that will be used to make the combination of chlorpyrifos and terbuthylazine.

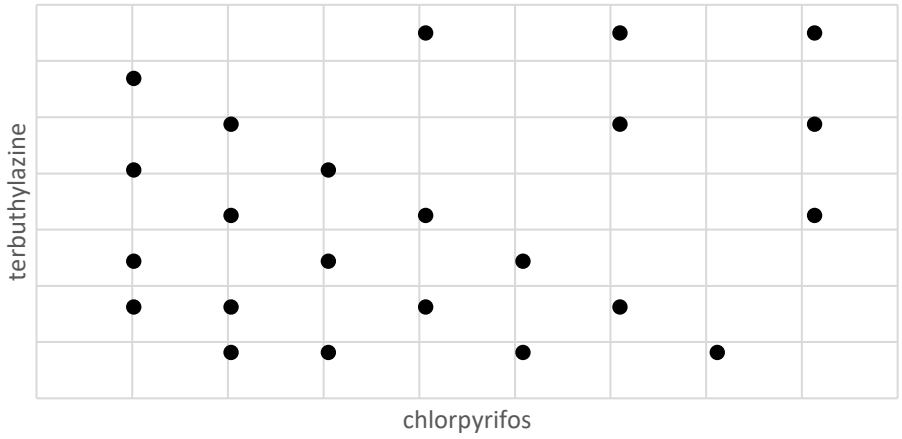

Fig 1. Scheme for the fixed ray design of the combinations used for chlorpyrifos-terbuthylazine for *D. magna* and *R. subcapitata*.

2.4.Data analysis

The values of the EC_{50s}, and slope were derived, for single exposures, using the same dose-response-curve formula used within the MIXTOX model (Jonker et al., 2005) namely a three-parameter logistic curve (Equation. 1), using the software SigmaPlot 13 (Systat, 2016).

$$Y_i = \frac{max}{1+\left(\frac{C_i}{EC_{50i}}\right)^{\beta_i}} \text{ (Equation 1)}$$

Where Yi is the response of a given parameter at a concentration (Ci) of a chemical (i) that was calculated using the maximum response value (max) for that parameter, the EC_{50i}, and the slope (b_i) for the pesticide. The three-parameter logistic curve can be used for endpoints that decrease or increase with the increasing of the dose, depending on the slope (Jonker et al., 2005).

To analyse the results obtained for the mixture exposures was used the MIXTOX model of Jonker et al. (2005), that compared the observed data with the expected mixture effects from both reference models. The second step was to extend both the CA and IA models, with deviation functions to describe synergistic/antagonistic interactions, dose-level, and dose-ratio dependency according to the methodology presented by Jonker et al. (2005). The parameters of the deviations were needed to build a nested framework. It was possible to fit the data to the models using the method of maximum likelihood and, as they are nested, the adjusted model can be statistically compared through likelihood testing (Neter et al., 1996). Where a statistically more descriptive deviation model was identified, the effects pattern was deduced directly from the parameter values as described below, and the maximum deviation was calculated in effect concentration (CA) or effect level (IA) terms to assess the biological significance (Loureiro et al., 2010).

For the synergy/antagonism deviation model (S/A model), the extra parameter a can become negative or positive, respectively, for both reference models. When a=0, the S/A model reduces to the CA or IA. A second parameter bDL con be included in addition

to a, in order to generate the dose-level (DL) deviation model. In this case the value of a indicates the deviation at low doses (i.e., a>0=antagonism, and a<0=synergism) and the value of bDL indicates at what dose level the deviation changes (i.e., from synergism to antagonism or vice versa). For CA/DL, the dose level where the deviation change occurs can be calculated using the follow expression: $1/bDL \cdot EC_{50}$; e.g., bDL=1 means that the switch occurs at the EC_{50} isobole. When bDL=0, the equation reduces to the S/A model. If bDL<0, the magnitude of synergism/antagonism (a) becomes dose-level dependent, but does not switch. In IA/DL deviation function, the switching can be estimated directly from 1/bDL; the switching occurs at mixture doses that cause a specific level of effect. If bDL=2, the switching occurs at doses where effect level is 50%. If bDL=0, the deviation function again reduces to the S/A model. When bDL<1, the magnitude of synergism/antagonism becomes response-dependent, but does not switch (Loureiro et al., 2010).

For dose-ratio (DR)-dependency, again a second parameter bDR is included in addition to a. The extra parameter bDR express the dependency of the reference models on the composition of the mixture. In a binary mixture, antagonism can be observed where the toxicity of the mixture is caused mainly by toxicant 1, whereas synergism can be observed where the toxicity is mainly caused by toxicant 2. Therefore, the bDR relates to the lead chemical of the mixture (i.e., the one mentioned and modelled first). In DR model, the parameter a quantifies the degree of antagonism (a>0) or synergism (a<0) and a significant bDR quantifies the degree of reduced (bDR>0) or increased (bDR<0) toxicity due to the lead chemical. When a and bDR have opposing signs, occurs a switch between antagonism and synergism within the response surface; whereas, if they have the same sign, the magnitude of the antagonism or synergism will vary with the ratio of chemicals, but not switch (Loureiro et al., 2010).

Effects on the growth inhibition of *R. subcapitata*, and on the mortality and/or immobility of *D. magna* from exposures to mixtures with the pesticides chlorpyrifos and terbuthylazine were fit in a first step to the IA model as pesticides with different mode of actions, but the adjustment data was also performed for the model CA. Both models were tested in order to evaluate which model predict better the effects, and deviations evaluated.

The nested deviations were compared using the method of maximum likelihood and the best fit chosen using 0.05 as the significance level. In addition, the lowest residual sum of square (SS) was preferred when comparing conceptual models and deviations. For full details on the derivation of these deviation functions, refer to Jonker et al. (2005).

In the statistical tests, differences were considered significant when p-value ≤ 0.05. The statistical analysis was performed with the assistance of software SigmaPlot 13 (Systat, 2016).

3. Results and Discussion

3.1.Individual toxicity tests

The 48-h immobility with the single pesticides showed that the insecticide chlorpyrifos and the herbicide terbuthylazine were very toxic ($EC_{50} \leq 1$ mg/L; EC, 2001) at effective median concentrations to daphnid species, respectively. The 72-h growth inhibition tests results with the single exposures of the two pesticides showed that terbuthylazine was also very toxic to the microalgae, and chlorpyrifos was classified as toxic ($1 \leq EC_{50} \leq 10$ mg/L, EC, 2001).

The EC_{50} values obtained after the 48-h and 72 h of exposure were used to calculate the TU values for the mixture experimental setup. EC_{50} for 48 and 72 h obtained directly from the bioassays, as well the EC_{50} values in the literature are depicted in Table 1.

The EC_{50} values for crustacean *D. magna* are in the same order of magnitude as those reported in studies present in the table above for the chlorpyrifos. For terbuthylazine the value calculated in this study is lower than the literature, being more sensitive. The microalgae showed to have results in the same order of magnitude than in literature.

Table 1 EC_{50} values (in µg/L) in the present study and literature

Pesticide	*D. magna*		*R. subcapitata*	
	In this study	Literature	In this study	Literature
CPF	0.24 (0.20-0.30)[1]	0.12-9.07[2]; 0.1 µg/L[7]	4067 (2780-7880)[1]	6600-53000[5]; 480[7]
TBZ	950 (800-1130)[1]	5000-21200[3]; ≥ 69300[4]; 21200[7]	65 (46-100)[1]	24-55[6]; 16[4]; 1.2[7]

[1]EC_{50} values with 95% confidence interval
[2]ECOTOX, 2016; Kikuchi *et al.*, 2000; Gaizick *et al.*, 2001; Palma *et al.*, 2008; Antunes *et al.*, 2010; Rubach *et al.*, 2011; Liu *et al.*, 2012
[3]Marchini *et al.*, 1988; ECOTOX, 2016
[4]McBean, C., 2012
[5]Antunes *et al.*, 2010
[6]Okamura *et al.*, 2000; Cedergreen and Streinig, 2005; Pérez *et al.*, 2011
[7]IUPAC, 2016

The EC_{50} values for crustacean *D. magna* are in the same order of magnitude as those reported in studies present in the table above for the chlorpyrifos. For terbuthylazine the value calculated in this study is lower than the literature, being more sensitive. The microalgae showed to have results in the same order of magnitude than in literature.

3.2.Binary mixture toxicity tests

The results obtained from fitting the data to the MIXTOX model are showed in Table 2 and 3, for immobilization and growth inhibition tests, respectively. The most important values are the SS, that quantify the model fit, and the value of $p(\chi^2)$, which indicates the significance of the deviations that can occur from the reference models.

For the fit of the CA model to the binary mixture data, for the immobilization test of *D. magna*, it was obtained an SS value of 218.1 (r^2 = 0.605; Figure 2). Adding the extra parameter a, to describe synergism/antagonism, the SS value decreased a little, but not significantly ($p[\chi^2]$ = 0.176), so the data showed no indication of synergism/antagonism. Adding to parameter a, parameters bDL and bTBZ the SS value decreased, but again not in significantly (both $p[\chi^2] > 0.05$), which indicates that there are no deviations from the reference model (Table 2). This is shown in the isobole diagram of the Figure 3A.

Comparing this data to de IA model, the SS value obtained was 228.7 (r^2 = 0.586). Adding the parameter a to the IA model, the SS value decreased slightly, not significantly ($p[\chi^2]$ = 0.569). Adding parameters a and bTBZ through the model, the SS decreased a little but again not significantly ($p[\chi^2]$ = 0.404). However, adding parameters a and bDL the SS value decreased significantly to 213.6 (r^2 = 0.613; $p[\chi^2]$ = 0.0005; Figure 2), and a dose level-dependent deviation from independent action was concluded. The positive value of a (3.529) in the deviation model, indicates that occurs antagonism at low dose levels and synergism at high dose levels. Parameter bDL being positive and approximately 2, indicating a shift between antagonism and synergism at the EC_{50} value (1/2=0.5) (Table 2). This is shown in the isobole diagram of the Figure 3B. The statistical analyses revealed that the DL deviation model explain more variance in the data than S/A model (χ^2 = 14.871; $p[\chi^2]$ = 0.0001).

Table 2 Summary of the analysis of the effect of the mixture on *D. magna*, using the MIXTOX model

	Concentration Addition				**Independent Action**			
	Reference	**S/A**	**DR**	**DL**	**Reference**	**S/A**	**DR**	**DL**
μ_{max}	0.91	0.91	0.91	0.91	0.90	0.90	0.90	0.90
β_{TBZ}	3.9	3.9	3.9	3.9	3.0	3.0	3.0	3.0
β_{CPF}	5.3	5.3	5.3	5.3	3.0	3.0	3.0	3.0
EC_{50TBZ}	1.18	1.18	1.18	1.18	0.90	0.90	0.90	0.90
EC_{50CPF}	0.00025	0.00025	0.00025	0.00025	0.0002	0.0002	0.0002	0.0002
a	NA	0.209	-0.343	-0.00081	NA	-0.212	-1.496	3.529
b_{DL}	NA	NA	NA	199.553	NA	NA	NA	2.152
b_{TBZ}	NA	NA	1.266	NA	NA	NA	2.757	NA
SS	218.1	216.3	214.6	216.7	228.7	228.4	226.9	213.6
χ^2	334.16	NA	NA	NA	NA	0.324	NA	15.195
df	NA	1	2	2	NA	1	2	2
$p(\chi^2)$	4.62×10^{-71}	0.176	0.173	0.484	NA	0.569	0.404	0.0005

Equations used to derive these results are detailed in Jonker *et al.* (2005).
μmax is the control response (maximum immobility); β is the slope of the individual dose-response; EC_{50} is the median effect concentration (mg/L); *a*, b_{DL}, and b_{TBZ} are parameters in the deviation functions; SS is the residuals sum of squares; χ^2 is the test statistics; df is the degrees of freedom; and $p(\chi^2)$ indicates the outcome of the likelihood ratio test (significance level $p < 0.05$). The abbreviation NA means quantity is not applicable.

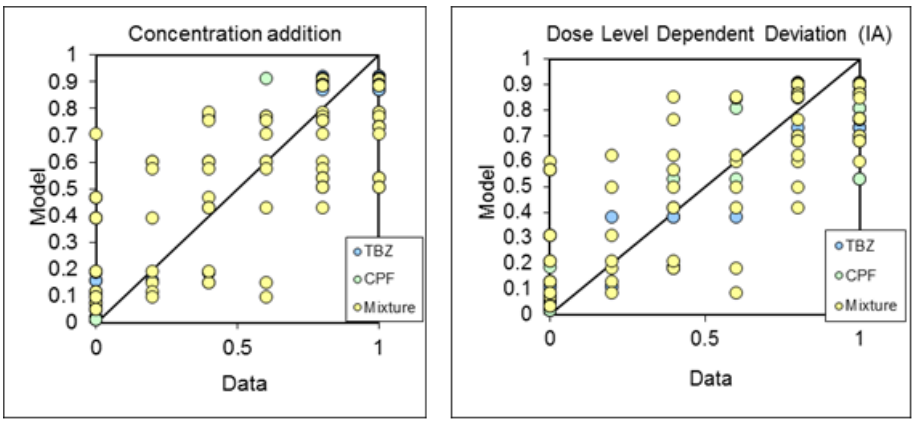

Fig. 2 Relationship between observed data from *D. magna* exposures and the modelled values. Left column: data vs modelled values using the CA reference model; right column: data vs modelled values using the IA model deviation.

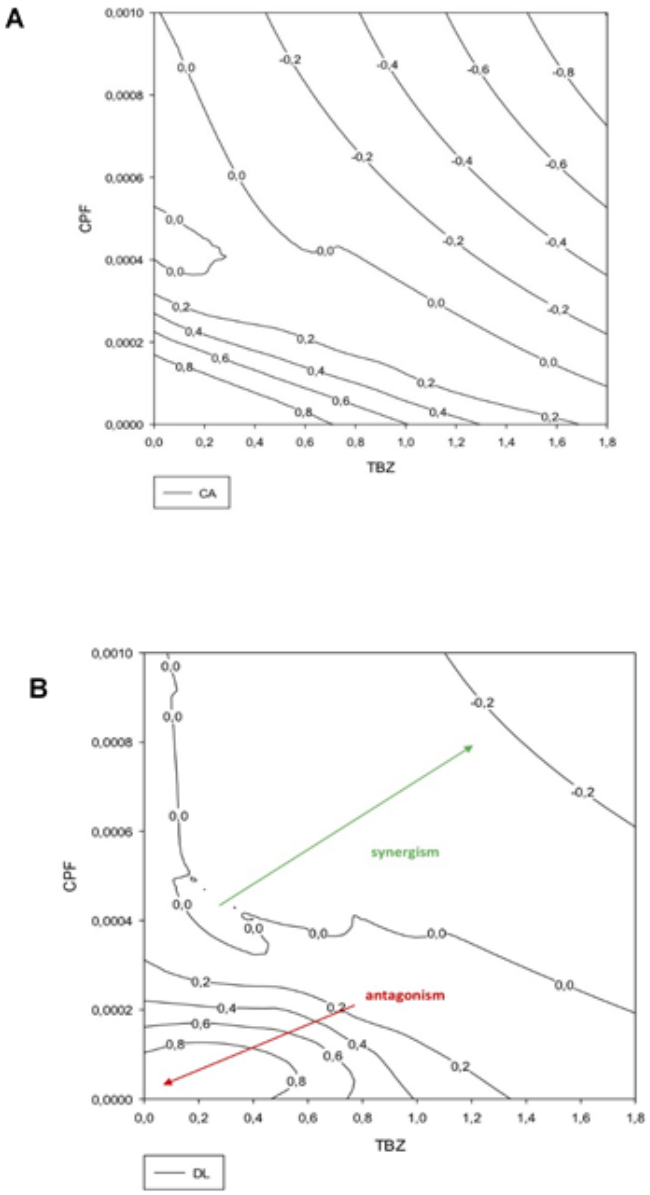

Fig. 3 Concentration-response relationship for the binary mixture of terbuthylazine and chlorpyrifos (2D isobolic surfaces) of the survival of *D. magna*: (A) Concentration Addition model fits, (B) Dose-level deviation after the Independent Action model fits.

During this study the main question was how well the reference models predict the joint effects of the mixture chosen, for both organism, and how the conceptual models becomes useful having a priori knowledge of the MOA of both pesticides. It is known that chlorpyrifos and terbuthylazine have a different molecular MOA. Theoretically, the IA model should be the preferred reference model. For *D. magna*, the higher proportions of the total variation explained by both reference models fits of 60.5% versus 58.6% to CA and IA model, respectively. This comparison between reference models showed that CA described a slightly higher proportion of the total variance than the IA, contrary to what would be predicted. However, a dose level-dependency was detected in the fit of the IA model that justified 61.3% of the total variance, slightly higher than the CA model.

In the study of Loureiro et al. (2010) with *D. magna*, the combined effects of pesticides and nickel were adjustable both to the IA and CA models, however the IA model can be chosen since the modes of action are dissimilar. Loureiro et al. (2009) studied other crustacean, where *Porcellionides pruinosus* exposed to atrazine and dimethoate (an organophosphate insecticide) showed a significant dose level dependent deviation from the IA model, showing antagonism at low dose levels and synergism at high dose levels, with no deviation for CA model.

Other studies provided examples where IA is not the best model to explain the data, as shifts for synergism and/or antagonism might occur depending on the dominant chemical present. Synergistic deviations from the conceptual models of mixtures have been frequently found in previous studies with invertebrates, showing that there may be an interaction between chemicals rather than an additive or independent response. Species such as *Chironomus tentans*, *Hyalella azteca* and *Ceriodaphnia dubia* exposed to atrazine and organophosphate insecticide mixtures have shown greater than additive toxicity (Pape-Lindstrom and Lydy, 1997; Anderson and Lydy, 2002; Belden and Lydy, 2000; Banks et al., 2005; Schuler et al., 2005; Jin-Clark et al., 2002; Lydy and Austin, 2004; Trimble and Lydy, 2006). The combined effects of dimethoate and atrazine showed mainly synergistic patterns in *Folsomia candida* (Amorim et al., 2011). A standard Organization for Economic Cooperation and Development (OECD) filter paper test was used to assess the acute toxicity of chlorpyrifos, atrazine, cyanazine, and simazine to the earthworm *Eisenia fetida*. Atrazine and cyanazine also increased the toxicity of

chlorpyrifos 7.9- and 2.2-fold, respectively. However, simazine caused no toxicity to the worms and did not affect chlorpyrifos toxicity in binary mixture experiments. Possible mechanisms for the greater-than-additive toxicity for the binary combinations of atrazine and cyanazine with chlorpyrifos were investigated, including changes in uptake and biotransformation rates of chlorpyrifos in the presence of atrazine. Uptake of chlorpyrifos into the worms decreased slightly when atrazine was present in the system, therefore eliminating increased uptake as a possible explanation for the increased toxicity. Body residue analysis of worms indicated increased metabolite formation, suggesting the greater-than-additive response may be due to increased biotransformation to more toxic oxon metabolites (Lydy and Linck, 2003). Yang et al. (2015) showed that the binary mixture of chlorpyrifos and atrazine was antagonistic toward *E. fetida* at all fa levels in an artificial soil test. For the *Enchytraeus albidus* the exposure to the mixture atrazine and dimethoate showed a significant deviation from the IA model fit for antagonism (Loureiro et al., 2009). Wacksman et al. (2006) examined the interactions between atrazine and chlorpyrifos in four aquatic vertebrate species, and the presence of atrazine at 1.000 µg/L resulted in a significant increase in the acute toxicity of chlorpyrifos in the African clawed frog (*Xenopus laevis*). For the fish Pimephales promelas a lack of a clear toxicity pattern was observed, since that some bioassays results showed greater than additive toxicity, while others showed an additive response. In the other organisms studied (*Lepomis macrochirus* and *Rana clamitans*), no effect of atrazine on chlorpyrifos toxicity was observed (Wacksman et al., 2006). Xing et al. (2015) results also suggest that exposure to atrazine, chlorpyrifos or their combination promotes oxidative stress and autophagic responses in the brain of the common carp (*Cyprinus carpio L*.).

A study with *Danio rerio* in early-life stages, using the binary combinations of atrazine and terbuthylazine with chlorpyrifos, suggest that the s-triazine herbicides potentiated the chlorpyrifos toxicity. Changes in swimming behaviour and the inhibition of AChE were related and synergistic patterns were observed when zebrafish larvae were exposed to the binary mixtures. The increased of the chlorpyrifos toxicity with the presence of these herbicides it happens possibly due to the effect of s-triazines to accelerated the transformation of chlorpyrifos in its oxon form, increasing therefore toxicity by inhibiting AChE activity (Pérez et al., 2013a). Pérez et al. (2013b), also studied

this mixture in the *Chironomus riparius* larvae, when combined with both s-triazine herbicides, chlorpyrifos toxicity was enhanced by approximately 2-fold when tested in a binary mixture experimental setup, at the 50% effective concentration levels. Atrazine and terbuthylazine are not effective inhibitors of AChE, however they potentiate chlorpyrifos toxicity; both s-triazine herbicides at 200 μg/L increased the inhibition of the AChE activity by 7 and 8-fold, respectively.

These patterns were not coincident with the ones described here, showing dose-level deviations (antagonism at low concentrations and synergism at high concentrations) for the crustacean *D. magna*. Such differences could be due to species and endpoint specificity. Only the study with *Porcellionides pruinosus* (Loureiro et al., 2009) presents similar deviation patterns to our study.

To evaluate the joint effects of the mixture on the growth of the algae R. subcapitata, both reference models, CA and IA, were also used. In the fit of the CA model to the data the SS value obtained was 7.281 (r^2 = 0.64; Figure 4). With the adding of the parameters a and b the decrease of the SS value was not significant in either case, so was concluded that the data fits to the CA model ($p \leq 0.05$) (Table 3). This is shown in the isobole diagram of the Figure 5A.

In the IA model, the fit provided a SS value of 8.098 (r^2 = 0.60; Figure 4). Again, the adding of the parameters a and b do not provided a significant decreased of the SS values, concluding that do not occur deviations from this model. Therefore, the data fits to both reference models, however the CA models explains slightly better the proportion of the total variance than the IA model (Table 3). This is shown in the isobole diagram of the Figure 5B.

Table 3 Summary of the analysis of the effect of the mixture on *R. subcapitata*, using the MIXTOX model

	Concentration Addition				Independent Action			
	Reference	**S/A**	**DR**	**DL**	**Reference**	**S/A**	**DR**	**DL**
μ_{max}	0.772	0.771	0.769	0.772	0.737	0.776	0.774	0.764
β_{TBZ}	0.833	0.826	0.808	0.833	0.896	0.764	0,764	1.021
β_{CPF}	0.765	0.787	0.808	0.765	0.732	0.641	0.641	1.092
EC_{50TBZ}	0.056	0.053	0.056	0.056	0.084	0.049	0.049	0.058
EC_{50CPF}	3.809	3.55	3.81	3.809	6.154	4.567	4.567	3.764
a	NA	0.420	0	0	NA	1.367	0.079	-3.951
b_{DL}	NA	NA	NA	1	NA	NA	NA	2.073
b_{TBZ}	NA	NA	0.919	NA	NA	NA	2.233	NA
SS	7.281	7.26	7.26	7.28	8.098	7.62	7.58	6.3
χ^2	13.0	NA	NA	NA	12.187	NA	NA	NA
df	NA	1	2	2	NA	1	2	2
$P(\chi^2)$	0.011	0.893	0.988	1	0.016	0.490	0.772	0.406

Equations used to derive these results are detailed in Jonker et al. (2005).
μmax is the control response (growth rate); β is the slope of the individual dose-response; EC_{50} is the median effect concentration (mg/L); a, bDL, and bTBZ are parameters in the deviation functions; SS is the residuals sum of squares; χ^2 is the test statistics; df is the degrees of freedom; and $p(\chi^2)$ indicates the outcome of the likelihood ratio test (significance level $p < 0.05$). The abbreviation NA means quantity is not applicable.

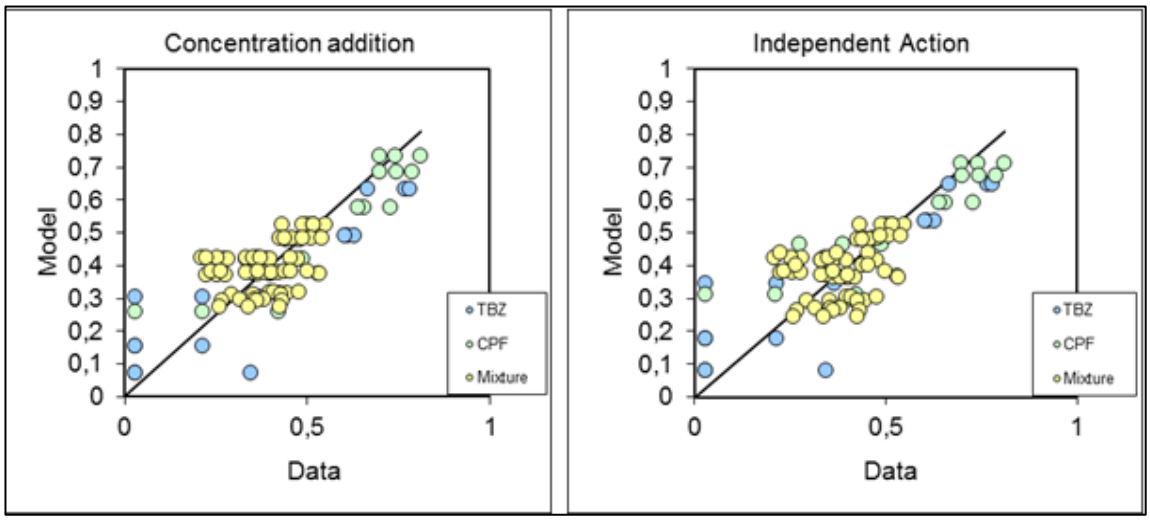

Fig. 4 Relationship between observed data from *R. subcapitata* exposures and the modelled values. Left column: data vs modelled values using the CA reference model; right column: data vs modelled values using the IA reference model.

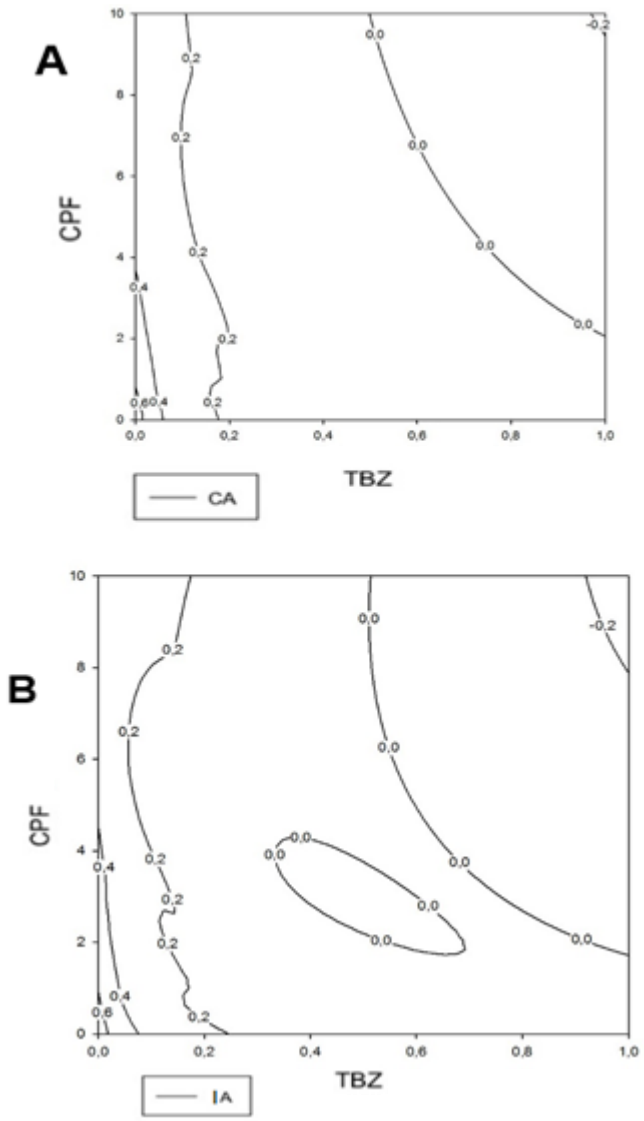

Fig. 5 Concentration-response relationship for the binary mixture of terbuthylazine and chlorpyrifos (2D isobolic surfaces) of the growth of *R. subcapitata*: (A) Concentration Addition model fits, (B) Independent Action model fits

Regarding chemicals with different molecular target sites, previous studies with the unicellular green freshwater algae *Scenedemus vacuolatos* demonstrated that the IA conceptual model presented a better prediction when compared to the CA model when testing the mixtures of 16 biocides (Faust et al., 2003). The mixture toxicity of different pollutants with unclear modes of action was also accurately predicted by IA at individual NOECs on the growth of the algae *S. vacuolatus* (Walter et al., 2002).

In addition, Backhaus et al. (2004) employed this IA model to predict the toxicity of six dissimilarly acting substances on the natural algae communities.

In the study performed by DeLourenzo and Serrano (2003), the mixture of atrazine and chlorpyrifos had additive toxicity to *Dunaliella tertiolecta* (*Chlorophyta*, green algae). However, Belden and Lydy (2000) found that atrazine and chlorpyrifos in mixture exhibited synergistic toxicity to the midge larvae *Chironomus tentans*. Atrazine was found to increase the biotransformation of the organophosphate compound, converting it into a more toxic metabolite. While this mechanism enables atrazine and chlorpyrifos to be synergistic in mixture to an invertebrate species, there is no comparable mechanism for chlorpyrifos toxicity in phytoplankton.

The study with the test organisms *R. subcapitata* and *Lemna minor* shows no indications of synergistic interactions between the tested pesticides, confirming the applicability of CA as a reference model predicting mixture effects of pesticides for aquatic plants and algae (Munkegaard et al., 2008). These pesticides in mixture displayed additive toxicity, which are in accordance with the results of our study.

3.3.Toxicity from the agricultural exposure scenario

When chlorpyrifos and terbuthylazine are present at their measured concentrations in field surface waters, the mobility on *D. magna* was affected by 45% (mixture 1: chlorpyrifos 0.17 and terbuthylazine 8.5 µg/L) and 75% (mixture 2: chlorpyrifos 0.17 and terbuthylazine 85 µg/L). The MDR values obtained with IA for mixtures 1 and 2 were comprised between 1 and 2 while with CA presented values greater than 2. For the two experiments with pesticides with different modes of action, IA more accurately predicted effects compared to CA, indicating the potential of synergistic interactions. The switch from antagonism to synergism was observed at the EC_{50} value. This might be transposed to a synergistic pattern at the two experiment concentrations as a result of the model extrapolation. However, this finding should be interpreted with caution because our ray design did not cover all low dose levels.

The two pesticides, chlorpyrifos and terbuthylazine, were shown to cause a total effect on R. subcapitata of 31% (mixture 1) and 88% (mixture 2). Fairly good compliance with the effect predicted by concentration addition and independent action (35% and 34%, respectively) demonstrates a high predictive power of both concepts for mixture 1,

although observed mixture toxicity and both predictions differed by MDR values between 1 and 1.5, for mixture 2 with 85 µg/L terbuthylazine.

4.Conclusion

The exposure to chlorpyrifos and terbutylazine showed dissimilar patterns for both species. Whereas binary mixture showed an accurate fit to the conceptual models (CA or IA) for *R. subcapitata*, a pattern for a toxicity level dependent on the dose area was observed for the *D. magna* case study.

In previous studies with *Danio rerio* and *Chironomus riparius* larvae, where the same set of chemical mixtures used in this study were tested, the patterns were not coincident with the ones described here, showing synergistic patterns for both. This confirms that for an adequate ecological risk assessment several groups of organisms and endpoints should be included. Similarity, using the MDR approach comparing observed effects to CA and IA predicted effects, binary mixtures of chlorpyrifos and terbuthylazine at environmentally realistic concentrations indicated either an additive or a synergistic result depending on the concentrations combination and the test species.

This study highlights the importance to assess the probability that the two reference models fail to correctly describe the joint effects of environmentally realistic chemicals, which is relevant information for risk assessors, e.g. when deriving a safety factor for mixture toxicity, and to investigate whether deviation patterns are conserved over different taxonomic groups or endpoints.

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Toxicity of environmentally realistic concentrations of chlorpyrifos and terbuthylazine in indoor microcosms

Abstract

Few studies have been conducted into the evaluation of environmental-realistic pesticide mixtures using model ecosystems. In the present study, the effects of single and combined environmentally realistic concentrations of the herbicide terbuthylazine and the insecticide chlorpyrifos were evaluated using laboratory microcosms. Terbuthylazine potentiated the effect of chlorpyrifos on feeding rates, presumably by triggering the transformation of chlorpyrifos to more toxic oxon-analogs. In addition, food-web interactions resulting from both indirect effects of the test compounds and recovery of affected populations were also recorded. If the ecological recovery option is to be adopted as the protection goal, possible food-web interactions between chemical (and other) stressors likely to be present in edge-of-field water bodies need to be further evaluated.

Keywords: Model ecosystems; Mixture toxicity; Pesticides; Ecological risk assessment; Fate models; Food web interactions

1. Introduction

With the modernization and intensification of agricultural practices in the past century, the use of pesticides was initiated to increase productivity of yields. As a consequence of pesticide use, water bodies near agricultural areas may become contaminated with pesticide residues through spray drift, drainage, run-off and/or accidental spills (Capri and Trevisan, 1998). Given the variety of pests, diseases and weeds that may need to be combated, it is common practice for several different pesticides to be applied during the growing season to protect crops. Subsequently, freshwater life in edge-of-field water bodies is likely to be subjected to a mixture of compounds. Environmental risk assessment (ERA) of chemicals like pesticides, however, mainly focuses on exposure to individual chemicals, although a number of guidance documents on how to deal with chemical mixtures have been published in the last years (e.g. EFSA, 2013; Bunke et al., 2014; ECHA, 2014; Kienzler et al., 2016).

Most scientific studies into mixture toxicity have been conducted using single species tests evaluating concentration series chosen to order to predict/evaluate in a more realistic way the behaviour of contaminants when they occur in the environment (independent action, concentration addition, and deviations of these). Such concentrations, however, may be considerably above concentrations most often monitored in the environment (Cedergreen et al., 2014). Only few studies have evaluated the mixture toxicity of compounds at concentrations likely to occur under real-world conditions (e.g. Banks et al., 2005; Junghans et al., 2006; Laetz et al., 2009; Silva et al., 2015). In addition, the laboratory bioassays that have most often been used in such studies may underestimate the effects of pesticide mixtures in aquatic environments since they do not consider potentially effects in top-down and bottom-up regulation of trophic interactions (Relyea and Hoverman, 2006; Bjergager et al., 2011; Choung et al., 2013).

Model ecosystems (microcosms and mesocosms) are experimental ecosystems that are constructed by collecting parts of natural ecosystems and bringing them together into an artificial housing or by enclosing parts of existing ecosystems in the field (Van den Brink and Daam, 2014). They provide a greater ecological realism than single species

tests and since they consider species interactions, top down and bottom up trophic effects may be studied. After reviewing available model ecosystem studies evaluating pesticide mixtures, Verbruggen and Van den Brink (2010) concluded that when pesticides affect the same biological groups, synergistic mixture effects are not to be expected. When mixtures of pesticides that affect different biological endpoints (e.g., insecticides and herbicides) are evaluated, increased indirect effects are often noted due to food web interactions (Verbruggen and Van den Brink, 2010).

Given the above, there is a clear need for model ecosystem studies that evaluate environmentally realistic mixtures of pesticides, especially for mixtures containing pesticides with different modes of action. Three studies previously evaluated the mixture toxicity of terbuthylazine and chlorpyrifos in laboratory bioassays with the cladoceran *Daphnia magna* and the green algae *Raphidocelis subcapitata* (Pérez et al., 2013a,b; Munkegaard et al., 2008). To the best of our knowledge, however, this mixture has hence never been evaluated at the community level neither at environmental-realistic concentrations. The aim of the present study was therefore to evaluate the effects of the herbicide terbuthylazine and the insectide chlorpyrifos using indoor model ecosystems. The two pesticides were evaluated individually and in two mixtures using concentrations measured or likely to occur in a Portuguese agricultural area. The ecological interactions between the two compounds and implications for their risk to aquatic life are discussed.

2. Material and methods

2.1. Experimental design

Fourteen microcosms were situated in a laboratory devoid of daylight and maintained at 24-28°C with a photoperiod (fluorescent lamp) of 12 h to simulate Mediterranean conditions (Van Wijngaarden et al., 2005). Each microcosm consisted of a glass cylinder (diameter 20 cm; height 50 cm), filled with 13 L water obtained from an uncontaminated pond at Instituto Superior de Agronomia (Lisbon, Portugal). Additional zooplankton was

collected from the same pond by passing pond water through a zooplankton net (mesh size, 55 µm; Hydrobios, Kiel) and equally distributed (500 mL) over the microcosms. The microcosms were also inoculated with less then 24-h old *D. magna* obtained from ephippia (Microbiotests, Ghent, Belgium). Microcosms were allowed to stabilise for 1 week, after which treatments were assigned randomly to the microcosms. Subsequently, the systems were monitored for several endpoints (see below) during an experimental period of four weeks. Water losses due to evaporation were replenished once a week with demineralized water throughout the experiment.

2.2. Pesticide treatments and analyses

Terbuthylazine (TBZ; Chemical Abstracts Service [CAS] number 5915-41-3; purity 98.6%) and chlorpyrifos (CPF; CAS number 2921-88-2; purity 98%) were purchased from Sigma–Aldrich. Treatment levels of terbuthylazine (8.5 µg/L) and chlorpyrifos (0.17 µg/L), individually and as a binary mixture, were selected from concentrations measured simultaneously in the "Lezíria Grande de Vila Franca de Xira" agricultural area, situated in the vicinity of the River Tagus Estuary Natural Reserve (Portugal). In line with the concentration of 8.5 µg/L terbuthylazine measured at this field site, similar (maximum) concentrations have been reported in several other studies (5.6 – 9.6 µg/L; Baillie, 2016; Knauer, 2016; Tsaboula et al., 2016). However, based on the predicted environmental concentrations reported in the draft assessment report of terbuthylazine, concentrations up to 31 µg/L may be expected for application scenarios in South Europe (EC, 2007). In line with this, Otto et al. (1999) reported a maximum terbuthylazine concentration of 47 µg/L in surface waters following its application in an Italian field trial. Wenneker et al. (2010) showed that concentrations of terbuthylazine in local surface water due to point sources linked to use of sprayers in arable farming were even 100 µg/L or higher. A concentration level of 85 µg terbuthylazine/L was therefore also included to represent a realistic worst-case exposure scenario. Subsequently, the following six treatments were made:

Control (CTR): no pesticide treatment

0.17 µg chlorpyrifos/L (CPF 0.17)

8.5 µg terbuthylazine/L (TBZ 8.5)

85 µg terbuthylazine/L (TBZ 85)

0.17 µg chlorpyrifos/L + 8.5 µg terbuthylazine/L (MIX 8.5)

0.17 µg chlorpyrifos/L + 85 µg terbuthylazine/L (MIX 85)

Single applications of the different pesticide treatments were made to two microcosms for each treatment, while four other systems were untreated to serve as controls. Before application, sub-samples were taken from the stock solutions for determination of nominal concentrations. Acetonitrile was used as a solvent for both stock solutions and kept below 0.1 mL/L as recommended in OECD (2002). Applications were made by evenly distributing appropriate aliquots of these stock solutions over the water surface of the microcosms, followed by gentle stirring of the water layer with a glass rod.

Concentrations of the pesticides in the water were determined 2 days before and 0.25 (6 hours), 1, 4, 7, 14 and 28 days after application of the test substances. Depth-integrated water samples of approximately 50 mL were taken from the microcosms by means of a glass pipette. Subsamples of 10 mL were placed in vials and extracted by immersion of a SPME fiber (PDMS/DVB; Supelco, Bellefonte, PA, USA) and analysed by GC-MS as detailed in Silva et al. (2012). The detection limits for CPF and TBZ were 23 and 42 ng/L, respectively, whereas the limit of quantification was 50 ng/L for both compounds.

2.3. Zooplankton

Zooplankton was sampled from each microcosm -2, 1, 7, 14 and 28 days post application. To this end, depth-integrated water subsamples were taken from different spots in the microcosms with a Perspex tube (length, 44 cm; diameter, 2.8 cm) until a final volume of 1.5 L was achieved. After stirring, 1 L of this sample was filtered through a zooplankton net (mesh size, 55 µm; Hydrobios, Kiel, Germany), whereas the remaining 0.5 L was used for chlorophyll-a determination (see section 2.4). The filtered water was poured back into the corresponding microcosm and the collected zooplankton was preserved with formalin (final volume, 4%). Zooplankton identification and counts were made using an inverted and a binocular microscope. Rotifers and cladocerans were identified to the

lowest taxonomic level possible. Copepods were identified to gender, and a distinction was made between nauplii and mature copepod stages, whereas ostracods were identified to gender.

2.4. Chlorophyll-a, nutrients and community metabolism

The chlorophyll-a measurements were made using 0.3 L of the 1.5 L sample taken as described above for zooplankton. Water was filtered through a glass-fiber filter (e.g., GF/C; diameter, 4.7 cm; mesh size, 1.2 µm) using a vacuum pump. The filter was then air-dried, wrapped in aluminium foil and stored below -20°C for a maximum period of 5 weeks. Extraction of the pigments was performed according to the method described by Moed and Hallegraeff (1978). Subsamples of the filtrate were transferred to centrifuge tubes and stored at 4°C before analyses for ammonium, nitrate and orthophosphate. Nutrient analyses were performed by molecular absorption spectrophotometry in a Skalar segmented flow analyser using the Berthelot and sulphanilamide methods (Houba *et al*., 1998). The remaining filtrate and unfiltered water sample were returned to their corresponding microcosms.

Dissolved oxygen (DO), electrical conductivity (EC), temperature and pH were measured 7 days and 1 day before pesticide application, as well as twice a week after treatments were made. Measurements were performed at mid-water depth using a WTW Multiline F/set-3 multiprobe both in the morning (at the start of the photoperiod) as well as in the afternoon (8 h after the start of the photoperiod).

2.5. In-situ bioassays and post-exposure feeding rate

The in-situ bioassay and post-exposure feeding rate determinations were conducted based on the methods decribed by Barata et al. (2007) and McWilliam and Baird (2002a). Chambers were constructed from clear polyvinyl chloride cylindrical piping (10 cm long, 5 cm external diameter). Each chamber had two rectangular windows (5 cm x 3.5 cm) cut into either side of the cage, covered with 150 µm nylon mesh. Pipe ends were sealed

with polypropylene caps. Just after application of the pesticides, one test chamber was placed in each microcosm, each containing 10 (less than 24-h old) *D. magna* neonates (Microbiotests, Ghent, Belgium).

Animals were retrieved from the chambers 24 h after deployment. Five surviving juveniles were transferred to 60ml screw-capped glass jars containing 50 mL Standard Freshwater (ISO medium, formula according to ISO 6341) with *Raphidocelis subcapitata* at a density of $5x10^5$ cells/mL (Mc William and Baird, 2002b). Three jars containing no animals were used to establish initial algal densities. Post-exposure feeding experiments were conducted in under controlled temperature (20 ± 2 ºC) in darkness to avoid algal growth. Animals were allowed to feed for 4 h in darkness, after which individual feeding rates (cells/individual/h) were determined according to the method described in McWilliam and Baird (2002b). Cell density was estimated from absorbance measurements at 650nm using a Hitachi U-2000 spectrophotometer UV-Vis (Hitachi, Ltd., Tokyo, Japan) and standard calibration curves based on at least 20 data points, with an $r^2 > 0.98$.

2.6. Data analysis

Water quality variables were analysed using analysis of variance (ANOVA) followed by a Fisher's LSD test to assess whether there was a significant response to the treatments over time. Levene's test and the Kolmogorov-Smirnov test were used to test variance homogeneity and normality.

The effects of the different treatments on the zooplankton communities were analysed by the principal response curve (PRC) method (Van den Brink and ter Braak, 1998), which was performed using the multivariate analysis statistical program CANOCO (Ter Braak, 2009). The canonical coefficients calculated by PRC express the part of the variance in community structure that can be attributed to treatment. By plotting the community-level multivariate response against time (x-axis), treatment effects are separated from temporal changes in community structure and therefore easy to interpret. Treatment effects are expressed as deviations from the control so that control becomes a straight line over time to which all treatments are contrasted. With the PRC, calculated species

weights can be interpreted as the affinity of the taxon to the principal response curve. Species with a high positive weight are indicated to show a response similar to that indicated by the PRC, whereas those with a negative weight show a response opposite to that indicated by the PRC. Species with a near-zero weight are indicated to show either a response very dissimilar to that indicated by the PRC or no response at all. To assess significant differences between the biological communities of the different treatments and the control microcosms for each sampling date and differences within them, a one-way analysis of variance (ANOVA) was performed using the sample scores on axis 1 from each RDA analysis, followed by multiple comparison tests post-hoc testing. Before running the ANOVAs, homogeneity of the variance was tested for each sampling date using Levene's test. Comparisons between treatments were carried out by Fisher's LSD test when significant differences were found by ANOVA.

Post exposure feeding rates of among treatments and controls were compared by one-way ANOVA followed by post-hoc Fisher's LSD test multiple-comparison test. Before analysis, data were checked to meet ANOVA assumptions of normality and variance homoscedasticity and, if required, log-transformed. Statistical analyses were performed using STATISTICA 7.0 (Stat Soft Inc., 2004).

3. Results and discussion

3.1. Pesticide dissipation

Six hours after application, chlorpyrifos could only be quantified (0.07 µg/L) in one of the six microcosms that had received a chlorpyrifos treatment (CPF 0.17; TBZ 8.5; TBZ 85; n = 2). In the other five chlorpyrifos-treated microcosms, concentrations had already dropped below the limit of quantification (0.05 µg/L) at that time. Although chlorpyrifos is indeed known to dissipate fast from the water column, Van Wijngaarden et al. (2005) reported a DT50 of 30-32 h in a relatively similar laboratory set-up under simulated Mediterranean conditions. The decrease in chlorpyrifos water concentrations from approximately day 1 to day 10 is governed by partitioning processes that vary

substantially between experiments depending on their experimental designs and conditions (e.g. Leeuwangh, 1994; Daam and Van den Brink, 2007; Daam et al., 2008a,b). The fact that the applied nominal chlorpyrifos concentration was close to the limit of quantification evidently will also have played a role. In the terbuthylazine-applied microcosms (TBZ 8.5; TBZ 85; MIX 8.5; MIX 85), 63% to 68% of the applied dose was still detected at the end of the 28-d experimental period. Terbuthylazine is indeed known to degrade very slowly under aerobic conditions and being stable against hydrolysis and photolysis (DT50 > 56 d; Coors et al., 2006).

3.2. Effects on chlorophyll-a and water quality

Chlorophyll-a levels in microcosms treated with 85 µg/L (TBZ 85 and MIX 85) and, to a lesser extent, 8.5 µg/L (TBZ 8.5 and MIX 8.5) terbuthylazine decreased immediately after application ($p < 0.05$; Figure 1A).

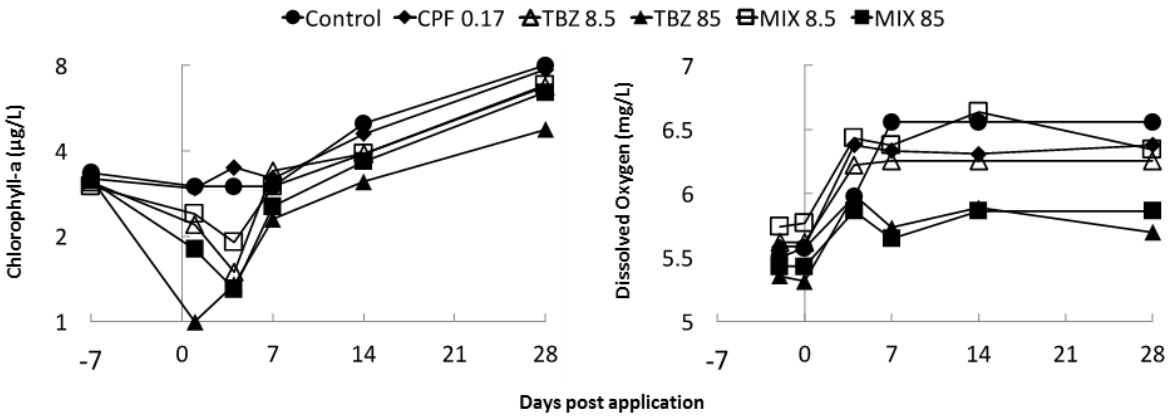

Fig. 1. Dynamics of chlorophyll-a (A; in µg/L) and dissolved oxygen (B; in mg/L) throughout the course of the experiment.

Although in the first week following application chlorophyll-a levels in these treatments increased, they remained lower than controls throughout the rest of the experiment. In the highest TBZ treatments, this was accompanied with rather constant DO concentrations thoughout the experimental period, whereas the other treatments showed increasing DO levels (Figure 1B). Although the increasing chlorophyll-a levels in

the high TBZ treatments 7 days post application allowed DO levels be maintained they did not follow the increasing trend in DO levels observed in the other treatments. Fiori and Pistocchi (2014) demonstrated that intermediate TBZ concentrations led to increased cell chlorophyll levels in the diatom *Skeletonema marinoi*, and that photosynthetic efficiency was determined by lower TBZ levels than those affecting cell growth. Subsequently, it is likely that the increasing chlorophyll-a concentrations in the first week following the high TBZ treatments had lower photosynthetic efficiency and hence did not allow for DO concentrations to increase. However, it did allow avoiding a drop in DO to anoxic conditions, which may also be related with the relatively low maximum suppression of photochemical efficiency in response to TBZ when compared to other herbicides (Choi et al., 2012). This may suggest that binding of TBZ is less effective than for other herbicides or that the toxic effect is limited by cell uptake capacity (Weiner et al., 2004; Choi et al., 2012).

No effects were noted on any other water quality parameters (T, EC, pH, nutrients; $p > 0.05$). In addition, no effect of the single chlorpyrifos treatment on chlorophyll-a or an additional effect of CPF on the toxicity of terbuthylazine was noted (effect TBZ 85 = MIX 85 and effect TBZ 8.5 = MIX 8.5; Figure 1). In line with this, mixture experiments with the green algae *Raphidocelis subcapitata* and the floating plant *Lemna minor* did not show a synergetic effect of chlorpyrifos on terbuthylazine toxicity, nor did any of the other insecticide (malathion, endosulfan and chlorpyrifos) - herbicide (metsulfuron-methyl, terbutylazine and bentazone) mixtures evaluated (Munkegaard et al., 2008).

3.3. Post exposure feeding inhibition

The mean percentage of animals recovered (dead and alive) from the chambers after 24 h deployment was greater than 90%. Feeding rates were significantly reduced in the TBZ 85 and both MIX treatments, whereas feeding rates in CPF 0.17 and TBZ 8.5 were comparable to that of the control (Figure 2A). The absence of a significant effect in the CPF treatment is in line with the toxicity values of chlorpyrifos for feeding rate inhibition reported by Loureiro et al. (2010; NOEC = 0.3 µg/L; EC50 = 0.45 µg/L).

Post-exposure feeding rate inhibition may be expected to occur at lower concentrations than immobility or mortality in daphnids, depending on the compound of concern (e.g. McWilliam and Baird, 2002a). The effect of terbuthylazine on the feeding rate of *D. magna* or other invertebrates, however, has to the best of our knowledge never been evaluated before. A comparison was therefore made between EC50 values for feeding rate available in the US Environmental Protection Agency (US-EPA) ECOTOX database (http://cfpub.epa.gov/ecotox/) for pesticides with their respective toxicity values for immobility/mortality. Feeding rate appeared on average to be four to five times more sensitive than immobility/mortality for fungicides and insecticides and even 26 times for herbicides (Figure 2B). The 48h-EC50 (immobility) as established in a *D. magna* bioassay at our laboratory was 950 µg terbuthylazine/L (unpublished data). The absence and occurrence of effects on feeding rate as observed in the TBZ 8.5 and TBZ 85 microcosms, respectively, are hence in line with the discussed above. The absence of food in the bioassay (in accordance with OECD, 2004) and presence of food in the laboratory microcosms may also have played a role. Toxicity at sublethal concentrations of chemicals may be greater in the presence than in the absence of food since in the former animals are also exposed through ingestion of particle-bound contamination (e.g. Taylor et al., 1998). This may especially have been significant in the present study since the TBZ treatment of 85 µg/L exceeded the reported water solubility of 11.5 µg/L, which indicates that a substantial amount of TBZ was associated with dissolved or particulate organic matter in the water phase (Coors et al., 2006).

Although both the individual treatments with chlorpyrifos and 8.5 µg terbuthylazine/L did not result in significant effects on feeding rate, they did when applied as a mixture (Figure 2A). Previous studies have indeed demonstrated that s-triazines, even at lower concentrations considered not ecologically harmful, can increase the expected toxicity of certain organophosphate insecticides to several invertebrate species (Banks et al., 2005; Choung et al., 2010; Pérez et al., 2013b; Cedergreen et al., 2014). For example, Pérez et al. (2013a,b) demonstrated that terbuthylazine potentiated the toxicity of chlorpyrifos to zebrafish and chironomid larvae. These authors hypothesised that this increased toxicity was due to the induction of the cytochrome P450 activity by terbuthylazine, which subsequently increased the conversion of chlorpyrifos to the more potent AChE inhibitor oxon-analogs (Pérez et al., 2013a,b and references therein).

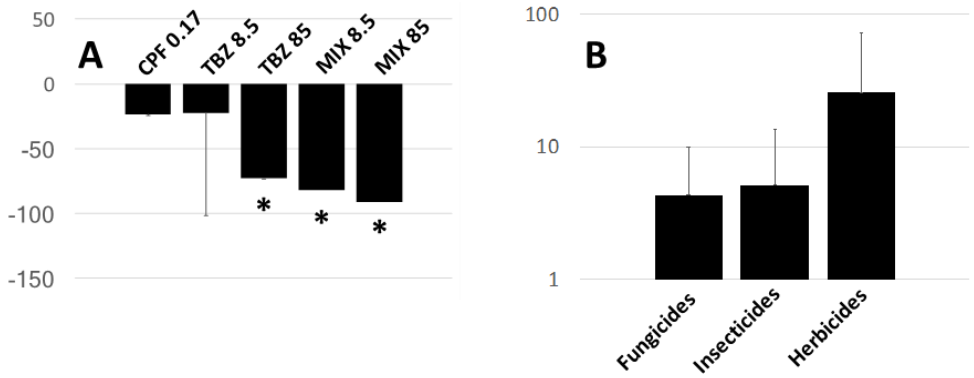

Fig. 2. (A) Relative post-exposure feeding rates (% of control) and (B) ratio of 24h-48h EC50 based on immobility or mortality and EC50 values based on feeding rate, grouped by main pesticide group (fungicides, insecticides and herbicides). Laboratory toxicity values were obtained from the US Environmental Protection Agency (US-EPA) ECOTOX database (http://cfpub.epa.gov/ecotox/). Asterisks indicate significant differences between treatments and the control (ANOVA; $p < 0.05$).

3.4. Zooplankton community effects

A total number of 33 taxa were identified from the zooplankton samples taken from the 14 microcosms throughout the experiment. Rotifers were the most diverse in terms of numbers of taxa but cladocerans dominated in terms of total abundances. Ostracods mostly appeared on individual sampling dates and in low numbers. Only immature stages of copepods (nauplii) were sampled from the microcosms, and their numbers were low but relatively stable in the control microcosms throughout the experiment.

From the PRC of the zooplankton dataset, it appears that all pesticide treatments deviated from the control in the first weeks following applications, but the TBZ 8.5 treatment was not statistically significant ($p > 0.05$; Figure 3). Of all variance, 29% could be attributed to sampling date; this is displayed on the horizontal axis. Differences due to treatments accounted for 55% of all variance. A Monte-Carlo test indicated that 35% of the variance captured by the treatment regime is displayed in the diagram, which was statistically significant ($p < 0.005$). The highest species weights (> 1.5) were obtained for several rotifers (*Lophocharis oxysternon*, *Cephalodella gibba* and *Polyartha* sp.), copepod nauplii and the cladoceran *D. magna* (Figure 3), which decreased in abundance in response to the treatments. Negative species weights were all between 0 and -1 and

the species with the lowest species weights (*Lecane closterocerca* and *Lecane quadridentata*; Figure 3) were rare species: they never occurred on two consecutive sampling dates in the experimental period and at abundances between 15 and 90/L. This thus indicates that none of the taxa may be expected to have increased in abundance due to the pesticide treatments.

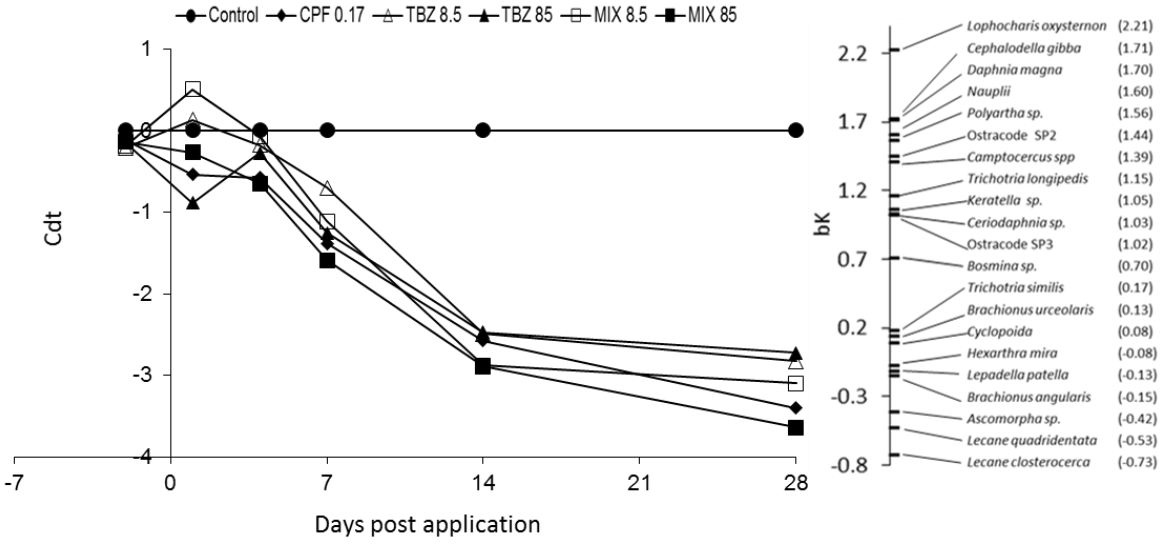

Fig. 3. Principal response curves (PRCs) resulting from analysis of the zooplankton data set, indicating the treatment effects of the pesticide treatments on the zooplankton community. The lines represent the course of the treatment levels over time. The species weight (bk) can be interpreted as the affinity of the taxon with the PRCs. The PRC diagram displays a significant amount of the treatment variance ($p < 0.005$).

One day after pesticide treatments, daphnids in the control had tripled the abundance counted 2 days before application (Figure 4A). Of all other treatments, only the TBZ 8.5 treatment also showed increased numbers over this period (20%). The microcosms that only received a chlorpyrifos treatment (i.e. CPF 0.17) showed a decrease in abundances of 57%, which is in line with the EC50 (48 h) established in our laboratory (0.24 µg/L). Since no effect of this treatment was observed on feeding rate (see previous section), this indicates that immobility was a more sensitive test endpoint for chlorpyrifos than was post-exposure feeding rate. Chlorpyrifos is known to have a contact/stomach action, low bioaccumulation and bioconcentration potential and high elimination rates (Rubach et al., 2010; Giddings et al., 2014). These toxicodynamic-toxicokinetic features may hence be related with this parameter-specific sensitivity. The application of terbuthylazine in combination with chlorpyrifos, i.e. Mix 8.5 and Mix 85, led to slightly higher decreases in daphnid numbers between days -2 and 1 (75% and 80%,

respectively) when compared to chlorpyrifos alone (57%), although this difference was not statistically significant ($p > 0.05$). Greater effects of the mixture relative to their individual compounds may indeed be expected through increased conversion of chlorpyrifos to more potent AChE inhibitor oxon-analogs by terbuthylazine as explained in the previous section. Interestingly, the greatest reduction in daphnids (100%) was found for the TBZ 85 treatment, although one week after application numbers already equalled those in controls again (Figure 4A). This short but strong effect on *D. magna* at this treatment is most likely due to the almost complete absence of algae (indicated by chlorophyll-a levels; Figure 1A). Other factors that may also have played an additional role include: i) removal of individuals from already small populations in the pre-treatment when compared to the other treatments (Kennedy et al., 2002); ii) quality of the few algae remaining may have been reduced (c.f. Bessa da Silva et al., 2016); iii) increased toxicity through ingestion of food particles (see previous section); and iv) reduced population growth through change to a male-dominated sex ratio as has been demonstrated for daphnids exposed to s-triazine (e.g. Juttner at al., 1995; Dodson et al., 1999).

Copepod nauplii were eliminated in all microcosms that received a chlorpyrifos application (CPF 0.17, MIX 8.5 and MIX 85; Figure 4B). This is in accordance with several model ecosystem studies, which suggest that copepods may have chlorpyrifos-susceptible representatives (López-Mancisidor et al., 2008a,b and references therein). In addition, it is a common observation that early life stages of test organisms like nauplii are more sensitive to contaminant than older (mature) stages (Stark and Wennergren, 1995; Naddy et al., 2000; Daam et al., 2008b; López-Mancisidor et al., 2008a,b). As discussed above, the fact that nauppli populations were already small may also have played a role.

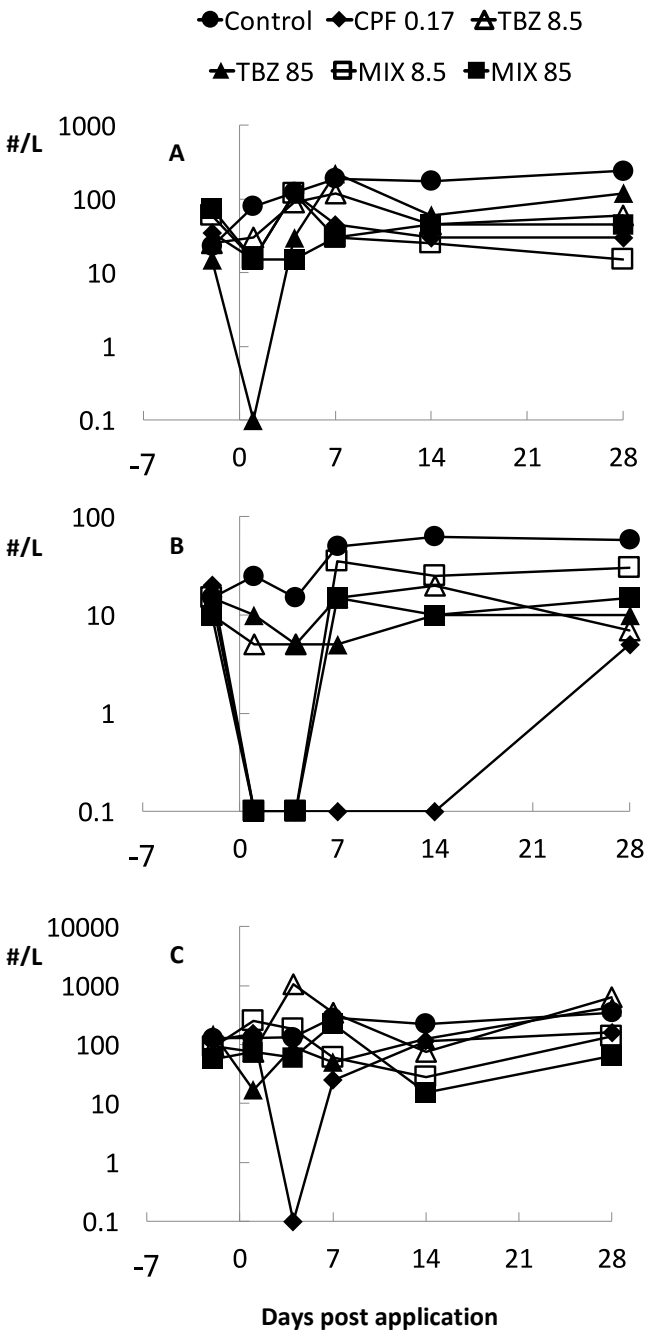

Fig. 4. Change over time in densities (#/L) of the most discriminative zooplankton taxa: *Daphnia magna* (**A**), copepod Nauplii (**B**), and rotifers (**C**). A value of 0.1 denotes absence of the taxon.

An increase in rotifer numbers has frequently been reported in model ecosystem experiments evaluating insecticides (e.g. Fleeger et al., 2003 and references therein; Daam et al., 2008a,b; López-Mancisidor et al., 2008a,b). Contrarily, a complete elimination of rotifers was noted four days after chlorpyrifos application in the CPF 0.17 treatment ($p < 0.05$; Figure 4C). It is unlikely that this was the result of a direct toxic action of chlorpyrifos given i) the low reported sensitivity of rotifers; ii) the fact that this

did not occur immediately following application; and iii) the absence of this effect in the MIX 8.5 and MIX 85 treatments. Rotifer numbers were low and dynamic in all treatments. In addition, numbers of *D. magna* increased eight-fold between day 1 and 4 in microcosms receiving the CPF 0.17 treatment. Rotifers may indeed be suppressed by increasing *Daphnia* spp. through increased competition for food resources and mechanical interference (e.g. Gibert, 1985; Fleeger et al., 2003; López-Mancisidor et al., 2008a,b).

Ecological effects chain and concluding remarks

Previous studies have demonstrated that single or few similarly acting compounds usually dominate the effect in environmentally realistic mixtures, even if the mixture includes substances with diverse and partly unknown mechanisms of action (Junghans et al., 2006). In model ecosystem studies mimicking the practical application of pesticides for a particular crop, Verbruggen and Van den Brink (2010) also noted that the effects were mostly no larger than those of the most toxic substance. These authors further indicated that when mixtures of pesticides that affect different biological endpoints (e.g., insecticides and herbicides) are evaluated, increased indirect effects are often observed due to food web interactions. In line with this, several interactions between terbuthylazine and chlorpyrifos were noted in the present study as visualized in Figure 5. In summary, direct toxicity of terbuthylazine was noted on phytoplankton (measured as chlorophyll-a), which was hypothesized to indirectly lead to effects on daphnids throught decreased food and DO levels, in combination with decreased feeding rates resulting from ingestion of terbuthylazine-containing particles. Terbuthylazine is also likely to have potentiated the effect of chlorpyrifos on feeding rates by triggering the transformation of chlorpyrifos to more toxic oxon-analogs. Direct toxic effects of chlorpyrifos were noted on copepod Nauplii and cladocerans, and the recovery of the latter is likely related with the decrease observed in rotifers (Figure 5).

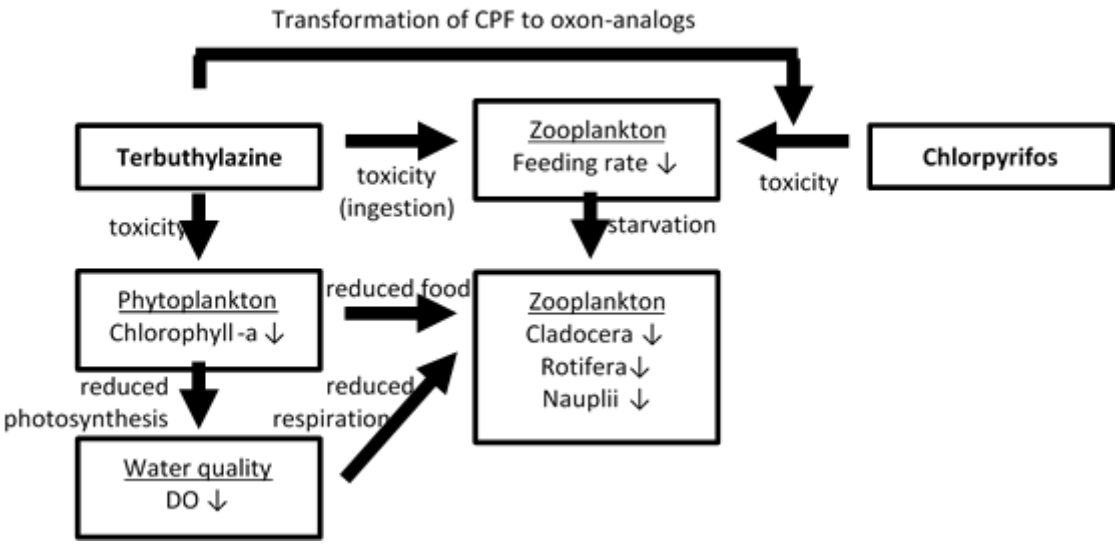

Fig. 5. Schematic overview of the hypothesized ecologic effect chain after single and combined applications of terbuthylazine and chlorpyrifos.

The contamination of surface waters by herbicides and insecticides has the potential to cause 'ecological synergism' in which top down and bottom up trophic effects interact (Relyea and Hoverman, 2006). Such indirect effects are mainly to be expected when concentrations of the pesticides in the mixture are likely to result in direct toxic effects. With an ecological threshold protection goal, i.e. accepting negligible population effects only, as has traditionally been applied in environmental risk assessments of pesticides, this is likely to be prevented with the evaluation of the individual compounds. However, the ecological recovery option, i.e. accepting some population-level effects if ecological recovery takes place within an acceptable time period, has also recently been included as a valid possibility for use as protection goal (EFSA, 2013). If ecological recovery is to be set as the protection goal, possible interactions with other chemical stressors likely to be present in edge-of-field water bodies resulting in increased toxic effects through food web interactions hence also need to be evaluated.

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CHAPTER 6

AQUATIC COMMUNITY STRUCTURE CHANGES IN MEDITERRANEAN EDGE-OF-FIELD WATERBODIES AS EXPLAINED BY ENVIRONMENTAL FACTORS AND THE PRESENCE OF PESTICIDE MIXTURES

Based on the following manuscript:

Pereira AS, Dâmaso-Rodrigues ML, Daam MA, Cerejeira MJ Aquatic community structure changes in Mediterranean edge-of-field waterbodies as explained by environmental factors and the presence of pesticide mixtures (*accepted in Ecotoxicology)*

Abstract

Studies addressing the predicted effects of pesticides in combination with abiotic and biotic factors on aquatic biota in ditches associated with typical Mediterranean agroecosystems are scarce. The current study aimed to evaluate the predicted effects of pesticides along with environmental factors and biota interactions on macroinvertebrate, zooplankton and phytoplankton community compositions in ditches adjacent to Portuguese maize and tomato crop areas. Data was analysed with the variance partitioning procedure based on redundancy analysis (RDA). The total variance in biological community composition was divided into the variance explained by the multi-substance potentially affected fraction [(msPAF) arthropods and primary producers], environmental factors (water chemistry parameters), biotic interactions, shared variance, and unexplained variance. The total explained variance reached 39.4% and the largest proportion of this explained variance was attributed to msPAF (23.7%). When each group (phytoplankton, zooplankton and macroinvertebrates) was analysed separately, biota interactions and environmental factors explained the largest proportion of variance. Results of this study indicate that besides the presence of pesticide mixtures, environmental factors and biotic interactions also considerably influence field freshwater communities. Subsequently, to increase our understanding of the risk of pesticide mixtures on ecosystem communities in edge-of-field water bodies, variations in environmental and biological factors should also be considered.

Keywords: Agroecosystems; Freshwater community; Pesticide mixtures; multi-substance potentially affected fraction; redundancy analysis

1. Introduction

While a relatively large body of information exists on the biodiversity and management of many semi-natural habitats in farmlands, such as field margins and hedgerows, others, particularly small freshwater bodies, remain neglected (Shaw et al. 2015). Ditches are widespread in agricultural land and permanent and temporary ditches are often the first concentration point of water draining from agricultural land (Biggs et al. 2007; Shaw et al. 2015).

Edge-of-field ditches provide very different habitats compared to other farm habitats and may harbour a large diversity of aquatic macrophytes, algae and macroinvertebrate species characteristic of small freshwater bodies (Davies et al. 2008). They are particularly important as overwintering sites for aquatic invertebrates and as dispersal routes for amphibians and water birds (Williams et al. 2003; Manhoudt et al. 2005; Cushman 2006; Biggs et al. 2007; Herzon et al. 2008). These species groups are hence also rather poorly studied, yet are an important contribution to the overall species diversity at local and landscape scales (Thiere et al. 2009). Ditches and their margins may also function as corridors within the landscape for other important organisms (e.g. pollinators; Van Geert et al. 2010). The levels of organic compounds found in surface waters have increased in recent decades as a result of human activities. Of these organic compounds, pesticides are most commonly detected (Nakamura and Daishima 2005; Sáenz and Di Marzio 2009; Ricart et al. 2010; Malaj et al. 2014). Different agricultural practices may cause the presence of pesticide mixtures, which can vary in terms of their complexity (Altenburger et al. 2013). As cumulative stress of toxicants has been identified as one of the main pressures affecting ecological status, mixture risks have to be evaluated and reduced (Brock 2013). Subsequently, ecologists are challenged to understand and predict the impacts that these mixtures may have on natural communities (Relyea 2009).

Mixture risk assessment predictions have mostly been based on mathematical models that were validated primarily with single species laboratory studies and have hence rarely been confronted with biological changes observed under real-world environmental conditions (Gregorio et al. 2012). To protect aquatic biodiversity and its

ecosystem functions, however, it is important to understand the effects of chemicals on aquatic biota in the field. The reason for this is that in the real environment various abiotic and biotic factors influence the performance of aquatic organisms and affect the fate of pesticides in the aquatic environment (Ieromina et al. 2016). For example, organisms exposed to chemicals in their natural surroundings may be more (or less) sensitive to toxicants than organisms exposed in the laboratory, because of effects such as density dependence and stress induced by food shortage or competition (Wendt-Rasch et al. 2003; Mansano et al. 2016). Several studies have therefore emphasized the need and importance of considering ecological parameters in eco-toxicological studies (Liess et al. 2003; Peters et al. 2013) and a number of studies have already evaluated the influence of ecological factors in the assessment of pesticide effects on aquatic biota in the field (Berenzen et al. 2005; Martin et al. 2011; Schäfer et al. 2011; Gregorio et al. 2012; Schäfer et al. 2012; Ieromina et al. 2016).

The above may stress the need to confront predictions of mixture risk assessments with observations in the field or, to state it differently, to establish whether mixture toxicity is a parameter that can explain changes in a given ecosystem (Gregorio et al. 2012). Last year, the effects of pesticides on aquatic biota in combination with abiotic factors, biotic factors and time was studied for the first time in the field (Ieromina et al. 2016). To the best of our knowledge, no such study has ever been conducted under Mediterranean conditions. The aim of the present study was therefore to evaluate to what extent predicted pesticide mixture toxicity, abiotic conditions and species interactions can explain changes in macroinvertebrate and plankton community compositions in small freshwater bodies associated with typical Mediterranean agroecosystems. To this end, macroinvertebrate and plankton communities, water chemistry and pesticide concentrations were monitored in edge-of-field ditches at an intensive agricultural area of maize and tomato production in Portugal. We used classical statistical ecological methods such as partial redundancy analysis (Legendre 1998) to enable unravelling the individual importance of environmental factors, biota interactions and pesticide mixture toxicity on field community changes in time.

2. Materials and methods

2.1. Research area

The research area “Lezíria Grande de Vila Franca de Xira” located on the river Tagus lowlands, is an alluvial plain with approximately 13000 ha of irrigated farmland. It is bounded by two rivers, the Tagus and the Sorraia, and located in the highest part of the estuary of the River Tagus, about 25 km upstream from Lisbon. The climate is Mediterranean and the average annual rainfall is 700 mm, most of which falls between October and March. About 20 percent of the area is covered by light-to-medium-textured, mainly fluvial, deposits; the remaining 80 percent is heavy-textured marine deposits, most of which is moderately to very saline. The study area is located within one of the most important areas for Portuguese horticulture and cereal crops and is mainly dominated by rice, tomato and maize crops. Part of the research area lies in the Natural Reserve of the Tagus Estuary, a portion of the Tagus estuary that became a nature reserve by the Portuguese Decree Law 565/76 and has a high biotic diversity (Caçador et al. 2000, 2013) with a vast number of migratory birds using this estuary regularly (Delany et al. 2009). The reserve has an area of almost 15000 ha and includes estuarine waters, marshes, mudflats, salt pans, islands, channels, and agricultural land.

2.1.1. Sampling sites

Plankton, macroinvertebrates and water chemistry were monitored at 6 sites within the area (see Figure 1): one uncontaminated irrigation water ditch (R location), two ditches alongside maize agricultural areas (M1 and M2 Locations) and three ditches in tomato crop areas (T1, T2 and T3 locations). The locations were chosen based on the fact that different contamination levels could be expected from differences in intensity of agricultural activity at the different locations. Sampling was performed at nine moments during the main period of agricultural activities (May–August 2014) in the area to

account for possible seasonal fluctuations in pesticide concentrations, water chemistry and freshwater communities.

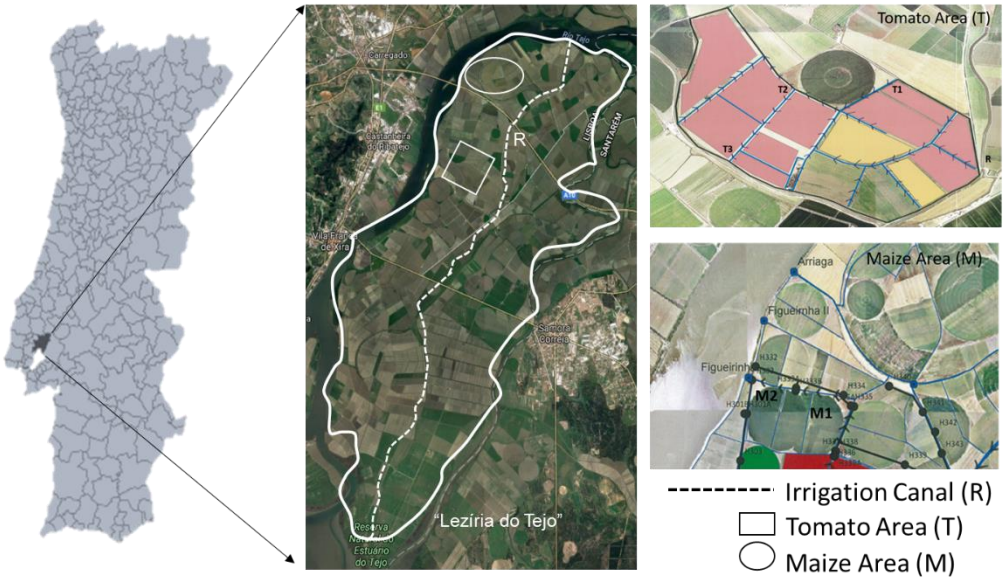

Fig. 1. Sampling sites at the "Lezíria do Tejo" agricultural area. Sampling sites M1, M2 were located in ditches on a maize area, whereas sampling sites T1, T2 and T3 were located in ditches on a tomato crop area. Sample site R were located in the main irrigation canal of the "Leziria do Tejo"

2.2. Environmental and chemical parameters

The main pesticides used in the agricultural area, as indicated by farmers and the local associations (personal communication), were monitored. Concentrations of these pesticides were measured through GC-MS and LC-MS/MS by an external Laboratory following standard guidelines in accordance with DIN EN ISO/IEC 17025:2005. Samples for water chemistry and pesticide analysis as well as plankton and macroinvertebrates (see next two sections) were collected on the same day. Water temperature, conductivity, pH and dissolved oxygen (DO) were evaluated in situ using field probes (WTW—Multiline F/7–3). Ammonia, nitrates and alkalinity were evaluated in the laboratory in accordance with standardized analytical methods (APHA 1998).

2.3. Plankton sampling and determination

Several depth-integrated sub-samples were collected using a perspex tube until a 15-L sample was obtained. From this bulk sample, a subsample of 1-L was taken to study the phytoplankton community and another 1-L for determination of the phytoplanktonic chlorophyll-a concentration. Then the bucket was emptied till a final volume of 5 L, which was concentrated through a plankton net (mesh size, 55 µm; Hydrobios Kiel, Germany). Three replicate samples were taken per sampling site. These samples were immediately preserved with formalin (ca. 4% vol.). Subsamples of the zooplankton sample were counted with an Olympus CH-2 compound microscope using the Sedgewick-Rafter Cell method (Serfling 1949).

The 1-L phytoplankton sample was stained with lugol and concentrated after sedimentation of 6 days. Additional lugol was added when needed to assure conservation of the samples. Subsamples of the phytoplankton samples were counted with an inverted microscope (Leica DM IL LED at a magnification 100 – 400) and numbers were recalculated to numbers per litre of sampled water.

For the chlorophyll-a determinations, the 1 L of the water sample (in parts of 100 mL until filter saturation) was concentrated over a Schleicher and Schuell glass fiber filter (GF52; diameter, 4.7 cm; mesh size, 1.2 lm), by means of a vacuum pump. The filter was stored in a labelled Petri dish wrapped in aluminium foil at a temperature below -20 °C for a maximum period of 3 months. Extraction of chlorophyll-a was performed using the method described by Parsons et al. (1984). Chlorophyll-a content was analysed by spectrophotometric measurement (following Lorenzen 1967).

2.4. Macroinvertebrate sampling and determination

Macroinvertebrate samples were collected using a dipping net with an opening of 25 cm and a mesh size of 500 μm. To this end, the dipping net was dragged through the entire

water column including the upper part of the sediment layer (depth 3–5 cm within the sediment layer) over a total length of 5 m of the ditches. Multiple samples were collected from dominating habitats according to the method described in Keizer-Vlek et al. (2011), resulting in a multi-habitat sampling strategy. Macroinvertebrate samples were rinsed and transferred to plastic sample jars. Samples were preserved with 70% ethanol (v/v) directly after field sampling. Samples were sorted and identified using a stereo-microscope (Olympus SZ X7 magnification: 50x). Whenever possible, macroinvertebrates were identified to species level.

2.5. Toxic risk calculation for pesticide mixtures

The present study used the Compendium of Pesticide Common Names (Alan Wood, available at www.alanwood.net/pesticides; last accessed on 2016-12-05) to categorize pesticides by their toxic mode of action (TMoA), following recommendations by de Zwart and Posthuma (2005). The quantification of the predicted risk of toxicant mixtures by msPAF (multiple-substance predicted affected fraction) consisted of site-specific exposure assessments and calculation of single-compound PAFs and mixture msPAFs. If the concentration of a pesticide was below its detection limit, half of the detection limit value was used in the data analysis (after Clarke 1998).

The combined toxic risk (msPAF) of the pesticide mixture residues as measured in the ditch water was calculated separately for each taxonomic group (i.e. arthropods and primary producers) following the methodology described in Traas et al. (2002) and de Zwart and Posthuma (2005) with modifications proposed by Rämö et al. (2016). Toxicity data was obtained from the U.S. EPA Ecotox database (USEPA 2016) and the E-toxBase (de Zwart 2002). In cases where median lethal (effective) concentrations (L(E)C50) were not available for at least two species in each of the two taxonomic groups (i.e., primary producers and arthropods), the database was complemented with a variety of other data sources: draft assessment reports (EFSA 2014), EU review reports (EC 2014) 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).

Hazard units (HUs) were calculated for each species group-pesticide combination as the geometric mean of literature toxicity data (similar to the HC50). These HUs were used to scale toxicity data and measured environmental concentrations (MECs) of pesticides to dimensionless HU values to adjust for differences in the potency of pesticides. Mean (α) and standard deviation (σ) of log toxicity data (expressed in HU units) were calculated for each pesticide using equal weight of species for α but taking intra-species variance into account for σ (Table 3). Each pesticide was assigned a TMoA based on molecular activity following de Zwart et al. (2009). All the samples contained compounds with different TMoA, so that the risk of the mixtures was calculated using the response-addition model.

2.6. Statistical analysis

The aim of the statistical analysis was to evaluate to what extent changes in biological community (phytoplankton, zooplankton and macroinvertebrates) compositions were related with changes in the potential risk predictions (msPAF), environmental descriptors (i.e., physico-chemical parameters and chlorophyll–a) and biota interactions. For the gradient analysis, Redundancy Analysis (RDA) (linear method) was applied on the data following Legendre and Legendre (1998), as DCA revealed that the dominant gradient length was below 3 (Lepš and Šmilauer 2003). RDA allows evaluating how a matrix of explanatory variables could explain a matrix of response variables and is commonly employed in ecology (Gilbert and Bennet 2010).

Furthermore, partial redundancy (pRDA) was employed to underline the changes in plankton and macroinvertebrate community structure due to the msPAF gradient only. The response variable dataset consisted of the total species composition. The variance in total community composition was divided into four components: variance explained by msPAF gradient (MS|E), environmental factors (E|MS), shared variance between msPAF and environmental factors (MS∩E), and residual (unexplained) variance. The

same approach was also used to underline the changes in each biota group (phytoplankton, zooplankton and macroinvertebrates) due to msPAF, environmental factors and the presence of other biota. The variance in group composition was divided into six components: variance explained by msPAF (MS|E∪B), environmental factors (E|MS∪B), biota interactions (B|MS∪E), shared variance between msPAF, environmental factors and biota (E∩MS∩B), and residual (unexplained) variance.

Prior to analysis, all biological data were transformed using the Hellinger transformation (Legendre and Gallagher, 2001). Variance Inflation Factors (VIFs) were used to identify multicollinearity between explanatory variables. A common rule is that VIF values over ten indicate redundant constraints (Chatterjee and Hadi 2006) and such variables were excluded from the canonical analysis. Finally, significance tests of constraints for RDA and pRDA were carried out using permutation tests (Legendre and Legendre 1998). Multivariate analyses were performed with the Canoco software version 4.5 (Lepš and Šmilauer 2003).

3. Results

3.1. Pesticide exposure and mixture risk prediction

Throughout the study period, nineteen pesticides from fourteen different chemical classes (8 herbicides, 8 insecticides and 3 fungicides) were detected in the surface water samples taken at the different sites and sampling moments. These included the glyphosate and terbuthylazine metabolites aminomethylphosphonic acid (AMPA) and desethyl-terbuthylazine, respectively. Detection frequencies varied from 1 to 48 occurrences per pesticide in a total of 54 samples. At the site R, pesticide residues were always below the limit of detection, with the exception of one occasion (samples of May 2014). Maximum and average amounts of pesticide residues detected throughout the study are presented in Table 1. Sites showing particularly high levels of certain pesticide classes were M2 (organophosphates and triazines), M1 (pyrethroids) and T1 (anthranilic diamides and phosphonoglycines; see Fig. 1 and Table 1). Triazines were the most

constantly detected pesticides in all the sampling sites with the highest value of 8.5 µg/L for terbuthylazine (Table 1). From the organophosphates family, chlorpyrifos was the pesticide with the highest detected concentration, with a maximum of 12 µg/L. In the case of the pyrethroids, cypermethrin had the highest concentration (up to 10 µg/L) and the highest individual concentration found was 16 µg/L for the metabolite AMPA.

Based on calculated msPAF values, we found a low to high risk for toxic effects on primary producers and arthropods for all the sample locations and dates (Table 2) with the higher toxic effects predicted for the water samples associated with the maize area for both communities. However, not all the pesticides had the same potency in the mixture and it appeared that the toxicity of the mixture was mostly driven by only a few pesticides. Following the approach described in Rämö et al. (2016), the fraction of risk contributed by each pesticide to each species group over the study period was determined. A pesticide in this system may be ranked among the top risk contributors when posing a frequent but low risk to the environment or when posing an infrequent but high risk. The most influential pesticides were terbuthylazine (37%), metolachlor (20%) and rimsulfuron (10%) for the $msPAF_{primary\ producers}$ and chlorpyrifos (35%), cypermethrin (21%), lambda-cyalothrin (10%), chlorantraniliprole (6%) and imidacloprid (4%) for $msPAF_{arthropods}$.

Table 1. Measured environmental concentrations (MECs) of pesticides in the 54 samples taken at the six sites in the "Lezíria do Tejo" in 2014

CAS	Common name	Type[a]	Chemical Group	Avg. MEC (µg/L)	σ	Max. MEC (µg/L)
1066-51-9	aminomethylphosphonic acid	M	organophosphate	0.78	3.91	16.0
131860-33-8	azoxystrobin	I	methoxyacrylate strobilurin	0.03	0.00	0.03
500008-45-7	chlorantraniliprole	I	diamide	0.83	1.27	4.50
1897-45-6	chlorothalonil	F	aromatic	0.03	0.00	0.03
2921-88-2	chlorpyrifos	I	pyridine organothiophosphate	0.56	2.27	12.00
57966-95-7	cymoxanil	F	aliphatic nitrogen	0.03	0.02	0.03
52315-07-8	cypermethrin	I	pyrethroid ester	0.43	1.92	10.00
30125-63-4	desethyl-terbuthylazine	M	chlorotriazine	0.24	0.34	1.10
13194-48-4	ethoprophos	I	aliphatic organothiophosphate	0.02	0.01	0.03
133-07-3	folpet	F	phthalamide	0.04	0.02	0.05
51276-47-2	glufosinate	H	organophosphate	0.02	0.01	0.03
1071-83-6	glyphosate	H	organophosphate	1.05	1.24	3.90
138261-41-2	imidacloprid	I	neonicotinoid	0.50	0.94	3.00
173584-44-6	indoxacarb	I	oxadiazine	0.03	0.00	0.03
91465-08-6	lambda-cyhalothrin	I	pyrethroid ester	0.04	0.01	0.05
51218-45-2	metolachlor	H	chloroacetanilide	0.38	0.85	2.80
21087-64-9	metribuzin	H	triazinone	0.05	0.14	0.96
122931-48-0	rimsulfuron	H	pyrimiddinylsulfonylurea	0.03	0.00	0.03
5915-41-3	terbuthylazine	H	chlorotriazine	0.33	1.23	8.50

[a] F = fungicide; H = herbicide; I = insecticide; M = metabolite

Table 2. Mean (±σ) values of environmental variables and multi-substance potentially affected fraction (msPAF) for the six sampling sites along the nine sampling dates in 2014.

Parameter	**Sampling sites**					
	R	**T1**	**T2**	**T3**	**M1**	**M2**
Ammonia (mg NH4/l)	2.95±0.90	4.09±1.60	0.84±0.60	2.08±1.84	0.77±0.48	2.65±1.27
Nitrates (mg NO3/l)	0.97±0.68	0.02±0.03	0.02±0.03	0.03±0.07	0.56±0.95	0.57±1.53
Phosphates (mg P2O5/l)	0.09±0.17	0.66±0.38	0.01±0.01	0.01±0.01	0.02±0.01	1.64±0.14
pH	7.0±0.36	7.0±0.30	6.92±0.37	6.98±0.36	6.9±0.20	7.01±0.39
Dissolved oxygen (mgO2/l)	10.1±0.5	9.5±0.5	8.4±1.11	8.7±0.44	8.5±0.32	8.7±1.6
Water temperature (°C)	21.4±1.44	20.9±1.49	21.7±1.19	22.1±1.10	21.2±1.59	21.5±1.65
Conductivity (µs/cm)	284±56	271±63	251±61	267±69	213±74	209±83
Alkalinity (mg HCO3/l)	87.2±43.7	93.8±47.1	79.0±25.9	69.6±19.2	90.4±18.6	86.8±17.1
Chlorophyll a	10.39±3.90	7.94±3.93	8.36±3.68	7.33±4.61	7.67±3.68	7.81±3.73
msPAF primary producers	0.003±0.002	0.013±0.01	0.01±0.02	0.01±0.01	0.184±0.26	0.153±0.12
msPAFarthropods	0.005±0.003	0.008±0.01	0.01±0.02	0.01±0.01	0.418±0.17	0.484±0.14
msPAF primary producers (max.value)	0.005	0.05	0.03	0.03	0.86	0.36
msPAFarthropods (max.value)	0.01	0.05	0.02	0.02	0.87	0.75

Table 3. Mean (μ) and standard deviation (σ) of log-transformed toxicity data (expressed in HU units) as calculated for each pesticide and used for the msPAF calculations.

Pesticide	Primary producers		Arthropods	
	μ	**σ**	**μ**	**σ**
aminomethylphosphonic acid	5.4031	0.5624	5.4031	0.5624
azoxystrobine	3.1222	0.8293	2.5568	0.7017
chlorantraniliprole	2.5734	1.1936	2.0203	0.8389
chlorathalonil	2.0068	0.8109	2.2162	0.7637
chlorpyrifos	2.3142	0.8767	-0.3572	1.3089
cymoxanil	3.8985	0.459	3.7787	0.6978
cypermethrin	0.1714	0.7195	-0.3572	1.3089
desethyl-terbuthylazine	1.6134	0.7866	2.8573	0.8956
ethoprophos	2.4893	0.6064	2.0612	0.9043
folpet	2.9793	0.7053	3.3219	0.9655
glufosinat	5.3784	0.8047	5.3784	0.4201
glyphosate	5.2477	0.7585	4.7564	1.1393
imidacloprid	4.5553	1.2167	4.5553	1.2167
indoxacarb	2.906	0.6157	2.906	0.6157
lambda-cyhalothrin	-0.0534	0.7195	-0.4297	1.3089
metolachlor	3.834	1.1849	3.8793	0.4773
metribuzin	1.9787	0.6097	4.5336	0.8502
rimsulfuron	2.0769	1.2737	3.9549	0.2369
terbuthylazine	1.6134	0.7866	2.8573	0.8956

3.2. Community compositions and relation with risk predictions

The biological samples comprised in total 82 taxa (37 zooplankton taxa, 33 macroinvertebrates and 12 phytoplankton taxa). In terms of number of taxa, the macroinvertebrate communities were dominated by Diptera (8 taxa), Trichoptera (7 taxa) and Oligochaeta (5 taxa; see Table 4). The abundance of Ephemeroptera-Plecoptera-Trichoptera (EPT) in the samples with lower values of msPAF $_{arthropods}$ (irrigation canal and tomato area samples) was higher than in the areas with higher msPAF $_{arthropods}$ values. In the locations with the lowest average values of msPAF $_{arthropods}$ (R and T1), Trichoptera dominated this group. Freshwater zooplankton was dominated by rotifers in all locations. In locations with the highest values of msPAF $_{arthropods}$ (M1 and M2) the rotifer abundances increased whereas cladocera were absent in these locations (Table 5). In terms of total abundances throughout the sampling period, phytoplankton was highly dominated by diatoms and blue-green algae in all the sample locations (Table 6). Presence of green algae could be associated with sample locations with lower values of msPAF $_{primary\ producers}$ (with the exception of the sample location M1). Environmental factors including water temperature, pH, alkalinity and dissolved oxygen exhibited only slight variation (up to 10% of total standard deviation) among the sampling sites in each study area (Table 2).

Table 4. Relative abundances (in %) of macroinvertebrate taxa averaged over the sampling period at the tomato (T), maize (M) and irrigation (R) ditches.

Taxa	Sample Locations					
	T1	**T2**	**T3**	**M1**	**M2**	**R**
Turbellaria						
Dugesia sp.	7.7	0.3	47.5	-	-	2.0
Oligochaeta						
Nais sp.	24.0	0.4	3.8	-	-	3.3
Stylaria sp.	3.8	0.0	1.1	3.1	0.5	4.3
Lumbriculidae sp.	-	47.3	-	-	-	3.0
Eiseniella sp.	0.4	1.2	0.3	6.1	-	2.1
Eiseniella tetraedra	-	4.2	2.4	-	-	1.9
Decapoda						
Atyaephyra sp.	-	-	-	1.0	0.5	3.0
Astacus sp.	0.0	0.3	0.9	0.0	-	0.1
Hirudinea						
Erpobdella monostriata	7.6	9.7	-	-	-	0.8
Gastropoda						
Physella acuta	17.5	0.1	8.2	9.2	0.5	5.9
Viviparus sp.	-	2.9	0.1	-	0.5	2.0
Coleoptera						
Riolus sp. (larvae)	-	-	0.1	-	1.6	1.5
Diptera						
Culex sp.	-	-	0.3	-	-	6.3
Phalacrocera sp.	-	-	-	-	0.2	1.0
tr. Chironomini	-	16.8	26.7	46.2	12.8	1.0
Chironomus gr. plumosus (pupae)	0.9	-	-	-	6.1	-
Chironumus gr. Thumni (pupae)	18.0	3.8	0.2	-	2.3	-
tr. Chrironomini (nymphs)	0.2	0.6	0.2	33.8	17.0	
tr. Tanytarsini (nymphs)	-	3.8	3.2	-	57.5	-
Corynoneura sp.	-	-	0.1	-	-	2.4
Odonota						
Libellula sp.	0.6	-	-	-	0.2	-
Hemipetera						
Gerris thoracius	-	-	-	-	0.2	0.3
Corixidae	-	0.1	0.1	-	-	12.4
Ephemeroptera						
Cloen dipetrum	11.6	0.2	-	-	-	4.0
Plecoptera						
Nemoura sp.	-	0.4	0.1	-	-	3.0
Capnia sp.	0.1	-	0.2	-	0.1	5.0
Trichoptera						
Glossossoma sp.	2.4	-	0.1	0.6	-	1.0
Agapetus sp	2.0	1.3	2.0	-	-	14.2
Limnephilus sp.	3.1	-	0.2	-	-	1.0
Limnophora sp.	-	-	0.1	-	-	2.1
Antichaeta sp. (pupae)	-	2.0	-	-	-	3.0
Antichaeta sp. (larvae)	0.1	4.2	0.7	-	-	3.3
Hydropsyche exocellata	-	0.1	1.6	-	-	10.0

Table 5. Relative abundances (in %) of zooplankton taxa averaged over the sampling period at the tomato (T), maize (M) and irrigation (R) ditches.

Taxa	Sample Locations					
	T1	**T2**	**T3**	**M1**	**M2**	**R**
Ploima						
Brachionus rotundiformis	0.1	0.2	1.4	11.8	96.1	27.2
Brachionus calyciflorus	1.1	0.9	0.1	-	-	11.0
Brachionus urceolaris	0.7	0.2	0.5	-	-	1.7
Brachionus angularis	-	0.4	0.2	0.8	-	13.4
Brachionus quadridentatus	-	-	-	1.5	-	1.3
Keratella cochlearis	0.6	-	-	-	-	2.0
Hexartha sp.	-	-	-	-	-	-
Ascomorpha sp.	-	-	-	0.7	-	3.7
Filinia terminalis	-	-	-	-	-	2.6
Filinia cornuta	-	0.1	-	-	-	0.6
Filinia brachiata	5.5	12.6	0.4	0.2	0.1	1.1
Mytilina ventralis sp.	26.2	1.8	3.1	-	-	1.1
Trichocerca sp.	-	12.6	2.7	3.0	0.5	0.3
Cephalodella sp.	11.2	-	-	-	-	-
Cephalodella forficula	-	-	-	0.1	-	0.2
Lecane quadridentata	3.2	1.0	1.5	-	-	-
Lecane sp	16.3	4.1	4.4	0.2	-	-
Polyartha sp.	-	0.6	-	-	-	6.3
Synchaeta sp.	-	-	-	-	-	6.9
Lepadella patella	5.5	13.1	63.9	-	-	-
Colurella sp.	-	1.2	0.6	0.8	-	-
Rotifer sp1 (morphospecies)	0.8	6.1	3.5	1.6	1.7	0.5
Rotifer sp2 (morphospecies)	-	5.0	0.2	2.1	-	0.5
Rotifer sp3 (morphospecies)	13.4	0.5	1.1	4.4	-	0.7
Rotifer sp4 (morphospecies)	-	1.9	0.2	-	-	0.5
Rotifer sp5 (morphospecies)	-	1.5	0.7	0.5	-	0.7
Rotifer sp6 (morphospecies)	-	-	-	4.7	-	0.2
Rotifer sp7 (morphospecies)	-	-	-	-	-	0.2
Copepoda						
Nauplii Copepoda	3.6	-	-	1.2	1.4	-
Copepodite / Adult *Acartia clausi*	1.3	-	1.3	-	-	2.0
Copepodite / Adult *Acartia tonsa*	0.5	-	0.5	65.2	0.1	4.0
Copepodite / Adult *Centropages* sp.	-	15.6	3.0	-	-	7.0
Copepodite / Adult *Centropages typicus*	-	-	6.5	-	-	0.6
Copepodite /Adult *Centropages chierchiae*	1.3	-	1.5	-	-	1.0
					-	
Cladocera						
Daphnia sp.	-	-	0.9	-	-	0.1
Bosmina longirostris	0.1	0.3	-	-	-	-
Podon leuckartii	-	-	-	-	-	2.4
Ostracoda	8.6	20.3	1.9	1.1	0.1	0.4

Table 6. Relative abundances (in %) of phytoplankton taxa averaged over the sampling period at the tomato (T), maize (M) and irrigation (R) ditches.

Taxa	Sample Locations					
	T1	**T2**	**T3**	**M1**	**M2**	**R**
Blue-green algae						
Cyanophyta filamentous	11.32	8.73	18.75	4.01	2.12	48.23
Diatoms						
Bacillariophyta pennales	82.96	81.82	72.63	48.41	12.58	3.33
Bacillariophyta centric	4.97	8.87	-	47.58	58.15	1.11
Fragilaria	-	-	-	-	15.43	-
Euglenoids						
Euglenophyta	-	-	-	3.32	1.78	-
Green-algae						
Chlorophyta	-	-	-	-	0.01	-
Scenedesmus	-	-	-	-	4.34	8.44
Monoraphidium	-	-	-	-	0.03	-
Pediastrum	0.04	0.03	-	-	2.36	-
Desmidiaceae	-	-	0.63	-	-	-
Staurastrum	-	-	-	-	2.06	-
Dinoflagellates						
Dynophyceae	0.72	0.54	7.99	-	2.92	38.90

3.3 Identification of variables influencing community composition fluctuations and variance partitioning

The RDA analysis showed that temperature ($p<0.01$), nitrates ($p<0.01$), msPAF $_{\text{primary producers}}$ ($p<0.01$) and msPAF $_{\text{arthropods}}$ ($p<0.001$) significantly influenced the community structure. Selected environmental variables and msPAF values in the redundancy analysis collectively explained 39.4% of the variation in the community composition, with a high statistical significance ($p=0.0010$, Monte Carlo permutations).

The first constrained axis is significant and accounts for 33.5% of the variance, which is principally correlated with msPAF $_{\text{arthropods}}$ (Fig 2a). Along this first axis there is a clear distinction between ditches of the maize agroecosystem (M1 and M2) on the one side

and the irrigation canal and tomato area on the other (Fig 2b). Rotifers and copepods are closely associated with the highest values of msPAF $_{arthropods}$ and the remaining taxa were related to low msPAF $_{arthropods}$ values (Fig 2a).

The second axis is also significant and accounts for 27.2% of the variance. This axis is principally positively correlated with msPAF $_{primary\ producers}$ and msPAF $_{arthropods}$ and negatively correlated with temperature. The second axis represents mainly a gradient over time and separates the samples from the highest msPAF and lower temperature values from the samples with the lowest values for these parameters in the left side of diagram (Fig 2b). Euglenoids and diatoms were the taxonomic groups that were most closely associated with the higher msPAF $_{primary\ producers}$ values. The class Insecta, the class Ciliata and the order Mollusca were associated with low msPAF values and higher temperature values. Further constrained axes were not significant.

The results of the partial RDA showed that msPAF predictions contributed mostly to the explained variance (23.7%) and after accounting for other covariables it is still significant with a permutation test (P=0.001). Indeed, all covariables other than msPAF explained only 10.3% of the variance with a shared variance between the factors of 5.4% (Fig. 3).

From all groups of biological communities analysed, the percentage of total explained variance was the highest for the macroinvertebrate community (55.6%). All factors explained between 5.8 % and 22% of the total variance in this community composition (Table 7).

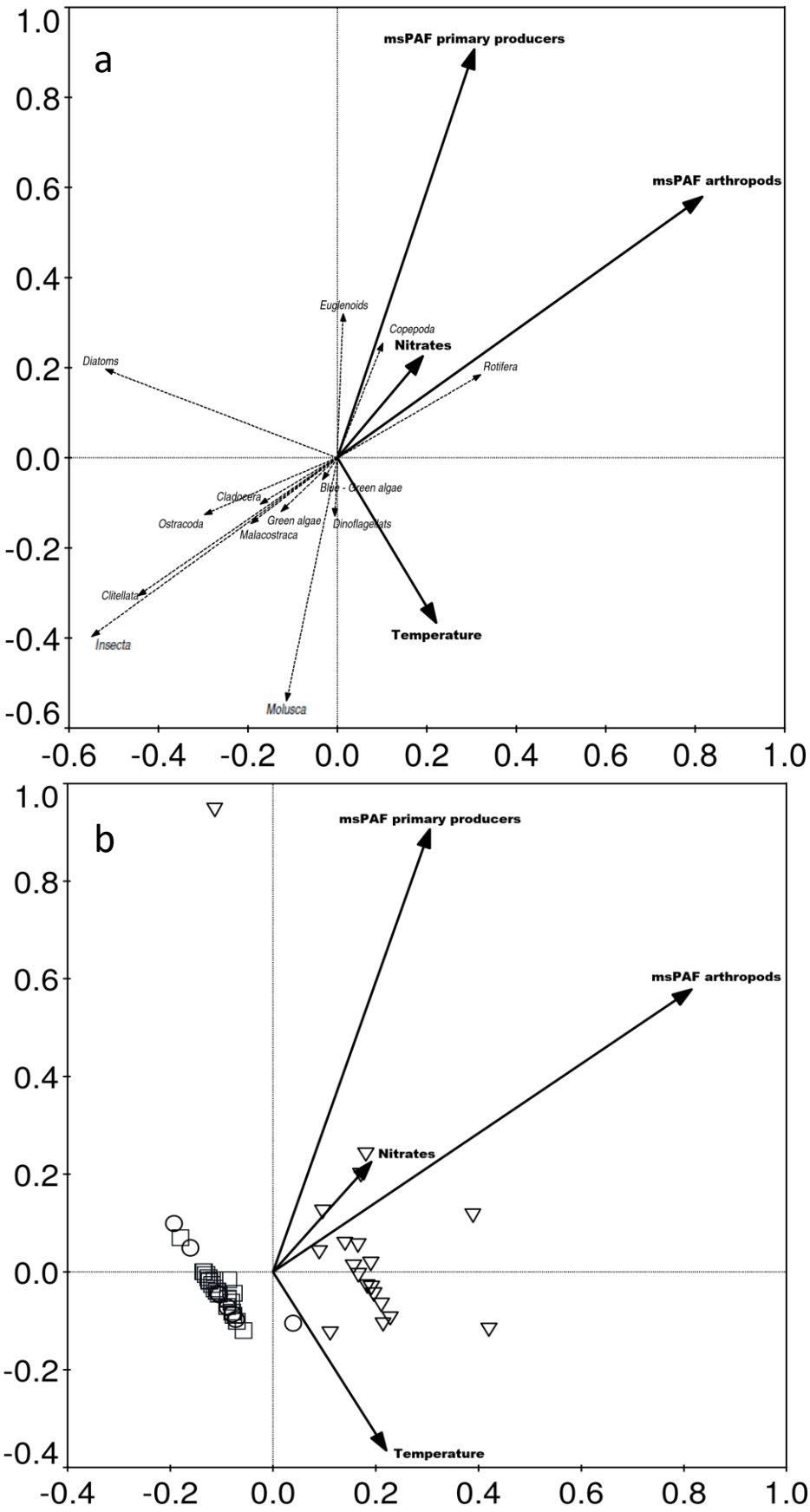

Fig. 2. Biplot based on redundancy analysis of the macroinvertebrate, zooplankton and phytoplankton datasets: (A) ordination of species groups and (B) ordination of samples sites. Inverted triangles: samples (M1 and M2) from the maize area; Circles: samples (R) from the irrigation canal; Squares: samples (T1, T2, T3) from the tomato area.

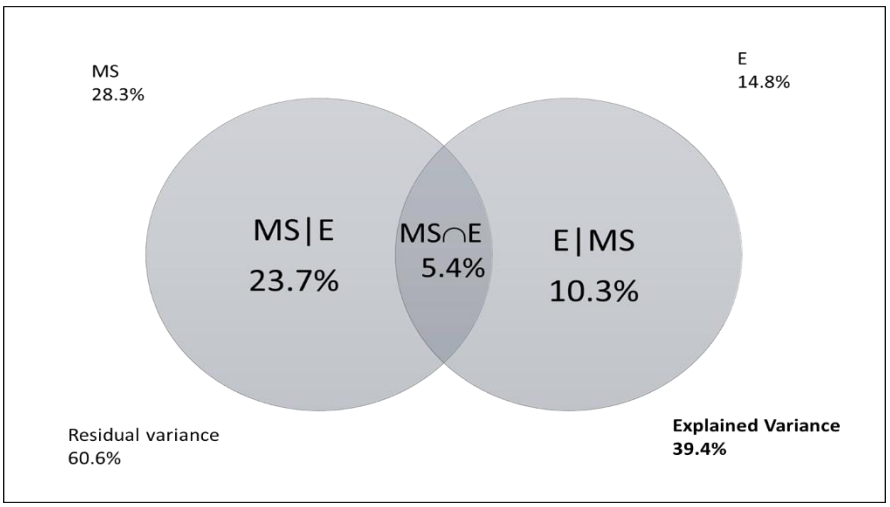

Fig. 3. Components of variance estimated for total freshwater communities (in %): total explained variance (MS∪E), residual variance, variance explained only by msPAF values (MS|E), only by environmental factors (E|MS); all variance explained by msPAF values (MS) (i.e. including other explanatory variables as co-variables), all variance explained by environmental factors (E); and shared variance between environmental variables and msPAF values.

Table 7. Components of variance estimated for macroinvertebrates, zooplankton and phytoplankton groups introducing the other components of biota as components of variance.: total explained variance (MS∪E∪B), residual variance, variance explained only by msPAF values (MS|E∪B), only by environmental factors (E|MS∪B), only by biota (B|MS∪E) (i.e. including other explanatory variables as co-variables) ; all variance explained by msPAF values (MS), by environmental factors (E) and biota (B); shared variance between environmental variables, msPAF values and biota Presented are the percentages of explained variance.

Response Group	MS∪E∪B	Residual variance	MS|E∪B	E|MS∪B	B|MS∪E	MS	E	B	E∩MS∩B
Macroinvertebrates community	55.6	44.4	8.1	18.5	22.1	33.3	32.7	29.5	6.9
Zooplankton community	46.1	53.9	5.8	15.5	17.6	8	22.8	18.7	7.2
Phytoplankton community	49.7	50.3	7.1	19.2	15.9	12.7	25.6	21.3	7.5

4. Discussion

4.1. Pesticide mixture toxicity

We found a low to high risk for toxic effects on primary producers and arthropods depending on the sampling site and moment (Table 2). The percentage of species potentially affected by the mixture was higher in the locations M1 and M2 associated with the maize agroecosystem, reaching a maximum of 86% (for arthropods) and 87%

(for primary producers) for the M1 location. The group of organophosphates and pyretroids contributed around 60% to the mixture toxicity (in M1 and M2 locations) over the whole period. The top three risk contributors identified in the msPAF for arthropods are chlorpyrifos (35%), cypermethrin (21%) and lambda-cyalothrin (10%) for all the samples. A field mixture toxicity study by Rämö et al. (2016) also found the insecticide chlorpyrifos to be in the top five pesticides contributing to 90% of the risks to fish and arthropods. Silva et al. (2015) provided a general environmental status of the Portuguese 'Mondego', 'Sado' and 'Tejo' river basins during the main periods of agricultural activity from 2002 to 2008 and ranked the relative contribution of the individual pesticide compounds (or TMoA class) to the total msPAF. These authors verified that acetylcholinesterase (AChE) inhibitors (chlorfenvinphos and chlorpyrifos) and the GABA-gated chloride channel antagonist endosulfan appeared to be the most hazardous for arthropods and fish species, respectively (Silva et al. 2015).

A clear distinction could be made in community structures between ditches of the maize agroecosystem (M1 and M2) from the irrigation canal and tomato area (Fig. 2b). The samples with higher values of msPAF (arthropods and primary producers) were closely associated with higher abundances of less sensitive taxa like rotifera, copepoda and diatoms, and lower msPAF values with more sensitive taxa including insects and green-algae (Fig. 2a). The fact that different taxa varied in their response to pesticides can be explained by the fact that the detected compounds act differently depending on their target organism (DeLorenzo et al. 2001). Maltby et al. (2005), for example, verified that all the 16 insecticides that they evaluated were more toxic to arthropods than vertebrates (fish and amphibians) and non-arthropod invertebrates (i.e., Mollusca, Annelida, Platyhelminthes, Rotifera, Protozoa). Diatoms have been reported to be generally more tolerant to PS II inhibiting herbicides than other algae, especially green-algae (Herman et al. 1986; Gurney and Robinson 1989; Molander and Blanck 1992; Hoagland et al. 1993; Bérard et al. 1999). For example, a model ecosystem study evaluating the PS II inhibiting herbicide linuron by Daam et al. (2009) indicated that chlorophytes belonging to the genera *Scenedesmus*, *Coelastrum* and *Pediastrum* were more sensitive than other chlorophytes, diatoms, and cryptophytes. Tolerant taxa appeared to be less digestible for several zooplankton taxa, which subsequently decreased in abundances (Daam et al. 2009). Subsequently, when high msPAFs for direct

toxic effects are obtained for a certain taxonomic group, indirect effects may be anticipated on other taxonomic groups. This may hence also illustrate the importance of biota interactions on the (indirect) effects of pesticides under field conditions.

EPT (Ephemeroptera, Plecoptera and Trichoptera) taxa were more abundant in locations with lower predicted mixture effects on arthropods, whereas in the locations with the lowest average values of msPAF $_{arthropods}$ (R and T1) Trichoptera dominated the invertebrate communities. This is in accordance with results from previous field studies demonstrating the general high sensitivity of EPT taxa to insecticides like chlorpyrifos and cypermethrin that were indicated to be the highest contributors to the msPAF $_{arthropods}$ (Leonard et al. 2000; Berenzen et al. 2003, Rico and Van den Brink 2015; Ieromina et al. 2016). Despite that annelids and snails are generally among the least sensitive taxa to insecticide pollution (e.g. Maltby et al. 2005), higher abundances were associated with samples with lower msPAF $_{arthropod}$ (Figure 2). However, several annelid taxa have been reported to be sensitive to the neonicotinoid imidacloprid, one of the main contributors to msPAF$_{arthropod}$, and the relatively high vulnerability of snails may be explained by their low dispersal abilities, regardless of their medium to low pesticide sensitivity (Rico and Van den Brink 2015).

4.2. Variance partitioning of community compositions

The multivariate analysis showed that the pesticide mixture toxicity explained a significant part of the variance in species abundance (23.7%) after accounting for co-variables (Fig. 3). Those results are in agreement with the results of other large freshwater field monitoring studies, in which agricultural pesticides are usually identified as the main chemical stressors for invertebrate communities (e.g. Liess and Von der Ohe 2005; Kuzmanović et al. 2015). Changes in the community structure due to priority and emerging pollutants have previously been reported in Mediterranean rivers (Muñoz et al. 2009; Ricart et al. 2010; Brix et al. 2012; Kuzmanović et al. 2016), indicating the general biological impairment in relation to pollution. Other field studies reported similar percentages of variance in biological communities explained by different factors. In a study by de Zwart et al. (2006), toxicants explained 3% of the total variance in fish

communities in rivers, relative to 28% of variance explained by water chemistry parameters and 16% of variance explained by habitat characteristics. Zuellig et al. (2012) concluded that the total variance in freshwater algae, fish, and invertebrate communities explained by between-site variance and time was also ∼30%. The variance in macroinvertebrate community explained by environmental and spatial factors reached ∼ 25% in a study by Heino et al (2012). Also Ieromina et al. (2016) found that total variance of macroinvertebrates communities explained by pesticides concentrations, environmental factors and time reached ∼23% and the largest proportion of the variance (10.1%) was attributed to environmental factors, followed by pesticides (5.4%), and time (4.8%).

In our study, biota explained the largest percentage of variance in the different groups (macroinvertebrates, zooplankton and phytoplankton), followed by environmental factors, msPAF predictions and shared variance (Table 7). From all groups of biological communities analysed, the percentage of total explained variance was the highest for the macroinvertebrate community (55.6%). These results demonstrate the importance of biotic interactions and site-specific environmental conditions in structuring community compositions. Previous studies have also emphasized the importance of environmental factors in shaping the community compositions of aquatic biota. For example, in a study by Larsen et al. (2012), environmental factors were found to be more important than species interactions in structuring fish and invertebrate communities. In a study by Zuellig et al. (2012), environmental factors dominated the inter-annual variance in shaping invertebrate community. Friberg et al. (2003) concluded that some of the effects found on the macroinvertebrate community composition could be indirectly mediated through changed biotic interactions within the lotic food web. Research of Schulz and Dabrowski (2001) demonstrated that pesticides may influence such biotic interactions. For example, the authors found that the mortality of mayflies increased in a synergistical manner when both fish and sublethal concentrations of two pesticides (azinphos-methyl and fenvalerate) were present (Schulz and Dabrowski 2001).

Mechanisms such as competition and predation are important in structuring ecosystem communities, and the relative strength of these interactions will likely influence how communities respond to anthropogenic disturbance (Clements and Rohr 2009). A

reduction in abundances of primary producers leading to a decrease in herbivore populations as noted for example in Daam et al. (2009) is a common phenomenon and has been attributed to food limitation and/or habitat loss (Schäfer et al. 2011). In this regard, Thompson et al. (2015) suggested that compositional differences in zooplankton communities have a larger impact on ecosystem function than local environmental conditions.

Since the variation in ecosystem communities is highly influenced by factors such as habitat and physical-chemical conditions as discussed above, establishing causality between chemical pollution and community compositions in the field is not straightforward (Sabater et al. 2016). The correlational findings could also be the result of cumulative or synergistic effects caused by the stressors or by other stressors that co-occur in the system. For example, various studies have reported on the joint effects caused by triazine herbicide mixtures on benthic diatom communities (e.g. Faust et al. 2003; Gregorio et al. 2012). However, it has also been shown that environmental factors governing diatom assemblages are associated with a variety of physical-chemical characteristics so that the predictability of the detected variables is diminished under highly variable environmental conditions (Potapova and Charles 2002).

Conclusions

The analysis of the data revealed that the plankton and invertebrate communities had similar responses to the stressors, with a decrease in biodiversity and associated simplification of the biological structure in the presence of higher predicted risk to arthropods and primary producers. Most of the sampling sites and moments with higher predicted PAF for mixtures were indeed associated with a decrease in pesticide-vulnerable taxa. Thus, the observed losses in taxonomic diversity can at least partly be associated with the loss of those taxa specifically vulnerable to pesticides. The RDA and pRDA analysis further indicated that environmental factors and biotic interactions also influenced the freshwater communities considerably.

More work is needed to better understand and unravel the effects of co-occurring chemical, environmental and biological stressors in aquatic ecosystems. This should

include multi-stressor assessments in (semi) field studies, taking abiotic factors, habitat features, biotic interactions, as well as differences in responses of taxa due to their varying ecological preferences into account. The appropriate combination of different community indicators and endpoints (e.g. behaviour or functioning) will aid in improving the realism of ecological risk assessments in aquatic ecosystems.

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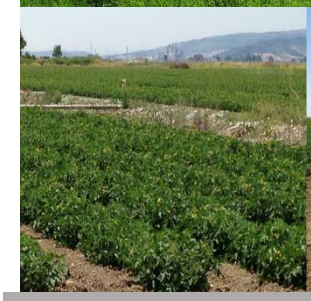


CHAPTER 7

GENERAL DISCUSSION AND CONCLUDING REMARKS

1. General Discussion and conclusions

Several conclusions can be drawn based on the results from the approaches developed and applied to increase our understanding of the linkage between pesticide mixture exposure and effects under relevant South European conditions. These conclusions are listed below and are discussed in relation to the specific aims of this thesis that were outlined in Chapter 1.

1.1. Accurateness of the models and scenarios developed by the FOCUS group and used in the European ERA of pesticides to predict environmental concentrations measured under specific Mediterranean conditions

1.1.1. Accurateness and representability of pesticide fate models/scenarios for rice fields

In Chapter 3, concentrations measured in experimental rice plots following aereal application of the neonicotinoid insecticide imidacloprid were compared with those predicted by first-tier (MED-Rice) and higher-tier (RICEWQ) predicted concentrations. **Although first-tier assessments may be expected to lead to worst-case predictions, the peak concentrations measured in the imidacloprid-treated rice plots (52-60 µg/L) were approximately two times higher than the simulated first-tier concentration using the MED-Rice** methodology (30 µg/L). The underestimation of the insecticide concentrations was verified for all the different scenarios evaluated, i.e. the default scenarios proposed by MED-RICE as well as site-specific scenarios (Chapter 3, sections 3.1. and 3.2). On the other hand, when comparing the first-tier PECs using the model proposed by US-EPA (78 µg/L), the actual peaks concentrations were lower than the simulated values. This difference between the two models is probably due to differences in how the PEC is calculated and assumptions that are made in this. For example, the sediment depth used by MED-Rice and EPA is five and one cm, respectively. Given the same DT50 values (based on field data) and the lower peak-concentrations modelled by the MED-Rice method, TWA concentrations calculated over 28 days post application

from **MED-Rice simulations** (between 2 µg/L and 2.5 µg/L) were logically also lower than those obtained from actual paddy field measurements (8.0 µg/L). Similarly, the **modelled peak of imidacloprid concentration in the drainage canal water (0.43 µg/L) was more than an order of magnitude lower than that actually measured in the field (8.8 µg/L).**

The PEC of imidacloprid calculated with the higher-tier model **RICEWQ agreed well with concentrations measured in the field** (with good agreement of both the water balance calibration and of the overall exposure profile). Previous validation studies of the model under European conditions also showed a high agreement between observed and predicted pesticide concentrations (e.g. Capri and Miao 2002; Karpouzas et al. 2005; Karpouzas and Capri 2006; Christen et al. 2006; Infantino et al. 2008). Methodologies generally used in the EU and USA for lower-tier PEC calculation, however, underestimated actual field concentrations and appear to **need further evaluation and eventually amendments**.

1.1.2. Predictiveness/accurateness of FOCUS predictions and representativeness of South European surface water scenarios for the Mediterranean conditions

Predicted pesticide concentrations used in aquatic risk assessment should cover the range of realistic estimates of field concentrations. However, according to study presented in Chapter 2, significant differences between PEC_{gw} (predicted environmental concentrations in groundwater) simulated with the model PELMO and the measured maximum concentrations in groundwater (MEC_{gw}) as reported in the literature were observed (*vide* Table 2 and 3, Chapter 2). For example, the **highest MEC_{gw} value reported for dimethoate (110 µg/L) is approximately three order of magnitude higher than the highest simulated PEC_{gw} of 0.11 µg/L**. MECs reported in other studies for dimethoate in different sampling periods and localities were comparable to this PEC value (0.15 µg/L and 0.09 µg/L), although also slightly (0.9 µg/L) to clearly higher (2.3 µg/L and 10.9 µg/L) values were encountered. This could thus indicate that the **scenarios adopted to calculate groundwater PECs do not fully cover particular local agricultural practices** in case of diffuse pollution and/or a high occurrence of specific and punctual

pollution episodes with this insecticide. An in-depth consideration of irrigation efficiencies and practices, in particular in Southern Europe indicate that a revision of the FOCUS scenarios should be considered (e.g. the FOCUS Porto scenario does not consider irrigation, whereas intensive irrigation is usually applied in agricultural areas in Porto and the rest of Portugal). Calculations with groundwater pesticide fate models could also become more realistic if not only one application date is used per scenario, which is the current practice in the model scenarios used, but the actual range of application dates in different scenarios and years. **Future field monitoring studies are hence needed to validate and eventually calibrate the way PEC_{gw} values are currently calculated with the different models and scenarios currently** in use. Such studies would also aid to address the question to what extent the high MEC values may be attributed to diffuse or point-source pollution and/or limitations in the fate models and scenarios used.

Concerning the **predictiveness of FOCUS surface water ($FOCUS_{sw}$)** models under South European conditions, **44% of the concentration predictions** in tomato and maize edge-of-field water bodies discussed in Chapter 4 **underestimated the measured pesticide concentrations in surface waters** (MEC_{sw}), thus showing a **non–compliance** between the predictions **with the field data**. In spite of the low number of compounds that were analysed, a trend is pointed out with **herbicide concentrations being overpredicted** and **insecticides and fungicide concentrations underpredicted**. Thus, because values that are lower than the actual surface water concentrations are considered in the regulatory risk assessment, pesticides **might exhibit unacceptable ecological effects in realistic conditions**. Besides comparing PECs with maximum MECs, the calculated PECs were also compared with the 90^{th} percentile MEC_{sw} from the detected substances at each crop site (tomato and maize). This was done to exclude particularly low water-phase concentrations and to increase the chance that measured concentrations might be considered as realistic peak concentrations, because the FOCUS approach also aims at predicting maximum peak concentrations. However, this approach led to the same number of substances that underestimated and/or overestimated the MECs as it was obtained with the comparison using the maximum MECs. The best compliance was verified for the insecticides imidacloprid and indoxacarb with an “underestimation” of 0.25 and 0.22 times, respectively, when comparing with the 90^{th} percentile. In addition,

a reduction of the **percentages of underestimation was obtained for all substances when using the 90th** percentile method, reaching for example a **significant reduction for the pyrethroid cypermethrin (from 98 times up to 29 times)**. This result is especially relevant since pyrethroids have become increasingly important agricultural insecticides over the past decades (Hendley et al. 2008).

Mackay et al. (1996) stated that it is unlikely for exposure models to be universally valid but they may produce results that are deemed to be reliable with a level of accuracy that depends on the nature of the chemical, the environment, and regulatory requirements. **Regarding only the aspect of the regulatory requirements, the FOCUS approach failed to meet the demanded protection goal. On the other hand**, and as mentioned above, **the underestimation of MEC$_{sw}$ may also be the result of farmers' malpractice**, for example **the non-adherence to spray buffer zones, besides eventual model inaccuracies**.

An underestimation of actual pesticide concentrations by FOCUS$_{sw}$ models was also reported in studies by Knäbel et al. (2012, 2014). Using a literature dataset of measured concentrations of pesticides in mainly European but also American surface waters, these authors showed that 23% and 15% of the measured insecticide and fungicide field concentrations, respectively, were underpredicted by the step-3 PECs calculated with FOCUS using the exact methodology as it is applied within the regulatory risk assessment for pesticides. Based on their results, Knäbel et al. (2012, 2014) hence questioned the protectiveness of the FOCUS exposure assessment. Only a few other published studies have compared predicted environmental pesticide concentrations and field data and most of these studies only evaluated a very limited number of cases, did not use the FOCUS surface water approach, or used a dataset mainly based on MECs determined in locations outside Europe (Padovani and Capri 2005; Singh and Jones 2002; Jackson et al. 2005; Knäbel et al. 2012, 2014).

Overall **our results showed that the South European scenarios used for PEC$_{sw}$ step 3 predictions were not well adapted when compared to real-world surface water** situations. This could hence **reflect unacceptable ecological effects which are not assessed by the current Regulatory risk assessment**. The results of **this study reinforces that the predictions of FOCUS approach are not accurate and therefore not protective** predicting pesticide concentrations in the field in the context of European pesticide risk

assessment, taking into account the 90th percentile protection goal claimed by the FOCUS surface water group.

1.2. Increase our understanding of the risk evaluation of pesticides in Mediterranean freshwarter ecosystems

1.2.1. Provide a preliminary risk evaluation of predicted pesticides attending to those with trigger values lower than 0.1 µg/L and increase the knowledge concerning their potential underprotection of the risks to groundwater life

The potential **risks to groundwater life** was predicted by **comparing the trigger values** (TVs) for the pesticides for which Daam et al. (2010) calculated a TV lower than 0.1 µg/L **with** their respective calculated **PECs and MECs** obtained from the open literature (Chapter 2). The TVs were calculated using three different approaches: (1) first-tier (*Daphnia magna* and Vibrio fisheri); (2) species sensitivity distributions (SSDs), constructed for surrogate freshwater organisms for the truncated groundwater biodiversity; (3) the case-based model PERPEST. For the substances for which was identified a potential risk, the severity and probability of potential effects was accessed, through species sensitivity distributions (SSDs) and PERPEST model.

Based on simulated PEC values with an 80th percentile <0.01 µg/L, with exception of dimethoate, **no risks are expected** and the **trigger values calculated for groundwater thus appears to be sufficiently protective** for those PPPs (see Chapter 2, Table 2). Subsequently dimethoate is the only substance in the study that indicated risk (RQ = PEC/TVs) with high values for both short-term (RQ=112) and long-term (RQ=1123) risk. Nevertheless, our r**esults indicated a lack of concordance between calculated PEC$_{gw}$ with MEC$_{gw}$** encountered in literature. **Considering concentrations actually measured** in the field, **99.7% showed RQ values higher than 1** and 36.7% even higher than 100. Additionally based on the SSD curves constructed for the four pesticides for which the highest MECs values were found in the open literature (i.e. **chlorpyrifos, esfenvalerate, dimethoate and phosmet) the potentially affected fraction of the species assemblage**

was always greater than 20%, varying between 21% for dimethoate up to as high as 49% for esfenvalerate. Also for **chlorpyrifos, esfenvarelate and lambda-cyalothrin** pesticides included in the PERPEST model indicate a **large probability of clear effects on taxonomic groups** likely to be encountered **in groundwater ecosystems**. Moreover, it should be taken into account that water quality objectives for PPPs are frequently far below analytical detection limits and that the lack of any positive experimental finding does not necessarily mean absence of risk. **Based on our results unacceptable effects of pesticides can potential occur and were not totally covered by the current prospective PPPs environmental risk assessment** (first tier or higher tier). On the effect side, the use of toxicity data generated with surface water taxa for the sensitivity assessment of groundwater organisms should be evaluated by developing toxicity testing with true groundwater taxa and subsequently comparing results obtained with their surface water counterparts.

1.2.2. Evaluate the prospective and retrospective risk posed by pesticides in surface waters of different agricultural ecosystems

An integrated crop 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.

Concerning the **rice crop-based** approach, developed in Chapter 3, the **peak of imidacloprid concentration of 8.8 µg/L, as measured in the drainage canals, is more than an order of magnitude higher than the maximum ecological quality reference value of 0.2 µg/L** of imidacloprid in Europe, whereas annual-average benchmark values set for imidacloprid are even as low as 0.0083 to 0.067 µg/L. Subsequently based on our results, **a withholding period of at least 28 days would be needed to allow time for imidacloprid residues in the paddies to dissipate to levels that may be considered acceptable for (acute) environmental protection prior to being discharged from the field**. Therefore the **withholding periods currently used and recommended in the EU (0 to 7 days) are insufficient to avoid the spread of pesticides over watersheds** and hence need to be increased. Several invertebrates (**ostracods, dipterans and coleopterans)**

decreased in numbers following imidacloprid treatment. The increase in snails (***Physa acuta***), however, assured **ecosystem functioning through functional redundancy**, implying no unacceptable risks in the paddy if ecosystem function is set as the protection goal. In this case, however, care has to be taken to have sufficient adjacent non-agricultural wetlands to avoid declines in bird populations.

Regarding the crop based approach for **maize and tomato** (Chapter 4) **ten substances contributed at least once to an exceedance of a maximum acceptable environmental quality standard (MAC-EQS).** The most critical substances were cypermethrin and lambda-cyalothrin that exceeded the MAC-EQS in 50% of the samples taken in the maize crop area, followed by **DET (37%) and chlorpyrifos (25%). In the tomato crop area, rimsulfuron (50%), chlorantraniliprole (28%), metolachlor (16%), imidaclorid (15%) and indoxacarb (12%) exceeded the MAC-EQS** in the most frequent manner. Similarly, recent studies into the prioritization of pollutants in Mediterranean rivers concluded that pesticides and their derivates were the most important compounds in contributing to risk to aquatic ecosystems, with chlorpyrifos was identified as one of the most important compounds (Kuzmanović et al. 2015; López-Doval et al. 2012, Silva et al. 2015).

With **regards to the protectiveness of the prospective risk assessment, the MECs of the organophosphate insecticide chlorpyrifos and the pyrethroid insecticide cypermethrin MEC_{max} were up to 100 and 480 times higher than their respective regulatory RACsw**. Also the **pyrethroid lambda-cyalothrin, the neonicotinoid imidacloprid and the triazine terbuthylazine were detected in concentrations up to 23, 10 and 7 times higher than their RACsw**, respectively. Stehle and Schulz (2015) conducted a comprehensive meta-analysis in which they verified that 44.7% of the measured insecticide concentrations in 1566 samples of EU surface waters exceeded their respective RACs. Pyrethroids (n=108) appeared to be the pesticide class with the highest percentage of RAC_{SW} exceedances (70.4%); followed by organophosphorus insecticides (37.5%; n=1100) and neonicotinoids (24.2%; n=33) (Stehle and Schulz 2015).

Our results highlights that especially insecticides may form an important threat to freshwater biodiversity in edge-of-field water bodies in 'Lezíria do Tejo', as insecticide levels above their RACs may lead to severe biodiversity reductions (Stehle and Schulz 2015). **The overall link between the regulatory risk assessment and the actual situation**

in the field should be considerably strengthened, and findings from this study and other field studies on pesticide exposure and effects should be used in prospective risk assessments validation. The **approach developed provides a feedback mechanism between the prospective and retrospective risk assessment.**

1.2.3. Provide insights in the risk assessment for environmental-realistic pesticide mixtures

Taking into account this objective, new integrated approaches for the ecological risk assessment of pesticide mixtures in surface waters were applied, according to the study presented in Chapter 4. **Risk was assessed by comparing the environmental quality standards (EQS) with their respective MECs assuming that concentration addition model** is applicable.

Overall, the study results **highlight that even in mixtures with a high number of components** (up to 14 in tomato and maize crop areas), **one pesticide** compound was responsible for **>50% of the toxicity**. **Insecticides (mainly pyrethroids and organophosphates) were the pesticide groups that accounted most to the highest risk** of toxicity in samples with RQmixture ratios above 1 in the "Lezíria do Tejo" study area. 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 are likely to dominate the potential for risk in water bodies of agricultural landscapes. As a general approach, the risk quotients applied in this study could be assumed as a first tier risk assessment procedure for pesticide mixtures. **If RQ (MEC/EQS) is above 1, then more sophisticated mixture toxicity models could be used to quantify overall ecotoxicological** pressure and expected local impacts in terms of predicted species loss, and to pinpoint the chemicals or the group of chemicals (considering their modes of action and targets) responsible for the identified risk .

The **pesticides with frequent co-occurrence and high potential for synergistic effects**, the triazine **terbuthylazine** and organophosphate **chlorpyrifos,** were evaluated individually and in two mixtures using concentrations measured or likely to occur in a

Portuguese agricultural **concerning there potencial side-effects on single-species and at zooplankton community** (microcosm-) level **at environmentally realistic concentrations**.

The effects of these pesticides singly and as a binary mixture on the immobility of *Daphnia magna* and on the growth rate of the microalgae *Raphidocelis subcapitata* were evaluated. Terbuthylazine and chlorpyrifos at single exposure caused a very toxic or toxic response in both organisms. The toxicity of the mixtures was evaluated in relation to the reference models CA and IA. For immobility endpoint, the data fits better to the IA model, due to different mode of action of the pesticides, however a specific pattern was showed; at low dose levels **the immobility was lower than modelled (antagonism), whereas at high dose levels the immobility was higher than modelled (synergism).** On the other hand, **no deviation was observed from independent action in algal tests.** This study represents an important step to understand the interactions among pesticides detected previously in our field monitoring (*vide* Chapter 4).

The potencial effects on phytoplankton and zooplankton community were evaluated by performing small indoor laboratory test systems, that are less complex and results are therefore easier to interpret. An in-situ bioassay and postexposure feeding rate was perfomed to better understand the potential effects. The ecological interactions between the two compounds and implications for their risk to aquatic life are discussed. **Direct toxicity of terbuthylazine was noted on phytoplankton** (measured as chlorophyll-a), which was hypothesized to **indirectly lead to effects on daphnids** through decreased food and dissolved oxygen levels, in combination with **decreased feeding rates resulting from ingestion of terbuthylazine-containing particle**. **Terbuthylazine potentiated the effect** of **chlorpyrifos on feeding rates** by **triggering the transformation of chlorpyrifos to more toxic oxon-analogs**. In addition, **food-web interactions resulting from both indirect effects of the test compounds and recovery of affected populations were also recorded**. If the **ecological recovery** option is to be adopted as the protection goal, possible **food-web interactions between chemical (and other) stressors likely to be present in edge-of-field water bodies need to be further evaluated**.

This **study highlights the need for a tiered approach** in order to **identify the chemicals** and/or type of chemicals **responsible for the identified risk and to focus on more**

problematic mixtures to assess the cumulative and synergistic effects in the aquatic environment. Adequate linking of fate and effects therefore needs the translation of complicated field-exposure patterns into representative and realistic worst-case exposure scenarios to be tested in mesocosms, to be evaluated by modelling and to be compared with ecological scenarios, improving therefore the prospective and retrospective risk assessment.

1.3. Assess how well effects of the obtained real-world exposure profiles may be predicted by comparing mixture effect predictions with those observed in the field.

Toxic substances occur in the environment as fairly complex mixtures and it is **impossible to test all existing combinations experimentally**. Therefore a **proper modelling** of their effects at various levels of biological organisation is highly appropriate and **may help to understand** and predict toxic effects of chemical mixtures on living biota. To **evaluate or validate their predictive power**, models predictions should be compared with effects observed in the field to assure they are used correctly. **Studies addressing the predicted effects of pesticides in combination with abiotic and biotic factors on aquatic biota in ditches associated with typical Mediterranean agroecosystems are scarce.** Results obtained in the study presented in Chapter 6, pointed out that the effects of pesticide mixtures in edge-of-field tomato and maize agroecosystems were predicted by the multi-substance PAF approach (msPAF) calculated with aggregation protocols based on fundamental theory on mixture toxicity and the TMoA of compounds in the mixture, quantifying the overall ecological risk of mixtures of pesticides measured in surface waters of 'Lezíria do Tejo' for different groups of species of the aquatic community. Subsequently, **to evaluate the predicted effects of pesticides along with environmental factors and biota interactions** on macroinvertebrate, zooplankton and phytoplankton community compositions in ditches adjacent to Portuguese maize and tomato crop areas the variance partitioning procedure based on redundancy analysis (**pRDA)** was used. The total variance in biological community composition was divided into the variance explained by the

msPAFarthropods and msPAFprimary producers, environmental factors (water chemistry parameters), biotic interactions, shared variance, and unexplained variance.

The analysis of the **data revealed that the plankton and invertebrate communities had similar responses to the stressors**, with a **decrease in biodiversity** and an **associated simplification of the biological structure** in the **presence of higher predicted risk** to arthropods and primary producers. Most of the sampling sites with **higher predicted PAF for mixtures were indeed associated with a decrease in pesticide-vulnerable taxa.** Thus, the observed losses in taxonomic diversity could at least partly be associated with the loss of those taxa specifically vulnerable to pesticides, indicating a **high correlation between effects of the obtained real-world exposure profiles** and there **prediction through the use of the msPAF** approach.

The pRDA analysis further indicated that **environmental factors (including the msPAF)** and **biotic interactions** also **influenced the freshwater communities considerably**. The pRDA showed that the **msPAF explained a significant part of the variance** in species abundance **(23.7%)** after accounting for co-variables (see Chapter 6, Figure 3). **When the biotic interactions were included** in the pRDA **biota explained the largest percentage of variance** in the different groups **followed by the msPAF** (see Chapter 6, Table 7). From all groups of biological communities analysed, the percentage of total explained variance was the highest for the macroinvertebrate community (55.6%). The **results demonstrated the importance of biotic interactions and site-specific environmental conditions in structuring community compositions**. Previous studies have also emphasized the importance of environmental factors in shaping the community compositions of aquatic biota (Friberg et al. 2003; Larsen et al. 2012; Zuellig et al 2012). Research of Schulz and Dabrowski (2001) demonstrated that pesticides may influence such biotic interactions. Mechanisms such as competition and predation are important in structuring ecosystem communities, and the relative strength of these interactions will likely influence how communities respond to anthropogenic disturbance (Clements and Rohr 2009). Thompson et al. (2015) suggested that compositional differences in zooplankton communities have a larger impact on ecosystem function than local environmental conditions. Retrospective causal analysis and in particular eco-epidemiological studies allow the contribution of chemical mixtures to be determined. These studies can show that mixture impacts can be spatially

quantified in aquatic ecosystems, and there is a need to understand site-specific stressor combinations in order to define effective measures to improve ecological status. **More work is needed to better understand and unravel the effects of co-occurring chemical, environmental and biological stressors in aquatic ecosystems**. This should include multi-stressor assessments in (semi) field studies, taking abiotic factors, habitat features, biotic interactions, as well as differences in responses of taxa due to their varying ecological preferences and traits into account. The a**ppropriate combination of different community indicators and endpoints** (e.g. behaviour or functioning) will aid in **improving the realism of ecological risk assessments** in aquatic ecosystems.

2. Concluding Remarks

The present research addresses some of the emerging challenges in risk assessment, particularly for an integrated assessment of pesticide stress in aquatic ecosystems, in order to **achieve a better link between (mixture) exposure and effects**. In these way provided an **important contribute** to the overall knowledge on the **adequacy of the actual environmental exposure assessment** and **showed that the actual risk** of pesticides **might be underestimated** already within the actual registration procedure. The **importance of chemical monitoring** studies for constructing exposure and effect **databases of frequently occurring pesticide mixtures** in surface waters is highlighted. The data generated in the present study contributed to i) the **derivation of optimized programs of measures** under the scope of European legislation; ii) **the identification of sites with the highest expected impacts of pesticide mixtures;** iii) the **evaluation of the major pesticide compounds** that contributed mostly to the **identified aquatic risks**. Furthermore our results contribute to **better understand and unravel the effects of co-occurring chemical, environmental and biological stressors in aquatic ecosystems** considering the effects of biotic and abiotic interactions at community and ecosystem levels.

The **improved tiered approach** developed in these study is **relevant contribute to deal with the complexity of environmental risk assessment**. The overall link between the regulatory risk assessment and the actual situation in the field should be considerably

strengthened, and findings from our and other field studies on pesticide exposure and effects should be used in prospective risk assessments. **Real-world exposure data** and **actual ecological risks** in the field should also **be considered** in **future identification** and **prioritization of WFD priority substances**.

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