Extrapolation of effects of pesticides on aquatic communities and ecosystems across different exposure patterns

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"When Ambition exceeds the Performance, The Gap is called Frustration. When the Performance exceeds Ambition, The Overlap is called Success….''

(Cullen Hightower)

Dedicated to My Beloved Mother Shamim Begum

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Chapter 1

General Introduction

What is there that is not poison?

All things are poison, and nothing is without poison

Solely the dose determines that a thing is not a poison

(Paracelsus, translation by Deichmann *et al*. 1986)

"Risk assessment is the product of a shotgun wedding between science and the law"

(William Ruckelshaus)

Pesticides

Almost all modern and traditional cultures rely on agriculture as a means of providing a steady food source to their people. In order to maximize the size and success of crop yield, farmers have turned to pesticides for the control of pests and diseases. Because of an intensification of agricultural practices as well as new technologies and developments, like the green revolution, the use of pesticides has increased considerably throughout the world over the past decades (Ecobichon, 2001; Berg, 2001). Technological advances, particularly in the form of chemical products, led to the creation of high efficiency pesticides and fertilizers, enabling a large increase in crop yield. After development, these technologies were quickly implemented globally. As a result, much of the agriculture in the world today relies heavily on the protection provided by pesticides.

Global use of pesticides

Globally, the amount of pesticides used in the agricultural sector exceeded 2.36 billion kilograms of active ingredient (a.i.) in 2007 and is still climbing (see Figure 1). Figure 1 indicates that the total amount of pesticide used in the world slightly increased in 2007 compared to 2001, and 2007 is the latest year for which figures are available. Herbicides (chemicals used to control plants, usually weeds) accounted for the largest portion (40%), followed by other[2], insecticides and fungicides which constitute 33%, 17% and 10% of the total pesticide world consumption, respectively (U.S. EPA Pesticide Market Estimates: Usage, 2001-2007; http://www.epa.gov/pesticides). Furthermore, total sales of pesticides worldwide had increased from $35.8 billion in 2006 to $39.4 billion in 2007. Expenditures on herbicides accounted for the largest portion of total expenditures (39%), followed by expenditures on insecticides (28%), fungicides (23%), and other pesticides (9%), respectively (U.S. EPA Pesticides Market Estimates: Sales, 2006-2007; http://www.epa.gov/pesticides).

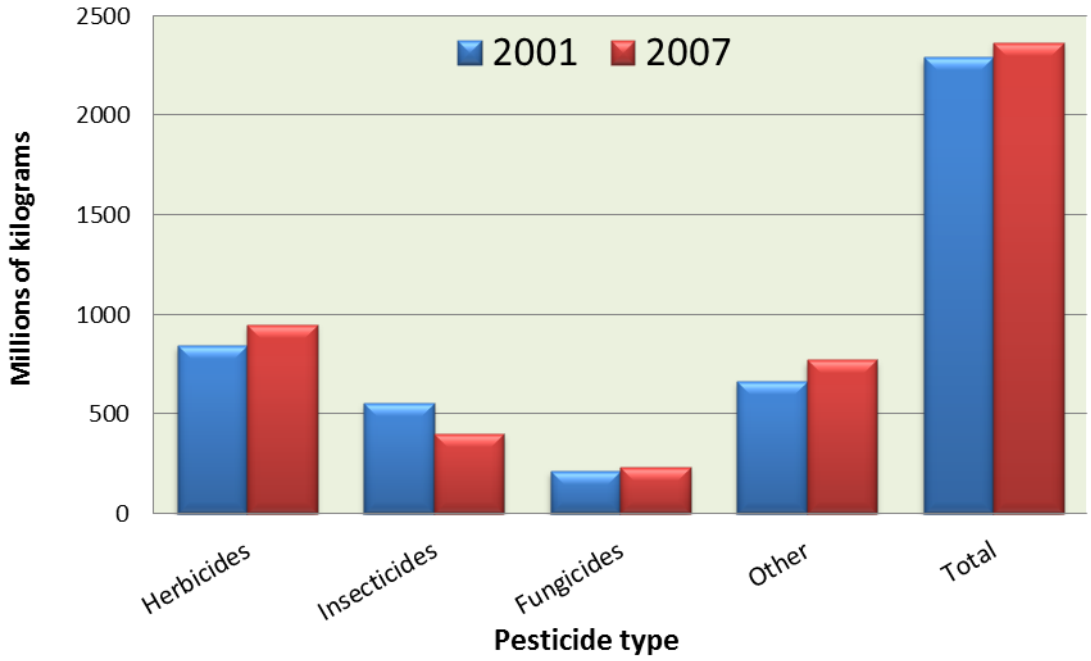

Figure 1: *World amount of pesticide active ingredients used by pesticide type, 2001-2007 estimates.*
Note: "Total" does not include wood preservatives, specialty biocides, and chlorine/hypochlorites.
Source: EPA estimates based on Croplife America annual surveys, USDA/NASS, and EPA proprietary data.

(1) *"Herbicides" include herbicides and plant growth regulators.*

(2) *"Other" includes nematicides, fumigants, rodenticides and other miscellaneous conventional pesticides, and other chemicals used as pesticides such as sulfur, petroleum oil, and sulphuric acid.*

Environmental impacts of pesticides

Since there are many terrestrial and aquatic non-target organisms which are closely related to the pest species that the pesticides aim to control, most pesticides may exert serious detrimental effects on ecosystems. Decades ago, these negative impacts included the thinning of egg shells of birds by DDT, for the terrestrial environment, leading to mass egg mortality rates and a drastic drop in population size for species like the bald eagle, which was consequently listed as an endangered species. There have also been incidences of high levels of mutations and sterility in agricultural run-off zones, altering the natural reproduction and survival rates of species, e.g. birds (Anthony *et al.* 1993). The publication of *Silent Spring* by Rachel Carson in 1962 raised public awareness about the dangers of pesticides, with a specific focus on persistent organochlorines such as DDT. Organochlorine pesticides were mostly used as insecticides. DDT was banned in many countries in the 1970s in response to public concern and mounting scientific evidence linking DDT with damage to wildlife. Many organochlorines have now been banned around the world because of concerns about environmental impacts and human health effects. Recent monitoring programs show that

since these organochlorine chemicals were banned, no such kind of adverse effects are detected anymore and concentrations of DDT in human milk have declined (Smith, 1999).

In developed countries, several persistent pesticides are banned, yet they are widely used in developing countries (Simonich and Hites, 1995). Also less persistent, but more toxic, pesticides are observed to be used under inadequate regulation in many developing counties. These latter pesticides are more harmful to human and ecological entities and their effects can be compared with those of DDT as was described by Rachel Carson in 1962 (Roth *et al*. 1994; Henriques *et al*. 1997; Castillo *et al*. 2000; Ecobichon, 2001; Murray *et al*. 2002; Wesseling *et al*. 2005).

Aquatic systems adjacent to agricultural fields not only support agricultural needs of e.g. irrigation and drainage, but also function as an important habitat for many water organisms. Aquatic organisms are subject to contamination by pesticides, particularly due to direct spray drift, leaching and runoff water from treated areas (Bretaud *et al*. 2000; Dabrowski *et al*. 2002; Brown *et al*. 2004; Liess *et al*. 2005). Therefore, in agricultural areas chronic and acute exposure to pesticide pollution may occur and may potentially affect aquatic flora and fauna.

Regulation of pesticides according to EU legislation

Protection of non-target organisms from the potential effects of agricultural pesticides is the aim of risk assessment procedures. To prevent unacceptable environmental effects, every pesticide has to undergo a risk assessment process before it is placed on the European market. To this end, the registration of pesticides is regulated in the context of the European Plant Protection Products directive 91/414/EEC, which has been replaced by the new regulation 1107/2009/EC in June 2011. While small edge-of-field water bodies are covered under this directive (European Commission, 2002), bigger water bodies and the respective risk assessment are covered by the water framework directive (WFD) 2000/60/EC that sets environmental quality standards for pesticides on the watershed level to ensure good ecological status (European Commission, 2000).

The first step in the aquatic risk assessment of pesticides in the European Union (tier 1) is based on the results of acute and chronic single-species toxicity studies performed with standard test species (*Daphnia*, algae and fish) (EC, 2002). The first tier data generally consist of acute median lethal/effective concentration (LC_{50}/EC_{50}) data estimated from short-term laboratory tests as well as no observed effect concentration (NOEC) data from long-term laboratory tests. Under directive 91/414/EEC and the new directive 1107/2009/EC, the risk of adverse effects is estimated through the calculation of a toxicity exposure ratio (TER), which is the toxic effect value (LC_{50}, EC_{50}, NOEC) divided by the predicted environmental concentration (PEC). These toxicity data for aquatic

organisms are compared with short-term and long-term exposure concentrations to generate TERs. If the TER for an acute test is larger than 100, or for the chronic test larger than 10, the risk to aquatic organisms is considered to be acceptable. In the regulatory framework of the EU for the registration of a new pesticide, it is stated that no authorisation will be granted if the pesticide does not pass the first tier "unless it is clearly established through an appropriate risk assessment that under field conditions no unacceptable impact on the viability of exposed species occurs - directly or indirectly - after use of the plant protection product according to the proposed conditions of use". When the first tier trigger value is exceeded, such a follow-up, more sophisticated, risk assessment may be applied to test whether adverse effects are to be expected under more realistic conditions. This tiered approach forms the basis of the environmental risk assessment schemes that support the registration of pesticides (e.g. Campbell *et al*. 1999; EC, 2002; Boesten *et al*. 2007; Solomon *et al*. 2008).

Aquatic microcosm and mesocosm experiments, also referred to as freshwater model ecosystems, have been frequently used as ultimate higher tier studies to assess the potential risks of pesticides to aquatic ecosystems (Maltby *et al*. 2005; Van Wijngaarden *et al*. 2005). This type of studies intends to simulate the responses of a semi-natural aquatic ecosystem to experimentally applied stress. Compared to lower tier laboratory single-species tests, these test systems provide robust ecological realism because the exposure and the biological and ecological responses to pesticide stress are more realistic. Model ecosystems allow for well-replicated experimental designs which are often not possible under field conditions, and have therefore been considered a bridge between laboratory and the field (Brock *et al*. 2000a; 2000b).

Over the years many cosm experiments have been performed and published in literature. Van Wijngaarden *et al*. (2005), Brock *et al*. (2006), Van den Brink *et al*. (2006a) and Maltby *et al*. (2009) performed literature reviews for evaluating the effects of pesticides observed in freshwater model ecosystem studies in order to explore the ecological threshold levels as well as to evaluate the ecological consequences of exceeding these threshold levels. These reviews are currently incorporated into the empirical database underlying the PERPEST model. PERPEST (Predicting the Ecological Risks of PESTicides in freshwater ecosystems) is a model that can be used to predict the effects of pesticides concentration using information across compounds and across mode of actions (Van den Brink *et al*. 2002; 2006b).

Linking exposure to effects in environmental risk assessment

Extrapolation of exposure patterns

Traditionally, ecotoxicological tests focused exclusively on assessing the risk of contaminants by using laboratory and semi-field experiments, evaluating a range of pesticide concentrations which have a peak exposure or are held constant for short periods of time. However, this situation contrasts with real-world pesticide applications also exposing aquatic systems, where organisms in nature frequently experience multiple applications of pesticides over time rather than a single constant concentration. It has been recognized by several authors that aquatic non-target organisms may be typically exposed to fluctuating concentrations or sequential pulses of pesticide contaminants (Handy, 1994; Reinert *et al*. 2002). In recent years, however, more attention has been paid to pulsed or intermittent exposure scenarios, which are typical for many spills, episodic runoff events, periodic agro-chemical applications and industrial releases. Consequently, prediction of pulsed or intermittent exposure effects on populations is emerging as an important issue to be resolved in ecotoxicology (Boesten *et al*. 2007; Brock *et al*. 2010).

In the European Union (EU), ecological risk assessment (ERA) of pesticide use is estimated following the measures, which are placed in the European Plant Protection Products directive 1107/2009/EC (European Commission, 2009). The risks of chemicals to aquatic ecosystems are often assessed by performing semi-field (micro-and mesocosm, hereafter referred to as cosms) experiments which evaluate the fate and effects of a pesticide after a single application (1-pulse). However, the resulting exposure pattern does not necessarily correspond with the exposure pattern which occurs in the ecosystems to be protected (Fig. 2). Concerns have been raised in order to allow an appropriate linkage of the exposure and effects components of the risk assessment. To do this, the results obtained from higher tier cosm experiments sometimes need extrapolation to another kind of exposure pattern than the one that was evaluated in a cosm experiment (Boesten *et al*. 2007). The discrepancy between exposure patterns observed in the field through chemical monitoring and the exposure regime used in experiments that underpin the effect assessment (e.g. cosm experiments) is one of the biggest challenges in contemporary ERA. In this light, this issue was high-lighted in a series of two EU workshops (ELINK I and II) on linking exposure and effects in the aquatic risk assessment procedures for pesticides under the directive 91/414/EEC (Brock *et al*. 2010), during which the extrapolation of effects across exposure patterns is a major issue and this plea is addressed in the present thesis.

One of the key challenges in chemical stress ecology is to develop methodologies, models, rules-of-thumb etc. that enable an extrapolation of effects and recovery patterns observed in one situation to another situation (Solomon *et al*. 2008; Van Wijngaarden and Brock, 2006; Brock *et al*. 2008a; b). A central question in this respect is, for example, if the effects observed in a microcosm experiment can be extrapolated to a real field situation of concern or can a sensitivity value for a European species be used for an Asian risk assessment. Van den Brink (2008) has reported three types of extrapolations that are considered to be most important for the ERA of chemicals: (1) extrapolation across exposure patterns, (2) habitat and ecosystem complexity and (3) season and geography. The research presented in this thesis aims to establish empirical, experimental and modelling approaches in support of the first type of extrapolation.

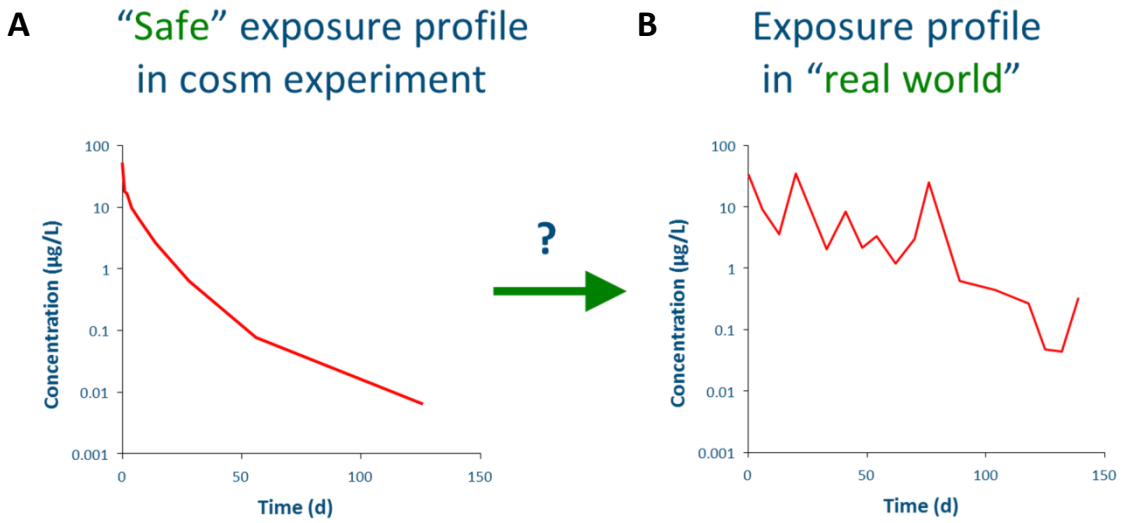

Figure 2*: Schematic, conceptual representation of the extrapolation of effects resulting from one type of exposure to another type of exposure.* ***A)*** *Exposure profile occurring in a "normal" cosm experiment using one application* ***B)*** *Exposure profile observed in a field situation.*

Peak versus time-weighted average concentrations

The proceedings of the ELINK workshop recommend some approaches for linking exposure and effects in the risk assessment of pesticides. One of the recommendations concerns the question whether, for the aquatic risk assessment of pesticides, the peak concentration or the time-weighted average (TWA) concentration should be applied in the risk assessment process whilst the predicted field exposure is variable in time (Fig. 3). Normally, peak values are used in acute risk assessments, while TWA concentrations may be used in chronic risk assessments. The ELINK workshop recommended further research to scientifically underpin the criteria that can be used to decide whether or not the TWA concentration approach is appropriate (Brock *et al*. 2010). To address this

plea and gain more insight, the present thesis focuses on evaluating the effects of different time-variable exposure regimes with the same TWA but different peak concentration, or vice versa.

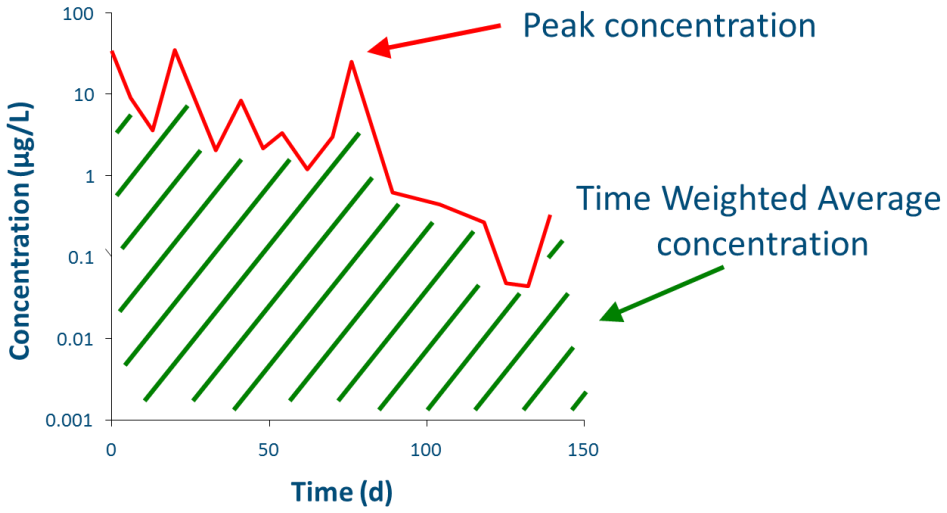

Figure 3*: Schematic depiction of peak and TWA concentration in the risk assessment process of pesticides.*

Time-variable exposure patterns and ecotoxicological effects

As stated above, current procedures for aquatic risk assessment have not been able to adequately address some of the uncertainties arising from time-variable surface water exposure profiles that are more often the rule rather than the exception in the field. At present, therefore, particular attention has been given to seeking a better understanding for addressing the time-varying exposure scenarios more realistically.

Time-variable exposure profiles of pesticides in surface water may vary considerably (FOCUS 2001). Figure 4 shows the key characteristic of exposure profiles, which are (1) the height of the peak concentration, (2) the area under the curve concentration (AUC), (3) the duration of peak exposure, (4) the interval between peaks, (5) the height of a possible long-term background concentration and (6) the frequency of peaks. A further parameter to characterise the exposure pattern is the half-life (DT_{50}) of a compound, which describes the decline of the peak. These metrics or parameters that describe exposure characteristics can be used to delineate exposure regimes for higher-tier effects studies. The parameters are supportive and provide information of exposure profiles in order to assess the risks of time-varying exposure patterns (Brock *et al*. 2010).

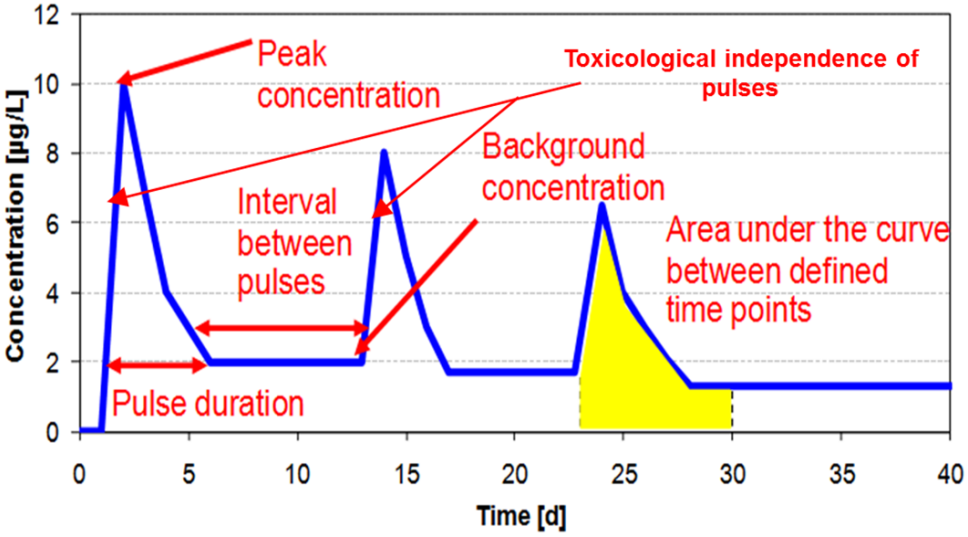

Figure 4*: Key features of an exposure profile that provide information for the risk assessment of time-variable exposure profiles either used in ecotoxicological effect studies or observed in modelled field situations. This figure is taken after slight modification from Brock et al. (2010).*

Toxicokinetics and toxicodynamics in ERA

A critical step in the prospective and retrospective ERA of toxicants is the linking of exposure and effects data. This interface is defined by EFSA (2005) and Boesten *et al*. (2007) as the type of concentration that gives an appropriate correlation to ecotoxicological effects, and is called the Ecotoxicologically Relevant Concentration (ERC). A lack of a clear conceptual basis for the interface between the environmental exposure and ecotoxicological effects may lead to an overall incorrect ERA (Brock *et al*. 2010). It was decided by the European Food Safe Authority (EFSA) Panel on Plant Protection Products and their Residues (PPR) that the ecotoxicological endpoint from a study with a time-varying exposure should be expressed in terms of the ERC (EFSA-PPR 2006). The ERC is the concentration that correlates strongest with observed treatment-related responses. Examples include the peak or the TWA concentration in surface water, in depth-integrated water samples, in interstitial water, or in the top layer of the total sediment. In risk assessment procedures, the ERC needs to be consistently applied so that exposure and effect estimates can be compared and extrapolated as readily as possible.

For a consistent linking of aquatic exposure and effects, toxicokinetic/toxicodynamic (TKTD) models may be used to assess the effects from time-variable exposure. TKTD models simulate the time-course of processes leading to toxic effects on individual organisms (also referred to as the individual level) (Ashauer *et al*. 2007a; b).

Toxicokinetics (TK) consider the time course of concentrations within an organism in relation to concentration in the external medium. Toxicodynamics (TD) describe the time-course of damage, subsequent effects induced by chemical and repair processes (Ashauer *et al*. 2006; Ashauer *et al*. 2007a). TKTD modelling has been identified as a tool to assess the toxicological independence of peaks, i.e. to evaluate whether the toxicity from the first peak has been repaired completely before the second peak of exposure occurs (Fig. 4). To demonstrate the toxicological independence of pulsed exposures, either specially designed pulsed exposure toxicity tests or parameterised TKTD-models for the relevant organisms and pesticides are required. Since evidence can be provided that different pulsed exposures are toxicologically independent, it may be important to also demonstrate their ecological independence. This will, for example, be necessary when recovery at the population level is taken into account (Galic, 2012). Peaks may be considered ecologically independent if peak intervals are greater than the relevant recovery time of the sensitive population of concern.

Exposure-response reciprocity (relationship) and the use of TWA concentration in long-term risk assessment

The use of the TWA concentration approach in the ERA of pesticides is based on the observation that effects of pesticides on aquatic organisms may be similar when exposed for a shorter time to a high concentration or for a longer time to a low concentration. This phenomenon is called reciprocity and relates to Harbers' law, which assumes that toxicity is a product of concentration and time (Giesy and Graney, 1989). For example, a 4-day exposure at 5 µg/L may cause the same effect as a 2-day exposure at 10 µg/L or a 1-day exposure at 20 µg/L. Linear reciprocity is the basis of the TWA approach where exposure concentration is integrated over time (Area Under the Curve = AUC) and then divided by the duration of the toxicity test. When this approach is applied, it is assumed that different exposure patterns with the same AUC or TWA have similar effects.

This thesis aims to contribute to answering the question whether or not the TWA concentration approach is appropriate for assessing the risks to aquatic communities. The semi-field experiments presented in this thesis are performed with different time-variable exposure regimes which have the same TWA concentrations and, thus, allow for the comparison of the effects on aquatic communities.

Overall aim of thesis

The aim of this thesis is to investigate the comparison of the effects between different time-variable exposure regimes of pesticides on aquatic species and communities, using laboratory experiments and semi-field experiments. Furthermore, the thesis also uses empirical approaches to establish rules-of-thumb for extrapolating from one type of exposure pattern to the other. The comparison of effects is made on the basis of different time-variable exposure regimes with the same TWA concentrations but different peak concentrations of pesticides towards freshwater communities. This is to evaluate whether the peak or the TWA is more important for extrapolation of effects across exposure regimes.

Research objectives

Following research objectives are discussed in this thesis

1. To review relevant published cosm experiments in order to refine the exposure part of the empirical PERPEST informatics model in order to allow an extrapolation of classified effects observed in cosm experiments across exposure regimes from which rules-of-thumb for the extrapolation of effects across exposure regimes can be extracted.
2. To perform single- and multi-species experiments testing the rules-of-thumb deduced from the empirical analysis.

Outline of thesis

Chapter 2 compares the effects as observed in cosm experiments with the peak exposure concentration of the exposure profile as well as with the TWA_{21d} concentration using different sensitivity endpoints. The intention was to evaluate whether the TWA_{21d} is a better predictor for long-term effects of insecticides than the measured peak concentration. Therefore, a review of freshwater model ecosystem studies evaluating the effects of insecticides was made on the basis of studies present in the PERPEST database (Van den Brink *et al*. 2002). PERPEST itself is a model that predicts the effects of a particular concentration of a pesticide on various community endpoints, based on empirical data extracted from published cosm experiments. In this chapter, I focus on insecticides with three main mode-of-actions, namely acetylcholinesterase inhibitors (organophosphates and carbamates), sodium channel modulators (pyrethroids), and moulting inhibitors (benzoylurea and insect growth regulators) and the potentially sensitive endpoints for these pesticides, microcrustaceans, macrocrustaceans and insects. Within insecticide groups and within toxicological modes-of-action this comparison is done for the separate chemicals. Using this

comparison, an effort is made to derive rules-of-thumb on which type of concentration should be used to extrapolate effects across exposure patterns and also whether these rules-of-thumb are unique for individual chemicals or can be generalized over groups of chemicals with the same toxicological mode-of-action. From this experience, I aimed to design empirical approaches to validate these rules-of-thumb to extrapolate from one type of exposure pattern to another.

Chapter 3 compares effects of different time-variable exposure regimes with the same TWA concentrations and different peak concentrations of the organophosphate insecticide chlorpyrifos towards freshwater invertebrate communities. The aim of this study was to enable the extrapolation of effects across exposure regimes. I performed this study in outdoor microcosms and introduced three different treatment regimens. Effects on macroinvertebrates, zooplankton, and community metabolism were observed. All treatment regimes showed the same effect magnitude at the end of the experimental period. This indicates that the TWA concentration seems to be more important than the peak concentration for assessing long-term risks of chlorpyrifos on arthropod communities. However, this general observation is not true for individual species. In case of the mayfly *Cloeon dipterum* the peak concentration was a better predictor for effects than the TWA approach. This could be explained by the toxicokinetics-toxicodynamics of chlorpyrifos for this species (subject of Chapter 4).

Chapter 4 in order to explain the difference in responses observed between species as found in Chapter 3, long-term survival experiments were performed with four species by applying pulsed exposures using different time intervals in laboratory experiments. These experiments were designed to estimate toxicodynamic parameters (Ashauer *et al*. 2007a, b). Chapter 4 aims to parameterise the toxicodynamic part (TD) of the Threshold Damage Model (TDM) for the insecticide chlorpyrifos in several aquatic macroinvertebrates and to compare recovery abilities among species. The toxicodynamic parameters killing rate constant (k_k), recovery rate constant (k_r), threshold (*threshold*) and background mortality are evaluated for the four freshwater arthropod species *Chaoborus obscuripes, Cloeon dipterum, Plea minutissima* and *Daphnia magna*. Furthermore, I evaluate how these arthropod species, with different sensitivities, respond to time-varying exposures of chlorpyrifos in terms of survival and mobility. *C. obscuripes* and *D. magna* showed direct decrease in mobility and a delayed effect in survival, whereas *C. dipterum and P. minutissima* responded directly to the exposure for both endpoints. *C. obscuripes* was the only species showing no recovery. In general, the effect of the pulses was smaller when more time was given for elimination and potential recovery, as was intended by the experimental design. The TDM was able to fit the experimental data relatively well. However, not all parameters were estimated robustly

and the TDM does not provide consistent results, which makes difficult parameter comparison between species.

Chapter 5 is the follow-up on the results of Chapter 3 and evaluates the effects of different time-varying exposure patterns of the strobilurin-fungicide azoxystrobin on freshwater microsocosm communities. This chapter focuses on the comparison of effects of chronic exposures with the effects of a single peak exposure and a multiple peak scenario. The exposure patterns included two treatments with a similar peak but different TWA concentrations, and two treatments with similar TWA but different peak concentrations. Effects of azoxystobin on structural endpoints, i.e. macroinvertebrates, zooplankton, phytoplankton and macrophytes biomass, as well as functional endpoints, i.e. decomposition of particulate organic matter, community metabolism and water quality, were assessed. By the end of the experimental period, multivariate analysis indicated the same effects magnitude for the pulsed treatment regimes, which were placed in between the chronic treatment regimes. This indicates that for long-term effects the TWA could be a more adequate approach for the comparison of different exposure regimes for most zooplankton species than comparing peak concentrations.

Chapter 6 provides a summarizing discussion of the results and conclusions of this thesis. The findings of the thesis are brought together in this chapter and an overall discussion and conclusion on how to extrapolate the effects from one type of exposure regime to another is presented.

Chapter 2

Explanatory power of peak and time-weighted average concentrations for effects of insecticides as observed in semi-field experiments

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Manuscript in preparation

“A limited data base is no excuse for not conducting a sound risk assessment. On the contrary, with less knowledge of a system, the need for risk assessment and management becomes imperative”

(Haimes 1998)

Abstract

Laboratory and semi-field experiments usually do not provide insight in the effects of time-variable exposure of chemicals to aquatic organisms. Whether in the aquatic risk assessment of pesticides, the peak or time-weighted average (TWA) concentration should be used to predict the long-term effects of time-variable exposure patterns, is under discussion. The present study aims to compare the effects as observed in model ecosystem experiments using either peak or time-weighted average (TWA_{21d}) concentrations as a relevant predictor variable for different sensitivity endpoints. For this purpose, a literature review was performed using the empirical database associated with the PERPEST model. Peak exposures of single and multiple applications of pesticides were derived from the publications included in the PERPEST database and their corresponding TWA_{21d} concentrations were calculated. In order to allow the grouping of studies performed with different insecticides, we expressed the exposure concentrations as toxic units (TU). Based on TU, threshold values were assumed to be equivalent for compounds with a similar mode-of-action. Different grouped endpoints were selected from each model ecosystem study and responses were assigned to an effect class (no, slight and clear effects). When standardised on peak exposure concentrations in case of the insecticide chlorpyrifos, clear effects were reported for all endpoints at exposure concentrations of 0.1 µg/L and higher. When expressed as TWA_{21d} concentrations, clear responses were reported at concentrations of 0.05 µg/L and higher. On the basis of these comparisons between peak and TWA_{21d} concentrations we found that when applied once, direct effects became apparent at TWA_{21d} exposure concentrations which were a factor of 5 lower than their corresponding peak exposure concentrations. For acetylcholinesterase inhibitor insecticides, TWA_{21d} concentrations can be used as good predictors for predicting long-term effects on sensitive endpoint groups in the risk assessment process. Therefore, we recommend an extrapolation factor of 5 to be used to assess the long-term risks of time-variable exposure profiles in peak exposures due to a single application for organophosphates (mainly based on studies with chlorpyrifos). The data presented for sensitive endpoints and acetylcholinesterase inhibitors show a clearer dose-response relationship when expressed using TWA_{21d} exposures, compared to peak exposures. In case of rapidly acting compounds like pyrethroids, both the peak as the TWA_{21d} concentrations did not show a clear dose-response relationship. For moulting inhibiting insecticides, the peak and TWA_{21d} concentrations may have equal importance in order to evaluate the effects.

Introduction

Areas with intensive agriculture are often highly integrated with aquatic ecosystems because of their dependence on water supply and/or drainage. When pesticides are applied with the prevailing application methods used for crop protection, however, it is inevitable that a portion of sprayed pesticides will reach such untargeted edge-of-field surface waters (e.g., Hung and Thiemann 2002; Osano *et al*. 2003; Sarkar *et al*. 2008). Since aquatic ecosystems contain species related to the target organisms of pesticides, unintended repercussions are to be expected when these ecosystems become contaminated (Liess *et al*. 2005). Evaluation of the potential adverse effects of pesticide stress on non-target aquatic organisms in aquatic ecosystems is considered to be a major challenge (European Commission, 2002). Therefore, governmental authorities have set criteria to protect aquatic life from pesticide-stress (e.g. European Commission, 2009). Consequently, the ecological relevance of estimated risk levels is an important subject in recent ecotoxicological research with pesticides (Van den Brink, 2008).

Traditionally, the domain of ecotoxicological research has focused almost exclusively on assessing the risk of contaminants by using laboratory and model ecosystem experiments characterised by testing a range of pesticide concentrations which either include a peak exposure or a constant exposure for short periods of time. Hence, these tests usually do not provide insight in the effects of time-variable exposures to aquatic organisms. From these experiments, one can estimate which concentration of a contaminant will cause 50% mortality/sub-lethal effects (LC50/EC50), and which concentration will cause no observable effect (NOEC), for example, on reproduction. However, these evaluated exposure profiles are not representative from those expected from real-world pesticide applications in aquatic systems, where organisms in nature frequently experience time-variable exposure regimes (due to multiple exposure routes or applications) of pesticides over time rather than from a single constant concentration (Brock *et al*. 2010). In realistic exposure scenarios, pesticides can not only vary in concentration, but also in the timing of the application, and the frequency of repeated applications (Viant *et al*. 2006). In addition, repeated (multiple) applications may cause species to be exposed to a larger amount of a pesticide and may also trigger larger impacts than a single pulse application (e.g. Ashauer *et al*. 2007c). Existing procedures for aquatic risk assessment are not fully adequate to characterize the potential uncertainties arising from the time-variable surface water exposure profiles (Brock *et al*. 2010). Therefore, current ecotoxicology tests in the context of ecological risk assessment should include efforts to understand the impact of time-variable exposures on the effects of the toxicants on

species. This objective can be achieved by studying the effects of time-varying or intermittent exposures.

Recently, the above mentioned issue was the focus of two workshops (known as ELINK I and II) on linking the aquatic exposure with potential effects in the aquatic risk assessment procedures for pesticides under the European Union (EU) plant-protection registration directive (Brock *et al*. 2010). One of the recommendations of the ELINK workshops is to study whether in the aquatic risk assessment for pesticides the peak concentration or the Time-Weighted Average (TWA) concentration should be used when the predicted and/or measured field exposure is variable in time. It was anticipated in the ELINK workshops that further experimental work would be required to underpin whether or not the TWA concentration approach is appropriate to be used in long-term risk assessment (Brock *et al*. 2010). To contribute to the knowledge on this subject, two microcosm experiments have been performed which compared the effects of exposure profiles with similar TWA concentrations, but different peak exposure concentrations (Zafar *et al*. 2011; 2012).

Van Wijngaarden *et al*. (2005), Brock *et al*. (2006), Van den Brink *et al*. (2006a) and Maltby *et al*. (2009) performed literature reviews for evaluating the effects of pesticides observed in freshwater model ecosystem studies (microcosms or mesocosms, hereafter referred to as cosms) in order to explore the ecological threshold levels as well as to evaluate the ecological consequences of exceeding these threshold levels. The largest limitation found in these reviews is that only the nominal peak concentration, not the TWA concentration, is taken as a reference when evaluating the effects of pesticides. Considering this data gap, it is prudent to evaluate the effects observed in these freshwater model ecosystems in relation to not only the peak exposure but also the TWA exposure. The vast majority of studies have examined the effects of single applications of insecticides (Brock *et al*. 2006; Van Wijngaarden *et al*. 2005a). Fewer model ecosystem studies have directly compared the impacts of single versus multiple pesticide applications on aquatic communities (Daam *et al*. 2008; Hanazato and Yasuno, 1990). With the exception of Zafar *et al*. (2011; 2012), none of the beforehand published cosm studies evaluated the ecological effects of different time-variable exposure regimes (single application, multiple application and chronic exposure regime) having the same TWA, which is critical in predicting population responses resulting from different TWA exposures regimes.

The present study focused on evaluating whether the TWA concentration is a better predictor for long-term effects of pesticides than the peak concentration. The aim of the present study was to compare the effects as observed in cosm experiments to the peak concentration of the exposure profile as well as to the TWA_{21d} concentration using different sensitivity endpoints. This comparison is performed for a number of chemical substances separately, and also for groups of

chemicals with the same toxicological mode-of-action. By doing so, we hoped to establish empirical approaches to extrapolate from one type of exposure pattern to the other.

Materials and Methods

The PERPEST database

The empirical data base which is the basis of the PERPEST (Predicting the Ecological Risks of PESTicides in freshwater ecosystems) model that predicts the effects of a particular concentration of a pesticide on various (community) endpoints, was used as a starting point (Van den Brink *et al*. 2002; 2006b). This database has been built by performing a review of cosm studies evaluating the effects of pesticides (Van Wijngaarden *et al*. 2005; Van den Brink *et al*. 2006a; Brock *et al*. 2006; Maltby *et al*. 2009). The PERPEST model can predict the effects of single and multiple applications on the basis of the highest peak concentration and 7-day and 21-day TWA concentrations.

The reviews mentioned above yielded 136 experiments, that were incorporated in the PERPEST database (www.perpest.wur.nl). These experiments resulted in a total of 573 evaluated pesticide-concentration combinations (cases). The studies reported were published between 1980 and 2006. The PERPEST database includes 253 cases for insecticides, 252 for herbicides, and 68 for fungicides. This paper focused on the effects of insecticides, which were further divided based on the mode-of-action or molecular group. Acetylcholinesterase inhibitors (carbamates and organophosphates) accounted for 103 cases, sodium channel modulators (synthetic pyrethroids) for 77 and moulting inhibitors for 38 cases. The ecological risks of 21 insecticides for freshwater ecosystems are discussed in this paper.

As this study aimed to establish empirical approaches to extrapolate from one type of exposure pattern to another, additional data than yet available in the PERPEST database were collected. Firstly, the exposure description needed in more detail since only the peak concentration and whether the exposure was resulting from a single or repeated/chronic application was available. To this information (1) the shape (peak versus constant), (2) the measured peak concentration, (3) the dissipation rate of the pesticide (DT50) in the water column of the systems and (4) the number of applications along with the time interval between application were gathered by re-reviewing all 136 experiments. From this information, subsequently the peak and TWA_{21d} exposure concentrations were calculated for every case. From this overview, graphs were made to illustrate the categorised effects on different endpoints against the peak and TWA_{21d} exposure concentrations.

The exposure concentrations were expressed as toxic units (TU) by scaling them on the median laboratory acute toxicity value for the expected most sensitive group (see Rubach *et al*. 2010b for rationale). In case of insecticides, arthropods were expected to be the most sensitive group, and the median toxicity of each insecticide for arthropods was calculated (Maltby *et al*. 2005). This was done in order to allow comparisons of effects between chemicals. This comparison is done for chemicals separately (e.g. chlorpyrifos) and within group of chemicals sharing the same toxicological mode-of-actions (e.g. acetylcholinesterase inhibitors).

Peak concentration and TWA_{21d} concentration

The time window of 21d for the TWA exposure calculation was selected based on recommendations by the ELINK workshops, which states that the time-window of the TWA should be equal to or smaller than the length of the relevant chronic toxicity test that triggers the risk (Brock *et al*. 2010). In this case, the chronic 21d test with *Daphnia* is most relevant. Another potentially sensitive group is that of fish, however, it was hardly studied in cosm experiments.

Peak exposures were derived from the concentrations of selected studies which were measured shortly after application. If the peak concentration was not measured in the study the nominal intended concentration was used. The peak concentration from a number of applications with fixed time intervals between applications was calculated using the equations provided in Peeters *et al*. (2008), for details see supporting information (SI) and equation used for it.

A pesticide application at t = 0 results in a peak concentration, which decreases over time. The TWA concentration of a single application results from integration over t = 0 to $t = t_{TWA}$, which can be computed by the equations 20 and 21 provided in Peeters *et al*. (2008), see SI for the equations and its parameters.

The TWA concentration of a multiple applications scenario depends on the following parameters: (i) the number of applications, (ii) the time interval between applications and (iii) the number of applications within the TWA period. Four different situations determining the TWA may be distinguished, called case 1 to case 4, which are provided in Peeters *et al*. (2008), while further details on equations and their parameters are described in SI. The TWA_{21d} of the cosm studies under consideration were calculated with the help of the PERPEST software, version 2.0.0.0.

The TWA concentrations for short-term pulsed applications in flow-through systems or artificial stream microcosms were calculated without considering dissipation using Equation (1):

$$TWA_{21d} = \frac{Peak^1 * T}{24\ (\text{hours}) * 21\ (\text{days})} \quad (1)$$

where:

TWA_{21d} is time Weighted Average concentration for period with length t of 21 days (µg/L)

$Peak^1$ is peak concentration resulting from a single loading (µg/L)

T is time of exposure duration (hours)

Calculation of the dissipation time 50%

In order to calculate the TWA_{21d} concentrations, it was needed to derive the dissipation time 50% (DT50) from the cosm studies. The dissipation rate constant (*k*) was derived from the dynamics in the water concentration as measured in cosm studies using Equation (2).

$$C_t = C_{t=0}\ \exp(-k^* t) \quad (2)$$

where:

C_t is concentration of pesticide dissolved in water at time *t* (µg/L)

$C_{t=0}$ is concentration of pesticide at time 0 (µg/L)

T is time (d)

K is dissipation rate constant (d^{-1})

Finally, when *k* was derived from Equation 2, the half-life of the chemical was calculated using Equation (3):

$$DT50 = \ln(2)/k \quad (3)$$

where:

DT50 is half live for dissipation in water (d)

K is dissipation rate constant (d^{-1})

Calculation of Toxic Unit (TU) based on HC50

In order to allow comparing between studies performed with different insecticides, we expressed the exposure concentrations as toxic units (TU). To this end, we divided the studied exposure concentration (usually the measured peak concentration of the insecticide in the water column and its corresponding TWA_{21d} concentrations) by the Hazardous Concentration 50% (HC50) which is the median laboratory acute toxicity value based on the expected most sensitive group, i.e. arthropods for insecticides (see Rubach *et al*. 2010b for rationale). Hence, 1 TU_{msg} equals the median laboratory toxicity 50% (HC50) value for the most sensitive group (msg) for insecticides (i.e. arthropods). HC50 values obtained from Maltby *et al*. (2005) were used for the transformation of treatment concentrations for the insecticides into TUs (Table 1).

Classification of effects on sensitive endpoints

The endpoints evaluated in the cosm experiments were classified for three different ecological endpoints, microcrustaceans, macrocrustaceans, and insects, which were considered to be sensitive for insecticides (Maltby *et al*. 2005). Within each of the ecological endpoint, the most sensitive taxon was selected for assignment to an effect class. The responses observed for these ecological endpoints were assigned to one of the three effect classes according to their magnitude (based on the concept of Brock *et al*. (2006) :

0- Endpoint not evaluated in the study.

1- ***No effects demonstrated***: no consistent adverse effects are observed as a result of the treatment; observed differences between treated test systems and controls do not show a clear causality.

2- ***Slight effects***: confined responses on sensitive endpoints (e.g., partial reduction in abundance); effects observed on individual sampling dates only and/or of very short duration directly after treatment.

3- ***Clear effects***: convincing reductions on sensitive endpoints; effects observed on consecutive sampling dates.

Table 1: Insecticides with different mode-of-action, hazard concentration based on the most sensitive taxonomic group and half-life values of used insecticides.

Mode-of-action / chemical group	Chemical name	HC50 (µg/L)	48-96h geometric mean EC50 (µg/L) *D. magna*	DT50 (days)
1- Acetylcholinesterase inhibitor				
1a- Organophosphate insecticides				
	Azinphos-methyl	4.1	1.1	2.0
	Chlorpyrifos	1.6	0.4	2.2
	Diazinon	9.7	1.0	9.5
	Fenitrothion	7.8	17	1.8
	Parathion-ethyl	5.3	1.6	3.5
	Parathion-methyl	10	7.3	13
	Phorate	3.4	4.0	3.2
1b- Carbamate insecticides				
	Bendiocarb	68	30	2.0
	Carbaryl	76	5.5	2.5
	Carbofuran	22	29	2.4
2- Sodium channel modulator				
	Cyfluthrin	0.28	0.16	1.3
	Cypermethrin	0.099	0.68	1.0
	Deltamethrin	0.15	0.064	0.45
	Esfenvalerate	0.20	0.65	0.42
	Fenvalerate	0.42	0.76	4.1
	Lambda-cyhalothrin	0.046	0.39	0.67
	Permethrin	2.5	0.60	3.5
	Tralomethrin	0.67	0.038	0.53
3- Moulting inhibitor				
	Azadirachtin	4309	3540	30
	Diflubenzuron	8.4	7.2	3.6
	Tebufenozide	1541	3800	34

Results and discussion

Acetylcholinesterase inhibiting insecticides

Within the insecticide group, the selected semi-field experiments for the evaluation of the ecological impact of acetylcholinesterase inhibiting insecticides comprised 30 studies (103 cases) performed with seven organophosphates, viz., chlorpyrifos (13 studies), azinphos-methyl (5), fenitrothion (2), parathion-methyl (2), parathion-ethyl (1), diazinon (1) and phorate (1), and with three carbamates, viz., carbaryl (3), bendiocarb (1), and carbofuran (1). The majority of cosm experiments evaluated a single application (23) while multiple applications (3) and chronic exposure (4) of insecticides were studied less frequent. The overall responses of the most sensitive endpoint observed in the test systems stressed by cholinesterase inhibiting insecticides are presented in Figure 1.

Figure 1 illustrates at which concentration the onset of different classes of effect occurred for microcrustaceans (1A, 1B), macrocrustaceans (1C, 1D) and insects (1E, 1F) in various studies. The graphs on the left express exposure on the basis of peak concentrations (Peak-TU), whereas the graphs on the right express exposure on the basis of time-weighted average concentrations (TWA-TU). When expressed using the peak concentrations, clear effects (effect class 3) were reported for all endpoint categories at exposure concentrations of acetylcholinesterase inhibiting insecticides from about 0.05 Peak-TU and higher (Fig. 1A, C, E). Slight effects (effect class 2) on microcrustacean endpoints were observed at exposure concentrations in the range of 0.01-1 Peak-TU (Fig. 1A), and for macrocrustaceans and insects, they were mostly found in the range of 0.1-1 Peak-TU (Fig. 1C, E). When expressed as TWA concentrations, clear effects start to become apparent in the categories microcrustaceans and insects at exposure concentrations higher than 0.02 TWA-TU (Fig. 1B, F), while for macrocrustaceans clear effects are reported at exposure concentrations from a little higher than 0.06 TWA-TU (Fig. 1D). Within the concentration range 0.005-0.1 TWA-TU slight effects are reported for the microcrustacean and macrocrustacean endpoints while for insects they are predominantly reported in the range 0.1-1 TWA-TU (Fig. 1B, D, F).

However, in a few cases, results deviated from a more general concentration-response relationship that was obtained for the sensitive endpoint categories, microcrustaceans, macrocrustaceans and insects (Fig. 1A, C, E). Three studies that evaluated chronic exposure to chlorpyrifos reported effects below the 0.1 Peak-and TWA-TU level (Van den Brink *et al*. 1995; Ward et al. 1995; Zafar *et al*. 2011). In addition, clear effects on microcrustaceans were also reported below the 0.1 Peak-TU concentration in a study that evaluated a repeated application of chlorpyrifos (Lopez-Mancisidor *et al*. 2008) at an exposure concentration of 0.071 Peak-TU. Dortland (1980)

found no effects of chronic exposure of parathion-ethyl at concentrations of 0.038 Peak-TU and 0.095 Peak-TU on insects but clear effects are reported at chronic exposure of 0.19 Peak-TU. For microcrustaceans, clear effects were observed in the same study at chronic exposure concentrations of 0.095 Peak-TU and 0.19 Peak-TU (Fig. 1E, F). We incorporated also those studies of acetylcholinesterase inhibitors which have been conducted in flow-through or stream systems evaluating pulse and constant exposures. For example, in the study of Schulz *et al*. (2002) with azinphos-methyl, two continuous exposures of 1 h duration with concentrations of 0.050 Peak-TU and 0.25 Peak-TU had no effects on insect populations in stream microcosms, while clear effects were observed at concentrations of 1.2 Peak-TU and 4.8 Peak-TU. In this study effects observed at relatively high peak concentrations were most likely due to the briefness of exposure. This is also consistent with another study by Pusey *et al*. (1994), who found no effects on the abundance of insects when chlorpyrifos was applied as a pulse of 6 h with a concentration of 0.038 Peak-TU in artificial stream systems. Clear effects were observed in the same study at a concentration of 1.6 Peak-TU. Knuth *et al*. (1992) and Sierszen and Lozano (1998) found slight effects on macrocrustaceans at a pulse concentration of 0.050 Peak-TU azinphos-methyl, in contrast Stay and Jarvinen (1995) found no effects at the same exposure concentration with the same chemical.

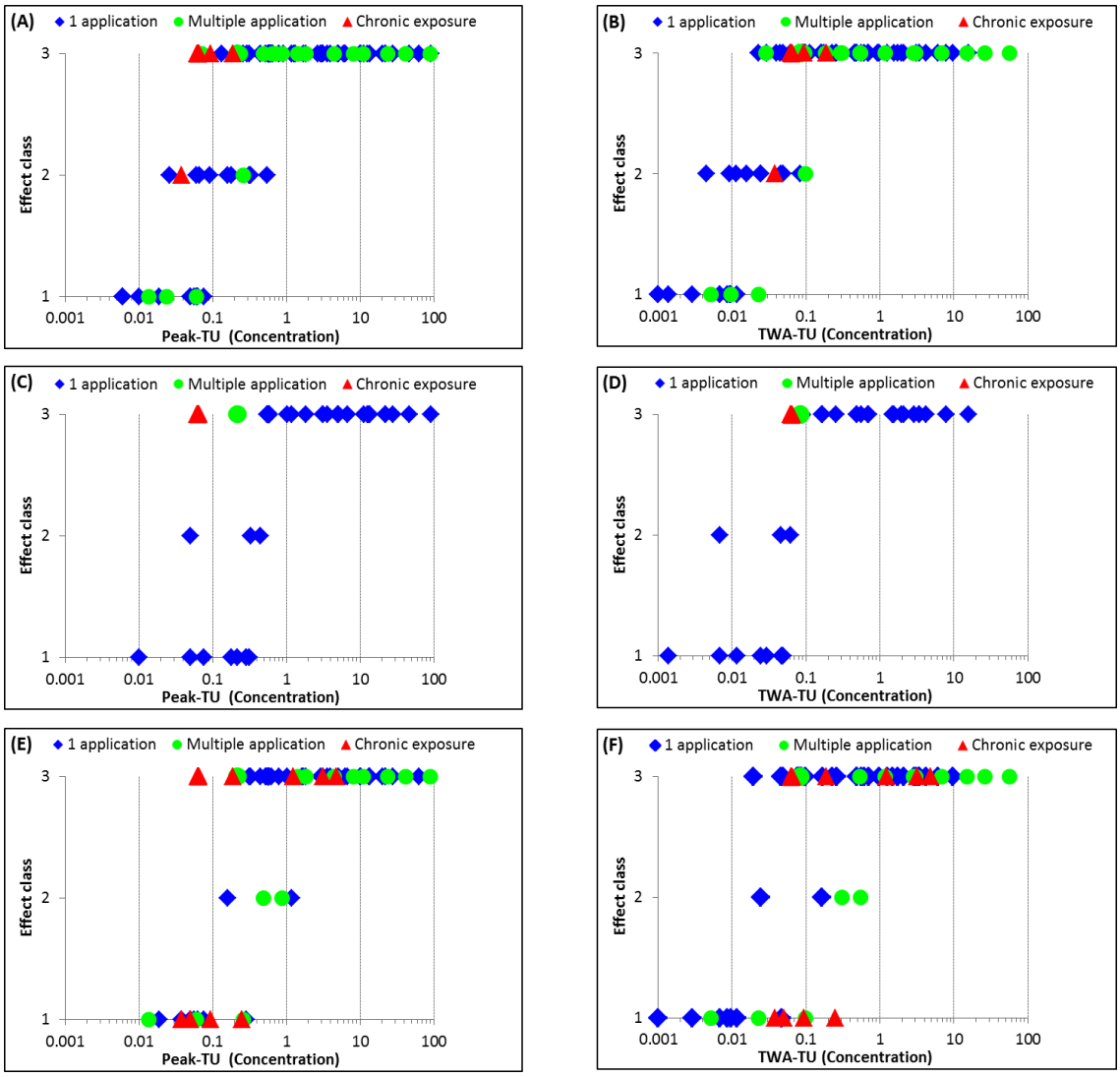

Figure 1: Classified effects of insecticides with an acetylcholinesterase inhibiting mode-of- action as observed for sensitive ecological endpoints in freshwater model ecosystem studies. The figure includes observations of studies for single and multiple applications along with chronic exposure regimes in stagnant as well as running water test systems. The effects are classified according to magnitude: **1** = no effect, **2** = slight effect, **3** = clear effect; (see text for detailed explanation). The x-axis displays the exposure concentration of the insecticides evaluated in cosm studies expressed in toxic units (TU) corresponding to peak **(left panels)** and TWA **(right panels)** concentrations. Effects are classified for three potentially sensitive endpoints: microcrustaceans **(A-B)**, macrocrustaceans **(C-D)** and insects **(E-F).**

Clear effects became apparent at exposure concentrations that were a factor of 5 lower when expressed as TWA-TU concentrations compared to Peak-TU concentrations (Fig. 1; compare left and right panel). As a result, the threshold values based on TWA-TU were approximately lower by a factor of 5 than those based on the peak exposure concentrations. The factor is around 5 in the case of single applications, while the factor is slightly lower (3) for multiple applications and 1 in case of chronic applications (Fig. 1). This means that for an adequate risk analysis it is at least desirable to distinguish between exposure regimes resulting from single applications on the one hand, and that of multiple/chronic applications on the other.

Our findings with the acetylcholinesterase inhibitor insecticides indicate that the threshold of effects is clearer when concentrations are expressed as TWA concentrations, compared to peak concentrations. This means that for these type of insecticides TWA concentrations can be used as good predictors for long-term effects on sensitive endpoints. On the basis of effect data presented in Figure 1, it appears that microcrustaceans are somewhat more sensitive than other endpoints (macrocrustaceans and insects).

In addition to mode-of-action, this following section presents the ecological impact of organophosphorous insecticide chlorpyrifos, separately. Chlorpyrifos was chosen because this compound is studied most intensively in cosm studies (Van Wijngaarden *et al*. 2005a). The effects of chlorpyrifos on microcrustaceans, macrocrustaceans and insects as observed in 46 cases were classified into the three effect classes (Fig. 2). When standardised on peak exposure concentrations, clear effects were reported for all endpoints at concentrations of 0.1 µg/L and higher (Fig. 2A, C, E). All effects observed at insecticide concentrations lower than 0.15 µg/L related to chronic exposure studies. Between 0.1 and 1 µg/L, slight effects were reported for microcrustaceans (Fig. 2A). When TWA_{21d} concentrations were used, clear responses of microcrustacean and insect endpoints were reported at concentrations higher than 0.05 µg/L (Fig. 2B, F), whereas for macrocrustaceans they were observed at concentrations of 0.1 µg/L and higher (Fig. 2D). At concentrations in the range between 0.015 and 0.15 µg/L, slight effects were observed on microcrustaceans (Fig. 2B). Based on the comparisons between single application studies evaluated using TWA_{21d} concentrations, we found that clear effects became apparent at TWA_{21d} exposure concentrations that were lower by a factor of 5 than evaluated based on the peak concentrations (Fig. 2; left and right), which is in accordance with the findings of Zafar *et al*. (2011). It is clear that the concentration–response relationship of chlorpyrifos does not deviate from that of the acetylcholinesterase inhibiting insecticides (compare Fig. 2 with 1).

Applying the EU registration criteria (Uniform Principles), the first tier water quality standards (acceptable concentration) for chlorpyrifos is 0.004 µg/L (48-96h L(E)C_{50} = 0.4 µg/L divided by 100) on the basis of acute toxicity E(L)C_{50} for the most sensitive standard test species *Daphnia magna* or 0.005 µg/L (chronic NOEC = 0.05 µg/L divided by 10) based on chronic NOEC of *Daphnia* sp. (21 days) (European Commission, 2009; Brock and Van Wijngaarden, 2012). The criteria as set by the Uniform Principles appear to provide sufficient protection for freshwater ecosystems when exposed to the cholinesterase inhibiting insecticide chlorpyrifos, even in the case of chronic exposure.

The threshold values obtained from single application studies (0.1 µg/L; expressed as (nominal) peak concentration) are remarkably similar between studies, as was also demonstrated by Brock *et al*. (2006). However, in a repeated pulse exposure experiment (Lopez-Mancisidor *et al*. 2008) the threshold level of 0.033 µg/L (expressed in terms of nominal concentration) is lower than the reported threshold levels (0.1 µg/L) of the single application studies, and higher than that the threshold (0.01 µg/L) of the constant exposure study reported by Cuppen *et al*. (2002). Effects on the sensitive endpoint groups (microcrustaceans, macrocrustaceans and insects) and chlorpyrifos show a distinct dose-response relationship when effects are expressed against TWA_{21d} exposure concentrations instead of peak exposure concentrations (Fig. 2A-H).

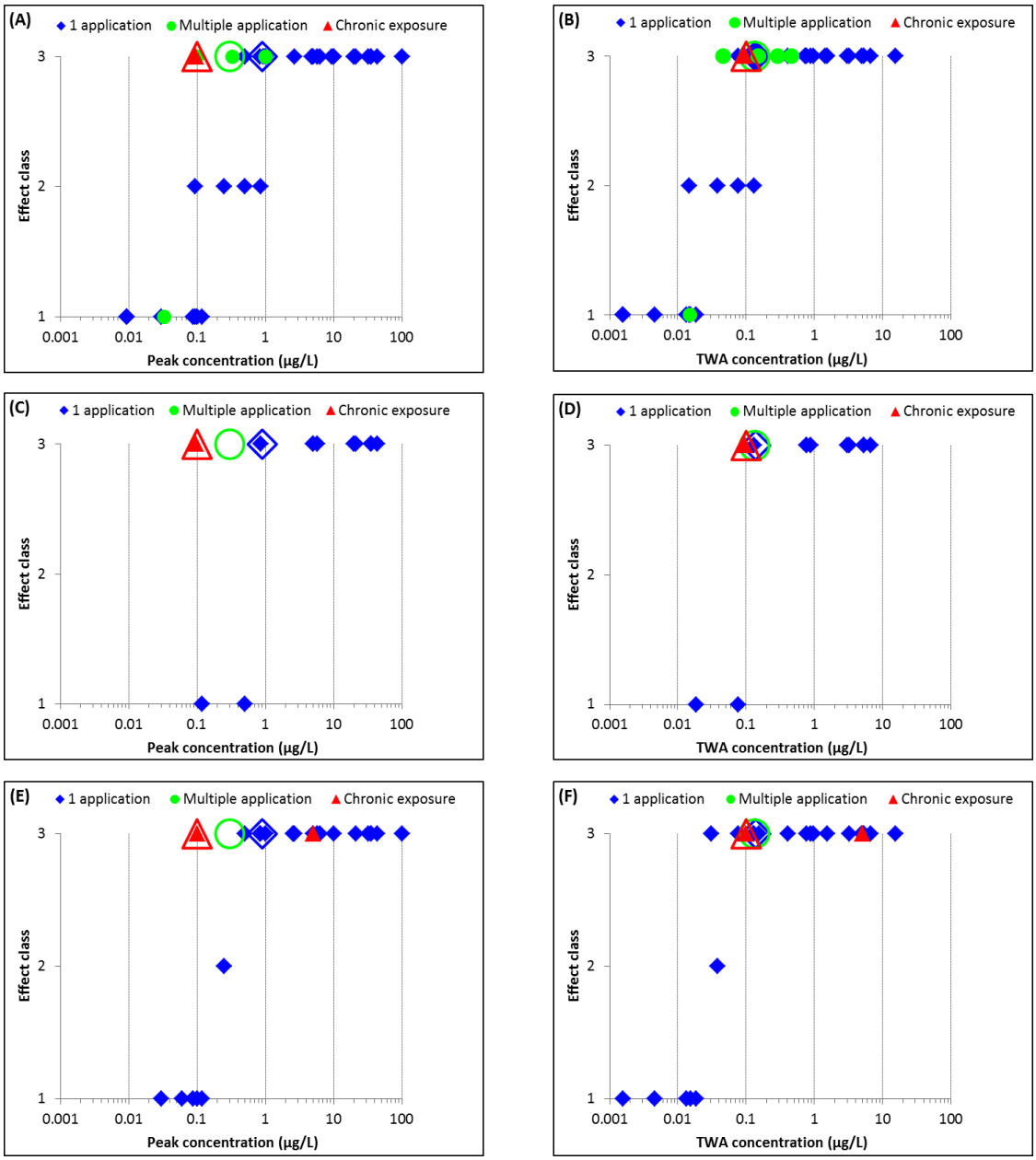

Figure 2: Classified effects of the organophosphate insecticide chlorpyrifos as observed for sensitive ecological endpoints in freshwater model ecosystem studies. The figure includes observations of studies for single and multiple applications along with chronic exposure regimes in stagnant as well as running water test systems. The effects are classified according to magnitude: **1** = no effect, **2** = slight effect, **3** = clear effect (see text for detailed explanation). The x-axis displays the exposure concentration of chlorpyrifos evaluated in the cosm studies expressed as peak **(left panels)** and TWA **(right panels)** concentrations. Effects are classified into potentially sensitive endpoints: microcrustaceans **(A-B)**, macrocrustaceans **(C-D)** and insects **(E-F).** The observed effects obtained from the study of Zafar *et al*. (2011) are highlighted by large symbols. These effects are indicated with empty triangle, circles and diamonds symbols following same colour corresponding exposure patterns.

Sodium-channel modulator insecticides

Of the insecticides group, the selected coms experiments that evaluated the ecological impact of sodium channel modulator insecticides comprised of 20 studies (77 cases) performed with 8 active ingredients of synthetic pyrethroids. The selected studies were mainly conducted on esfenverlate (5 studies), cypermethrin (4), and lambda-cyhalothrin (4) while other studies included fenvalerate (2), cyfluthrin (1), deltamethrin (2), permethrin (1), and tralomethrion (1). Mimicking the normal agricultural use, the majority of these coms experiments evaluated multiple applications (11), followed by single application (7) and chronic exposure regime (1). They provide adequate information on the ecological risks of the active ingredients belonging to the pyrethroid group of insecticides. The overall responses of the most sensitive endpoint categories in the semi-field tests stressed by pyrethroid insecticides were presented after classification into the three effect classes (Figure 3).

For the peak concentrations, clear effects were reported for all endpoints at pyrethroid insecticide concentrations higher than 0.01 Peak-TU. Within the concentration range 0.001-10 Peak-TU, slight effects (Class 2) were reported for these categories (Fig. 3A, C, E; left panel). For example, in the study of Mayasich *et al*. (1994), slight effects on microcrustaceans were observed at concentrations of 0.0027 and 0.0078 Peak-TU (most extreme observations of Fig. 3A). For insects, slight effects were reported at 0.0027 Peak-TU and clear effects at 0.0078 Peak-TU concentrations (most extreme observations of Fig. 3E). Unlike as for the acetylcholinesterase inhibitor insecticides, no clear dose-response relationship was found when summarising the effects of sodium channel modulator insecticides based on their Peak-TU concentrations (Fig. 1 and 3).

In the case of TWA exposure concentrations, clear effects were reported for all endpoints at exposure concentrations around 0.001 TWA-TU and higher. In the range between 0.001 and 1 TWA-TU, slight effects were recorded (Fig. 3B, D, F). Again, the lowest effect concentrations of clear and slight effects were found by the study of Mayasich *et al*. (1994). For example, slight effects for microcrustaceans were found at 0.00030 TWA-TU and 0.00085 TWA-TU. For insects, clear effects were found at exposure concentrations of 0.00085 TWA-TU and slight effects at 0.00030 TWA-TU (Fig. 3B and F). As for the Peak-TU standardised effects also for the TWA-TU standardised effects, no clear dose-response relationship is present (Fig. 3). This was probably because either the standardisation procedure (based on the HC50) was not correct for pyrethroids or the concentrations that needed to be expressed in a different way (neither peak nor TWA_{21d}). The standardisation on the HC50 may be incorrect because it seems that there might be a few arthropod species which are much more sensitive to pyrethroids than other arthropod species (see Fig. 6 of Maltby *et al*. 2005 for lambda-cyhalothrin) so a standardisation on the HC5 could work better.

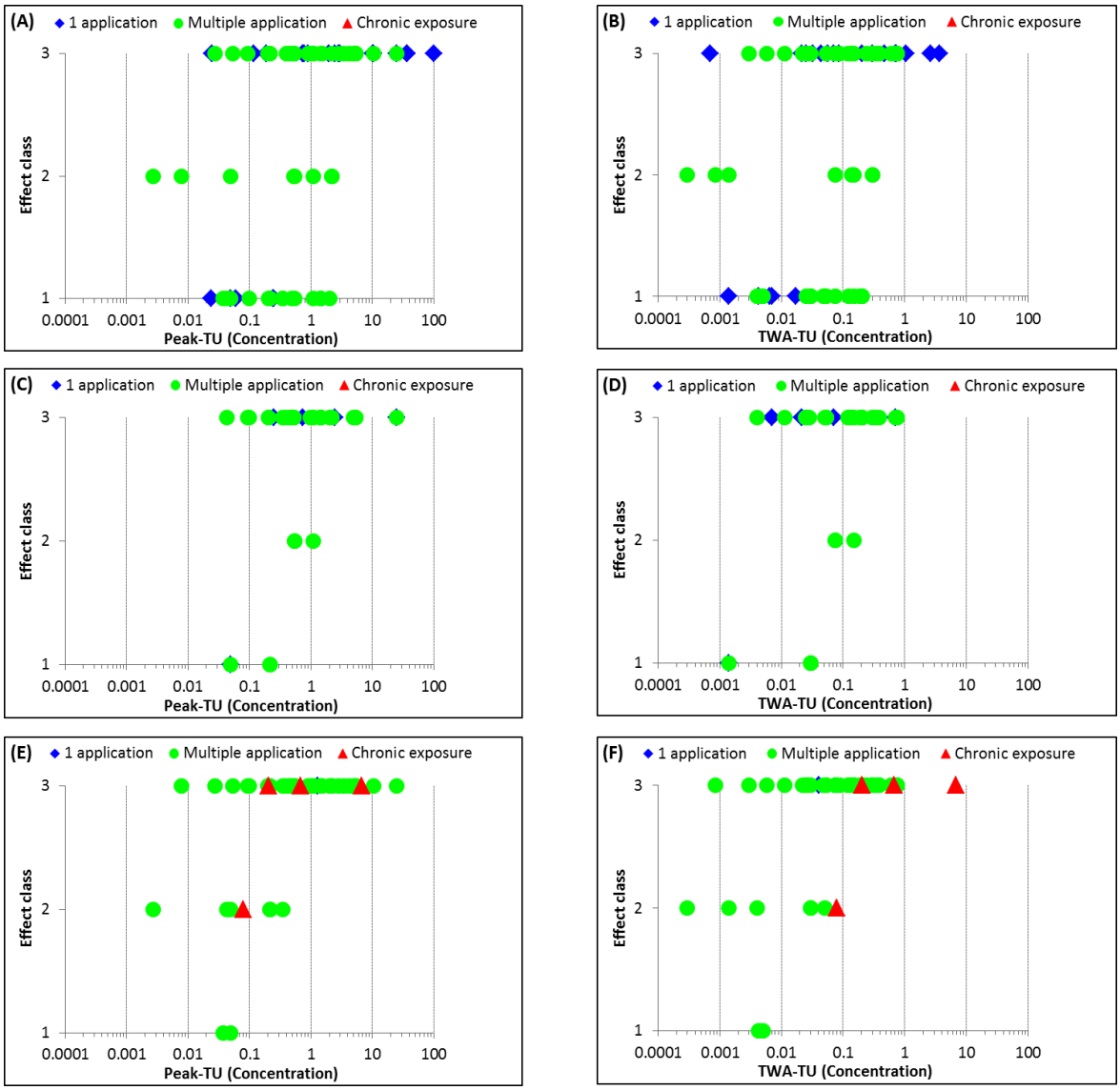

Figure 3: Classified effects of insecticides with a sodium channel modulator mode-of-action (synthetic pyrethroids) as observed for sensitive ecological endpoints in freshwater model ecosystem studies. The figure includes observations of studies for single and multiple applications along with chronic exposure regimes in stagnant as well as running water test systems. The effects are classified according to magnitude: **1** = no effect, **2** = slight effect, **3** = clear effect; (see text for detailed explanation). The x-axis displays the exposure concentration of the insecticides evaluated in cosm studies expressed in toxic units (TU) corresponding to peak **(left panels)** and TWA **(right panels)** concentrations. Effects are classified for three potentially sensitive endpoints: microcrustaceans **(A-B),** macrocrustaceans **(C-D)** and insects **(E-F).**

Standardising the concentrations on the EC50 value of *D. magna* instead of the HC50 would not improve the dose-response relationship (*results not shown*). Also, if only a few arthropod species show much higher sensitivity than other arthropod species, the presence of these species in the cosm studies is determining whether effects are found at low concentrations.

In contrast to the above, the responses recorded for microcrustaceans in cosms evaluating the effects of the pyrethroid insecticide cypermethrin (12 cases) showed a better dose response relationship (Fig. 4). In case of single peak concentrations of cypermethrin in selected cosm experiments, clear effects were usually observed for microcrustaceans at exposure concentrations of 0.08 µg/L and higher. Nevertheless, in case of TWA concentrations, clear effects are reported at exposure concentrations of 0.006 µg/L and higher (Fig. 4A, B).

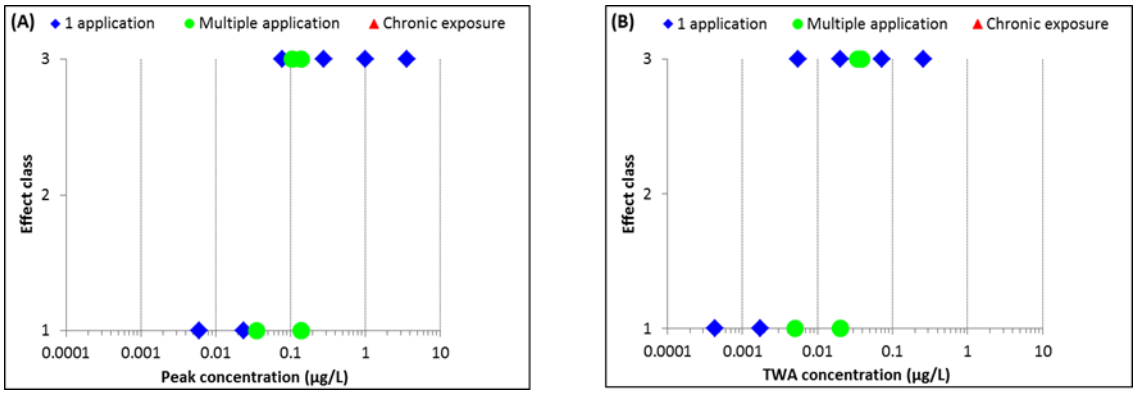

Figure 4: Classified effects of the pyrethroid insecticide cypermethrin as observed for the most sensitive ecological endpoint in freshwater model ecosystem studies. The figure includes observations of studies for single and multiple applications along with chronic exposure regimes in stagnant as well as running water test systems. The effects are classified according to magnitude: **1** = no effect, **2** = slight effect, **3**= clear effect; (see text for detailed explanation). The x-axis displays the exposure concentration of the insecticides evaluated in cosm studies expressed in toxic units (TU) corresponding to peak **(left panels)** and TWA **(right panels)** concentrations. Effects are classified into one potentially sensitive endpoint: microcrustaceans **(A-B).**

Moulting inhibiting insecticides

The molecular groups benzoylurea and hydrazine make up the moulting inhibitor insecticides. Their mode of action is disruption of, or interference with, any of the hormones inactivating the moulting process. Some insecticides, called insect growth regulators, target the insect's growth and development processes by interfering with hormones, and others, called chitin synthesis inhibitors, through blocking the production of a structural component of the exoskeleton (chitin) (Oetken *et al*. 2004; Soin *et al* 2009; Merzendorfer, 2012; Adel, 2012). Of the total insecticide group, the selected cosm experiments for the evaluation of the ecological impact of moulting inhibiting insecticides comprised 9 studies (38 cases) performed with three active ingredients benzoylurea (5 studies), azadirachtin (3) and tebufenozide (1). Six studies were included that evaluated a single application, 2 studies using multiple applications and 1 study using a chronic exposure regime. The effects of insecticides with a moulting inhibiting toxicological mode of action on sensitive endpoints (microcrustaceans, macrocrustaceans and insects) were classified into the three effect classes (Figure 5).

Overall, when related to peak concentrations, clear responses of microcrustaceans, macrocrustaceans and insects were reported at concentrations from about 0.1 Peak-TU and higher (Fig. 5A, C, D). One exception is a study that evaluated the effects of a single pulse of azadirachtin (Kreutzweiser *et al*. 2004), where effects were observed below 0.01 Peak-TU (Fig. 5). When standardised on TWA concentrations, most clear effects on microcrustaceans, macrocrustaceans and insects were observed at exposure concentrations higher than 0.01 TWA-TU, again with the exception of the study of Kreutzweiser *et al*. (2004). Slight effects were found in the range 0.01-0.1 TWA-TU (Fig. 5B, D, F).

The deviating study of Kreutzweiser *et al*. (2004) performed with azadirachtin, reports clear effects below 0.01 Peak/TWA-TU for microcrustaceans. In this study, microcrustaceans responded by a factor 10-100 times more sensitive than observed in other studies (Fig. 5A, B). The concentrations tested in the study of Kreutzweiser *et al*. (2004) corresponded to a very low TU resulting in effect class 3 compared to the other TUs from other studies assigned to this effect class due to high sensitivity of the copepod group for the neem-based insecticide Neemix (Fig. 5A). When the study of Kreutzweiser *et al*. (2004) was not taken into account, the data presented for moulting inhibitor insecticides in Figure 5 suggested TWA-TU based threshold values for sensitive endpoints being lower by a factor of 5 than those based on Peak-TU. The population responses of microcrustaceans seemed a bit more sensitive than those of macrocrustaceans and insects for this group of insecticides (Fig. 5). When excluding the Kreutzweiser *et al*. (2004) study, the dose-response relationship of microcustaceans appeared slightly better when effects on microcrustaceans were related to Peak-TU compared to TWA-TU (Fig. 5A and B), although the reverse was true for insects (Fig. 5E and F)

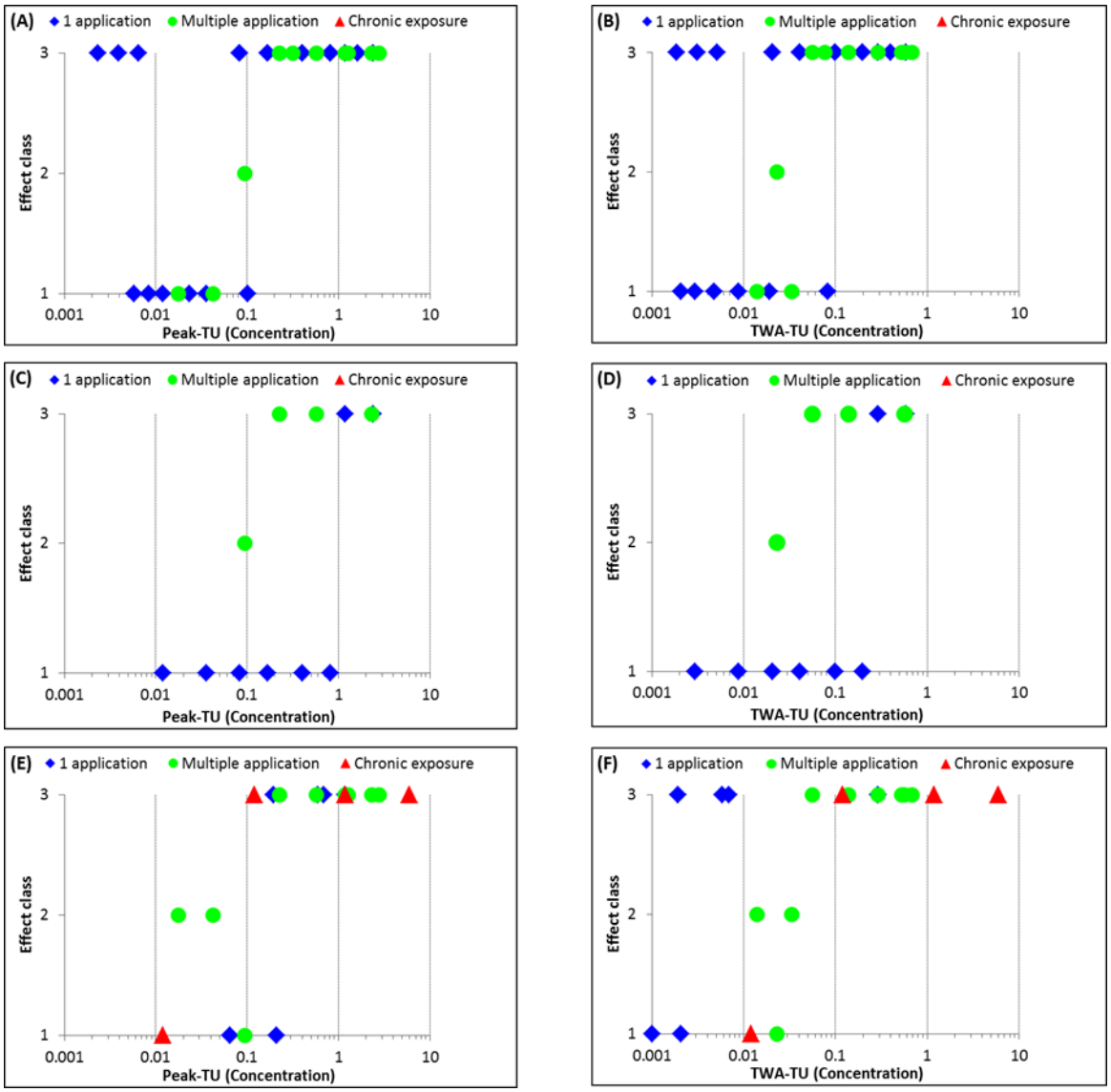

Figure 5: Classified effects of insecticides with a moulting-inhibiting mode-of-action as observed for sensitive ecological endpoints in freshwater model ecosystem studies. The figure includes observations of studies for single and multiple applications along with chronic exposure regimes in stagnant as well as running water test systems. The effects are classified according to magnitude: **1** = no effect, **2** = slight effect, **3** = clear effect; (see text for detailed explanation). The x-axis displays the exposure concentration of the insecticides evaluated in cosm studies expressed in toxic units (TU) corresponding to peak **(left panels)** and TWA **(right panels)** concentrations. Effects are classified for three potentially sensitive endpoints: microcrustaceans **(A-B)**, macrocrustaceans **(C-D)** and insects **(E-F).**

The ecological impact of the moulting inhibiting insecticide (urea) diflubenzuron on sensitive taxonomic groups was compared as classified by the help of peak and TWA_{21d} concentrations. Effects of the urea insecticide diflubenzuron (25 cases) as observed in cosm studies were also analysed (Figure 6). The cosm studies with diflubenzuron indicated clear effects on all endpoints for peak concentrations of 0.7 µg/L and higher (Fig. 6A, C, E). On the other hand, when standardising on TWA_{21d} concentrations, clear effects are reported at exposure concentrations higher than 0.1 µg/L (Fig. 6B, D, F). A clear dose-response relationship is present for both standardisations (Peak-TU and TWA-TU), with no preference for either of them (Fig. 6).

Conclusions and outlook

The present paper focused on the issue of comparing the interpretation of effects as observed in cosms on the basis of either the peak or the TWA_{21d} concentrations. The comparison was made including a wide range of acetylcholinesterase inhibitors, sodium channel modulators and some moulting inhibitors. On the basis of findings from the present study, several of these observations can be generalized so as to obtain rules-of-thumb that may be suitable for extrapolation. After comparing peak and TWA_{21d} exposure concentrations and based on the discussed consistency in the model ecosystem-generated threshold values, we recommend that in case of a single application an extrapolation factor of 5 is reasonable for the risk assessment of time-variable exposure to extrapolate effects from peak exposures to chronic TWA concentrations. These results also show the importance to distinguish between exposure regimes; for a single application of non-persistent insecticides, exposure concentrations expressed as Peak-TU are possibly a factor of 5 higher than when the same concentrations are expressed as TWA_{21d}-TU concentrations for repeated and chronic exposures to the same chemicals. We conclude that for most insecticides that were evaluated in this paper (except for pyrethroids), the TWA_{21d} concentration is a better predictor for long-term effects, especially for acetylcholine esterase inhibiting insecticides (Fig. 1). A microcosm experiment has already been performed to verify these rules-of-thumb evaluating different exposure patterns which consisted of different time-varying exposure profiles with the same TWA concentration for chlorpyrifos (Zafar *et al*. 2011), which also indicated that TWA_{21d} based concentrations are a better descriptor of long-term effects than peak concentrations. Therefore, peak exposure concentration divided by 5 may be used to protect against adverse ecological effects arising from long-term exposure (expressed as TWA concentrations) to pesticides. Therefore, in the risk assessment the TWA_{21d} concentration threshold values may be considered as ecotoxicological relevant concentration for organophosphate insecticides (Boesten *et al*. 2007; Fig. 2).

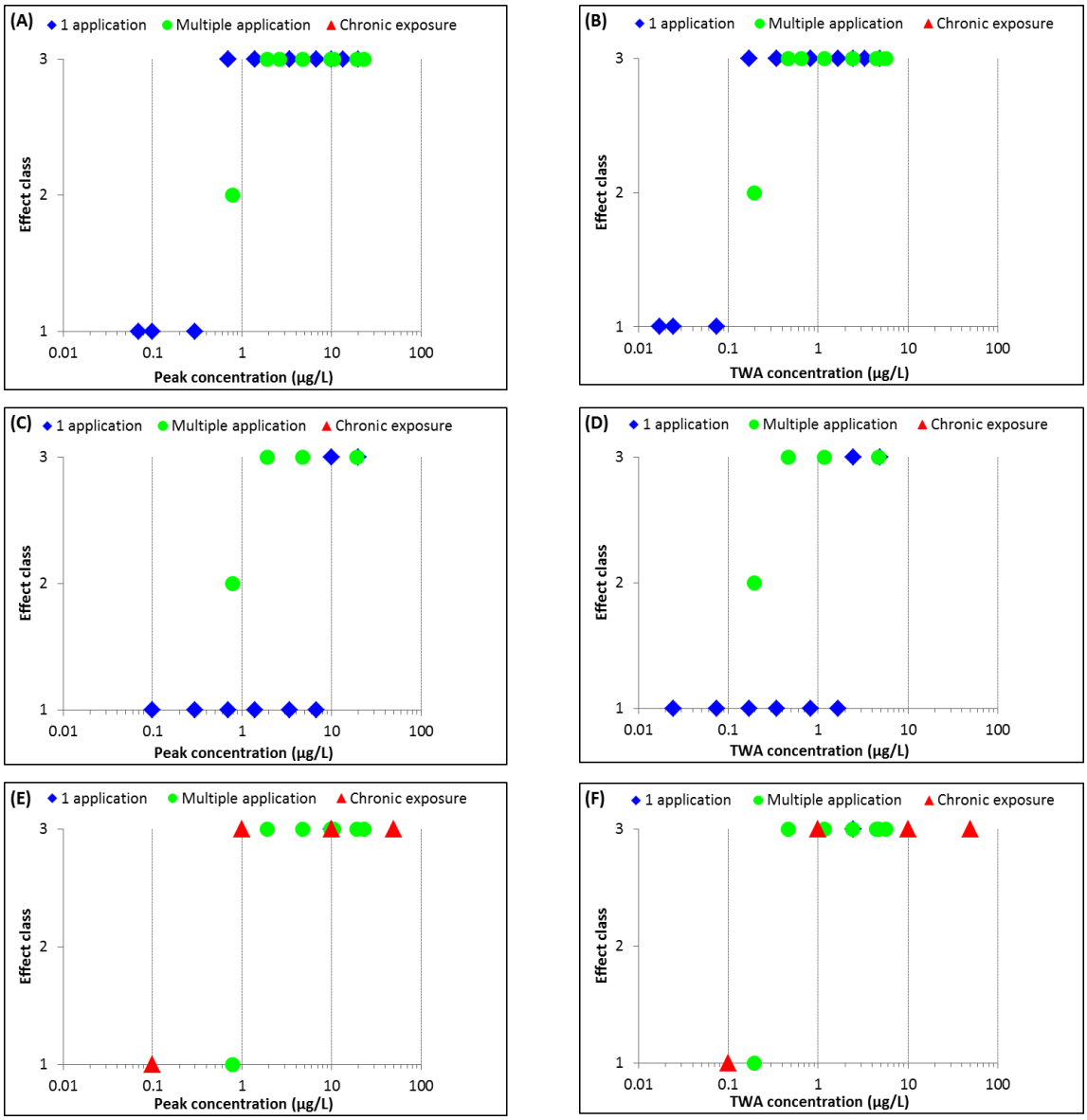

Figure 6: Classified effects of the urea insecticide diflubenzuron as observed for sensitive ecological endpoints in freshwater model ecosystem studies. The figure includes observations of studies for single and multiple applications along with chronic exposure regimes in stagnant as well as running water test systems. The effects are classified according to magnitude: **1** = no effect, **2** = slight effect, **3** = clear effect (see text for detailed explanation). The x-axis displays the exposure concentration of diflubenzuron evaluated in the cosm studies expressed as peak **(left panels)** and TWA **(right panels)** concentrations. Effects are classified into three potentially sensitive endpoints: microcrustaceans **(A-B)**, macrocrustaceans **(C-D)** and insects **(E-F)**.

Based on our findings no consistent pattern was observed for the pyrethroid insecticides. This finding indicates that when effects tend to be of an acute nature and dissipation and degradation of a compound is rapid, the TWA_{21d} is not suitable to evaluate the effects because it might underestimate the effects. Therefore, in case of rapidly acting compounds like pyrethroids, maximum or peak concentrations might be more important than the TWA, although also for Peak-TU no clear dose-response relationship was found (Fig. 3). For moulting inhibitor insecticides, peak and TWA can have equal importance in order to evaluate the effects (Fig. 5). Either of them can be used for evaluating the effects, however, this should be further evaluated and verified by performing experiments with moulting inhibitor insecticides similar to those of Zafar *et al*. (2011; 2012) in order to get more experimental evidence.

In addition, it may be necessary to evaluate whether the exposure–response relationships observed for insecticides can be extrapolated to other types of pesticides like herbicides and fungicides by performing a similar exercise for these types of pesticides.

Acknowledgments

We would like to thank John Deneer and Rene Van Wijngaarden with their help with the review of the cosm studies.

Supporting Information for Chapter 2

Calculation of peak concentration and Time-Weighted Average (TWA_{21d}) concentration

1- Calculation of peak PEC^n for multiple applications

The PEC (Predicted Exposure Concentration) from a series of *n* applications with fixed time interval between applications is calculated via using:

$$PEC^{n} = PEC^{1}\frac{1-e^{-n\cdot k\cdot \Delta t}}{1-e^{-k\cdot \Delta t}} \qquad (1)$$

where:

PEC^1 = momentary water concentration from a single application (µg/L)

PEC^n = momentary water concentration from '*n*' applications (µg/L)

n = number of applications (-)

K = overall dissipation rate coefficient accounting for degradation, volatilization, and dilution (1/d)

Δt = time interval between applications (d)

2- Calculation of TWA_{21d} from single application of pesticide

A pesticide loading at t = 0, results after instantaneous linear equilibrium sorption to suspended solids and aquatic macrophytes in a Predicted Exposure Concentration PEC (Peeters *et al*. 2008). The concentration as a function of time after the loading is given by Eq (2):

$$C(t) = PEC^{1}\exp(-k*t) \qquad (2)$$

where:

C(t) = concentration of pesticide dissolved in water at time *t* (µg/L)

t = time (d)

PEC^1 = PEC resulting from a single loading (µg/L)

k = total dissipation rate constant, accounting for transformation, volatilization and dilution (d^{-1})

The TWA concentration of single application resulting from integration over t = 0 to $t = t_{TWA}$, can be computed by Eq. (3):

$$TWA_t = \frac{PEC^1}{k * t_{TWA}} \left[1 - \exp\left(-k * t_{TWA}\right)\right] \quad (3)$$

where:

TWA_t = Time Weighted Average concentration for period with length t_{TWA} (µg/L)

PEC^1 = PEC resulting from a single loading (µg/L)

k = total dissipation rate constant, accounting for transformation, volatilization and dilution (d^{-1})

t_{TWA} = length of period for TWA (d)

3- Calculation of TWA_{21d} from multiple (repeated) application of pesticides

Case 1

The period in which application occurs is shorter than the TWA period, hence $n.\Delta t < t_{TWA.}$

$$TWA_t = \frac{PEC^1_{water}}{K \bullet t_{TWA}} \left[n - \left(\frac{1 - \exp(nk \bullet \Delta t)}{1 - \exp(k \bullet \Delta t)} \right) \exp(-nk \bullet \Delta t) + \left(\frac{1 - \exp(-nk \bullet \Delta t)}{1 - \exp(-k \bullet \Delta t)} \right) \exp(-k \bullet \Delta t)(1 - \exp(-k \bullet (t_{TWA} - n\Delta t)) \right] \quad (4)$$

where:

TWA_t = Time Weighted Average concentration for period with length tTWA (µg/L)

PEC^1_{water} = momentary water concentration from a single application (µg/L)

t_{TWA} = length of period for TWA (d)

k = overall dissipation rate coefficient accounting for degradation, volatilization and dilution (1/d)

n = number of applications (-)

Δt = time interval between applications (d)

Case 2

Concentrations in the Δt interval after the final application are all higher in the period before the final application; $c(t_n+\Delta t) > PEC^{n-1}$. Hence, the condition for use of the Case 2 solution is:

$$(1 - \exp(-nk*\Delta t)) \exp(-k*\Delta t) + \exp(-(n-1)k* \Delta t > 1 \quad (5)$$

The solution for case 2 is given by:

$$TWA_t = \frac{PEC^1{}_{water}}{K \bullet t_{TWA}} \left[\left(\frac{1-\exp(-nk \bullet \Delta t)}{1-\exp(-k \bullet \Delta t)} \right) (1-\exp(-k \bullet t_{TWA})) \right] \quad (6)$$

Case 3

Concentrations of the final m applications determine the highest TWA;

C ($t = t_{n-m} + \Delta t$) < c ($t = t_n + \Delta t + t_{rest}$). Hence, the condition for use of the Case 3 solution is:

$$(1-\exp(-nk*\Delta t)) \exp(nk*\Delta t\ t_{rest}) + \exp(-(n-m)\ k*\Delta t) > 1 \quad (7)$$

The solution of case 3 is given by:

$$TWA_t = \frac{PEC^1{}_{water}}{K \bullet t_{TWA}} \left[m - \left(\frac{1-\exp(mk \bullet \Delta t)}{1-\exp(k \bullet \Delta t)} \right) \exp(-nk \bullet \Delta t) + \left(\frac{1-\exp(-nk \bullet \Delta t)}{1-\exp(-k \bullet \Delta t)} \right) \exp(-k \bullet \Delta t)(1-\exp(-k \bullet t_{rest})) \right] \quad (8)$$

m = whole number of application intervals within TWA period (-)

t_{rest} = time remaining from TWA period (= t_{TWA} – m. Δt) (d)

Case 4

Concentrations of the final m+1 applications determine the highest TWA; PECn-m-1 > c($t = t_n + t_{rest}$). Hence, the condition for use of the Case 4 solution is:

$$(1-\exp(-nk*\Delta t)) \exp(-k*\ t_{rest}) + \exp(-(n-m)\ k*\Delta t) < 1 \quad (9)$$

The solution for case 4 is given by:

$$TWA_t = \frac{PEC^1{}_{water}}{K \bullet t_{TWA}} \left[m - \left(\frac{1-\exp(mk \bullet \Delta t)}{1-\exp(k \bullet \Delta t)} \right) \exp(-(n-1)k \bullet \Delta t) + \left(\frac{1-\exp(-nk \bullet \Delta t)}{1-\exp(-k \bullet \Delta t)} \right) (1-\exp(-k \bullet t_{rest})) \right] \quad (10)$$

Chapter 3

Effects of time-variable exposure regimes of the insecticide chlorpyrifos on freshwater invertebrate communities in microcosms

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"There is an enormous disparity between the types of data available for assessment and the types of responses of ultimate interest. The toxicological data usually have been obtained from short-term toxicity tests performed using standard protocols and test species. In contrast, the effects of concern to ecologists performing assessments are those of long-term exposures on the persistence, abundance, and /or production of populations"

(Barnthouse *et al*. 1987)

Abstract

The present study compared the effects of different time-variable exposure regimes having the same Time-Weighted Average (TWA) concentration of the organophosphate insecticide chlorpyrifos on freshwater invertebrate communities, to enable extrapolation of effects across exposure regimes. The experiment was performed in outdoor microcosms by introducing three different regimes: (1) a single application of 0.9 µg a.i./L, (2) three applications of 0.3 µg a.i./L, with a time interval of 7 d and (3) continuous exposure to 0.1 µg a.i./L for 21 d. Measurements showed that the TWA-21d concentration in the continuous-exposure treatment (0.098 µg/L) was slightly lower than in the three-applications (0.116 µg/L) and single-application (0.126 µg/L) treatments. The application of chlorpyrifos resulted in decreased abundances in the arthropods community, with the largest adverse effects reported for the mayfly *Cloeon dipterum* and cladocerans *Daphnia* gr. *longispina* and *Alona* sp., while smaller effects were observed for other insects, copepods and amphipods. At the population level, however, the mayfly *C. dipterum* only responded to the single-application treatment, which could be explained by the toxicokinetics of chlorpyrifos in this species. At the end of the experimental period, the invertebrate community showed approximately the same effect magnitude for all treatment regimes. These results suggest that for this combination of concentrations and duration of the TWA, the TWA concentration is more important for most species than the peak concentration for the assessment of long-term risks of chlorpyrifos.

Introduction

Pesticides used for crop protection in agriculture and horticulture may enter ditches, ponds, lakes and rivers in various ways, such as direct overspray, spray drift, leaching to surface and ground water, run-off from land and/or accidental spills (Brown *et al*. 2004). Consequently, these hazardous chemicals may affect the non-target biotic communities of such freshwater ecosystems (Van den Brink, 2008). Protecting the biological integrity of these waters requires assessing the potential risks associated with the pesticide stress to aquatic ecosystems. Freshwater model ecosystems such as microcosms and mesocosms have been widely recommended as surrogate tools for the (higher-tier) ecological risk assessment of pesticides (European Commission, 2002). Microcosms and mesocosms are most useful in the advanced phases of an ecological risk assessment, where they provide information that cannot be derived from laboratory studies, like indirect effects and recovery of affected populations (Van den Brink, 2008). A major advantage of these experimental systems is their realistic simulation of both chemical exposure and ecological effects (Van Wijngaarden *et al*. 2005a; Brock *et al*. 2006).

Contamination of surface waters with pesticides may occur by single or repeated pulses through various emission routes. A single pulse input typically results in a period of high concentration followed by a decline in concentration due to hydrological dilution, degradation, or partitioning from water to other compartments (air, sediment and/or macrophytes) in the ecosystem. With repeated pulses, the first pulse is followed by, at least, a second pulse due to another spray event or run-off after a rain or irrigation event within a matter of days (Schulz, 2004). Standard laboratory toxicity tests do not investigate the toxicity of time-variable exposures of aquatic organisms to substances, even though estimating the effects of realistic time-variable exposure regimes is often an important source of uncertainty in the ecological risk assessment of pesticides (Brock *et al*. 2010).

Models have been developed to predict effects on aquatic organisms resulting from time-variable exposure to pesticide (Ashauer *et al*. 2006). Toxicokinetic-toxicodynamic (TK/TD) models describe the processes that mechanistically link exposure to effects in an individual organism and can therefore be used to understand differences in response to the same time-variable exposure between different species. Toxicokinetics (e.g., uptake and elimination dynamics, bioconcentration) predict the time course of concentrations within an organism in relation to concentrations in the external medium. Toxicodynamics describe the time course of damage, subsequent effects and repair processes in the target organisms based on specific pattern(s) of exposure to the test compound. At present, TK/TD modelling is especially highly developed for aquatic invertebrates. The

toxicokinetics of chlorpyrifos in several freshwater arthropods have been investigated previously (Rubach *et al*. 2010), allowing differences in species responses to time-variable exposure in field settings to be linked to differences in the toxicokinetics and toxicodynamics of chlorpyrifos in species, which may facilitate a better understanding of the resulting effects.

In Europe, environmental risks associated with appropriate pesticide use are estimated using procedures described in the European Plant Protection Products directive 91/414/EEC (European Union, 1997) employing a tiered approach. In the higher tiers, the risks of pesticides to aquatic ecosystems are often assessed by performing microcosm or mesocosm experiments evaluating a particular exposure regime (e.g. a pulse application), which does not necessarily correspond to the exposure component of the risk assessment procedure (which may involve e.g. multiple applications). To allow an appropriate linkage of the exposure and effects components of the risk assessment, the results of these microcosm or mesocosms experiments therefore sometimes need to be extrapolated to a different exposure pattern than the one evaluated in the cosm experiment itself (Boesten *et al*. 2007). Since time-variable surface water exposure profiles are the rule rather than the exception in the field, two European Union (EU) workshops were convened in 2007 (ELINK I and II) to discuss how to link exposure and effects in the aquatic risk assessment procedures for pesticides under EU directive 91/414/EEC (Brock *et al*. 2010). One of the recommendations of the ELINK workshops was to determine when to use the peak or a time-weighted average (TWA) concentration in the risk assessment process. Normally, peak values are used in acute risk assessment, while TWA concentrations may be used in chronic risk assessment. The ELINK workshop proposed that further research was required to provide a scientific basis for criteria that can be used to decide whether or not the TWA concentration approach is appropriate to use in chronic risk assessment, and which time window the TWA should be based upon (Brock *et al*. 2010).

To address this question, as well as to gather empirical evidence for the use of either of these concentrations, the present study aimed to compare the effects of different time-variable exposure regimes having the same TWA of 21 days (TWA_{21d}), but different peak concentrations of a pesticide. The 21d time-interval is based, as recommended by ELINK, on the relevant chronic toxicity test (i.e. with *Daphnia magna*) (Brock *et al*. 2010). The pesticide used to compare the responses of freshwater invertebrate communities in outdoor microcosms was chlorpyrifos, a broad-spectrum organophosphorous insecticide extensively investigated in microcosm and mesocosm experiments (Daam and Van den Brink, 2010). Several model ecosystem studies have shown it to cause significant changes in sensitive macroinvertebrate and zooplankton assemblages at peak levels between 0.3 and 1 µg/L, while higher concentrations can also result in indirect responses relating to functional endpoints and primary producers (Van Wijngaarden *et al*. 2005b; Daam *et al*. 2008; Van den Brink *et*

al. 1996. Since effects of chronic exposure are observed at 0.1 µg/L (Van den Brink *et al*. 1995), we hypothesise that the ecological effects of chlorpyrifos are more strongly determined by the TWA_{21d} concentration than by the peak of the exposure.

Materials and Methods

Experimental set-up

The experiment was performed in 16 outdoor microcosms situated at the Sinderhoeve experimental station (www.sinderhoeve.org) in Renkum, The Netherlands. The characteristics of each circular microcosm were as follows: diameter 1.8 m, total depth 0.8 m, water depth 0.5 m and total volume approximately 1270 L. The microcosms were lined with a water-tight, nontoxic layer of black polyethylene to prevent exchange of water with the surroundings. Each microcosm contained an 8 cm sediment layer (fine clay), obtained from a mesotrophic *Elodea nuttallii*-dominated lake. The water was obtained from the station's water supply reservoir and introduced 6 months prior to the start of the experiment. This water has low nutrient concentrations.

One hundred shoots of *Elodea nuttallii* were planted in each microcosm 6 months before the experiment, evenly distributed over the sediment. Other macrophyte species (*Chara sp.*) developed from diaspores in the sediment. About 3 months prior to the insecticide treatment, macroinvertebrates, zooplankton and phytoplankton, collected from uncontaminated drainage ditches (Sinderhoeve Experimental Station, Renkum, and Veenkampen, an experimental field site of Wageningen University, Wageningen, The Netherlands) were introduced to develop a macrophyte-dominated freshwater community in the systems. Macroinvertebrates collected from Veenkampen were cleaned and washed very thoroughly to avoid fish entering the systems. The macroinvertebrates introduced comprised several taxonomic groups, especially insects (*Cloeon dipterum* and *Chaoborus* sp.) and crustaceans (*Gammarus pulex* and *Daphnia* sp.) because these taxa are known to be particularly sensitive to chlorpyrifos.

During an acclimatization period of approximately 3 months, the ecological community was allowed to mature in the microcosms. Meanwhile, all microcosms were interconnected by tubes (internal diameter 2.4 cm) and the water was circulated using a pump to maximize the similarity between the communities in the systems. The circulation of water was stopped three weeks before the start of the experiment. In order to maintain some water movement, the microcosms were lightly aerated. The aquatic community in the microcosms resembled that of macrophyte-dominated Dutch drainage ditches.

Calculation of chlorpyrifos concentrations and its application and sampling

The concentrations used were based on the knowledge that the 0.1 µg/L treatment level is the no-observed-effect-concentration (NOEC) for a single application at the species and community level obtained from microcosm and mesocosm experiments. Effects from a continuous-exposure regime were to be expected at this treatment level (Daam and Van den Brink, 2010; Van den Brink *et al*. 1995). The 0.3 µg/L treatment level is considered to be the Lowest Observed Effect Concentration (LOEC) of a single application, producing slight effects in microcosm and mesocosm experiments (Biever *et al*. 1994). The 0.9 µg/L treatment level is expected to cause clear effects on aquatic ecosystems based on mesocosm experiments (Van den Brink *et al*. 1996).

Time Weighted Average (TWA) concentrations of the single-application treatment were derived by integrating over t = 0 to t_{TWA}, and dividing by the length of the TWA period t_{TWA}, and calculated using this equation:

$$TWA_t = \frac{PEC^1}{k^* t_{TWA}} \left[1 - \exp\left(-k^* t_{TWA}\right)\right] \qquad \text{Eqn (1)}$$

Where

K^* = total dissipation rate constant, accounting for degradation, volatilization and dilution (d^{-1})

PEC^1 = predicted exposure concentration resulting from a single loading (µg/L)

TWA_t = Time Weighted Average concentration for period with length t_{TWA} (µg/L)

t_{TWA} = length of period for TWA (d)

The equation used to calculate the TWA for three-application treatment was:

$$TWA_{21d} = \frac{PEC^2}{k^* t_{TWA}} \left[3 - \exp\left(-7k^*\right) - \exp\left(-14k^*\right) - \exp\left(-21k^*\right)\right] \qquad \text{Eqn (2)}$$

Where

PEC^2 = predicted exposure concentration resulting from three-applications (µg/L).

The treatment level of continuous exposure was aimed to be equal to the TWA_{21d} concentration in the single-application and three-application treatment regimes. All three treatment regimes were intended to result in same TWA_{21d}.

On August 25, 2008, chlorpyrifos (Experimental sample, Lot no. OF 13272055, 480 g a.i./L EC,Dow AgroSciences, UK) was introduced into the microcosms using three different treatment regimes: a single application of 0.9 µg a.i./L; three repeated applications of 0.3 µg a.i./L with a time interval of 7 d; and a continuous exposure of 0.1 µg a.i./L using a pump for 21 d (Van den Brink *et al*. 1995). Concentrations in stock and dose solutions were checked to establish nominal initial concentrations.

All treatments, including controls, were quadruplicated and were assigned randomly to the microcosms. All test systems were dosed with the same volume of dosing solution while the control microcosms received water only. The applications were made by pouring a quantified volume of treatment solution into the microcosms, after which the water volume was gently stirred to mix the compound through the water column, but without disturbing the sediment and submerged macrophytes.

Exposure concentrations were measured by collecting water samples from each of the microcosms 1 h before, and 1 h, 6 h, 1 d, 2 d, 4 d and 7 d after each application, while the single application treatment was also sampled 14 d and 21 d after application. In the continuous–exposure regime, sampling and analysis of chlorpyrifos and additional dosing (when necessary) took place daily.

Chemical Analysis

Chlorpyrifos was extracted from the water samples by liquid/liquid extraction method. Depth-integrated water samples (average volume of 0.164 L) were collected by means of a stainless steel tubing system connected to a pre-weighed borosilicate glass flask. Sampling of each enclosure was done in triplicate. After sampling, flasks were weighed to determine the exact mass of water samples. A known volume of n-hexane (approximately 20 ml) was then added. Water and hexane were mixed thoroughly on an orbital shaker (approx. 175 rpm) for at least 15 min to extract chlorpyrifos into the hexane layer. A quantified amount of the hexane was collected separately in a tube. The extract was further concentrated by evaporating the hexane volume to 1 ml in a water bath (40 °C) with air. Hexane was added to this concentrated sample to achieve an end-volume of 2 ml, which was shaken on a vortex mixer. Chlorpyrifos was determined by splitless injection (5 µl) on a HP 5890 gas chromatograph equipped with an electron-capture detector (ECD) and an HP 6890 autosampler. Specifications for the gas chromatography electron capture detector (GC-ECD) analysis of chlorpyrifos were in accordance with the study by Rubach *et al.* (2011).

The Dissipation Time 50% (DT_{50}), i.e. the time during which 50% of the initially dosed compound had dissipated from the water phase, was based on the single-application treatment and calculated assuming first order kinetics by applying linear regression on ln-transformed concentrations versus time.

Macroinvertebrates

Benthic macroinvertebrates were sampled from each microcosm at -7, 3, 10, 17 and 24 d after the first chlorpyrifos application by means of litterbags and artificial substrates (pebble baskets) as described in Brock *et al*. (1992). Two litterbags and two pebble baskets were incubated in each system. On each sampling day, the artificial substrates were gently retrieved from the microcosm using a net to prevent organisms from escaping. Pebble baskets were first washed in a container to remove invertebrates from the pebbles after which the baskets were returned to the cosms at same position, together with a new set of litterbags. Subsequently, the macroinvertebrates obtained from substrates and litter bags were sorted manually, identified, counted alive, and then released again into their original microcosms. Identification of the macroinvertebrates was done to the lowest practical taxonomic level. Counted numbers of macroinvertebrates from artificial substrates and litter bags were pooled for further analysis.

Zooplankton sampling and identification

Zooplankton was sampled from each microcosm on weeks -1, 0, 1, 2 and 3 using a perspex tube (sampling volume: approximately 1.8 L). Several depth-integrated sub-samples were collected from each microcosm, evenly distributed over the cosms, until a 6 L sample had been obtained, and 5 L of each sample was used for zooplankton analysis. The 5 L sample was concentrated through a plankton net (mesh size 55 µm) and was preserved with an acetate-buffered lugol solution in a 100 ml sampling vial. The filtered water was poured back into its original microcosm. Micro-zooplankton (i.e. rotifera and copepod nauplii) was counted and identified under an inverted microscope (Carl Zeiss, Axiovert 10, magnification 100x) using a subsample of known volume. On average the subsample constituted of 20% of the original sample. Macro-zooplankton (i.e. cladocera, adult and subadult copepods) was quantified by counting the entire sample using a stereo microscope (Nikon SMZ-10, Japan, magnification 25x). Rotifers and cladocerans were identified to the lowest practical taxonomic level (genus/species), whereas copepods were classified as calanoids and cyclopoids. The abundance of each group (individuals/L) was calculated using a correction factor to account for the fraction of the total sample that was counted.

Phytoplankton chlorophyll-*a*

The phytoplankton chlorophyll-*a* content was determined 1 d before chlorpyrifos was applied, and thereafter on a weekly basis after the application. The remaining 1 L from the 6 L sample collected in the zooplankton sampling procedure was concentrated on a Whatman glass-fiber filter (GF/C; diameter 4.7 cm, mesh size 1.2 μm; Maidstone, UK) using a vacuum pump. The filters were transferred into Petri dishes, wrapped in aluminium foil, and stored at –20 ˚C (for a maximum of 1.5 months) awaiting further analysis (Dutch Organization for Standardization, 1981). After ethanol extraction of the pigments, chlorophyll-*a* content was measured using a Shimadzu 1601 PC UV-visible spectrophotometer, following the method described in Moed and Hallegraeff (1978).

Community metabolism

Temperature (T), dissolved oxygen (DO), pH, and electrical conductivity were measured in each microcosm just before the zooplankton and phytoplankton samplings, between 8 and 10 am, at a depth of approximately 25 cm. Temperature, pH and oxygen were measured using an HQ40D multimeter (Hach-Lange, The Netherlands). Electrical conductivity was measured with an Eijkelkamp 18.28 conductivity meter. Alkalinity was measured prior to the first application of the test substance (d -4) and at the end of the experiment (d 23), using 100-ml water samples taken at a depth of 10 cm by titration with 0.02 N HCl until a pH of 4.2 was reached (pH meter: WTW 323).

In addition, nutrients (ammonium, nitrate, nitrite, total nitrogen, orthophosphate and total phosphate) were measured one week before the first application and at d 23 of experiment. For this purpose, water samples were obtained from the filtered water (Whatman GF/C; 1.2 μm pore-size) collected for phytoplankton chlorophyll-*a* samples, and were transferred into 100-ml polyethylene flasks which were stored at temperatures below -18 °C until analysis. Total nitrogen, N-(NO_2^- + NO_3^-), NH_4^+, ortho-phosphate and total phosphate were analysed using a Skalar 5100 Autoanalyser.

Decomposition

Decomposition of particulate organic matter (POM) was studied by means of a leaf litter bags technique, using *populus* leaves. The *populus* leaves were soaked three times for a period of 2 days to remove the more easily soluble humic compounds. The material was dried in an oven for 72 h at 60 ˚C to allow storage. In the decomposition assessment, 2 g dry weight of *populus* leaves were enclosed in each litter bag, consisting of a glass Petri-dish (diameter: 11.6 cm), closed with a cover of stainless-steel wire (mesh size: 0.7 x 0.7 mm), in which 2 holes (diameter: 0.5 cm) were punched to allow the passage of most invertebrates.

In each microcosm, two litter bags were incubated at the sediment surface in an almost upright position for a period of approximately 2 weeks, and replaced when sampled. At the end of each 2-week incubation period, retrieved litter bags were emptied into a white tray to separate POM from adhering sediment particles and macroinvertebrates by rinsing with tap water. After a set of litter bags had been retrieved on a sampling day, a new set was incubated. The organic plant material was dried in aluminium foil at 105 °C for 48 h to obtain dry weight. The decomposition over a 2-week period was expressed as % remaining organic material.

Macrophytes and sediment

The macrophyte-dominated microcosms were populated primarily by *Elodea nutallii*. After the termination of the experiment (day 31), the above-sediment macrophyte biomass of two representative 0.5 x 0.5 m sample squares within the microcosms were harvested. The plant material harvested was rinsed under tap water to remove sediment particles and macroinvertebrates, and then dried in an oven in pre-weighed aluminium foil at 105 ˚C for 48 h to determine the dry weight of *Elodea*.

At the end of experiment, sediment samples were taken from 4 microcosms to determine the organic matter content of the sediment. On this sampling day, 3 upper sediment samples per cosm (height 5 cm, core diameter 3.9 cm) were taken to the laboratory, where water content and weight loss on ignition were determined. Organic matter content was calculated by dividing the weight loss on ignition by the dry weight.

Data analysis

Univariate analysis

Prior to univariate and multivariate analyses, abundance data of macroinvertebrates and zooplankton were Ln(*A*x+1) transformed, where x stands for the abundance value and the value of *A* is chosen in such a way that *A*x makes 2 by taking the lowest abundance value higher than zero for *x* (see Van den Brink *et al*. (2000) for rationale). This was done to downweigh high abundance values and to approximate a log-normal distribution of the data. The macroinvertebrate data were Ln(2x+1) transformed and the zooplankton data Ln(10x+1) transformed before analysis. All other variables were tested using untransformed values. Statistically significant differences between the treatments as well as against controls were assessed for all parameters or taxon levels at each time point, using analysis of variance (ANOVA) with multiple comparison tests. ANOVA was followed by Tukey range

test ($p \leq 0.05$), testing all treatments against the controls but also against each other. The analyses were carried out with the Genstat computer programme (v11.1) (Rubach *et al*. 2011).

Multivariate analysis

The effects of the chlorpyrifos treatment at the community level of macroinvertebrates and zooplankton were analysed by the Principal Response Curves (PRC) method using the CANOCO software package, version 4.5 (Ter Braak and Smilauer, 2002; Van den Brink and Ter Braak, 1999). The analysis results in a diagram showing sampling day on the *x*-axis and the first Principal Component of the treatment effects on the community on the *y*-axis (e.g. Fig. 3). The PRC method yields a diagram showing the most dominant community response to the treatment present in the data set. The species weights are shown in a separate diagram, and indicate the degree of affinity the species have with this dominant response. The results of the PRC analysis can also be evaluated in terms of the fractions of variance explained by the factors time and treatment, and the PRC diagram shows the fraction of the variance that is explained by the treatment.

In the CANOCO computer program, redundancy analysis is accompanied by Monte Carlo permutation to assess the statistical significance of the effects of the treatments on the species composition of the samples. The significance of the PRC diagram, in terms of displayed treatment variance, was tested by Monte Carlo permutation of microcosms, using an F-type test statistic based on the eigenvalue of the component (Van den Brink and Ter Braak, 1999). For each sampling date, all treatments were also tested against the controls using Monte Carlo permutation tests to assess the significance of treatment effects in time.

Results

Chlorpyrifos exposure

Figure 1 summarizes the chlorpyrifos exposure dynamics in the three treatments. The 21d-TWA concentrations (± SD) of the single-application, three-applications, and continuous-exposure treatments were 0.126 (± 0.008), 0.116 (± 0.015) and 0.098 (± 0.018) µg/L, respectively. The Dissipation Time 50% (DT50) was approximately 3 d for the single-application treatment and 2.5 d for the three-application treatment. Verification of the concentration in the dosing solution indicated that the intended concentrations were met (*data not shown*). One hour after application, concentrations of 83 and 81% of the initially applied concentration were found in the water phase of the single-application and three-application treatments, respectively. In the continuous-exposure treatment, the highest measured concentration was 0.131 µg/L, while the lowest was 0.062 µg/L. Quantification and detection limits of chlorpyrifos were 0.054 µg/L (Limit of Quantification, LOQ) and 0.016 µg/L (Limit of Detection, LOD), respectively.

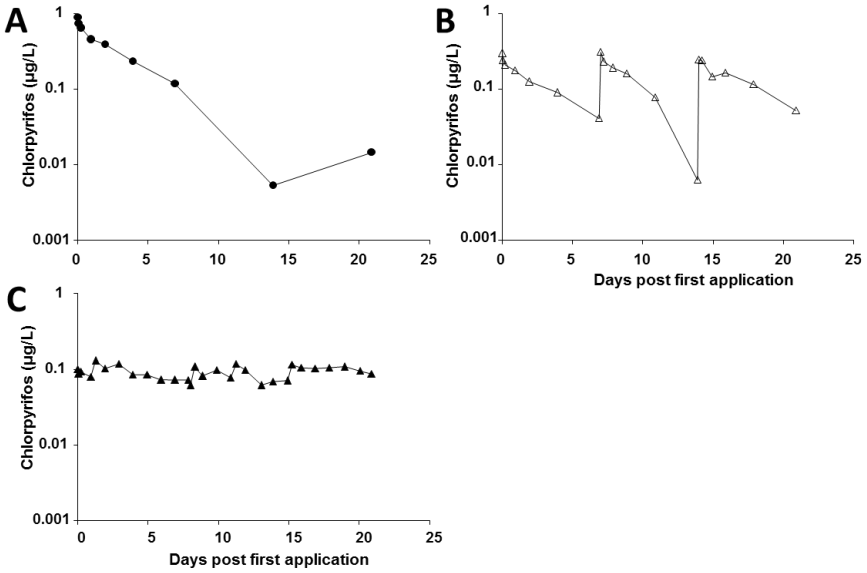

Figure 1: Dynamics of chlorpyrifos concentrations in the (**A**) single-application, (**B**) three-application, and (**C**) continuous-exposure treatments. The three applications took place on days 0, 7 and 14.

Macroinvertebrates

A total of 72 macroinvertebrate taxa were recorded in the study. During the experimental period, the macroinvertebrate community consisted of 36 insect taxa, 4 crustacean taxa and 32 non-arthropod taxa belonging to 14 different orders. Insects were the most diverse group, with 25 families belonging to six different taxonomic orders.

Ephemeropterans and dipterans accounted for 26 and 34% of the total macroinvertebrate abundance, with *Cloeon dipterum* and *Chaoborus* sp., respectively, as the most abundant taxa. Hirudinea, Turbellaria, and Gastropoda were also numerically dominant. *Erpobdella* sp. and *Mesostoma sp.* were dominant taxa of Hirudinea and Turbellaria, while *Lymnaea* sp. and *Radix* sp. were abundant Gastropoda.

A treatment-related decline in the total number of arthropod taxa was observed immediately after the single application (Fig. 2A and B). A non-significant increase in the number of non-arthropod taxa was found for all treatments at the end of the experimental period, while (non-significant) reduced numbers of arthropod species were evident in the single-application and three-applications treatments (Fig. 2A and B).

The effects of the chlorpyrifos treatments on the macroinvertebrate community are also visualized in the PRC diagram excluding *C. dipterum*, presented in Figure 3. *C. dipterum* was excluded because when included, it dominated the PRC diagram by having a species weight (b_k) score two and a half times higher than that of the second species, being *G. pulex*.

Cloeon dipterum was the only taxon that showed a disproportionally large response to the single-application treatment relative to the other treatments and so obscured the overall community response. Since the response of *C. dipterum* was also observed in the univariate analysis at the species level, this species was deleted from the PRC analysis, resulting in a PRC diagram that provided a good summary of the response of the rest of the community. Before exposure, the PRC diagram depicts little variation in macroinvertebrate community composition between the treatments (Fig. 3).

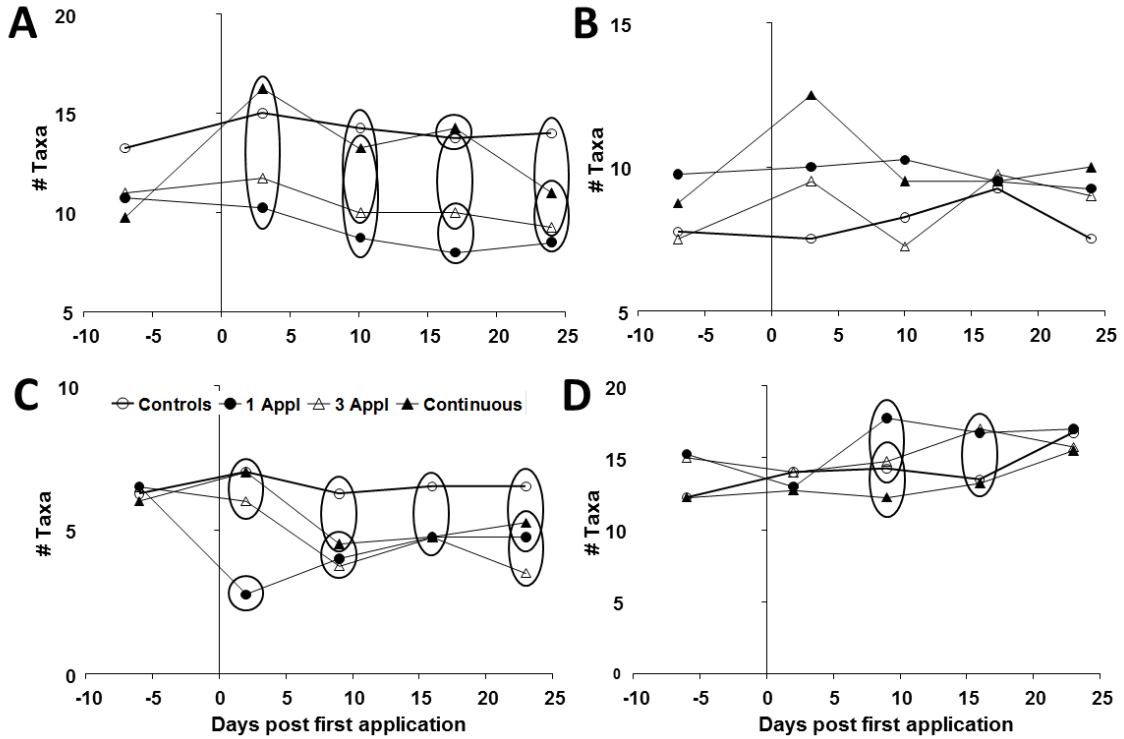

Figure 2: Dynamics of numbers of macroinvertebrate arthropod (**A**) and non-arthropod (**B**) and zooplankton arthropod (**C**) and non-arthropod (**D**) species. Numbers represent averages per treatment. Significant differences are indicated by the circles, when circles are absent no significant differences were found for that sampling date. Treatments present in the same circle did not differ significantly from each other, while those not sharing the same circle did differ significantly. If all treatments are in one circle, significance is indicated by the analysis of variance (ANOVA), but could not be attributed towards particular treatments by the Tukey range test.

Table 1: Results of Monte Carlo permutation tests for macroinvertebrate and zooplankton communities, testing all treatments against the controls.

	Days relative to first application				
*Macroinvertebrates**	-7	3	10	17	24
1 Application	0.047		0.023	0.091	0.050
3 Applications				0.081	0.080
Continuous					0.049
Zooplankton	-7	3	10	17	24
1 Application		0.023	0.023	0.023	0.023
3 Applications				0.023	0.054
Continuous			0.054	0.047	0.023

Empty cells denote *p*-values > 0.10. * Macroinvertebrates without *C. dipterum*

After the first application, the diagram shows large effects of the single application of 0.9 µg/L, while the effects of the other two treatments progressed over time (Fig. 3). The deviations of the treatments from the controls as depicted in the PRC were confirmed by the results of the Monte Carlo tests, which detected significant treatment effects for all regimes at the end of the experimental period, when 0.10 is taken as the critical *p*-value (Table 1).

The PRC analysis indicated that, apart from *C. dipterum* (Ephemeroptera), the largest effects were found on taxa belonging to the Trichoptera (Phryganaidae) and Diptera (*Chaoborus* sp., Ceratopogonidae and *Chironomini*) and on the macrocrustacean *G. pulex*, since they had the highest species weight (b_k) with the PRC diagram (Fig. 3). This is confirmed by most of the population responses of the taxa that showed significant effects of the treatments, as shown in Figures 4A through D. Pronounced effects of the single-application treatment were found immediately after application for *C. dipterum*, *Chaoborus* sp., and *G. pulex*, while smaller effects were recorded for Phryganaidae. At the end of the experimental period, effects on Phryganaidae and *G. pulex* occurred in all treatments, while *C. dipterum* and *Chaoborus* sp. were only significantly affected at the single-application treatment (Fig. 4). Snails were represented by three families: Lymnaeidae (*Lymnaea* sp. and *Radix* sp.), Valvatidae (*Valvata* sp.) and Planorbidae (*Planorbis* sp.). Fig. 3 indicates that several non-arthropod taxa belonging to the Hirudinea (*Alboglossiphonia* sp. and *Erpobdella* sp.) and Turbellaria (*Mesostoma* sp. and *Dugesia lugubris*) were more abundant in (some) treatments compared to the controls. Statistical testing at the population level indicated that these increases were non-significant.

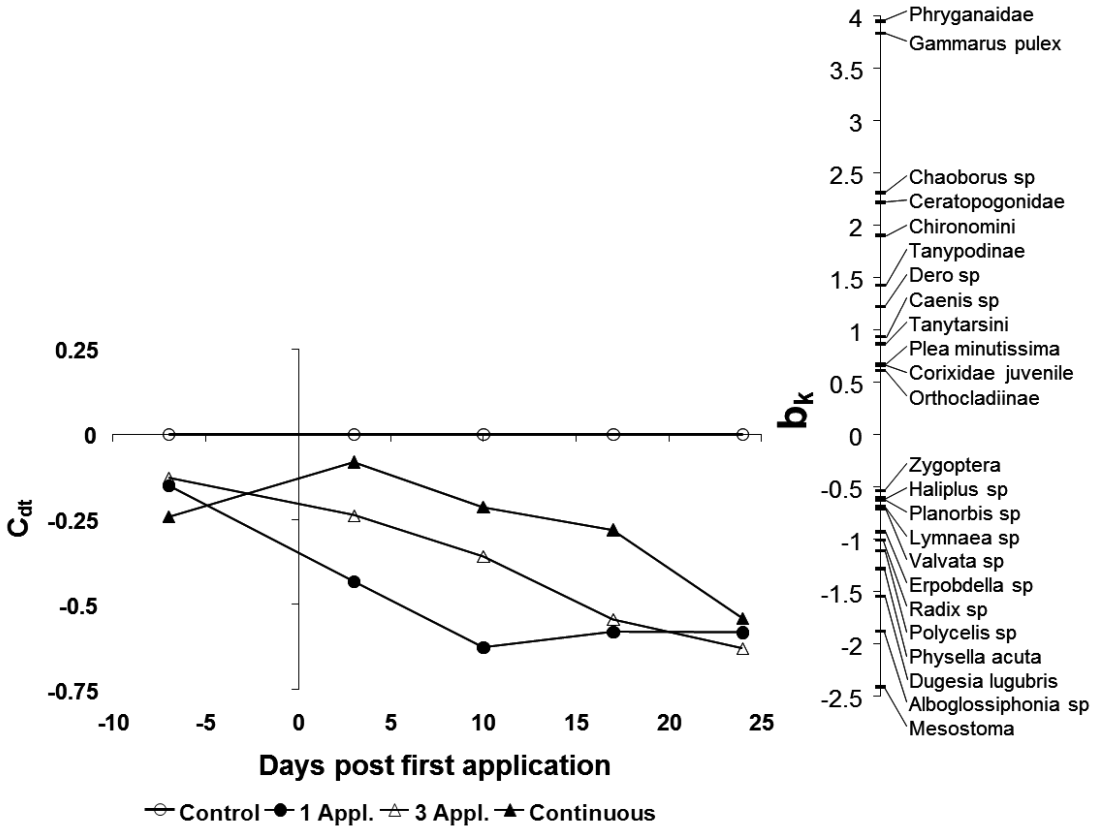

Figure 3: Principal Response Curves resulting from the analysis of the macroinvertebrate data set excluding *Cloeon dipterum*, indicating the effects of different chlorpyrifos treatments. Eight percent of all variance could be attributed to the sampling date; this is displayed on the horizontal axis. Twenty-two percent of all variance could be attributed to treatment level, 38% of which is displayed on the vertical axis. The lines represent the development of the treatments in time. The species weight (b_k) can be interpreted as the affinity of a taxon with the Principal Response Curves (c_{dt}). Taxa with a species weight between 0.5 and -0.5 are not shown. A Monte Carlo permutation test indicated that the diagram displays a significant amount of the variance explained by the treatment (p = 0.008).

Cloeon dipterum was the most severely affected species; it was completely eliminated by the single application of 0.9 µg/L chlorpyrifos, and remained significantly different from all other treatments on all post-treatment sampling dates (Fig. 4A). Effects of the other treatments did not appear until day 10, with the three-application treatment remaining significantly different from the controls until the end of the experiment. Phryganaidae showed a significant reduction in the single-application and three-applications treatments from day 10 onwards, while its abundance in the continuous-exposure treatment was significantly different from that in the controls from day 17 onwards (Fig. 4B). *Chaoborus* sp. showed the largest decrease in the single-application treatment on day 10 and 17 after the application, followed by the three-application treatment. *Chaoborus* sp.

recovered at least partially before the end of the experiment (Fig. 4C). *Gammarus pulex* was strongly affected in the single-application treatment from day 10 onwards (Fig. 4D). Significant effects in the three-application treatment were observed from day 17 onwards, and only at the end of the experiment in the continuous-exposure treatment (Fig. 4D).

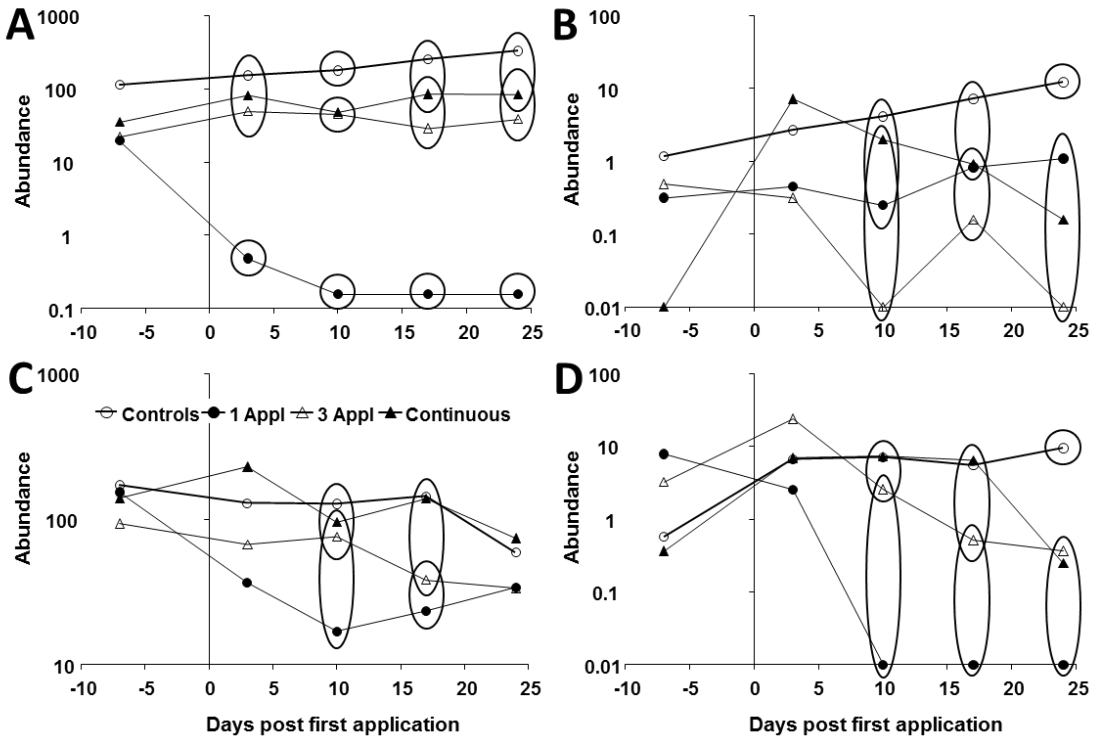

Figure 4: Dynamics of most important macroinvertebrate populations showing significant treatment-related responses to the chlorpyrifos treatments. Numbers are geometric mean abundance numbers of **(A)** *Cloeon dipterum*, **(B)** Phryganaidae, **(C)** *Chaoborus* sp, and **(D)** *Gammarus pulex*. In the figures, an abundance value of 0.01 denotes the absence of the taxon. When significant differences were found they are indicated by the circles; when circles are absent no significant differences were found for that sampling date. Treatments present in the same circle did not differ significantly from each other, while those not sharing the same circle did differ significantly. Only species for which significant differences for at least two sampling dates were indicated are shown. If all treatments are in one circle, significance is indicated by the analysis of variance (ANOVA), but could not be attributed towards particular treatments by the Tukey range test.

Zooplankton

A total of 35 different zooplankton taxa were identified in the microcosms during the study. The majority of taxa belonged to the Rotifera (28), followed by Cladocera (4) and Copepoda (3). During the experimental period, the total zooplankton abundance in the microcosms was dominated by Rotifera (83%), Copepoda (11%) and Cladocera (5%). The most abundant rotifer taxon was *Polyarthra remata* (36%), followed by *Anureopsis fissa (*16%), and *Hexartha* sp *(*10%), while other abundant taxa included copepod nauplii (8%). Most taxa increased in abundance over time in the control cosms.

The number of arthropod taxa decreased significantly in the single-application treatment, while the number of non-arthropod taxa increased non-significantly (Fig. 2C and D).

The PRC diagram of the zooplankton data set shows little variation in the pre-treatment period and large treatment-dependent differences from the controls after the start of the treatments (Fig. 5). Effects on the zooplankton community structure were first observed in the single-application treatment, followed by the three-applications and continuous-exposure treatments. The effects in the latter two treatments progressed over time (Fig. 5). The visual differences were confirmed by the results of the Monte Carlo permutation tests (Table 1). In contrast to the macroinvertebrate response to chlorpyrifos treatments shown in Figure 3, the treatments do not all fully converge in the zooplankton PRC diagram (Fig. 5). At the end of the experimental period, small differences in effect size remained between the continuous-exposure treatment and the other two treatments.

Cladoceran taxa like *Daphnia.* gr. *longispina*, *Alona* sp., and *Alonella nana* have a high positive weight with the PRC diagram, indicating that they were the most responsive zooplankton species (Fig. 5). *Simocephalus vetulus*, copepod nauplii and cyclopodia had lower positive b_k scores, indicating lower decreases in abundance. In contrast, several rotifer taxa like *Keratella cochlearis*, *Mytilinia ventralis*, and *Trichocerca similis* had a relatively high negative species weight, indicating an increase in abundance in the treatments compared to the controls (Fig. 5).

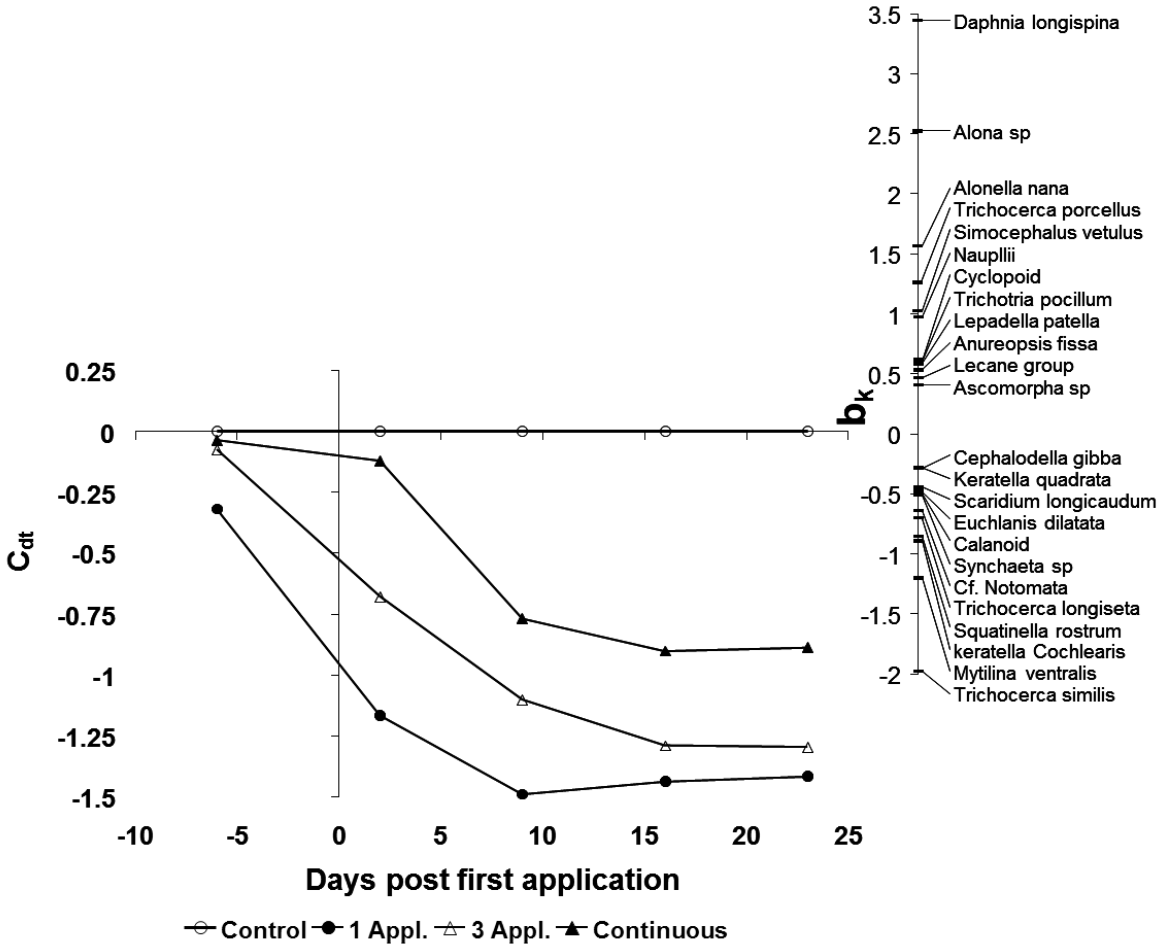

Figure 5: Principal Response Curves resulting from the analysis of the zooplankton dataset, indicating the effects of different chlorpyrifos treatments. Eighteen percent of all variance could be attributed to the sampling date; this is displayed on the horizontal axis. Twenty-two percent of all variance could be attributed to treatment level, 38% of which is displayed on the vertical axis. The lines represent the development of the treatments in time. The species weight (b_k) can be interpreted as the affinity of a taxon with the Principal Response Curves (c_{dt}). Taxa with a species weight between 0.5 and -0.5 are not shown. A Monte Carlo permutation test indicated that the diagram displays a significant amount of the variance explained by the treatment (p = 0.004).

At the population level, a significant decrease in abundance was observed for *D*. gr. *longispina*, *Alona* sp., *A. nana* and nauplii (Fig. 6A-D). The cladoceran species immediately decreased in abundance after the single-application treatment and showed increasing effects in the other treatments towards the end of experiment. Copepod nauplii population densities were especially reduced in the single-application treatment and to a lesser extent also in the other treatments (Fig. 6D). Significant increases in abundance due to the chlorpyrifos treatments were observed for the

rotifer *T. similis* (Fig. 6E). The largest increases in abundance were generally observed in the single-application treatment, followed by the three-applications and continuous-exposure treatments.

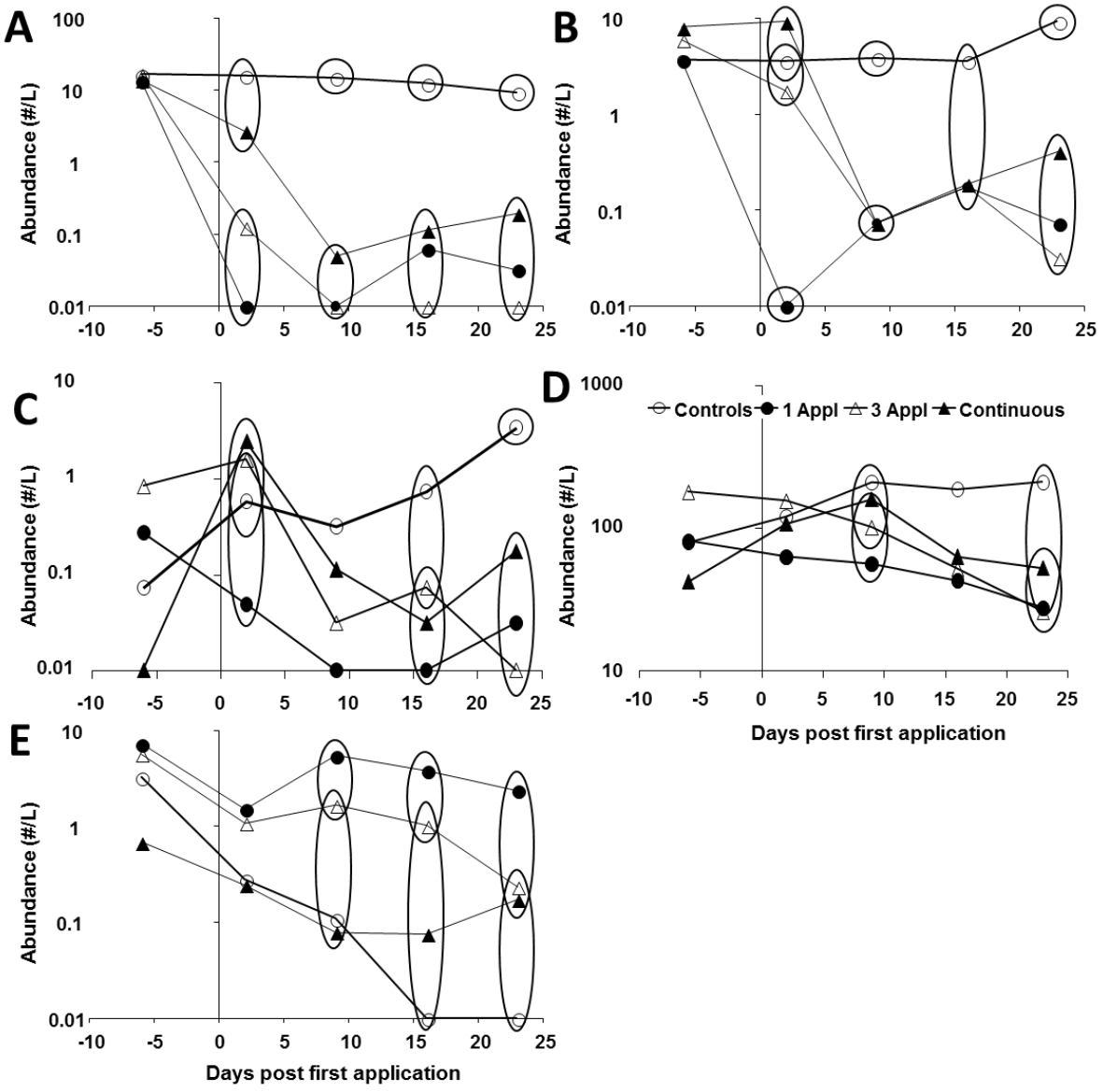

Figure 6: Dynamics of most important zooplankton populations showing significant treatment-related response to the chlorpyrifos treatments. Numbers are geometric mean abundance numbers of (**A**) *Daphnia* gr. *longispina*, (**B**) *Alona* sp, (**C**) *Alonella nana*, (**D**) nauplii and (**E**) *Trichocerca similis*. In the figures, an abundance value of 0.01 denotes the absence of taxon. When significant differences were found they are indicated by the circles; when circles are absent no significant differences were found for that sampling date. Treatments present in the same circle did not differ significantly from each other, while those not sharing the same circle did differ significantly. Only species for which significant differences for at least two sampling dates were indicated are shown. If all treatments are in one circle, significance is indicated by the analysis of variance (ANOVA), but could not be attributed towards particular treatments by the Tukey range test.

Other endpoints

Primary producers were evaluated by the concentration of chlorophyll-*a* in water samples to provide an indicator of phytoplankton biomass. Levels range between 2 and 41 µg/L while significant differences in phytoplankton chlorophyll-*a* levels were only observed between the single-application and continuous-exposure treatments on d 2, immediately after the start of treatment.

The physico-chemical parameters pH, electrical conductivity and dissolved oxygen revealed isolated significant treatment-related responses to chlorpyrifos treatments, but temperature did not show any significant effects. Levels of pH ranged between 7.8 and 10.1 and significant effects were only observed after the single-application treatment. The chlorpyrifos treatments did not result in pronounced impacts on oxygen levels which ranged between 8.1 and 12.9 mg/L. Only at the end of the experimental period were statistically significant differences in electrical conductivity values observed (levels ranged between 85 and 152 µS/cm). Alkalinity ranged between 0.8 and 1.2 meq/L and did not show any treatment-related effects. The concentration levels of total phosphate, ammonia, nitrate and nitrite (NO3+NO2)-N, and ortho-phosphate were below the detection limit (LOD: 0.02, 0.04, 0.03 and 0.02 mg/L, respectively). We found no effects of the treatments on total soluble nitrogen levels (concentrations between 0.38 and 0.74 mg/ L).

No significant effects of the treatments were detected on the decomposition of particulate organic matter (POM). The percentage dry weight of populus leaves remaining in the litterbags over the whole experimental period across all microcosms was 89 ± 2 % (mean value ± SD). Vegetation harvested in all the microcosms at the end of experiment did not differ statistically between the treatment levels. The overall coverage of aquatic vegetation in most microcosms was dominated by *Chara globularis* (> 80% coverage), while *Elodea nuttallii* showed a low coverage (≈ 5%). Few of the microcosms were completely dominated (i.e. coverage > 80%) by *Elodea nuttallii*. Dry matter content, water content and organic matter content (%) of the sediment as determined in the upper 5 cm layer were 44%, 56% and 10%, respectively.

Discussion

Fate of chlorpyrifos

As expected, chlorpyrifos disappeared rapidly from the water column in all treatments due to losses by uptake, sorption, volatilization, photolysis, hydrolysis and biodegradation (Fig. 1). The dissipation rates were similar to those reported by other studies (Lopez-Mancisidor *et al*. 2008; Van Wijngaarden *et al*. 1996; Racke, 1993). In our three-application treatment, all concentrations dropped below the detection limits before the following application occurred. The concentrations of chlorpyrifos we measured generally corresponded to the intended exposure levels.

Effects of chlorpyrifos on invertebrates

The addition of chlorpyrifos resulted in rapid changes in the macroinvertebrate assemblages of the microcosms, which persisted throughout the experiment (Fig. 3). The changes observed were mostly due to a reduction in the abundance of dominant taxa, rather than a loss or change in taxa richness (Fig. 2). Over the entire duration of the experiment, representatives of the arthropod community responded more sensitively to chlorpyrifos than representatives of the non-arthropod community (Figs. 2). This has also been observed previously for this compound, in both laboratory (Maltby *et al*. 2005; Van Wijngaarden *et al*. 1993) and field studies (Van den Brink *et al*. 1996; Van den Brink *et al*. 1995; Lopez-Mancisidor *et al*. 2008) and is most likely due to differences in intrinsic sensitivity between arthropods and non-arthropods in their response to an acetylcholinesterase inhibiting insecticide (Rubach *et al*. 2010). As a consequence, some non-arthropod species may experience no effects or even favourable effects (Figs. 3 and 5), i.e. they may increase in abundance due to indirect effects (less competition), which has also been observed in previous studies (Van den Brink *et al*. 1996). This is supported by the finding that hardly any significant effect could be detected on phytoplankton (Chl-*a*) and physico-chemical parameters, which is in agreement with Van den Brink *et al*. (1995).

The near extinction of C. dipterum, the complete extinction of G. pulex and also the strong response of Chaoborus sp. in the 0.9 µg/L single-application treatment is in agreement with previous findings from laboratory and field studies (Van den Brink *et al*.1996; Van Wijngaarden *et al*. 1996; Van Wijngaarden *et al*. 1993). Similar patterns were found for chlorpyrifos-induced changes in the zooplankton community (Figs. 5 and 6). The PRC plot (Fig. 5) clearly indicates a pronounced treatment-dependent negative impact of chlorpyrifos on the arthropod zooplankton species. This is not only in line with the findings for arthropods and non-arthropods of the macroinvertebrate community in the present study, but also with previous findings reported in the literature (e.g. Van

Wijngaarden *et al*. 2005b; Lopez-Mancisidor *et al*. 2008; Van Wijngaarden *et al*. 1993). Significant effects on *D.* gr. *longispina* were observed just after the first 0.3 µg/ L application, which can be explained by the 96h-EC50 (effective concentration 50%) and 96h-EC10 (effective concentration 10%) values for *D.* gr. *longispina* of 0.3 and 0.2 µg/L, respectively, as determined by (Van Wijngaarden *et al*. 1993. The effects could have been enhanced by the additional predatory stress by the abundant phantom midge Chaoborus sp. during the first days after application, which may have increase the susceptibility of the *D.* gr. *longispina* population to chlorpyrifos, as has been shown before (Coors *et al*. 2008). Our observations are generally in accordance with (Lopez-Mancisidor *et al*. 2007 and Van den Brink *et al*. 1995), which reported effects at a continuous level of 0.1 µg/L chlorpyrifos on the macroinvertebrate and zooplankton communities of artificial streams and cosms, respectively.

We found nauplii to be more sensitive to chlorpyrifos than adult or subadult cyclopoids, which is in accordance with several model ecosystem studies (Lopez-Mancisidor *et al*. 2008; Ward *et al*. 1995; Brock *et al*. 1992). As in previous studies (Lopez-Mancisidor *et al*. 2008), Cyclopoida and Calanoida showed contrasting responses to chlorpyrifos. The Calanoida population significantly increased in numbers in the single-application treatment, indicating indirect effects (Siefert *et al*. 1989). The above-mentioned increase in Rotifera abundance after elimination of Cladocera by insecticides is also a well-known phenomenon (Van Wijngaarden *et al*. 2005b; Ward *et al*. 1995; Hurlbert *et al*. 1972). Previous micro/mesocosm experiments with non-persistent herbicides and insecticides have reported longer persistence of the indirect effects among the plankton community (Van Wijngaarden *et al*. 2005a; Fleeger *et al*. 2003).

Comparison of time-variable exposure regimes

Because the PRCs for both the zooplankton as the macroinvertebrate communities show more or less same effect magnitude at the end of the experimental period for all treatment regimes, they indicate that for most species, the TWA concentration could be more important than the peak concentration when it comes to assessing long-term risks. In the case of the zooplankton PRC, the effect magnitude was slightly lower in the continuous-exposure treatment than in the others (Fig. 5), but this may be attributed to the slightly lower TWA in this treatment (see *Results* section) or that for some populations the peak exposure is more important. This finding, however, does not hold true for all invertebrate populations. Several macroinvertebrate species, such as the mayfly *C. dipterum*, the crustacean *G. pulex* and the phantom midge *Chaoborus* sp., clearly showed different survival responses to the different treatment regimes (Fig. 4A-D), with (near) extinction only in the 0.9 µg/L single-application treatment. This result can be explained by the high, but not too high,

intrinsic sensitivities measured for these species in laboratory studies (Rubach *et al*. 2011). Intrinsic sensitivity is a product of the toxicokinetics (uptake, biotransformation and elimination of the compound) and toxicodynamics (internal damage, recovery and threshold) of a compound (Rubach *et al*. 2010). Therefore, differences in field responses of species to time-variable exposure may relate to differences in the toxicokinetics and toxicodynamics of chlorpyrifos in these species. The toxicokinetics of chlorpyrifos in several freshwater arthropods have been characterized previously and displayed a high variation (Rubach *et al*. 2010).

The mayfly *C. dipterum* is considered to be an average species in terms of uptake (K_{in}= 349 l/(kg_{ww}*d)) and elimination (K_{out} = 0.196 d^{-1}) rate constants, as well as in terms of bioconcentration factors (BCF_{ww}= 1782 l/kg_{ww} ; BCF_{lipid}= 24699 l/kg_{lipid}) and depuration time (t_{95} = 15.3 d); all values from Rubach *et al*. (2010). The almost complete loss of *C. dipterum* from the microcosms after dosing in the 0.9 µg/L treatment could be explained from the laboratory and mesocosm tests (Van Wijngaarden *et al*. 1996; Van Wijngaarden *et al*. 1993. The non-significant effect observed for the continuous-exposure regime (0.1 µg/L) is consistent with previous studies (Van den Brink *et al*. 1996; Van den Brink *et al*. 1995). Since the depuration time is twice as long as the interval between the applications in the three-application treatment, accrual of effects were expected after the second and third application. However, only small effects of chlorpyrifos were observed on the benthic populations of *C. dipterum* after the second and third application (Fig. 4A). This may be the result of a high damage repair rate. This may be further investigated by performing toxicodynamic experiments with this species to obtain estimations of toxicodynamic parameters.

The significantly lower number of *G. pulex* just after the treatment in the 0.9 µg/L treatment and at the end of the experiment in the 0.1 µg/L treatment compared to the controls (Fig. 4D), can be explained from its high susceptibility to chlorpyrifos (Van Wijngaarden *et al*. 1993) and are consistent with those found in previous microcosm studies (Van den Brink *et al*. 1996; Van den Brink *et al*. 1995). Since its t_{95} is almost equal to the time interval between the 0.3 µg/L pulses (t_{95} = 7.5d, (Rubach *et al*. 2010)), the effects of the subsequent pulses of 0.3 µg/L can be explained by the long time *G. pulex* takes to internally recover from damage caused by chlorpyrifos, i.e. by its toxicodynamics (Ashauer *et al*. 2007).

The decrease in abundance of *Chaoborus* sp. in the microcosms receiving 0.9 µg/L, is in line with Van den Brink *et al*. (1996). By contrast, *Chaoborus* sp. showed no reduction in abundance in microcosms exposed chronically to 0.1 µg/L, which can be explained by its laboratory sensitivity (96h-EC50 = 0.7 µg/L, (Van Wijngaarden *et al*. 1993)). The first application of 0.3 µg/L did not result in effects, but the subsequent applications did, although the difference was not significant (Fig. 4C). These effects can be explained by its t_{95} of 22.9d (Rubach *et al*. 2010). Since the depuration time is

considerably longer than the application interval, the organisms were not able to remove the accumulated toxicant from their body before being exposed to the next pulse of 0.3 µg/L. In the 0.9 µg/L treatment population, recovery started at the end of the experiment (Fig. 4C), which is in agreement with observations in Van den Brink *et al*. (1996).

Recovery and indirect effects

Table 2 provides an overview of the magnitude and duration of effects in terms of both structural and functional endpoints. In order to compare the results obtained in our experiment with those of previous experiments with chlorpyrifos, we summarized the observed effects into classes following the concept of (Brock *et al*. 2005). The following four effect classes were used: **Class 1** effects could not be determined; **Class 2** showed slight effects - effects only observed on individual samplings, especially shortly after the start of the treatment; **Class 3** showed clear short-term effects - effects observed at some subsequent sampling dates with full recovery occurred within the study period; and **Class 4** showed clear effects, no full recovery within study period - study was too short to observe recovery to control levels. Within each endpoint category, the most sensitive endpoint was used for the categorization into the four effect classes.

Class 4 effects were observed on both the macroinvertebrate and zooplankton communities for all chlorpyrifos treatment regimes (Table 2). No effects of the continuous/chronic exposure were recorded on the total abundance of Insecta (Class 1 effect; Table 2). This is probably a result of the absence of effects of this treatment regime on the second most abundant insect, the mayfly *C. dipterum*. The observed Class 4 effects are in accordance with Van den Brink *et al*. (1996) and Van den Brink *et al*. (1995) which also reported effects lasting longer than 3 weeks on zooplankton and macroinvertebrate communities after a single application of 0.9 µg/L and continuous exposure to 0.1 µg/L. One cosm experiment (Lopez-Mancisidor *et al*. 2008) is known to us that found the Class 4 effects on a zooplankton community after multiple applications of a concentration in the range of 0.3 µg/L. A report also exists (Biever *et al*. 1994) of slight effects after a single application of 0.3 µg/L, also confirming the response observed in our three-application treatment, just after the first application.

Table 2: Summary of effects observed in microcosms under different test exposures regimes with chlorpyrifos (single-application, three-application, and continuous-exposure treatments) but with similar TWA_{21d}.

Endpoint	Chlorpyrifos treatment		
	Single-application (0.9 µg/l)	Three-applications (3 * 0.3 µg/l)	Continuous-exposure (0.1 µg/l)
PRC macroinvertebrate community	4	4	4
Arthropods	4 ↓	4 ↓	4 ↓
Total abundance of insects (excluding *Chaoborus* sp.)	4 ↓	4 ↓	1[a]
Chaoborus sp.	3[b] ↓	1	1
Macrocrustaceans (*G. pulex*)	4 ↓	4 ↓	4[c] ↓
Non-arthropod macroinvertebrates (except *Planorbis* sp.)	3[d] ↑	1	3[d] ↑
PRC zooplankton community	4	4	4
Cladocera (except *S. vetulus*)	4 ↓	4 ↓	4 ↓
S. vetulus	2[e] ↓	1[f]	1[f]
Total abundance of copepods	4[g] ↓	4 ↓	1
Cyclopoid copepods	1[h]	1[i]	1[i]
Total abundance of rotifers	1[j]	1[j]	1
Tricocerca similis	4[k] ↑	1	1
Community metabolism	2[l, m] ↑	1	1
Chlorophyll-*a*	1	1	1

See text for explanation of effect classes.

TWA: Time-Weighted Average

PRC: Principal Response Curve analysis; ↓ = decrease in endpoint; ↑ = increase in endpoint

[a]Phryganaidae, class 4 effect observed at this exposure level (**Fig. 4B**)

[b]*C. obscuripes*, partial recovery observed at the end of the experiments (**Fig. 4C**)

[c]*G. pulex*, significant effects only observed at end of experiment (**Fig. 4D**)

[d]*Planorbis* sp., slight but statistically significant increase only on day 10

[e]*S. vetulus*, significant reduction only on day 3 shortly after application, recovery clearly evident but numbers remained lower than control

[f]*S. vetulus*, partial reduction immediately after application, though not statistically significant

[g]Calanoid, statistically significant increase on day 17

[h]Cyclopoid, partial reduction shortly after application, though not statistically significant

[i]Cyclopoid, transient reduction on days 17 and 24, but not statistically significant

[j]*M. ventralis*, significant transient increase on day 17

[k]*T. similis*, statistically significant increase from day 9 onwards (**Fig. 6E**)

[l]pH, two isolated significant deviations only on days 9 and 23

[m]Electrical conductivity, small significant decrease in electrical conductivity at end of experiment

In the present study, recovery was only observed for *Chaoborus* sp. (most likely *C. obscuripes*) in the single-application (Class 3 effect), which is in agreement with Van den Brink *et al*. (1996). The fast recovery may be explained by the multivoltine life cycle of *C. obscuripes* in The Netherlands (Van Wijngaarden *et al*. 2006).

In addition to direct effects on sensitive and responsive species, indirect effects can also be studied at the community or ecosystem level (Hurlbert *et al*. 1975). No treatment-related effects for cyclopoids were visible during the experimental period (class 1 effect), while calanoids showed a treatment-related increase over the course of the experiment (see footnote in Table 2). Similar responses have been observed in Siefert *et al*. (1989), who reported increases of Calanoida populations when Cyclopoida populations were reduced by application of an organophosphate insecticide in shallow ponds.

Non-sensitive herbivores have frequently been reported to increase in numbers in insecticide-stressed aquatic systems as a result of reduced competition and grazing pressure caused by the decline in sensitive herbivores (Van Wijngaarden *et al*. 2005; Hurlbert *et al*. 1972). In the present study we observed increases in the abundance of non-arthropod species belonging to the rotifers (Fig. 5 and 6E) and gastropods (Fig. 3), which after a chemical stress event is likely to be a result of the decreased competition with herbivores like cladocera and *C. dipterum*, respectively.

Our findings in the light of the ELINK guidance document

These results support the ELINK recommendation that the TWA concentration can be more relevant than the peak concentration for long-term effects (Brock *et al*. 2010). In long-term toxicity tests with *G. pulex*, Ashauer *et al*. (2007c) also verified that the TWA concentration approach can be used to predict effects of repeated pulses of chlorpyrifos and pentachlorophenol. For *C. dipterum*, however, the peak concentration is a better predictor of effects. Whether the peak or the TWA concentration is a better predictor of species-level effects depends on the toxicokinetics and toxicodynamics of chlorpyrifos in the species of concern, therefore recommendations for the community level can not be made. We support another recommendation of the SETAC ELINK workshop, to further develop toxicokinetic and toxicodynamic modelling, and increase their use in ERA to estimate the effects of time-variable exposures (Reinert *et al*. 2002).

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Chapter 4

Toxicodynamic experiment for different time-variable exposure regimes of the insecticide chlorpyrifos on freshwater arthropods

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"Models are, for the most part, caricatures of reality, but if they are good, then, like good caricatures, they portray, though perhaps in distorted manner, some of the features of the real world"

(Kac, 1969)

"Pollutants matter because of their effects on populations, and so, indirectly, on communities too, but pollutants act by their effects on individual organisms"

(Moriarty, 1983)

Abstract

One of the major dilemmas in environmental risk assessment is that exposure concentrations are maintained in standard laboratory tests whereas in fact exposure to chemicals in the aquatic environment occurs as time-varying pulses. Non-target organisms may be exposed to fluctuating concentrations or sequential pulses of pesticides in the environment. Currently, evaluation of the potential adverse effects of pulsed pesticide exposure on non-target aquatic organisms is considered to be a major challenge. Furthermore, recovery of individuals after being exposed to pesticides is not routinely taken into account in risk assessment. The Threshold Damage Model (TDM) is a process-based model to predict the acute effects of pulsed pesticide exposure concentrations on the survival of aquatic invertebrates and consists of a toxicokinetic part in which uptake and elimination are described and of a toxicodynamic part accounting for processes such as damage, individual recovery and internal thesholds. Here we present data from a series of laboratory experiments with the model substance chlorpyrifos, which were used to parameterize the toxicodynamic parameters of the TDM for different species. The experiment quantified mobility and survival of four freshwater species *Chaoborus obscuripes, Cloeon dipterum, Plea minutissima* and *Daphnia magna* in response to varying patterns of chlorpyrifos exposure. The killing rate constant, recovery rate constant and the threshold for damage were estimated by fitting the TDM to the experimentally observed survival data. The species *C. obscuripes* and *D. magna* showed an immediate decrease in mobility and a delayed effect in survival whereas *C. dipterum and P. minutissima* responded immediately to the exposure in both endpoints. *C. obscuripes* was the only species showing no individual recovery. In general, the effect of the pulses was smaller, if the intervals between pulses allowed for elimination and potential recovery. The experimental data were successfully fitted with the TDM, however, not all parameters were estimated robustly and the TDM does not provide consistent parameter estimates, which made it difficult to compare parameter values between species. This finding illustrates the need for further data collection and further advancement of toxicokinetics and toxicodynamics (TKTD) models for different species and compounds. Improved TKTD models could be combined with individual based models to provide more accurate and detailed information on effects such as population recovery and combine the different levels of biological organisation.

Introduction

Due to their increased use over the past decades pesticides have been invariably detected in small water bodies in the direct vicinity of agricultural areas and surrounding waterways throughout the world (Osano *et al*. 2003; Sarkar *et al*. 2008). As a result, non-target communities in aquatic ecosystems may be affected. The domain of ecotoxicology has traditionally assessed the risks of contaminants using standard laboratory toxicity tests experiments. These toxicity tests are performed at constant concentrations over a fixed duration. It has been recognized by several authors that the aquatic non-target organisms may be typically exposed to fluctuating concentrations or sequential pulses of pesticide contaminants as a result of spills, episodic drainage and runoff events and/or repeated pesticide applications (Handy, 1994; Reinert *et al*. 2002). Standard laboratory toxicity tests utilizing continuous exposure scenario usually do not investigate the effects of time-variable exposure or repeated exposure to aquatic organisms (Hickie *et al*. 1995; Parsons *et al*. 1991). In recent years, however, considerable attention has been given to the effects of pulsed or intermittent exposure scenarios, which has become a key issue in ecotoxicology (Brock *et al*. 2010).

Recovery from pesticide stress takes place at different levels of biological organisation and has been described by many authors especially at the population level (e.g. Galic *et al*. 2012). In addition, the importance of recovery periods between successive pulses for individuals, populations and communities has been recognized (Ashauer *et al*. 2007c; Kallander *et al*. 1997; Traas *et al*. 2004). Furthermore, the significance of the relation between effects and recovery at the sub-organismal (molecular, biochemical or cellular) level and the impact at higher biological level is poorly understood (Duquesne, 2006). The dynamics of individual recovery together with injury/damage at the individual level can be described by toxicodynamics (TD) modelling and can be linked with the mode of action of a compound (Ashauer *et al*. 2006; 2007a; b). Compounds with the same mode of action are assumed to show similar individual recovery rates and times (Ashauer *et al*. 2007a). However, the question remains if this is applicable to different species, since species specific traits play a role in species sensitivity and the toxicokinetics (TK) of pesticides (Rubach *et al*. in press; Baird and Van den Brink, 2007). Organophosphates, such as chlorpyrifos, are acetylcholinesterase (AChE) inhibitors and result in very slow recovery rates in organisms and are sometimes considered to induce irreversible effects (Legierse *et al*. 1999). However, in general cholinesterase can be reactivated by dephosphorylation (Roberts and Hutson, 1999). Conversely, for carbamate AChE faster recovery has been observed at the target site, since no reactivation and aging is needed (Ashauer *et al*. 2007c). Slow recovery at the target site highlights not only the importance of acute

internal concentrations but also that of the exposure history (Jager and Kooijman, 2005; see for example Ashauer *et al*. 2007c).

For an appropriate assessment of the risk associated to exposure profiles being characterised by repeated pulses, it is imperative to know whether or not the pulses are toxicologically independent of each other or not (EFSA, 2005). Modelling approaches may provide an alternative tool to investigate this process whether successive pulse exposures are toxicologically dependent or not. Toxicological dependence of repeated pulses may occur when the life span of a sensitive species is long enough to experience repeated pulsed exposures. If, for example, the predicted exposure profile consists of two pulse exposures, the second pulse can be considered toxicologically independent from the first pulse if between the two pulses: (i) internal exposure concentrations in the individuals of the sensitive species drop below critical threshold levels, and (ii) a complete repair of damage occurs. According to the proceedings of the ELINK workshop (Brock *et al*. 2010), the demonstration of toxicological independent pulsed exposures, requires either specifically designed pulsed exposure toxicity tests or toxicokinetic and toxicodynamic (TKTD) models for the relevant organisms and pesticides of concern.

In order to mechanistically link aquatic exposure and effects and their underlying processes, ecotoxicological models such as TKTD models have been developed (see Ashauer and Brown, 2008 for an overview). Toxicokinetics describe the time course of toxicant in the organism in terms of rate of uptake, biotransformation and elimination (i.e. what the organism does with the toxicant) and toxicodynamics describe the dynamics of injury and recovery as well as their link to the effect endpoint in the organism (i.e. what the toxicant does to the organism). TKTD models are tools for the environmental risk assessment of chemicals as well as for ecotoxicological research (Bedaux and Kooijman, 1994; Jager *et al*. 2006; Ashauer *et al*. 2007a; Jager and Kooijman, 2009. Because of their semi-mechanistic nature, TKTD models may be useful for extrapolation of effects to non-standard exposure patterns such as fluctuating or pulsed exposure patterns (Ashauer *et al*. 2006, 2007b). Recently, in the proceedings of the ELINK workshop recommendations for approaches to link exposure and effects for the risk assessment of pesticides were given, e.g. the utilisation of TKTD models.

TKTD models can be used to assess effects from time-variable exposure regimes of pesticides and can therefore deliver more realistic estimations of mortality dynamics than simple dose-response relationships (Galic, 2012; Ashauer *et al*. 2006). The parameterisation of TKTD models depends on experimental data. Currently, TKTD modelling is especially developed for aquatic invertebrates and fish and the parameterisation of these models facilitates a better understanding

of the causes for the differences in sensitivities between different species towards pesticides (Rubach *et al*. in press).

The Threshold Damage Model (TDM) is a process-based model to predict the acute effects of pulsed pesticide exposure concentrations on the survival of aquatic invertebrates (Ashauer *et al*. 2007a). However the TDM is only parameterized for a limited number of compounds/ species combinations (e.g. Ashauer *et al*. 2007b; Rubach, 2010), and partly extended to some other species (e.g. Rubach *et al*. 2010a). These parameterisations may not be representative for other species.

Several approaches to model the toxicokinetics and toxicodynamics exist, and they differ in their underlying hypotheses and assumptions. One major difference lies in the description of mortality, for which two different approaches/assumption in ecotoxicological survival modelling exist: (i) the Individual Tolerance distribution concept (IT) or Individual Effective Dose (IED) theory, and (ii) the Stochastic Death assumption (SD) (hazard models) (Newman and McCloskey, 2000). The Critical Body Residue (CBR) concept (McCarty and Mackay, 1993), the Critical Target Occupation (CTO) model (Legierse *et al*. 1999), the Critical Area Under the Curve (CAUC) model (Verhaar *et al*. 1999), and the Damage Assessment Model (DAM) (Lee *et al*. 2002) are all based on the assumption of instantaneous death when a certain damage threshold is exceeded at the target site. This threshold is assumed to differ among individuals; hence this approach is based on the assumption of an individual tolerance distribution. The model for receptor kinetics (Jager and Kooijman. 2005), the Threshold Hazard Model (THM) (Ashauer *et al*. 2006), the Dynamic Energy Budget (DEBtox) model (Bedaux and Kooijman, 1994) and the Threshold Damage Model (TDM) (Ashauer *et al*. 2007a) are hazard models. They assume that death is a stochastic event, described by the hazard rate. None of the aforementioned studies attempted to clarify the key issue of survival under both theories except the newly developed “General Unified Threshold model for Survival” (GUTS) model (Jager *et al*. 2011).

When a test population is repeatedly exposed to chemical concentrations equal to the 24h-LC50 for a duration of 24 h, assuming that the pulses are toxicologically independent, the stochastic theory predicts that every pulse would result in 50% mortality and, thus, that the population size would drop by 100% to 50% to 25% of the original size, and so on, during a series of such pulses. In contrast, the prediction with the Individual Tolerance (IT) theory postulates that animals with a threshold in the lower 50% of the distribution will die during the first pulse, and all the survivors will have higher threshold levels. Therefore, only a few individuals (or none) would die during successive pulses. In this case, the population size would drop by 100% to 50% to 50%, and then stay constant. The IT assumption therefore predicts that the population will persist much longer under a pulsed exposure scenario than the stochastic theory predicts. According to the IT theory, the IED is an

intrinsic characteristic of an individual, which represents a characteristic tolerance towards a toxicant; an individual will be dying only when this threshold is exceeded.

The first aim of present study was to parameterise the toxicodynamic part of the Threshold Damage Model (TDM) for the insecticide chlorpyrifos for several aquatic macroinvertebrates and to compare individual recovery potentials among species. For the species evaluated in this study, Rubach *et al*. (2010a) already parameterised the TK part of the TDM model. Second objective was to evaluate how these arthropods species respond to different time-varying exposure concentrations of chlorpyrifos in their survival and their mobility. This was achieved by performing long-term survival experiments, which were designed to estimate the toxicodynamic parameters of the TDM model (Ashauer *et al*. 2007a; b). Values for killing rate constant (k_k), recovery rate constant (k_r), threshold (*threshold*) and background mortality (h_b) were estimated for the four freshwater arthropod species *C. obscuripes, C. dipterum, P. minutissima* and *D. magna* using the experimental data with the model substance chlorpyrifos.

Materials and methods

Experiments

Test organisms and test medium

Four freshwater arthropod species were tested in the present study and some specific traits being relevant for their sensitivity and population recovery potential are given in Table 1 (Rubach *et al*. in press; Van den Brink *et al*. 1996). The mayfly *C. dipterum,* the water flea *D. magna* and *the phantom midge C. obscuripes* were selected because they showed different survival response to time-variable exposure regimes of chlorpyrifos and no complete recovery during a microcosm experiment (Zafar *et al*. 2011). Zafar *et al*. (2011) reported that for long-term effects, the TWA is more important than the peak concentration for most species. However, this does not hold true for *C. dipterum* for whom peak concentration seems to be a better predictor of effects. This is probably related to a difference in toxicokinetics or toxicodynamics of this compound in this species as compared to others.

As a representative of zooplankton in ecotoxicological tests water fleas *D. magna* is normally used as test organism and is known for its high sensitivity to pesticides. The pygmy backswimmer *P. minutissima* is not particularly sensitive to chlorpyrifos, but was nevertheless selected for testing due to availability of toxicokinetic information (uptake and elimination rates) (Rubach *et al*. 2010a).

D. magna individuals used in the present experiment were cultured from a newly established isofemale lineage of *D. magna* from the Aquatic Ecology and Water Quality Management Department laboratory, Wageningen University, The Netherlands. They were kept at room temperature (approx. 20° C) in glass jars with Ralph Tollrian (RT) medium with *S. obliquus* as food. The *C. dipterum, C. obscuripes and P. minutissima* individuals were collected from outdoor, uncontaminated ditches located at the "Sinderhoeve Experimental Station" of Wageningen University and Research centre (WUR) in Renkum, The Netherlands. Three days prior to the experiment, the organisms were kept in filtered and aerated groundwater in the laboratory for acclimatization with provision of sufficient food.

The toxicity tests were conducted in groundwater from the Sinderhoeve experimental station of WUR. To remove remaining solid particles and saturate the medium with oxygen, the groundwater was filtered through a 0.45 µm membrane using a pressure filtration and aerated for 24 h before usage. The filtered groundwater was stored at 16 ^{0}C before usage.

Table 1: Species traits of the four tested species, relevant for their sensitivity and individual and population recovery

Traits[1]	*C. obscuripes*	*C. dipterum*	*P. minutissima*	*D. magna*
Insect/Crustacean	Insect	Insect	Insect	crustacean
Respiration mode	[2]skin breather	[2]gill breather [2]skin breather	[3]air bubble	[2]gill breather [2]skin breather
Feeding type	Carnivore	herbivore, detritivore	carnivore	herbivore, detritivore
Compartments of life cycle	egg and pupa aquatic adult terrestrial	egg aquatic adult terrestrial	total in water	total in water
Life cycle (months)	1-12	1-12	n.a.	0-1
Life span (years)	0.6-1.9	0.6-1.9	0.6-1.9	0.1-0.5
Reproduction	Sexual	some asexual	Sexual	asexual sexual
Voltinism	univoltine, bivoltine	univoltine, bivoltine, multivoltine	univoltine	multivoltine
Reproduction (# juveniles)[4]	S 31-150; A 1-30	S, A 151-1500	Sp 1-30; S 31-150; A 1- 3	Sp, S, A 31-150
Colonization probability (%)	26-50	51-100	11-25	51-100
Colonization season[4]	S, A	All	Sp, S, A	All

[1]Trait data for *C. obscuripes, C. dipterum* and *P. minutissima* are at family level and for *D. magna* at class level (data obtained from http://ipmnet.org/PondFX/pondlife main.htm on 02-11-10; Heneghan *et al*. 1999)
[2]Skin breather and [2]gill breather = Dissolved oxygen breather; [3]Air bubble = Atmospheric oxygen
[4] Sp=spring, S=summer, A=autumn, W=winter

Test substance, preparation of dosing and test solution

Chlorpyrifos (*O,O- diethyl O-3,5,6-trichloro-2-pyridyl phosphoriothioate*, CAS nr. 2921-88-2, 99% purity, lot number 80711) was purchased from Dr. Ehrenstorfer GmbH, (Augsburg, Germany) and used as a model substance for survival experiment. Stock solutions were made by diluting chlorpyrifos (active ingredient) in 99% pure acetone and these solutions were used to prepare dosing solutions in 99% pure acetone for the pulsed applications of each species. The desired concentrations, containing 0.01% acetone in the test solutions were achieved by pipetting 50 µl of respective dosing solution into 499.95 mL groundwater in each test unit, after which the water was homogenised before transferring the test animals into the water. Nominal concentrations in the test media were 0.7 µg/L for *C. obscuripes*, 0.5 µg/L for *C. dipterum*, 5.3 µg/L for *P. minutissima* and 0.5 µg/L for *D. magna*. The 0.01% acetone concentration was also added to the acetone controls.

Sampling and chemical analysis

Water samples were taken regularly, from each test unit in order to verify nominal concentrations of chlorpyrifos. Water samples were collected before a pulse, directly after dosing the pulse and the next day before the transfer of the animals into non-contaminated medium and additional samples were taken between pulses. For analysis, 20 ml water samples were taken using a graduated glass pipette, placed in glass tubes and corresponding volumes of hexane 2 ml were added. After intense horizontal shaking (1 min), the samples rested for 10 minutes to separate into distinct layers of water and hexane, after which ~ 1 mL of the hexane (upper) layer was transferred into a GC-vial. All samples (~ 1 ml) were stored at -20 ^{0}C until further analysis. The chemical analysis was carried out by means of Gas Chromatography with an Electron Capture Detector (GC-ECD). Specifications for the GC-ECD analysis of chlorpyrifos were in accordance with the study by Rubach *et al*. (2010a). The limit of detection (LOD) for all species is 0.01 µg/L. The LOD is derived from the concentration factor of the extraction procedure and the limit of detection of the GC-ECD.

Design of the toxicodynamic (survival) experiment

The survival experiments comprised three different repeated pulsed exposure treatments with chlorpyrifos, each with different time intervals in which damage accrual, complete elimination or potential recovery was expected. All treatments, including the controls, were performed using five replicates. The exposure concentrations of chlorpyrifos were selected based on effective and lethal concentrations 24h-E(L)Cx of the species, which had been determined in previous study (Rubach *et al*. 2011). The exposure concentrations of the chlorpyrifos pulse used for *C. obscuripes* was based on its 24h-LC_{30} value of 0.7 µg/L, for *P. minutissima* on its 24h-EC_{50} of 5.35 µg/L, for *D.*

magna on its 48h-EC_{50} of 0.5 µg/L (corresponding with its EC_{17} 24h) and for *C. dipterum* on its 24-$LC_{0.1}$ of 0.5 µg/L (all values taken from Rubach *et al*. 2011). For *C. dipterum* a lower effect threshold was used since the late spring population of *C. dipterum* which was used in this study, appears to be more sensitive than the early spring population as tested by Rubach *et al*. (2010a) on which the 24h-LC_{30} (1 µg/L) was based. Therefore, a lower concentration of 0.5 µg/L was used corresponding to the 24h-$LC_{0.1}$ of the dose-response relationship as determined by log-logistic regression equation.

All treatments contained two pulses and each pulse endured for 24 h, however, the intervals between the pulses varied to allow for different organism recovery times. In between the pulses (i.e. after the end of a pulse) the test animals were transferred from contaminated water into clean uncontaminated water. For a new pulse, the test animals were transferred into contaminated water. The interval times between the pulses varied depending upon previously measured species specific elimination rates which were based on the 95%-depuration time (t_{95}) of chlorpyrifos, derived from $t_{95} = \ln(0.05)/k_{out}$ and previously determined in uptake and elimination studies (values taken from Rubach *et al*. 2010a) and upon the desired toxicodynamic treatment scenario (i.e. damage accrual, full elimination, potential recovery). The t_{95} is the 95%-depuration time that an organism would require to eliminate 95% of the accumulated toxin from the body when placed from contaminated water into clean water.

Chlorpyrifos was introduced into the test systems as described in the following three different treatment regimes. In treatment 1 (T1), the second pulse followed each other before $t = t_{95}$, thus accrual of damage is expected. In treatment 2 (T2), the interval between the pulses was equal to approximately the t_{95} and thus almost full elimination was possible, but no additional recovery time was given. In treatment 3 (T3), the interval between the pulses was longer than t_{95} and allowed potentially for a total physiological recovery. *C. obscuripes* were pulsed on day 0, 7, 21 and 26; *C. dipterum* on day 0, 5, 14 and, 21; *P. minutissima* on day 0, 7, 21, 29 and *D. magna* on day 0, 3, 6 and 12, respectively, for the three treatments (Table 2). The test medium in the controls and treatments during non-pulse periods were changed at latest after eight days.

The experiment was carried out under static conditions in 600-ml Pyrex beakers filled with 500 ml of the test solution. Each beaker contained 15 individuals of each species whereas 20 individuals were taken for *D. magna*. Furthermore, all the beakers were covered with parafilm for each species while nylon panties were used for the atmospheric breather *P. minutissima* in order to prevent the escape of test animals and cross contamination. The individuals were assumed to be a mixture of females and males with an estimated ratio of 1:1. The experiment was conducted at an average measured temperature (17.9 ± 0.8 °C) and light regime was a light: dark cycle of 14:10 h (Tropical daylight lamp: JBL Solar tropic, 30W T8). The animals were shaded from direct light

exposure to delay expected rapid pupation in case of *C. obscuripes* and *C. dipterum*. Table 2 shows the average physicochemical conditions during the entire experimental period. During non-pulse periods, *C. dipterum* was continuously aerated and provided with shoots of *Elodea nutalli* (0.015 g) every second day. These shoots contained algae and diatoms. The species *C. obscuripes* and *P. minutissima* were fed with *Daphnia sp.* every second day and no aeration was required. *Daphnia sp.* used for feeding were taken from an established Cladoceran culture at the Aquatic Ecology and Water Quality Management Department (AEW), Wageningen University. The *Elodea nutalli* shoots were collected from ditches situated at the Sinderhoeve. The species *D. magna* were fed with the green algae *Scenedesmus obliquus* and were not aerated. Medium (ground water) and food (10^7 μm^3/mL of *S. obliquus* cells, equivalent to ~ 5 mg C/L (Lürling, 2003)) were replaced every two or three days in experiment. Measurements of green algae were performed by CASY$^{(R)}$ technology, which is a cell counter model TT. Neonates released from the brood pouch during the experiment were directly discarded. During the pulses the animals were neither fed nor the water aerated. To avoid stress and cannibalism behaviour, two small stainless steel hook-shaped gauze pieces were placed in each beaker to provide shelter and structural elements for *C. dipterum* and *P. minutissima*.

The effects of the time-variable exposure on survival and immobilization were recorded every 24h. Besides this, emerging individuals were recorded and for *C. obscuripes* also pupation. Immobility was defined as abnormal movements compared to control animals and mortality as lack of body movement after 30 s of gentle stimulation with a forceps and for *D. magna* as a lack of heartbeat (if necessary evaluated under a binocular microscope). Dead animals were directly removed and placed in 70% ethanol for possible further identification. Recovery was defined as being an animal going from an immobile state to a mobile state.

Physicochemical parameters were measured before and after the application of the pulse and during the medium exchange (Table 2). Water temperature and pH were measured using a HQ40D meter (Hach-Lange, The Netherlands) and oxygen was measured with Oxi 330, WTW Germany.

Table 2: Experimental set up of the survival (toxicodynamic) experiments under pulsed exposure regimes

Species	T_{95}[1] (Day)	Nominal conc. (µg/L)	T1[2] (day)	T2[2] (day)	T3[2] (day)	pH (-)	Dissolved oxygen (mg/L)	Water temp. (^{0}C)
C. obscuripes	23.2	0.7	0, 7	0, 21	0, 26	7.76±0.20	8.60±0.64	18.1±0.8
C. dipterum	15.3	0.5	0, 5	0, 14	0, 21	7.95±0.22	9.22±0.74	18.4±1.1
P. minutissima	22.2	5.3	0, 7	0, 21	0, 29	7.93±0.27	9.71±0.96	17.7±0.7
D. magna	5.5	0.5	0, 3	0, 6	0, 12	8.05±0.18	9.97±0.78	17.4±0.6

[1]Taken from Rubach *et al*. (2010a)

[2]Day of the pulses after day 0 in each treatment based on the t_{95} from Rubach *et al*. (2010a)

T_{95} is the 95% depuration time

T1 is treatment 1 intended to cause damage accrual

T2 is treatment 2 intended to allow full elimination

T3 is treatment 3 intended to allow potential individual recovery

Data analysis

For modelling purposes, chlorpyrifos concentrations were converted to nmol/mL and averages of the replicates were calculated. Emerging individuals were taken into account in the total amount of individuals but recorded as missing and thus had no effect on the fractions of surviving or mobile individuals. The accumulated dead animals are included in the immobile counts. Accumulated survival and mobility were calculated in fraction per replicate and time point. Averages of the replicates per day per treatment were used for the TD modelling.

Modelling

Threshold Damage Model

TDM is a process-based model to predict the acute effects of pulsed pesticide exposure concentrations on the survival of aquatic invertebrates. The TDM combines various TKTD approached into one ecotoxicological model that simulates the time-course of processes leading to toxic effects at the level of organism over time (Ashauer *et al*. 2007a, b; Ashauer and Brown, 2008).

The toxicokinetics part describes the time course of internal concentration in relation to the water concentration surrounding the organism. Internal concentration acts as surrogate for the concentration at the target site. The toxicokinetic part of the model is the one-compartment first-order kinetic and given by Equation 1:

$$\frac{dC_{\mathrm{int}}(t)}{dt} = k_{in} * C_w(t) - k_{out} * C_{\mathrm{int}}(t) \qquad (1)$$

where

C_{int} is the internal concentration [amount*mass^{-1}]

C_w is the concentration in the water [amount*volume^{-1}]

k_{in} is the uptake rate constant [volume*mass-1*time^{-1}] and

k_{out} is the elimination rate constant [time^{-1}]

The toxicodynamics part describes the accrual of damage in time as a function of the internal concentration and the recovery from or repair of the damage and given by Equation 2.

$$\frac{dD(t)}{dt} = k_k * C_{\mathrm{int}}(t) - k_r * D(t) \qquad (2)$$

where

k_k is the killing rate constant [mass*amount^{-1}*time^{-1}]

k_r is the rate constant for the damage recovery/repair [time^{-1}] and

$D(t)$ is damage [-].

The differential of *H(t)* is the hazard rate which describes the probability of an individual to die at a given time *t* [time^{-1}], *Θ* is a probability constant without biological meaning (Equation 3). When the threshold for damage is exceeded the hazard rate turns positive.

$$\frac{dH(t)}{dt} = \theta * \max[\, D(t) - threshold, 0] \qquad (3)$$

where *threshold* is a dimensionless threshold parameter [-].

The hazard and survival rates are linked in Equation 4

$$S(t) = e^{-H(t)} * S_{background}(t) \qquad (4)$$

where

S(t) is the survival probability [-]being the probability that an organism survives until time t and $S_{background}$(t) is the survival probability from background mortality [-]

Background mortality is calculate by Equation 5

$$S_{background}(t) = e^{-h_b * t} \qquad (5)$$

where

h_b is background hazard rate [time^{-1}]

Parameterisation

The toxicokinetic part of the modelling requires parameter values for k_{in} and k_{out}. Respective values were taken from Rubach *et al*. (2010a) and kept constant during the parameterization of the toxicodynamics. The values for k_k, k_r and *threshold* were estimated by fitting the complete TDM to the experimentally observed survival data per species.

This was done with the least-squares method based on the Levenberg-Marquardt algorithm implemented in the program OpenModel v1.2 (Crout, 2008; University of Nottingham http://www.nottingham.ac.uk/environmental modelling/OpenModel.htm), see Appendix A for model settings. The background parameter h_b was estimated separately with its own control data set.

As initial starting values for all species, the toxicodynamic parameter values for *C. obscuripes* from Rubach (2010) and the robust fit parameters for *Gammarus pulex* from Ashauer *et al*. (2007b) (Table 4) were used. However, for *C. obscuripes* only the data from Rubach (2010) were used as starting values. To find the optimal fit, both initial starting values were varied with the factors 0.001, 0.05, 1, 10, 10000 and permuted (k_k had the extra variation factors 0.5, 1.5, 2, 3, 5 for the data of Rubach (2010) for *C. obscuripes* and *C. dipterum*). First, one parameter was changed with each factor while the others were kept at their initial value. Then ten fully randomized combinations were taken as starting values. In addition, using this information several extra combinations were tried. In the next step, good fits were selected. Firstly, the selection was based on the minimum residual sum of squares (RSS), Chi^2, R^2, mean % error and an additional check whether the model fit crossed the experimental data. Secondly, it was based on the parameter values and their standard deviation, see Appendix A for the range to include a fit. For the final parameters the average from the best estimations was taken and again fitted. In addition, a user constraint for k_r ($0.016 < k_r < 500$ d^{-1} for *D. magna* and $0.002 < k_r < 500$ d^{-1} for the other species) (see Rubach 2010 for more details) was used to make the data more comparable with other data from Rubach *et al* (2010a) and to create a narrow range for the parameter estimates which would limit the amount of local minima. However, these user constrains are not realistic since both shorter, at molecular level, and longer, e.g. for species without recovery abilities, recovery times are possible.

Results

Experiments

Chemical exposure

Measured concentrations of the time variable exposure regimes of chlorpyrifos during the experiments were meeting the intentions (Figure 1; lower twelve panels). None of the groundwater samples (n=20) taken from the storage containers for regular control measurements contained chlorpyrifos except for one sample (0.0298 µg/L on day 1 of the *P. minutissima* experiment). The average concentration of the *C. obscuripes* (n=72*)* and *C. dipterum* (n=65) control treatments were below the LOD (0.01 µg/L), expect for four samples, which were slightly higher than the LOD. In the control treatments for *P. minutissima* (n=29) and *D. magna* (n=23), no chlorpyrifos was detected. On average, *C. dipterum* was structurally over-exposed (125 ± 11% of the nominal concentrations), *C. obscuripes* under-exposed (93 ± 11% of the nominal concentrations), whereas *P. minutissima* was dosed according to the intended concentration (100 ± 16%) and *D. magna* was slightly over-exposed

(112 ± 24%). Overall, for the duration of the pulse the concentrations remained within 96%, 94%, 84% and 91% of the average concentration for *C. dipterum, C. obscuripes, P. minutissima* and *D. magna*, respectively. In the experiments performed with *C. dipterum* the concentration increased during the duration of the pulse on two occasions (Fig. 1). This occurred as well in the experiments with *C. obscuripes* (1 case) and *D. magna* (3 cases) (Fig. 1).

In some samples, immediately taken after transferring animals from the first pulse into clean water, concentrations above the LOD were found in T1 for *C. dipterum* (0.023 µg/L) and in T1 (0.085 µg/L), T2 (0.028 µg/L) and T3 (0.017 µg/L) for *P. minutissima*. During the non-pulse periods, no chlorpyrifos was found in the treatments for *D. magna*. For the other species measured concentrations were below LOD, except in samples of *C. obscuripes* after the first pulse in T1 (max. concentration 0.022 µg/L) and *C. dipterum* after the second pulse in T1 (0.0224 µg/L). In the experiments with *P. minutissima,* concentrations above the LOD were detected 4 days after the first exposure.

Effects

Data on the mobility (1st row in Figure 1) and on survival and TDM simulations (2nd row in Figure 1) of individuals during the experiments illustrate the effect of the different dosing regimens (using the final parameters combinations).

The survival and mobility in the control group of *P. minutissima* and *D. magna* stayed on average above 90% and 95% respectively (Fig. 1). In case of *C. obscuripes*, mobility and survival dropped to 80% after 24 days and to 76% at the end of the experiment. The control mobility and survival of *C. dipterum* strongly decreased already after day 15. The mortality was more than 20% after 18 days and increased to 52% survival by the end of the experiment (Fig. 1). Although the average mortality in the controls for the last two mentioned species was more than 20% (OECD, 1992) over the whole experimental period, the survival data were used for further calculations and model purposes, but all results for *C. obscuripes* and *C. dipterum* should be interpreted with care.

The survival for *C. obscuripes* decreased to the intended 70% in 7 to 9 days in all treatments, showing a delayed response in survival while effects on mobility were observed directly after the pulsed exposure (Fig. 1). After 11 days, T2 and T3 stabilized to background mortality. The first treatment received the second pulse (day 7), before it could stabilize and again there was a delayed effect on mortality. The intended mortality (30%) was reached after 13 days and thereafter the survival stabilized directly, i.e. did not increase. Mobility decreased directly after application to 40% 12 days post first application and stabilized at this rate. The second pulse in T2 caused a decrease in

survival after two days, which was smaller compared to T1 and never reached the intended mortality. Five days after the second pulse, the survival stabilized while mobility was 58% after 4 days. T3 showed hardly any decrease in mobility (3% in 5 days) and survival (1% after 7 days) after the second pulse (Fig. 1). In general, effects on both endpoints decreased with increasing time interval between the pulses. No recovery (from immobile to mobile) was observed except in one replicate of T3 where one animal recovered in the first treatment, five days after the first pulse.

A direct response in mobility and survival was observed for *C. dipterum* after the first chlorpyrifos pulse and within 1 day the intended mortality of 20% was reached (Fig. 1). Survival and mobility in T1 stabilized before the second pulse, however, T2 and T3 did not stabilize fully. After the second pulse, T1 showed a direct effect on both endpoints, being greater than the first pulse, without stabilizing completely. T2 decreased directly to the survival as observed in T1, after which immobility and survival stabilized for four days and sequentially dropped below the survival of T1. Again the second pulse had a stronger effect on the mobility and survival than the first pulse. The second pulse in T3 resulted in direct effects on immobility and mortality, though less severe compared to those resulting from the first pulse. Some animals were able to recover from the chlorpyrifos stress. After the first pulse, 1.3% of the animals recovered on average in each treatment after 3 to 5 days, thus going from immobile to mobile. After the second pulse in T1, 2.7% recovered after 3 days, 1.3% in T2 after 6 days and 1.8% in T3 after 2 days (Fig. 1).

The first pulse resulted in direct effects on the mobility (approximately 30 %) of *P. minutissima*, however not reaching the intended immobility of 50%, while effects on survival took some longer to be expressed (Fig. 1). The survival observed in T1 did not stabilize before the second pulse. The mortality in the other treatments did stabilize within 7 to 8 days, however both showing some mortality after that and finally stabilized after 14 to 15 days. The second pulse had a larger effect on T1 than the first pulse, after one day the intended immobility was achieved, whereas mortality decreased slowly to a steady level to 23% survival in seven days after the second pulse. In T2 and T3 the effect on mobility and survival were smaller than after the first pulse. Recovery was observed after every pulse in all treatments except for T2 after the second pulse. In general, 1.3 to 2.8% of the animals recovered within 1 to 9 days (Fig. 1).

After the first pulse, the mobility of *D. magna* decreased immediately and the intended immobility of 17% was attained after 1 day, while survival showed very prolonged delayed effects for *D. magna* (Fig. 1). In T1 there was hardly any additional mortality observed before the second pulse, while after this pulse the survival decreased and then stabilized at 57% on day nine of the experiment. After the first pulse, the survival in T2, started to stabilize on the day of the second pulse (day 6) and thereafter no high mortality rate was observed. However, exactly after the second

pulse a dip in mobility was seen but not reaching the intended effect size. This pattern was also observed in T3, direct effects on the mobility without achieving the intended effect size and small and delayed effects on the survival. Interestingly, all treatments ended with approximately the same survival and mobility rate (57-60%). Recovery was observed in T2 after the second pulse (5.6% on the second day after the pulse) and T3 after the both pulses (1.0% on the fourth day after pulse 1 and 3.0% on the second day after pulse 2). Most animals were only slightly immobile showing behaviour such as continuously spinning or lying cramped on the bottom. Thus, in two to four days post treatment 1.0 to 5.9% of the animals were able to recover (Fig. 1). During the experiment the animals increased in size, moulted and reproduced at a high rate (*results not shown*).

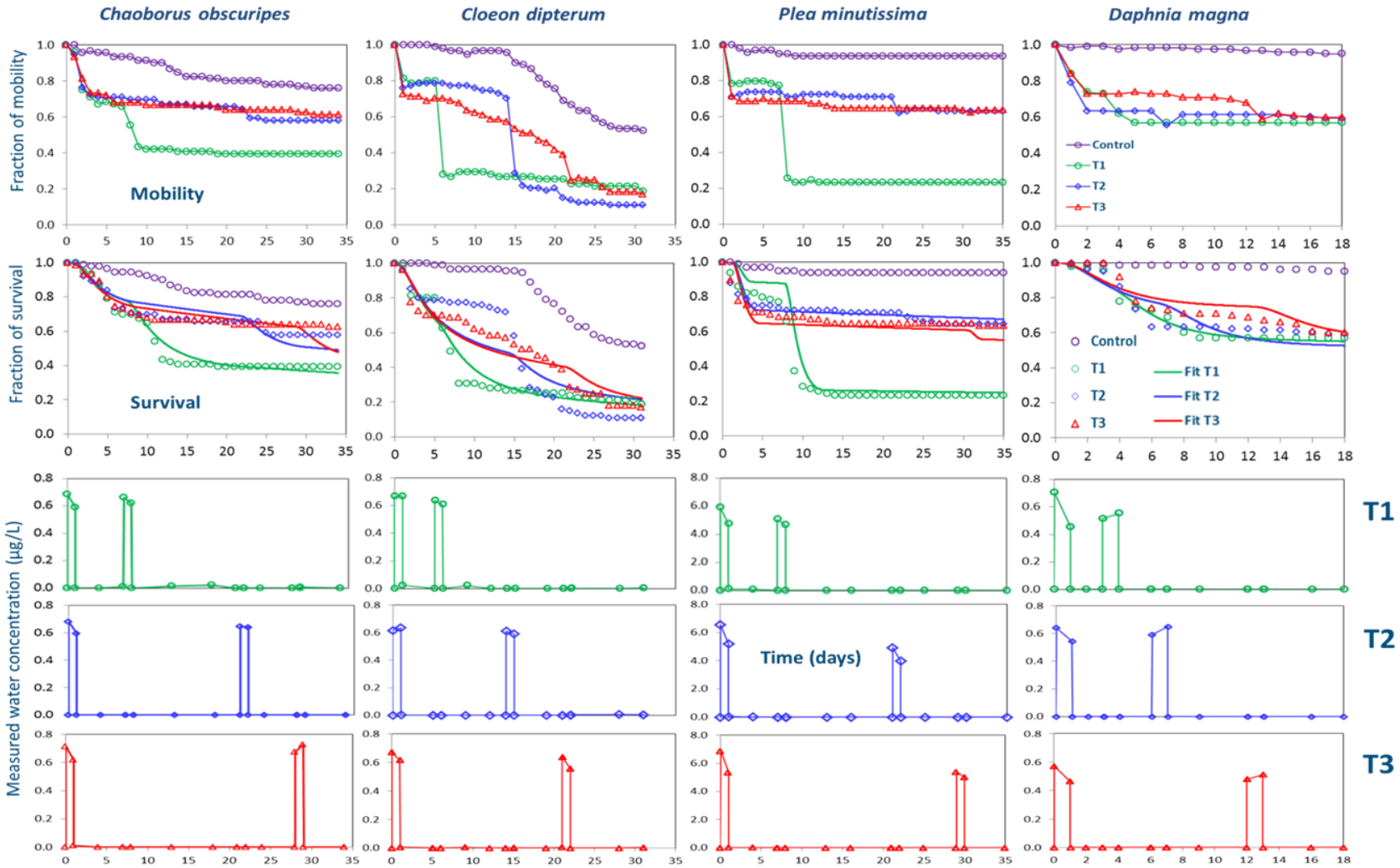

Figure 1: The experimental mobility and survival responses for *C. obscuripes*, *C. dipterum*, *P. minutissima* and *D. magna* under three pesticide exposure regimes damage accrual **(T1)**, full elimination **(T2)** and potential recovery **(T3)**. The mobility data **(1st panel)** also includes cumulative dead animals. Plots in the middle **(2nd panel)** show the TDM fits to survival. The **twelve lower panels** show the measured average concentrations in the different treatments (n=5 for treatments and n=6 for controls).

Modelling

The killing rate constant varied from 0.179 to 1.366 g_{ww}*$nmol^{-1}$*d^{-1} for *D. magna* and *P. minutissima*, respectively (Table 3). The lowest recovery rate constant was found for *D. magna* (0.572 d^{-1}) and the highest two parameter values were really close, with 1.544 d^{-1} for *C. obscuripes* and 1.575 d^{-1} for *P. minutissima*. The highest variability between species was seen for the threshold parameter with values ranging from 1.40E-8 to 0.844 for *C. dipterum* and *P. minutissima*, respectively. *P. minutissima* (2.47E-3) had the lowest value for the background death rate and *C. dipterum* the highest (1.56E-2).

After parameterisation, the model fitted the experimental data relatively good for the four species (Fig. 1; Table 3). The final parameters were the average values from those that were extracted from the selected best parameter fits, see Figure 2 and appendix B for the variability of these parameters.

Box plots in Figure 2 illustrated that the killing rate constant had a relatively low robustness, especially for *P. minutissima* where a large variability between model fits was observed for this parameter (see Appendix B for detailed data). The recovery rate constant showed a low variability for *C. obscuripes*, when outliers were excluded, but not for *C. dipterum* and *D. magna*. The threshold parameter showed a high variability in the data range again for *P*. *minutissima* and thus lacked robustness while the background death rate was robustly estimated for all species. The correlation between k_r and k_k is high for *C. dipterum* and *D. magna* (R^2= 0.99 and 0.99 respectively), medium for *P. minutissima* (R^2= 0.70) and low for *C. obscuripes* (R^2= 0.06). For *C. obscuripes* and *P. minutissima* the parameters were highly correlated before the final extraction of the best fits (Appendix C).

The time that an individual needs to recover from 95% of the internal damage (t_{95}) was calculated with the recovery rate parameter using the formula: t_{95} = -ln(0.05)/k_r by assuming full elimination, thus the internal concentration approximating zero (Ashauer, 2007b). *C. obscuripes* needed 0.45 d to recover to 50% of the initial internal damage and 1.94 d to 95%. For *C. dipterum* these values were 0.52 d and 2.25 d, for *P. minutissima* 0.44 d and 1.90 d and for *D. magna* 1.21 d and 5.24 d, respectively.

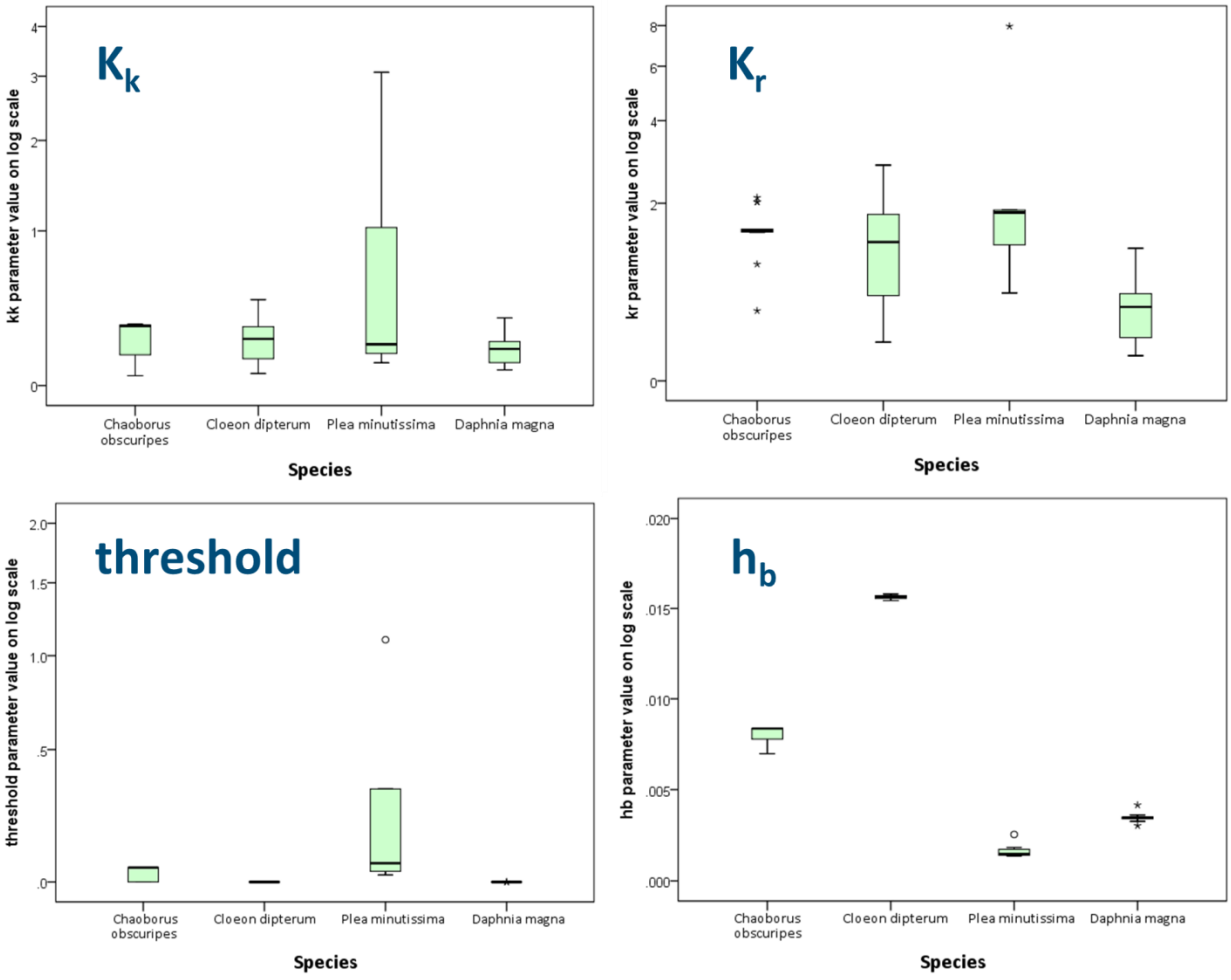

Figure 2: Box plots with outliers showing the variability of the parameters selected from the best fits. The variability indicates the robustness of the parameter estimates in the fits of the TDM model on the experimental data. Toxicodynamics parameters are $\mathbf{k_k}$ (killing rate constant), $\mathbf{k_r}$ (recovery rate constant), **threshold** and $\mathbf{h_b}$ (background mortality).

Table 3: Toxicokinetic and toxicodynamic parameters for several freshwater arthropod species for the insecticide chlorpyrifos as determined by this study and obtained from the literature.

Parameter	Symbol	*Gammarus pulex*[1]	*Asellus aquaticus*[2]	*Chaoborus obscuripes*		*Cloeon dipterum*	*Plea minutissima*	*Daphnia magna*
uptake rate constant	K_{in} ($ml*g_{ww}^{-1}*d^{-1}$)	747	596	318[2]		349[2]	88[2]	295[2]
elimination rate constant	K_{out} (d^{-1})	0.45	0.185	0.131[2]		0.196[2]	0.135[2]	0.546[2]
killing rate constant	k_k ($g_{ww}*nmol^{-1}*d^{-1}$)	0.047	7.072	0.088[2]	0.312[3]	0.228[3]	1.366[3]	0.179[3]
					(0.073)	(0.136)	(0.212)	(0.034)
recovery rate constant	k_r (d^{-1})	0.169	>494	0.518[2]	1.544	1.331	1.575	0.572
					(0.317)	(0.741)	(0.089)	(0.106)
threshold	*thr (-)*	0.022	0.048	3.3E-8[2]	4.528E-2	1.400E-8	0.844	2.060E-8 (4.113E-5)
					(7.812E-3)	(3.371E-5)	(0.113)	
background mortality	h_b *(-)*	0.0071	0.006	0.004[2]	8.359E-3	1.564E-2	2.467E-3	3.413E-3
					(3.183E-04)	(9.052E-4)	(2.766E-4)	(7.312E-4)
R^2			0.94	0.98[2]	0.94	0.91	0.95	0.93
mean error %			13.4	15.4[2]	5.8	22.3	8.8	4.6

[1]Data from Ashauer *et al*. 2007b; [2]Data from Rubach (2010); [3]Toxicodynamic parameter data from this study, numbers between brackets are the standard deviations of the parameters estimated.

Discussion

Experimental

Chemical exposure

In the experiments described in this study, the exposure to chlorpyrifos was the driving variable causing the effects. Therefore, the outcome of the experiment depends strongly on the correct dosing of the chemical. *C. obscuripes* was, however, under-exposed, while *C. dipterum* was over-exposed (Figure 1). Rubach (2010) found differences among species as well, in which *C. obscuripes* had also lower exposure values than intended. This was explained, apart from possible dosing errors, as differences in adsorption. In our case, this could still hold as an explanation for *C. obscuripes* and *C. dipterum*, while the latter has likely been structurally over-dosed. The concentration between the start of the pulse and the end after 24 h decreased for all species due to uptake and volatilisation of the compound. Interestingly, the decrease was the highest for *P. minutissima* while its uptake rate was the lowest of all species (Rubach *et al*. 2010a). The other species showed similar decrease in concentrations, probably due to the comparable uptake rates. In some cases, the concentration during the pulse increased over time (Figure 1). This could be caused by (cross) contamination, by incomplete mixing or by measurement errors. When transferring animals from exposed to clean water, some contamination will occur. This was observed for *C. dipterum* and *P. minutissima* since at the time of taking the water samples the animals did not have enough time to eliminate all chlorpyrifos from their bodies. Thus, for these species, concentration measured after the pulses in the non-pulse periods could be a result of contamination, while elimination by the animal could, however, also play a minor role.

Effects

After chlorpyrifos exposure, *C. obscuripes* showed a direct effect on mobility and delayed effect on mortality, without recovery. This delayed effect in survival and lack of recovery was also observed by Rubach (2010), who hypothesised that in general low chlorpyrifos concentrations might act on the hydrostatic vesicles with which these animals are equipped. This results in floating animals, which is assumed to be an irreversible effect and acts as an extra stressor, eventually leading to the death of the animal. Our results supported this hypothesis. The late occurrence of the intended effect could also be caused by the structural under-dosing of the systems. The effect on both mobility and survival decreased with increasing time interval between the pulses, which is in accordance with the results of Rubach (2010). The observed decrease in response with increasing time for elimination and recovery fitted the experimental design. In T1, the individuals had the

shortest time to eliminate chlorpyrifos from their body and therefore will suffer more from the second pulse through damage accrual. However, another hypothesis that could have contributed to the observed difference is that the individuals with the highest threshold will survive the first pulse and thus the group of individuals that is left is less sensitive to a second pulse, especially when some time is available for depuration and repair. The experimental results shown here support this individual effective dose theory (Gaddum, 1953), which is not included in the TDM model were stochastic death is assumed.

The reasons for the strong decrease in mobility and survival in the control units for *C. dipterum* after day 15 remain unclear; no contamination above the LOD was observed, physicochemical parameters did not change and no adjustments were made to the protocol. Some imposed stress due to the long time scale of the experiment and the lack of, for instance, a proper habitat seems to be the most probable explanation. *C. dipterum* showed direct effect on mobility, which was comparable to the response of *P. minutissima* and indicated acute inhibition of AChE. The survival response after exposure was observed faster than for the other species. This could be explained by the higher uptake rate for *C. dipterum* and/or by potentially fast distribution to the target enzyme and fast binding kinetics, which would result in a high killing rate constant. The lack of stabilization of the survival after the first and second pulses could partly be due to the background mortality as observed in the control treatment after day 15 (Fig. 1). After the second pulse, the mobility and survival observed in T2 dropped below T1, maybe as a result of the stress imposed by the test design. Another explanation for the survival rates in T2 to drop below T1 could be that the second pulse was applied before the animals eliminated 95% of the chlorpyrifos and thus 13 days elimination time between the pulses was not enough for mitigating the effects of the second pulse. This in turn, similar to the seasonality of sensitivity, highlights the potential seasonal dependence of the toxicokinetic parameter values, which themselves are a result of the species morphological, ecological and physiological traits at a given point in time (Rubach *et al*. 2010c). As intended, a smaller response was observed after the second pulse in the third treatment. This suggests that when an animal is given the time to fully eliminate the chemical and to recover by reactivating the inhibited enzyme, no accrual in effect is observed.

In general, 1.3 to 2.7% of the animals were able to recover from the chlorpyrifos exposure in all treatments within 2 to 6 days. We are not aware of other results available about the recovery of *C. dipterum* at the individual level. Therefore, no comparison can be made. In the experimental design potential recovery at molecular level was assumed after 21 days, which was based on the 95% elimination (day 14). However, in the experiment, recovery at individual level was observed within 2 to 6 days. All processes such as elimination and recovery occur simultaneously and in theory

recovery and repair may be completed within seconds or minutes at the target site, while recovery at the individual level as observed here may take days (Rubach, 2010). It still remains unclear if recovery times as calculated by the model relate to the molecular level or not. This question could be answered by including measurements of AChE inhibition over time in the experiment.

Direct effects on mobility and survival with some recovery after exposure were observed for *P. minutissima*. The intended immobility of 50% was not reached after any of the pulses except for the second pulse in T1, while no under-dosing was practiced (Fig. 1). Other possible reasons for not achieving the intended effects are differences in population sensitivities (the EC_{50} was originally based on a winter population while we used an autumn generation) and differences in laboratory practice. The T2 and T3 did not show much difference in mobility and survival after the second pulse, while T1 showed larger effects, most likely a result of damage accrual. These patterns are comparable with those observed for *C. obscuripes*. T2 showed a very prolonged and small effect on survival after the second pulse while a direct response was observed on mobility. This indicates that most animals were able to eliminate the chlorpyrifos from their body and thus had suffered less from the second pulse of T2 compared to those of T1. However, individual tolerance could also have played a role as was explained above for *C. obscuripes*. Again T2 and T3 do not show much difference in effects on mobility and survival and thus the time period between elimination and potential recovery is probably not enough to induce substantial differences in effect. Recover of animals in 1 to 9 days occurred after exposure, indicating that the animals are able to reactivate the inhibited enzyme.

Daphnia magna showed direct immobility and a very prolonged effect of several days on mortality. The intended immobility of 17% was reached in 1 to 2 days after the first pulse in all treatments, but not after the second pulse. This small effect on mobility and survival was not expected and could be due to the growth of the animals during the experiment. When growing, animals becoming potentially less sensitive and the chemical can be diluted by the increasing amount of body tissue, by moulting and by the release of offspring (Naddy *et al*. 2000). Another explanation could be again the individual tolerance principle. In the study of Naddy *et al*. (2000), exposure of *D. magna* to two chlorpyrifos pulses (0.5 µg/L; 12h) on day 0 and 3, 0 and7 and 0 and 14 showed a delayed effect on survival as well after the first pulse and direct and enlarged survival effect after the second pulse in every treatment. Our results show only high mortality after the second pulse in T1 and, controversially, lower mortality in the other treatments while exposure was even longer compared to Naddy *et al*. (2000; 24 vs. 12 hours). This and the delayed effect on survival could be explained by the definitions of the endpoint mortality, which is the lack of heartbeat in our study, while in the study of Naddy *et al*. (2000) and the other species in this experiment, the

endpoint was defined as lack of movement, which is a less accurate description for death of animals and thus individuals will be considered dead earlier. In T2 and T3, recovery was observed within two to four days. This is in agreement with the recovery period of one to three days for different exposure regimes found by Naddy and Klaine (2001). However, in their experiment more individuals were able to recover probably due to the shorter exposure time of only 6 hours. What could also contribute to a fast recovery is the high growth and reproduction rate and moulting, as observed in this experiment, which could causes excretion and dilution of chlorpyrifos.

After exposure, the initial effect of chlorpyrifos takes place at the molecular level, being the inhibition of AChE, which can result in immobility and finally death and possible recovery, as it was seen in the experimental data. As argued before by Rubach (2010), taking immobility into account provided us with information on AChE inhibition and recovery. However, measuring the AChE inhibition could even give more detailed information, specifically on individual recovery (see discussion on recovery for *C. dipterum* above and see Kalleander *et al*. 1997 for an example). This would allow us to observe effect at the molecular scale, which could not be observed otherwise. For example, it has been shown (Venkateswara Rao *et al*. 2005) that inhibition can be measured when fish were exposed to very low doses of chlorpyrifos while not showing any effects. Duquesne (2006) demonstrated that ChE inhibition is a useful predictor of long-term effects at physiological to individual levels of biological organisation. However, gaining more insight into these processes would on the other hand mean an increase in workload, costs and the complexity of the method (e.g. increase in test animals).

Modelling

One of the main aims of this research was to parameterise the toxicodynamic part of the TDM using the experimental survival data. Besides this, four additional data sets are now available for the TDM and other toxicodynamic models in order to further test, evaluate and improve such approaches.

The available datasets of chlorpyrifos and *G. pulex* (Ashauer *et al*. 2007b) and *C. obscuripes* (Rubach, 2010) served as good starting values for the fitting procedure for all species. For *C. dipterum* and *D. magna* the best predictions were given by varying the starting values of Rubach (2010) (Appendix B). *P. minutissima* showed equally well fits for both sets of original starting values and their variations. Although the original parameter values for *G. pulex* fitted well for *C. obscuripes,* no other combinations were tried because the original and combined parameters of *C. obscuripes* from Rubach (2010) showed many good fits.

The killing rate constant for *C. obscuripes* was less robust than found by Rubach (2010) for the same species. Compared with *Asellus aquaticus* and *Neocaridina denticulata* (Rubach, 2010) the

fits for all species were relatively robust, even for *P. minutissima,* who showed the lowest robustness in this research (Figure 2; Appendix B). The recovery rate constant was relatively robust for *C. obscuripes* and *P. minutissima*, but not so for *C. dipterum* and *D. magna* (Fig. 2). This is in accordance with the findings of Rubach (2010) who also found robust estimates for *C. obscuripes*. The best fits for the background mortality seemed to be constantly robust, which is not surprising since this is directly measured in the control units of the experiments. The threshold parameter lacked robustness for all species tested in this paper and by Rubach (2010), expect for *A. aquaticus* (Rubach 2010). Rubach (2010 see this thesis for a detailed discussion) touched upon two possible explanations for the lack of robustness in the threshold parameter. Firstly, the threshold parameter is not directly related to a biological process and it is dimensionless. Secondly, the TDM uses the stochastic death principal to predict mortality while in reality both the stochastic death principal and the individual tolerance principal could explain mortality (Rubach 2010; see also Zhao and Newman, 2007 for full discussion on this topic). The experimental data of the present study support both theories as possibilities but is more in favour of the individual tolerance principal (Fig. 1). Another problem for the low robustness is the experimental design. When more distinct treatments (e.g. different time periods between pulses) are used and thus more detailed results are obtained, parameters might become more robust and the model could better predict the survival.

For *C. dipterum* and *D. magna*, the killing rate and the recovery rate were highly correlated, which was also observed for *A. aquaticus* and *N. denticulate* by Rubach (2010) (Appendix C). This supports again the need for re-parameterization and model reformulation of the TDM, especially if such a model attempts to claim generality across species. However, *C. obscuripes* showed no correlation in the local minima (an average correlation was shown for the same species by Rubach, 2010) and *P. minutissima* showed an average correlation. Interestingly, before the last stage of best-fit selection, the parameters are highly correlated for both species. This indicates probably that selected fits are well chosen and it is likely that the local minimum is the global minimum.

Comparison of TD parameters per species

In this section the toxicodynamic parameters of the species are discussed and compared with each other and between other species in order to explore the link to species' characteristics and discuss the used model. Compared with the parameters from Rubach (2010), *C. obscuripes* showed a higher killing and recovery rate constant, each within a factor of 10, and the threshold differed extremely with an order of magnitude of six (Table 3). Possible reasons could be the differences in experimental design e.g. 2 vs. 3 pulses, the use of LC_{30} and LC_{50} doses instead of only LC_{30}, physiological variation between seasons as well as the failure of the model to fit the data. At

least, it illustrates the dependency of the model and the derived parameters on the available data. The killing rate was relatively high compared to the value found by Rubach (2010), and was more robust and not correlated with k_r and therefore more reliable to use. Based on the observed lack of ability to recover, it would be expected that *C. obscuripes* had a very low recovery rate constant, therefore, the recovery rate of 0.518 d^{-1} as determined by Rubach (2010) seems more realistic. This rate corresponds to6 days being needed to recover 95% of the damage. Even a smaller recovery rate or a rate of zero could theoretically be possible when the AChE inhibition is irreversible.

The killing and recovery rate constants of *C. dipterum* were in between the data for *D. magna* and *C. obscuripes* while the threshold had the lowest values of the four species (Table 3). The threshold for *C. dipterum* was in the same order of magnitude as for *C. obscuripes* (Rubach, 2010). This low threshold value could explain the relatively high sensitivity of this species. However, interpretation of the threshold values is difficult since this parameter does not represent a biological process and has no unit. The calculated 95% recovery of damage (2 days) fell within the experimental data where animals recovered between 2-6 days. Recovery in the model represents the internal recovery, which might need a different time than the recovery observed from the endpoint immobility to mobility during the experiments (see discussion about recovery for *C. dipterum* above).

P. minutissima showed a high killing and recovery rate constant and threshold (not robustly estimated). High recovery rate constant together with a high threshold could explain the relative insensitivity of this species. The low uptake rate constant (88 $ml*g_{ww}^{-1}*d^{-1}$) explains this as well. The calculated 95% recovery time (1.90 days) fell within the ranges of the observed 1 to 9 days for recovery, although in the experiment sometimes more time was needed.

The combination of a low recovery rate constant and threshold could explain the high sensitivity of *D. magna* for chlorpyrifos. However, the low killing rate might suggest the opposite but was probably influenced by the low recovery rate due to the high correlation between k_k and k_r. *D. magna* showed the highest recovery of all species in the experimental data, but still the lowest recovery parameter value was found for this species. However, this value was not estimated robustly and was therefore probably not reliable. Furthermore, the time to recover 95% of the damage (5.24 days) fell just outside of the observed range of 2 to 4 days. In an experiment with paraoxon-methyl (organophosphate compound) *D. magna* was able to recover the ChE activity within 2 days post application (Duquesne, 2006). This also indicates that this species is able to recovery relative rapid from OP exposure and therefore the recovery parameter could potentially be higher.

The TDM does not provide consistent TD parameter estimates, as was seen for *C. obscuripes* where parameter values varied heavily between two different experiments and many parameters were not robustly estimated, which makes these data hard to interpret and to be used with great care. Therefore, the comparison of parameter values between species is difficult and only general conclusions can be drawn.

Above problems illustrate again, as already indicated by Rubach (2010), the need to improve and develop TKTD models, such as the TDM, in terms of their use in ERA, for different species and compounds. The ultimate objective of such improvements is the ability of such model approaches to predict robust parameters for a wide variety of species, which would finally allow accurate comparison of species and evaluation of large range of exposure regimes. The various existing TKTD models for survival were unified and incorporated into the "General Unified Threshold model for Survival" (GUTS). GUTS is a TKTD framework for ecotoxicology, from which a large number of existing models can be derived as special cases (Jager *et al*. 2011). It is considered that GUTS can help to increase the application of TKTD models in ecotoxicological research as well as environmental risk assessment of chemicals. The unified framework serves as a reference model for survival and allows for quantitative interpretation of patterns in data that are best explained by various assumptions (e.g., stochastic death vs. individual tolerance) as compared to TDM (e.g. only assumes stochastic death). Therefore, the available data should be reanalysed by the GUTS model, and possibly a better estimation the parameters is obtained. This would facilitate explaining how the processes of toxicokinetic and toxicodynamic contribute to organism sensitivity.

Extrapolation to the field

Translating survival from simple toxicity test to the complexity of a field situation is already a difficult step and therefore safety factors are used in Environmental Risk Assessment. However, extrapolating individual recovery from the lab to population recovery in the field may be as difficult or even harder since many more process are involved (e.g. landscape features, biological traits and life cycles). Models could be used to address these problems and are thus important for predicting effects and recovery. When fully parameterized and validated, they could be highly valuable for risk assessors. Individual level TKTD models such as an improved version of the TDM, the DEBtox, or the GUTS model could be combined with population models such as the Metapopulation model for Assessing Spatial and Temporal Effects of Pesticides (MASTEP) to increase the realism for field relevant recovery times after time-variable exposure to pesticides (Ashauer, 2010; Galic, 2012).

Thus, in order to protect aquatic ecosystems, it is necessary to understand and predict adverse effects and recovery caused by time-varying exposure of PPPs at different biological levels. Before recovery of individuals and populations can be included in risk assessment, proper experimental methods must be established and models need to be developed and expanded to include different compounds, species and more realistic landscape characteristics and make them more standardized and user friendly for risk assessors.

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Appendices for Chapter 4

Appendix A: Model settings

Merit function

- Squared deviations
- Timing error 1
- Default Weight Value 0.1
- Fraction of Data Value
- Minimum Weight Value 0.1

Estimate Parameters: Classical Fitting

- Marquardt
- MCMC threshold 1E30
- Marquardt Options
    - Initial lambda value 1E-3
    - Number of convergent steps 8
    - Convergence Merit Function Change Threshold 1E-1
    - Fractional Change for Derivatives 1 E-2
    - Default Minimum Move 1E-6
    - Maximum Number of Iterations 20

Appendix B: The toxicodynamic parameter estimates for *C. obscuripes*, *C. dipterum, P. minutissima* and *D. magna*. The min and max parameter values indicate the parameter selection criteria. The average values were used to make a final fit, for the final parameters see Table 3.

		Parameters				**Goodness of fit**			
Species		k_k (g_{ww}*$nmol^{-1}$*d^{-1})	k_r (d^{-1})	*thr (-)*	h_b (-)	Chi^2	R^2	*min RSS*	*Mean error*
C. obscuripes	Min	0.045	0.543	3.30E-05	6.97E-03	0.298	0.84	0.528	9.8
*(n=14)**	Max	0.317	2.106	4.58E-02	8.37E-03	0.689	0.88	0.741	36.4
	average	0.24	1.538	2.93E-02	8.07E-03	0.414	0.87	0.6	19
	SD	0.101	0.394	2.25E-02	4.76E-04	0.162	0.01	0.062	7.1
C. dipterum (n=12)	Min	0.055	0.271	3.30E-11	1.54E-02	1.159	0.83	1.458	23.4
	Max	0.47	2.796	3.47E-05	1.58E-02	1.253	0.88	2.069	49.2
	average	0.228	1.331	1.34E-05	1.56E-02	1.184	0.85	1.732	34.9
	SD	0.119	0.726	1.60E-05	1.10E-04	0.031	0.02	0.183	9.3
P. minutissima (n=8)	Min	0.108	0.721	0.02	1.36E-3	0.325	0.62	2.834	32.0
	Max	3.072	7.964	1.10	2.45E-3	1.612	0.66	3.233	52.8
	average	0.783	2.354	0.25	1.63E-3	1.030	0.64	3.037	46.9
	SD	1.120	2.315	0.37	3.98E-4	0.502	0.02	0.177	7.0
D. magna (n=11)	Min	0.073	0.169	3.30E-11	3.02E-03	0.151	0.91	0.074	5.4
	Max	0.354	1.270	3.30E-05	4.16E-03	0.282	0.97	0.196	56.2
	average	0.177	0.573	5.38E-06	3.47E-03	0.185	0.93	0.164	29.6
	SD	0.086	0.332	1.12E-05	2.73E-04	0.05	0.02	0.039	21.1

*Indicates the number of best fits used for the statistics of this table

Appendix C: Correlations between the killing rate constant k_k and the recovery rate constant k_r for before the final selection of the best parameters and afterwards

Species	Correlation before final selection of the best parameters	Correlation after final selection of the best parameters
C. obscuripes	0.9998	0.0619
C. dipterum	0.9984	0.9996
P. minutissima	0.9502	0.6954
D. magna	0.9978	0.9978

Chapter 5

Ecological impacts of time-variable exposure regimes to the fungicide azoxystrobin on freshwater communities in outdoor microcosms

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"Life was certainly simpler in the old days…..

….. when we could evaluate risk with a safety factor"

(Doull 1984)

Abstract

This paper evaluates the effects of different time-varying exposure patterns of the strobilurin fungicide azoxystrobin on freshwater microsocosm communities. These exposure patterns included two treatments with a similar peak but different time-weighted average (TWA) concentrations, and two treatments with similar TWA but different peak concentrations. The experiment was carried out in outdoor microcosms under four different exposure regimes; (1) a continuous application treatment of 10 µg/L (CAT_{10}) for 42 days, (2) a continuous application treatment of 33 µg/L (CAT_{33}) for 42 days, (3) a single application treatment of 33 µg/L (SAT_{33}) and (4) a four application treatment of 16 µg/L (FAT_{16}), with a time interval of 10 days. Mean measured 42-d TWA concentrations in the different treatments were 9.4 µg/L (CAT_{10}), 32.8 µg/L (CAT_{33}), 14.9 µg/L (SAT_{33}) and 14.7 µg/L (FAT_{16}). Multivariate analyses demonstrated significant changes in zooplankton community structure in all but the CAT_{10} treated microcosms relative to that of controls. The largest adverse effects were reported for zooplankton taxa belonging to Copepoda and Cladocera. By the end of the experimental period (day 42 after treatment), community effects were of similar magnitude for the pulsed treatment regimes, although the magnitude of the initial effect was larger in the SAT_{33} treatment. This indicates that for long-term effects the TWA is more important for most zooplankton species in the test system than the peak concentration. Azoxystrobin only slightly affected some species of the macroinvertebrate, phytoplankton and macrophyte assemblages. The overall No Observed Ecologically Adverse Effect Concentrations (NOEAEC) in this study was 10 µg/L.

Introduction

In the European Union (EU), ecological risk assessment of pesticides follows a tiered approach, which is laid down in the European pesticide regulation (European Commission, 2009) and underlying guidance documents (European Commission, 2002). For non-target aquatic organisms, higher-tier risk assessments traditionally have incorporated the results of additional laboratory and semi-field experiments evaluating a range of pesticide concentrations that have a single or repeated pulse exposure, or that are held constant for a short period of time. It has been recognised, however, that under field conditions, aquatic non-target organisms may be exposed to fluctuating concentrations of pesticide contaminants (Reinert *et al*. 2002) and consequently, in recent years, more attention has been paid to pulsed or intermittent exposure scenarios. Prediction of effects of pulsed or intermittent exposure on populations is becoming an important issue in ecotoxicology (Boesten *et al*. 2007; Van den Brink, 2008). This issue was highlighted in a recent EU ELINK workshop (Brock *et al*. 2010), that resulted in recommendations for addressing time-variable exposures in aquatic risk assessment for pesticides, which developed guidance on when to use the peak or the time-weighted average (TWA) concentrations.

The present study evaluated the effects of azoxystrobin, a broad-spectrum, systemic fungicide belonging to the group β-methoxyacrylate strobilurins, with a biochemical mode of action that acts on respiration by inhibiting electron transport from cytochrome B to cytochrome C. It was first marketed in 1996 and has since then been registered worldwide for use on a wide range of crops (Bartlett *et al*. 2002). A range of laboratory, field, or semi-field toxicity data have been published for the fungicide azoxystrobin (Maltby *et al*. 2009; Warming *et al*. 2009; Cole *et al*. 2000; Gustafsson *et al*. 2010).

Fungicides can be toxic to a wide array of aquatic non-target organisms, and may affect the structure and function of biological communities (Maltby *et al*. 2009; Van den Brink *et al*. 2000; Slijkerman *et al*. 2004). According to our knowledge, no other information than that published in Cole *et al*. (2000) and Gustafsson *et al*. (2010) is available on the effects of strobilurin fungicides on aquatic systems of higher biological complexity than single species tests. Gustafsson *et al*. (2010) investigated the ecological effects of the fungicide azoxystrobin in outdoor brackish water microcosms and found that azoxystrobin is toxic to brackish water copepods at considerably lower concentration (≤ 3 µg/L) than previously reported for single species tests performed with freshwater crustaceans. Cole *et al*. (2000) tested the effects of a commercial formulation (YF9246, a 250 g/L suspension concentrate) of azoxystrobin on freshwater microcosms and found that zooplankton were more sensitive than other endpoints, with transient effects reported at 10 µg/L.

The present study was initiated to investigate, using azoxystrobin, which concentration profile, be it TWA or peak, is more appropriate for assessing the longer-term aquatic risks of this pesticide. This followed a similar microcosm study with the insecticide chlorpyrifos, which concluded that for most species, but not for all, the TWA concentration was more important than the peak concentration in explaining the longer-term effects (Zafar *et al*. 2011). The aim of the present study was to compare the effects of four different exposure regimes (two chronic, maintained exposure profiles, one repeated pulsed, and one single pulsed exposure regime) for the fungicide azoxystrobin. The high chronic and single pulse regimes had similar peak but different TWA concentrations, while the two pulsed regimes had different peak but similar TWA concentrations. The TWA concentrations of the two pulsed exposure regimes were intermediate relative to the two chronic regimes.

Material and methods

Experimental design

Sixteen outdoor microcosms (diameter 1.8 m, total depth 0.8 m, water depth 0.5 m, water volume ca. 1270 L) were used in the experiment. The microcosms were located at the Sinderhoeve Experimental Station (www.sinderhoeve.org) in Renkum near Wageningen, The Netherlands, and were lined with a watertight non-toxic layer of black polyethylene. Each microcosm was initially established with an 8 cm layer of sediment (fine clay) from a mesotrophic lake (dominated by the aquatic plants *Elodea nuttallii* and *Chara* sp.) and then filled with water, taken from the experimental station's water supply basin.

In the preparatory phase, one hundred shoots of *Elodea nuttallii* were planted on 75% of the sediment surface of each microcosm. In addition, other macrophytes (*Eleocharis acicularis*, *Spirodela polyrhiza*, *Potamogeton berchtoldii*, *Potamogeton pectinatus, Elodea canadensis, Potamogeton crispus* and *Ranunculus circinatus*) developed from diaspores in the sediment during the course of study. During the pre-treatment period (approximately 3 months), phytoplankton, zooplankton and macroinvertebrates were collected from uncontaminated mesotrophic ditches situated at the Sinderhoeve Experimental Station, and Veenkampen, an experimental field site of Wageningen University, Wageningen, The Netherlands and introduced into the systems in order to develop a freshwater community characteristic for lentic, edge-of-field surface water. The macroinvertebrates introduced comprised several taxonomic groups and they were representatives of various trophic levels. Dominant species included crustaceans (*Asellus aquaticus*, *Gammarus pulex* and *Daphnia* sp.),

insects (*Cloeon dipterum*, *Chaoborus* sp., *Plea minutissima*, Chironomidae, odonates and trichopterans), and the non-arthropods Hirudinea (*Erpobdella* sp.) and Gastropoda (*Valvata* sp.).

During the pre-treatment period all microcosms were interconnected by tubes and the water was circulated using a pump to achieve the development of a similar biocoenoses in the test systems. The circulation of water was stopped three weeks before the start of the experiment. The microcosms were investigated over a period of 7 weeks. One week prior to the first applications, all biological endpoints were sampled once to establish pre-treatment conditions, followed by a post treatment period of approximately 6 weeks.

Pesticide application and sampling

Azoxystrobin was provided by Syngenta Crop Protection AG, Switzerland as the formulated product AMISTAR® (Fluid) a 250 g a.i./L soluble concentrate formulation). There were four intended treatment regimes: (1) a continuous application treatment (CAT) of 10 µg/L (CAT_{10}) consisting of a continuous exposure to 10 µg a.i./L for 42 days (2) a continuous application treatment of 33 µg/L (CAT_{33}) consisting of a continuous exposure to 33 µg a.i./L for 42 days (3) a single application treatment (SAT_{33}) consisting of a single application of 33 µg a.i./L and (4) a four application treatment (FAT_{16}) consisting of four applications, each achieving a peak of 16 µg a.i./L with a time interval of 10 days . The treatment levels of the SAT_{33} and FAT_{16} applications were based on the 42d-TWA of 15 µg a.i./L, which fell in between the chronic exposure regimes of CAT_{10} and CAT_{33}. The concentrations in the chronic tests were kept constant between 80 and 120% of desired nominal concentrations by adding more azoxystrobin during exposure. To measure the exposure concentrations, water samples from all microcosm were collected regularly (see Fig. 1). In the continuous exposure treatments, sampling and analysis of azoxystrobin were performed every 1-2 days, with dosing as necessary in order to maintain the initial concentration. Approximately 1 h after the additional application, a water sample was taken and the concentration analysed as described below. Before application, concentrations in stock and dosing solutions were checked for establishing nominal initial concentrations. The first treatment day is referred to as day 0, the first sampling as day -7 while the post first treatment days run up to day 43.

The microcosms were randomly allocated to the different treatments. All treatments were performed in triplicate with four control replicates. Azoxystrobin was applied by pouring a defined volume of dosing solution into the microcosms. The control microcosms were treated with water only. The systems were gently stirred immediately after application to promote the mixing through the water column whilst avoiding any resuspension of sediment particles and disturbance of submerged macrophytes.

Calculation of treatment level for time-variable exposures

Azoxystrobin was selected as a compound for this study, as it has a measured waterphase DT_{50} of 13 days in an outdoor aquatic microcosm (Jones and Lake, 2000). In addition, with a log K_{ow} of 2.5 azoxystrobin would be expected to remain mainly in the water phase (Tomlin, 2011). Due to its relatively slow dissipation, exposures would be expected to be moderate to long-term. The concentrations of azoxystrobin chosen were based on the 10 µg/L NOEAEC (no observed ecologically adverse effect concentration (effect class 2: slight effects)) derived from a single application to an outdoor pond microcosm study (Cole *et al*. 2000). More pronounced effects may be expected when this concentration is maintained. Therefore, the intention was for the concentration in CAT_{10} to be equal to the 42d-TWA in SAT_{33} and FAT_{16} (calculations according to Zafar *et al*. (2011)). However, azoxystrobin proved to be more persistent in our microcosms and consequently the TWA concentration of the SAT_{33} and FAT_{16} were 15 µg/L instead of 10 µg/L, and therefore this TWA concentration fell in between CAT_{10} and CAT_{33}.

Azoxystrobin analysis

The concentrations of azoxystrobin were determined in the water samples by taking depth-integrated water samples from the microcosms by means of stainless steel suction tubes connected to glass flasks (Schott bottle, 250 mL) using a vacuum pump. Approximately 100 mL of water were sampled from each microcosm in duplicate. Duplicate 2 mL samples from the 100 mL-water sample were transferred into 4-mL WISP vials (borosilicate) containing 2 mL of acetonitrile. The exact mass of water added was calculated by weighing the vials. The vials were closed with a cap and thoroughly shaken manually. A 2 mL High Performance Liquid Chromatography (HPLC) vial was then filled with a portion of the sample, and was then sealed and analysed by Liquid Chromatography-Mass Spectrometry with Triple Quadrupole Systems (P2600 Agilent 6410 LC-MS/MS QQQ). The volume injected was 50 µL with an autosampler and the mobile phase (HPLC -water /acetonitrile ; (50/50, V/V) was set at a flow rate of 1.0 mL/min. The analytical column used was an Agilent Zorbax Eclipse XDB-C18 (diameter 4.6 mm; length 150 mm; 5 µm). Column was set at temperature 40 ^{0}C. Under these conditions, the retention time of azoxystrobin was approximately 2.40 min.

TWA concentrations of azoxystrobin were based on area under the curve (AUC) calculations, and the DT_{50} in SAT_{33} was estimated assuming first-order dissipation kinetics. Dissipation times were based on measurements for water samples above the limit of quantification (LOQ). The limit of Detection (LOD) and LOQ of the analysis were determined by adding a standard 0.01 µg/L of azoxystrobin in acetonitrile/water (v/v: 50/50) to each injection series. The concentration of this standard of 0.01 µg/L azoxystrobin was calculated from the calibration curve, while the standard

itself was not part of this calibration curve. In total, this standard was injected 105 times, yielding an average concentration of 0.0208 µg/L, with a standard deviation (SD) of 0.0048 µg/L. The LOD in water sample was defined as 3 * SD (3 * 0.0048 = 0.015 µg/L), the LOQ as 10 * SD (10 * 0.0048 = 0.05 µg/L). The DT_{50} was calculated by means of linear regression using ln-transformed measured pesticide concentrations versus time.

Macroinvertebrates

Artificial substrates, consisting of litter bags (see "Decomposition" section) and pebble baskets, were used to monitor the effects of azoxystrobin on the benthic macroinvertebrate assemblage. Two pebble baskets and two litter bags were placed on concrete tiles on the sediment in each microcosm two weeks before the initiation of the treatments in order to allow colonisation by macroinvertebrates (for a detailed description of methods see Brock *et al*. (1992)).

Macroinvertebrates were sampled 5 times from each microcosm at days -7, 3, 10, 17 and 43. Pebble baskets were gently retrieved using a net. The litter bags were collected by hand. The substrates were first washed in a container to remove invertebrates. The macroinvertebrates were identified and counted alive, and then released back into their original microcosms. The animals were identified to the lowest practical taxonomic level. From each microcosm abundances of macroinvertebrates from pebble baskets and litter bags were pooled prior to analysis of the data.

Phyto- and zooplankton sampling and identification

Zooplankton and phytoplankton were simultaneously sampled on days -5, 2, 9, 16, 23, 32 and 44 d by using a Perspex (Poly(methyl methacrylate)) tube (volume = 1.8 L). Depth-integrated water samples were collected from several spots in each microcosm until a bulk water sample of 12 L had been obtained in a bucket. From this bulk sample, 5 L was passed through a 55 µm mesh net to collect zooplankton. Another 5 L was passed through a 20 µm mesh net to collect phytoplankton, possibly missing the smaller phytoplankton taxa. The concentrated plankton samples were preserved with acetate buffered Lugol's solution in a 100 mL sampling vial. The filtered water was returned into its original microcosm.

Cladocerans, copepods and ostracods (macro-zooplankton) were counted using a stereo microscope (Nikon SMZ-10, magnification 25-x). Rotifers and copepod nauplii (micro-zooplankton) were quantified and identified with an inverted microscope (Carl Zeiss, Axiovert 10, magnification 100x), using a sub-sample of known volume. Rotifers and cladocerans were identified to the lowest practical taxonomic level (i.e., genus or species level), whereas copepods were identified to the

suborder by classifying as calanoids or cyclopoids. A distinction was also made between nauplii and the more mature stages of the copepods.

Phytoplankton species composition was studied by counting the number of cells of a known volume which were identified to the lowest practical taxonomic level. Taxa and number of cells were based on a maximum of 200 observations, consisting of a series of 20-40 counting fields of a single cuvette under an inverted microscope (magnification 400 x). Zooplankton and phytoplankton data were expressed as number of individuals per litre.

Chlorophyll-*a*

Phytoplankton chlorophyll-*a* was sampled in parallel with the phyto-and zooplankton sampling. One litre of the remaining from the bulk 12-L sample was used to determine the amount of chlorophyll-*a* of the phytoplankton. Samples were concentrated through a 1.2 µm pore size Whatmann glass-fibre filter (GF/C; diameter 4.7 cm; Maidstone, UK) using a vacuum pump. The filters containing phytoplankton were transferred into Petri dishes, wrapped in aluminium foil, and stored in a freezer at a temperature of –70 ˚C until analysis. After ethanol extraction of the pigments, measurements of chlorophyll-*a* content were performed using a HPLC with fluorescence detection (Webb *et al*. 1992).

As an estimate of periphytic algal biomass, chlorophyll-*a* was sampled on day -5 and on days 2, 9, 16, 23, 32 and 42. Periphyton was sampled from glass microscope slides (7.6 x 2.6 cm) that served as artificial substrates. The slides were positioned vertically in a stainless steel frame placed in the centre of all microcosms in the north - south position tied on a long rod, approximately 10 cm below the water surface of each microcosm, and incubated for 2 weeks. The placement of frame was kept the same in all test systems during whole experimental period. On each sampling day, 8 glass slides per microcosm (colonised for 14 days) were scraped visually clean with blades (Applo Ever-Sharp-Blades; Solingen-Germany) collecting the removed periphyton in tap water. New clean slides were then reintroduced in the microcosm. The chlorophyll-*a* content of the water periphyton solution was analysed as described above for the phytoplankton.

Water quality parameters

Dissolved oxygen (DO), pH, electrical conductivity (EC) and temperature (T) were measured in each microcosms on days -5, 2, 9, 16, 23, 32 and 42 to detect possible changes in community metabolism. On sampling days, measurements were carried out in the morning just around the start of photoperiod, at approximately 25 cm below the water surface. Together with DO, pH and T were

measured using a HQ40D multimeter (Hach-Lange, The Netherlands) and EC was measured with an Eijkelkamp 18.28 conductivity meter.

Alkalinity levels were determined in all microcosms prior to the initiation of the treatments (day -5) and at the end (day 43) of the experiment, using 100-mL water samples taken at a depth of 10 cm by titrating with 0.02N HCL until a pH of 4.2 was reached (pH meter: WTW 323).

Additionally, the concentration of ammonia, nitrate, nitrite, total nitrogen, orthophosphate and total phosphate were measured in the control microcosms at the start of the experiment and in all microcosms at end of experiment (day 42). For this purpose, water samples (approximately 100 mL) were obtained from the filtered water (Whattman GF/C; 1.2 µm pore-size) collected for phytoplankton chlorophyll-*a* samples. These samples were transferred into 100-mL polyethylene flasks which were stored at below -18 °C until analysis. Total soluble nitrogen, N-(NO_2^- + NO_3^-), NH_4^+, ortho-phosphate and total phosphate were analysed using a Skalar 5100 Autoanalyser.

Decomposition

Decomposition of particulate organic matter (POM) was determined using litter bags (Brock et al. 1982), containing *Populus x canadensis* (hybrid black poplar) leaves. In the decomposition assessment, a portion of 2 g dry weight (dried at 60 °C) of leaves were enclosed in each litter bag. The litter bags were made from a glass Petri-dish (diameter: 11.6 cm), closed with a cover of stainless-steel wire (mesh size: 0.7 x 0.7 mm), in which 2 holes (diameter: 0.5 cm) were punched to give invertebrates access to the leaves.

In each microcosm, two litter bags were placed at the sediment surface in an almost upright position for a 2-week incubation period. At the end of the incubation period, litter bags were emptied into a white tray to separate POM from adhering sediment particles and macroinvertebrates by rinsing with tap water. After sampling, a new set of litterbags was incubated. Remaining organic plant material was dried in pre-weighted aluminium foil at 105 °C for 48 h to obtain dry weight. The decomposition over a 2-week period was expressed as % remaining organic material.

Macrophyte cover, biomass and bioassay

Development of macrophyte species composition and macrophyte species cover was examined three times on days - 1, 14, and 44 d. Development of vegetation and the species-composition of macrophytes were investigated by monitoring macrophyte cover and abundance. The monitoring only involved the 75% of the sediment surface that was initially planted. Cover

values were estimated using ordinal scales of 1 (<1%), 2 (1-5%), 3 (5-12.5%), 4 (12.5-25%), 5 (25-50%), 6 (50-75%), 7 (75-100%).

At the last sampling date (day 42), aboveground biomass of all macrophyte species were harvested for each microcosm. The plant material harvested was rinsed under tap water to remove sediment particles and macroinvertebrates and then dried in an oven in pre-weighed aluminium foil at 105 ˚C for 48 h to determine the dry weight.

In addition to total macrophyte analysis, a *Myriophyllum spicatum* bioassay was performed. Flower pots (height 9.5 cm: 9 cm diameter) were filled with approximately 8.5 cm depth of sediment consisting of 86% peat, 8% sand, 6% clay and 3.73 kg fertiliser/m^3 (slow release). Each pot received three apical shoots of *M. spicatum* with a length of 10 cm and with at least one node in the sediment. Only unbranched, non-flowering apical *Myriophyllum* shoots without roots were selected. In the pre-treatment period at day -21, 500 pots were introduced into one of the ditches at the Sinderhoeve Experimental Station. At day -4, 12 pots per microcosm with healthy plants were placed in plastic trays on the macrophyte-free sediment section. On day -3, 16 *M. spicatum* pots (one from each test cosm) were sampled to characterise the plant material (i.e., shoot and root dry weight (105 °C for 24 hours), shoot length and shoot number) at the time of the first application. On days 14 and 42, 6 pots per microcosm were harvested. The plants were rinsed thoroughly to remove sediment particles. The endpoints (mean per shoot) measured were aboveground dry weight, belowground dry weight (roots), total length of shoots (length of main shoot and length of side shoots), mean length of shoots (total length of shoots/total # of side shoots), and number of side shoots. For each bioassay, belowground material (roots) was separated from the aboveground parts and plant samples were dried in aluminium foil (105 °C, 48 h) and weighed.

Data analysis

Univariate analysis

Prior to univariate and multivariate analyses, abundance data of macroinvertebrates and zooplankton were ln(ax+1) transformed, where x stands for the abundance value and ax makes 2 by taking the lowest abundance value higher than zero. We deviated from the usual ln(x+1) transformation because the data set frequently showed low or high abundance values (i.e. 1 individual per substratum for macroinvertebrates, 0.2 individuals per litre for the zooplankton and 2 individuals per litre for the phytoplankton community). We decided that the factor ax in the ln(ax+1) transformation should make 2 by taking the lowest abundance value higher than zero for x. A factor of two was chosen to avoid false discrepancy between zero abundance values and low abundance

values. Since, for instance, the lowest abundance value higher than zero in the zooplankton data sets was 0.2, a factor 10 was used (Van den Brink *et al*. 2000). All other variables were tested using untransformed values. Statistically significant differences between the treatments as well as against controls were assessed for all parameters or taxon levels at each time point, using analysis of variance (ANOVA) with multiple comparison tests. ANOVA was followed by Duncan's multiple-range test ($p < 0.05$), testing all treatments against the controls but also against each other. The analyses were carried out with the Genstat computer programme (v11.1, Laws Agricultural Trust, 2009 by VSN International Ltd). If the endpoint was measured more than 3 times after the initiation of the treatments, effects were only considered when they were consistent, i.e. occurring on at least two consecutive sampling dates.

Multivariate analysis

The effects of azoxystrobin treatment at the community level of macroinvertebrates, zooplankton and phytoplankton were analysed by the Principal Response Curves (PRC) method using the CANOCO software package, version 4.5 (Ter Braak and Smilauer, 2002; Van den Brink and Ter Braak, 1999). The analysis results in a diagram showing sampling day on the x-axis and the first Principal Component of the treatment effects on the community on the y-axis (e.g. Fig. 2). The PRC method yields a diagram showing the most dominant community response to the treatment present in the data set. The species weights are shown in a separate diagram, and indicate the degree of affinity the species have with this dominant response. The results of the PRC analysis can also be evaluated in terms of the fractions of variance explained by the factors time and treatment, and the PRC diagram shows the fraction of the variance that is explained by the treatment.

In the CANOCO computer programme, redundancy analysis is accompanied by Monte Carlo permutation tests to assess the statistical significance of the effects of the treatments on the species composition of the microcosms. The significance of the PRC diagram, in terms of displayed treatment variance, was tested by Monte Carlo permutation of microcosms, using an F-type test statistic based on the eigenvalue of the component (Van den Brink and Ter Braak, 1999). For each sampling date, all treatments were also tested against the controls using Monte Carlo permutation tests to assess the significance of treatment effects in time.

Results

Exposure to azoxystrobin

Dosing solutions corresponded well (108 ± 8%; mean ± SD) with the nominal concentrations, although measured peak concentrations of azoxystrobin exposure 4 hours after the applications were higher than nominal (Table 1). Table 1 shows that the TWA exposure concentrations in CAT_{10} and CAT_{33} were as planned and TWA concentrations in the SAT_{33} and FAT_{16}, at 14.9 and 14.7 µg/L, respectively, were almost identical over the period 0-42 days.

Table 1: Nominal, peak and time-weighted average (TWA) concentrations (µg/L) of azoxystrobin during the treatment periods (days 0-42).

Treatment Exposure regimes	Intended conc. (µg/L)	Nominal initial conc. (µg/L)	% of nominal initial conc. (%)	Measured peak conc. (4-h after application) (µg/L)	% of measured peak conc. (%)	TWA (0-42 days) (µg/L)	% of TWA conc. (%)
CAT_{10}	10	11.7	117	12.9	129	9.35	93.5
CAT_{33}	33	37.1	112	41.9	127	32.8	99.3
SAT_{33}	33	32.8	104	38.1	121	14.9	
FAT_{16}	15.8	15.7	99.3	18.4	121	14.7	

Nominal initial concentrations are based on the concentrations measured in treatment solutions.
All concentrations are presented as means of three replicates.

The dynamics in measured concentrations of azoxystrobin in microcosms treated with different application regimes are illustrated in Figure 1. In the continuous exposure treatment regimes, the highest measured concentration in CAT_{10} was 14.3 µg/L on day 2.2 and the lowest was 7.0 µg/L on day 16 (Fig. 1A), while the exposure concentration in CAT_{33} was rather higher than target concentration directly after application and fluctuated in the beginning, becoming less variable from day 7 to 42 (Fig. 1B). The highest measured concentrations in CAT_{33} were 41.9, 43.1, and 46.7 µg/L on days 0.17, 1.0 and 2.2, respectively and the lowest was 25.6 µg/L on day 16.

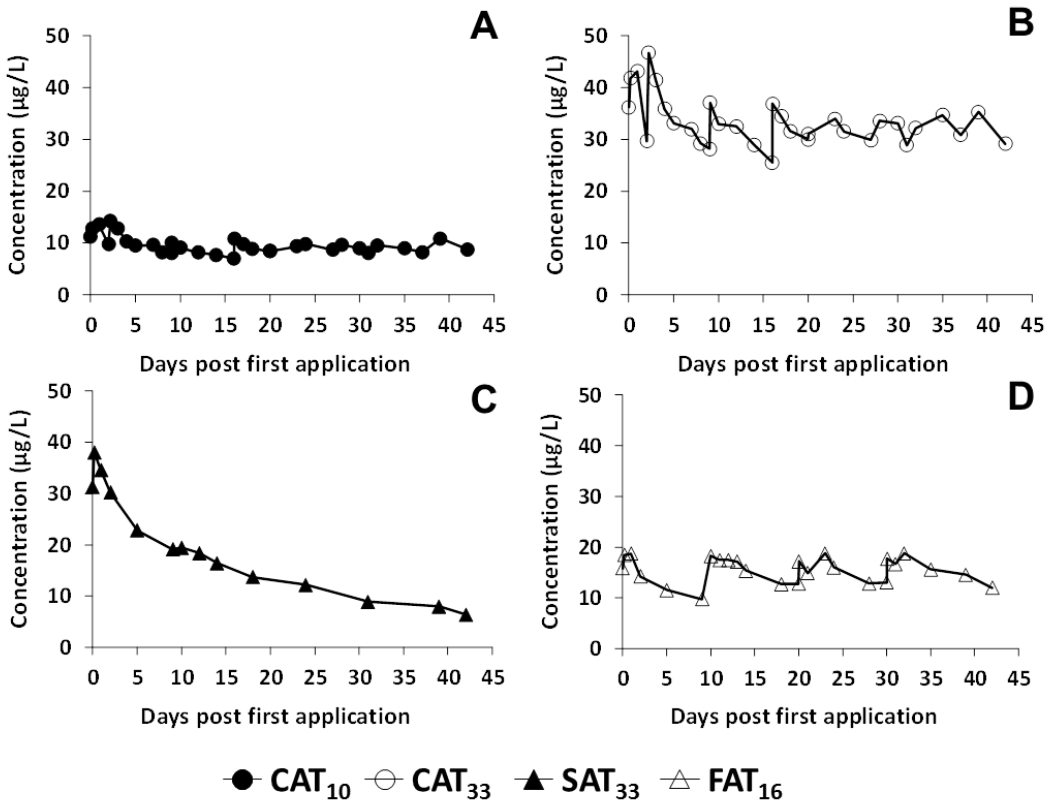

Figure 1: Dynamics of azoxystrobin concentrations in microcosms water during the 42 days period **(A)** continuous exposure of 10 µg/L, **(B)** continuous exposure of 33 µg/L, **(C)** single application and **(D)** four application treatments. The four applications took place on day 0, 10, 20 and 30.

At the end of experiment (day 42), the measured azoxystrobin concentration in the SAT_{33} was approximately 20% of the nominal applied (Fig. 1C).The DT_{50} from water-phase as calculated from the concentration dynamics in the SAT_{33} was 18 days. The FAT_{16} shows four pulses followed by slow dissipation between applications (Fig. 1D). After 9 days, exactly one day before the 2nd application in the FAT_{16} treatment, 53% of the initial measured test concentration was still present, while on day 20 and 30, just before the 3rd and 4th application 69% and 71% of subsequent applications were detected, respectively.

Zooplankton

Over the experimental period, a total of 46 different zooplankton taxa were identified in the microcosms. In terms of total abundance, the zooplankton community was dominated by Rotifera and Copepoda followed by Cladocera and Ostracoda. Rotifera were the most diverse taxonomic group with 33 taxa and 80% of total zooplankton abundance. Four taxa belonged to the *Trichocerca* family making it the most diverse genus, followed by *Lecane sp.* (two species) and *Keratella* sp. (two species). Among the rotifers, *Polyarthra remata* was the dominant species (37% of the total zooplankton abundance), followed by *Synchaeta* sp. (10%), *Keratella quadrata* (9%) and *Hexarthra* sp. (9%). Cladocera were represented by 9 taxa, Copepoda by 3 taxa (copepod nauplii, Cyclopoida, Calanoida) and Ostracoda by 1 taxon (not further identified). Copepod nauplii accounted for 15% of total zooplankton abundance and had a high abundance throughout the experimental period in the controls, with an average of 200 individuals/L.

The Monte Carlo permutation tests indicated that significant treatment-related effects were observed in CAT_{33}, SAT_{33} and FAT_{16} (Table 2). The effects of azoxystrobin application on zooplankton community structure are visualised in the PRC diagram presented in Figure 2. The PRC diagram of the zooplankton data set revealed small non-significant variation in the pre-treatment period but substantial treatment-dependent differences to the controls after the start of the treatments. The zooplankton community response was characterised by pronounced effects in all azoxystrobin treatments except CAT_{10}. Up until day 9, the treatment-related responses of the zooplankton community in the SAT_{33} and CAT_{33} treatments, characterised by more or less the same initial peak concentration, were similar, but after that the SAT_{33} treatment showed a trend of recovery in contrast to the CAT_{33} treatment (Figure 2). Initially (day 9), the treatment-related response of the zooplankton community was more pronounced in the SAT_{33} than in the FAT_{16} treatment, but later on, responses between these treatments (characterised by the same 42-d TWA concentration) were very similar (Figure 2). The deviation of treatments from the controls was consistent with the results of Monte Carlo tests (Table 2). The high positive species-weight ($b_k > 1.5$) of all Copepoda (copepod nauplii, Cyclopodia, Calanoida) and one Cladocera species (*Daphnia* gr. *longspina*) in the PRC diagram (Fig. 2) indicate that abundances of these taxa correlated best with the community response, herewith showing a treatment-related decline. Several taxa belonging to Rotifera, such as *Lecane* gr. *luna*, *Euchlanis dilatata*, *Synchaeta* sp. and *Scaridium longicaudum*, and the cladocerans *Alonella* sp. and *Alona* sp. had a weak negative species-weight score ($b_K < -1$; Fig. 2), suggesting a small treatment-related increase.

Table 2. Results of Monte Carlo permutation tests performed on the zooplankton data set.

Day	Control versus CAT_{10}	Control versus CAT_{33}	Control versus SAT_{33}	Control versus FAT_{16}	CAT_{10} versus CAT_{33}	CAT_{10} versus SAT_{33}	CAT_{10} versus FAT_{16}	CAT_{33} versus SAT_{33}	CAT_{33} versus FAT_{16}	SAT_{33} versus FAT_{16}
-5										
2		0.03	(0.10)	0.03						
9		0.03	0.03	0.03	(0.09)	(0.09)				
16		0.03		(0.07)						
23		0.03	0.03	0.03	(0.09)					
32		0.03			(0.09)					
43		0.03		0.03						

p-values between 0.05 and 0.10 are stated between brackets because they are only indicative for significant differences. Empty cells denote *p*-values larger than 0.100.

The dynamics of the four taxa that showed consistent statistically significant (Duncan test; $p < 0.05$) treatment related differences in the univariate analyses are shown in Figure 3. These responses at the taxon level are in accordance with their high species-weight in the PRC diagram. Treatment-related effects on nauplii became apparent soon after application, particularly for the CAT_{33} and SAT_{33} treatments, followed by FAT_{16} and CAT_{10}. The most pronounced effects in terms of magnitude and duration were observed for CAT_{33} which was significantly different from other treatments on day 32 and 43 (Fig. 3A). In the course of the experiment, mean densities of nauplii were somewhat lower in the CAT_{10} treatment when compared to controls but for CAT_{10}, statistically significant effects were only apparent on two isolated sampling days (day 2 and 43) (Fig. 3A). Again the responses of nauplii in SAT_{33} and CAT_{33} treatment (similar initial peak concentration) were similar up until day 9. At the end of the experiment, densities of nauplii were very similar in the SAT_{33} and FAT_{16} treatments (characterised by similar 42-d TWA concentration). Calanoida disappeared from the FAT_{16} microcosms two days after the first application, followed by SAT_{33}, CAT_{33} and CAT_{10}, respectively (Fig. 3B). Statistically significant differences relative to the controls remained apparent in all treatments throughout the experiment, except for the last sampling date (day 43), which was a result of a decrease of abundance of calanoids in the controls (Fig. 3B). Note that densities of Calanoida already were low in all test systems prior to fungicide application. Cyclopoida showed prominent effects in all treatments except for the treatment CAT_{10} (Fig. 3C).

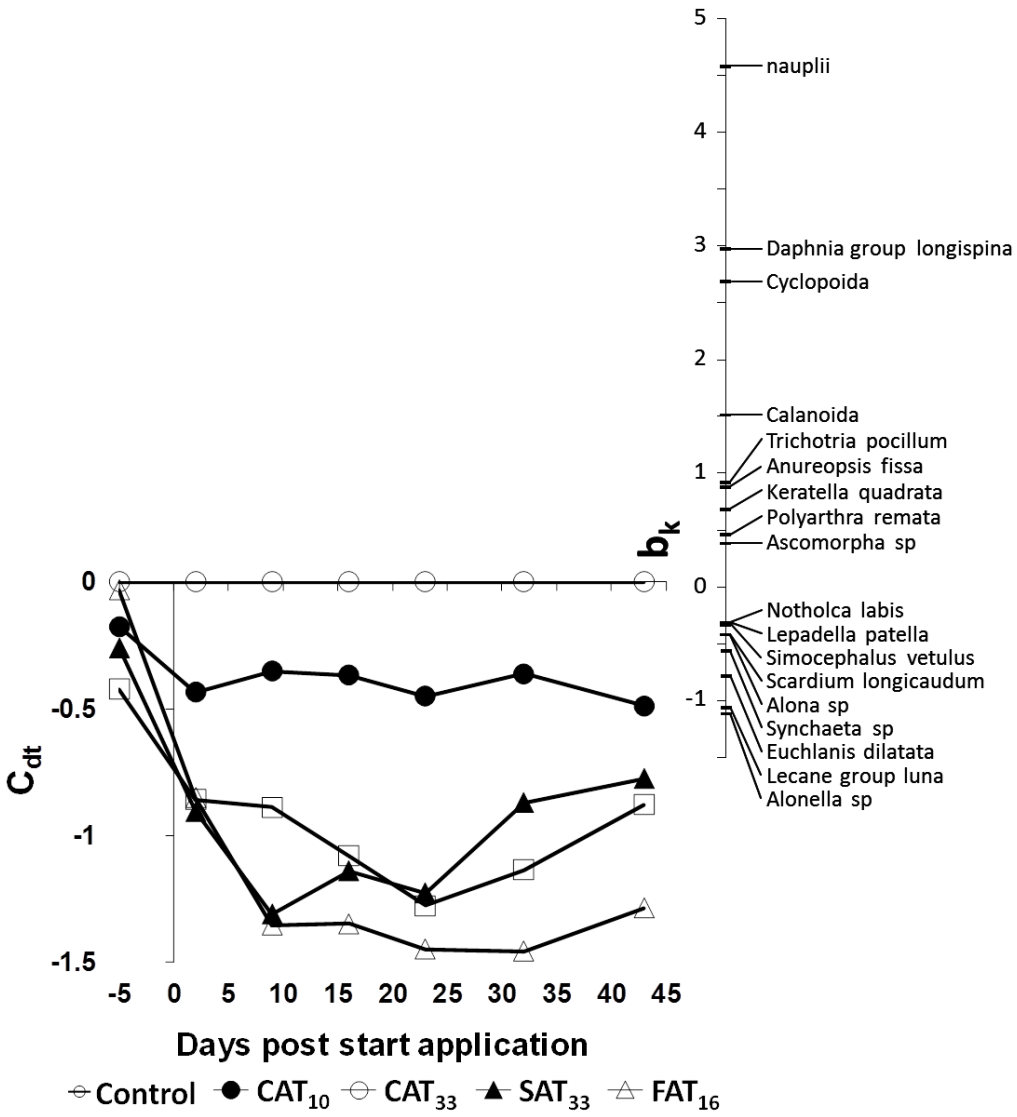

Figure 2: Principal Response Curves resulting from the analysis of the zooplankton dataset, indicating the effects of different azoxystrobin treatments. Nineteen percent of all variance could be attributed to the sampling date; this is displayed on the horizontal axis. Twenty-six percent of all variance could be attributed to treatment level, 35% of which is displayed on the vertical axis. The lines represent the development of the treatments in time. The species weight (b_k) can be interpreted as the affinity of a taxon with the Principal Response Curves (c_{dt}). Taxa with a species weight between 0.25 and -0.25 are not shown. A Monte Carlo permutation test indicated that the diagram displays a significant amount of the variance explained by the treatment (p = 0.004).

CAT_{33} was significantly different from all other treatments on days 17 and 43 and showed a decline in abundance until the last sampling date. Statistical analysis indicated that partial recovery had occurred in the SAT_{33} and FAT_{16} treatments by the end of the study. No consistent significant effects were detected for CAT_{10} (Fig. 3C). Azoxystrobin had adverse effects on the abundance of the *D.* gr. *longispina* populations in all treatments in the first week after application (Fig. 3D). *D.* gr.

longispina completely disappeared in CAT_{33} and FAT_{16} after 23 days. Increasing effects were observed after the second application on day 10 of FAT_{16} (Fig. 3D). No recovery was observed in CAT_{33}, while partial recovery was observed for SAT_{33} and FAT_{16}. At the end of the experiment, densities of *D*. gr. *longispina* were very similar in the SAT_{33} and FAT_{16} treatments (characterised by the same 42-d TWA concentration). For CAT_{10}, significant effects could be demonstrated only on day 2 (Fig. 3D).

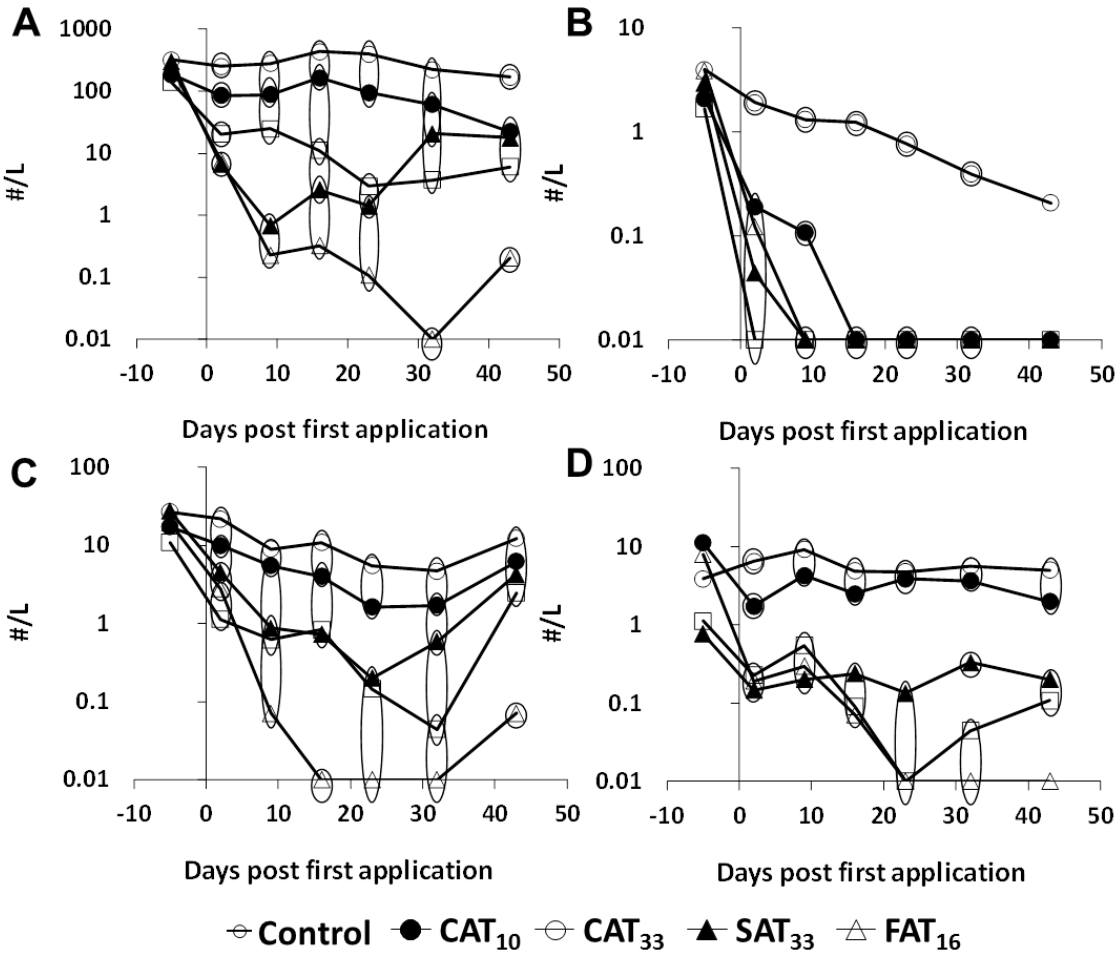

Figure 3: Zooplankton population dynamics, in numbers per litre (geometric mean), of taxa showing consistent responses to azoxystrobin treatments. Nauplii **(A),** Calanoida **(B),** Cyclopoida **(C)** and *Daphnia gr. longispina* **(D)**. Significant differences are indicated by the circles. Treatments present in the same circle did not differ significantly from each other, while those not sharing the same circle did differ significantly (Duncan test, $p < 0.05$). The value 0.01 denotes 0 numbers in the samples.

Macroinvertebrates

Over the experimental period, a total of 91 different macroinvertebrate taxa were found in the microcosms, which were dominated by Insecta (51 taxa), followed by Mollusca (19), Oligochaeta (7), Hirudinea (5), Turbellaria (5), Crustacea (3) and Hydracarina (1). Among the insects, Diptera and Ephemeroptera accounted for 36% and 14% of total abundance of macroinvertebrates, respectively, of which *Chaoborus obscuripes* and *Cloeon dipterum* were the most abundant taxa. Macrocrustaceans comprised 22% of the total macoinvertebrate abundance and were represented by *Gammarus pulex*, *Asellus aquaticus* and *Proasellus meridianus/coxalis.* Among the non-arthropods, the most abundant taxonomic groups were the Hirudinea and Gastropoda, which accounted for approximately 9% and 11% of total invertebrate abundance, respectively. *Erpobdella* sp. was the most abundant taxon in Hirudinea while *Valvata* sp. was the most abundant taxon in Gastropoda.

At the community level, the PRC analysis of the macroinvertebrate dataset indicated no effects, which was confirmed by the results of the Monte Carlo permutation tests ($p > 0.05$; results not shown). At the species level, statistically significant declines in abundance relative to controls were observed only for *Chaoborus obscuripes* (Fig. 4), in the FAT_{16} treatment in particular. For this treatment, recovery was observed at the end of the experiment (Fig. 4). A similar decline was not observed in the CAT_{33} treatment, characterised by both a higher peak concentration and a higher 42-d TWA concentration than the FAT_{16} treatment.

Phytoplankton and periphyton

Over the experimental period, a total of 201 different phytoplankton taxa were identified in the microcosms. In terms of numbers of taxa as well as total abundance, the most important taxonomic groups were Chlorophyta (green algae), Charophyta (green algae), Cyanobacteria (blue-green algae) and Bacillariophyta (diatoms). Among the Chlorophyta, the most abundant taxa were *Sphaerocystis* (Tetrasporales; Chlorophyceae) and Chlorophyta 2-5 µm, which accounted for 16% and 3% of the total phytoplankton abundance, respectively. For Cyanobacteria, the most abundant taxa were *Chroococcales* 2-5 µm colony (53%), *Chroococcales* 1-2 µm colony (7%), followed by *Pseudanabaena* (4%) and *Pannus planus* (3%).

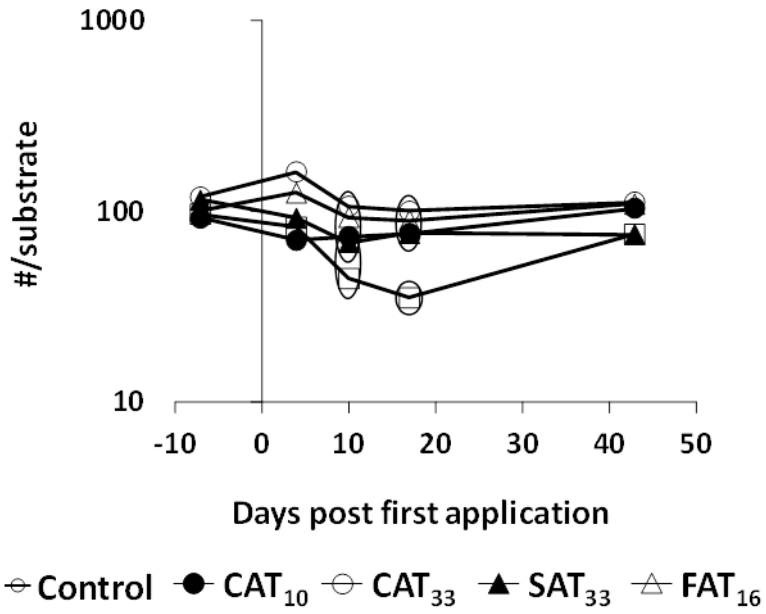

Figure 4: Population dynamics of *Chaoborus obscuripes* in numbers per substrate (geometric mean). Significant differences are indicated by the circles. Treatments present in the same circle did not differ significantly from each other, while those not sharing the same circle did differ significantly (Duncan test, $p < 0.05$).

The multivariate analysis showed no significant community-level responses to the treatment (Monte Carlo permutation test, $p > 0.05$). In addition, consistent and statistically significant effects at the population level were only detected for *Tetraedron minimum*, which showed a significant reduction in $CAT_{10,}$ SAT_{33} and FAT_{16} on the last two sampling dates relative to controls.

Chlorophyll-*a* content of phytoplankton ranged between 0.00 and 36.76 µg/L. For periphyton values ranged between 10.09 and 58.97 µg/cm^2. No statistical differences between the various treatments and the controls were detected (Duncan test, $p > 0.05$).

Macrophytes

Over the experimental period, a total of 14 different species of macrophyte were monitored in the microcosms. Rooted submerged macrophyte formed the majority of taxa, comprising of 3 *Potamogeton* species followed by 2 *Elodea* species. The multivariate statistical analysis indicated that the macrophyte community was not significantly affected by azoxystrobin (Monte Carlo

permutation test, $p > 0.05$). Univariate analysis of populations indicated statistically significant deviations (Duncan test, $p < 0.05$) for *Spirodela polyrhiza*, by the end of experiment.

For the bioassays, 14 days after the start of the azoxystrobin exposures, the number of shoots of *M. spicatum* was significantly higher in CAT_{10} and CAT_{33} compared to the controls (Fig. 5A). The mean length of the shoots was significantly reduced in CAT_{33} at the same sampling time (day 14, Fig. 5B). Significant effects on dry weight of roots were also detected 14 days after the azoxystrobin application in the SAT_{33} and CAT_{33} treatments (Fig. 5C). No consistent significant effects were detected in any treatment on other endpoints (i.e., dry weight of shoots and total length of shoots), nor on the final biomass of macrophyte species (mean dry weight for all cosms = 128 ± 27 g dw/m^2, mean ± SD).

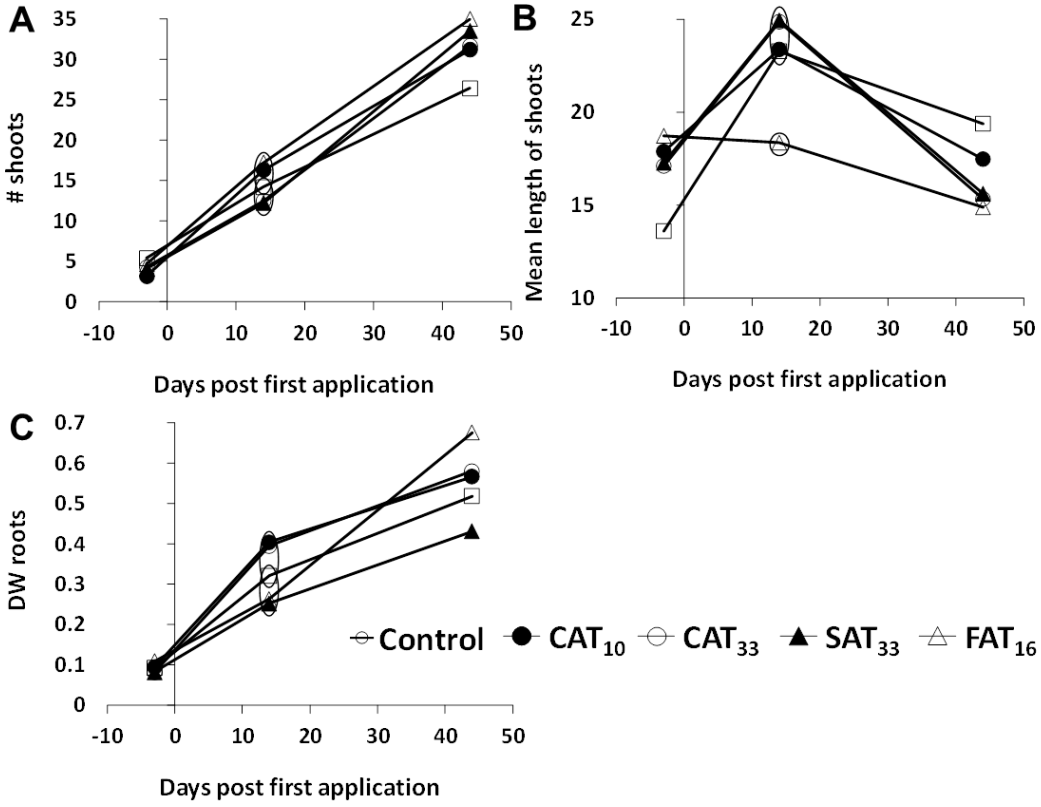

Figure 5: Results of the bioassays performed with *Myriophyllum spicatum*. Number of shoots **(A)**, mean length of shoots **(B)** and weight of roots **(C)**. Significant differences are indicated by the circles (Duncan test, $p < 0.05$). Treatments present in the same circle did not differ significantly from each other, while those not sharing the same circle did differ significantly.

Decomposition

No significant effects of the azoxystrobin treatments (Duncan test, $p > 0.05$) were detected in the breakdown of particulate organic matter (POM). The remaining dry weight of leaves in litterbag over the whole experimental period including all microcosms was 80 ± 7% (mean ± SD).

Water quality analysis

The water quality variables DO, EC, T and alkalinity did not reveal consistent treatment-related responses and mean values in all microcosms during the entire experimental period were 10.6 ± 1.0 mg/L; 115 ± 14 µS/cm^2; 19 ± 0.8 °C, and 0.84 ± 0.05 meq/L, respectively. An increase in pH was observed for most treatment levels, but kept within one pH unit (Fig. 6). At day 16, pH values were statistically significantly elevated in CAT_{10}, SAT_{33} and FAT_{16} while on day 23, in CAT_{10}, CAT_{33} and SAT_{33}. Notably, deviations of these treatments were statistically significant relative to control rather than from each other (Fig. 6). All treatment regime pH values were significantly different to the control at the end of experiment (Fig. 6).

The concentrations of the ammonia, nitrate and nitrite, and ortho-phosphate were below the LODs of 0.04, 0.03 and 0.02 mg/l, respectively. No significant effects were found on the total phosphate and total soluble nitrogen levels (concentrations between 0.60 and 1.01 mg/L; < LOD and 0.14 mg/L, respectively).

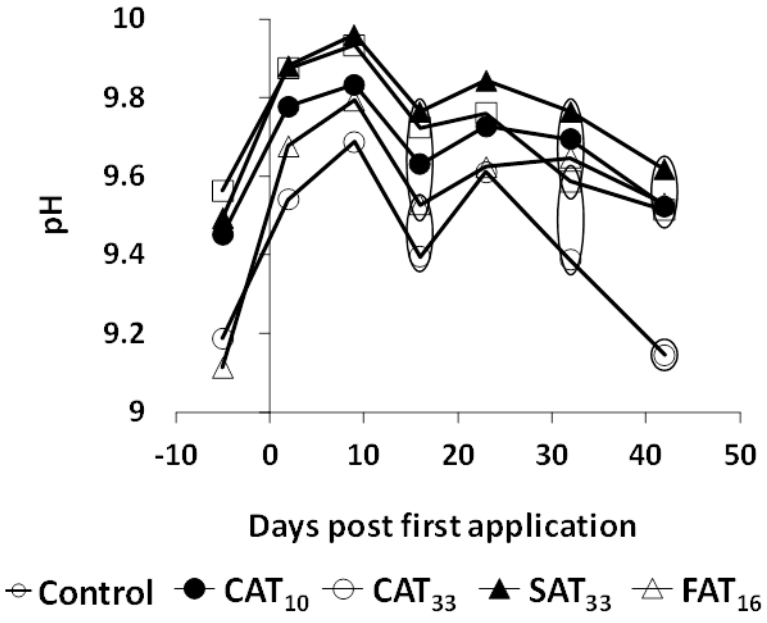

Figure 6: Dynamics of pH in the different treatments. Significant differences are indicated by the circles. Treatments present in the same circle did not differ significantly from each other, while those not sharing the same circle did differ significantly.

Discussion

Comparison across time-variable exposure regimes (similar Peak versus similar 42-d TWA)

To determine which effects can be assessed by the peak and TWA_{42d} exposure concentrations, treatment-related impacts of SAT_{33} and CAT_{33} were compared because they had a similar initial peak concentration (different TWA), and those of SAT_{33} and FAT_{16} are compared because they had similar TWA concentrations (but different peak concentrations). At the community level, the PRC diagram resulting from analysis of zooplankton data-set (Fig. 2) elucidates small and similar sized magnitude of effects among different time-variable exposure profiles (SAT_{33}, FAT_{16} and CAT_{33}) shortly after the start of the experiment (day 2), while the effects in the SAT_{33} and CAT_{33} increased in magnitude on day 9. The time-variable exposure regimes that have similar initial peak concentrations (SAT_{33} and CAT_{33}) resulted in comparable effects on the zooplankton community until day 9, but not afterwards. This indicates that the peak concentration is a good predictor of short-term effects only. The magnitude of effect in SAT_{33} was pronounced relative to FAT_{16} until day 9, after which the magnitude of effects increased in FAT_{16} to become similar to that in SAT_{33} (from day 16 onwards). Since both these pulsed treatment regimes are characterised by the same 42-d TWA concentration, it can be concluded that the similar TWA concentrations cause a comparable effects on the zooplankton community in the long-term. These results support the ELINK recommendation that for long term effects the TWA concentration can be more relevant than the peak concentration (Brock *et al*. 2010). During the last few years, several workshops and projects have proposed using the TWA concentration approach, instead of the nominal peak concentration for assessing effects of repeated exposures (ECOFRAM, 1999; Boxall *et al*. 2001; Reinert *et al*. 2002; Boesten *et al*. 2007; Brock *et al*. 2010).

The finding that TWA is a better predictor of effects also holds true at the taxon level. Several taxa such as nauplii, adult Cyclopoida and *Daphnia* gr. *longispina* clearly show similar survival responses to the different time-variable exposure regimes (Fig. 3A, C and D), i.e. similar effect magnitude for the SAT_{33} and CAT_{33} treatments (similar peak concentration) during the first 1.5 week after the first application and similar effect magnitudes for the FAT_{16} and CAT_{33} treatments (similar TWA concentrations) at the end of the experiment.

For the risk assessment of azoxystrobin, it is not so surprising that the short-term effects observed due to different peak concentrations in the microcosm experiment can be related to measured or predicted peak concentrations. The results, suggest that long-term effects can be assessed by comparing TWA rather than peak concentrations, even if the dynamics of the pulses are different. Zafar *et al*. (2011) also found similar relationships between long-term effects and TWA

concentrations for most invertebrate species exposed to chlorpyrifos in microcosm under different exposure profiles with the same TWA concentration. They also concluded that for the applied combination of concentration dynamics, the TWA concentration was a more adequate predictor for long-term risks of chlorpyrifos for most species than the peak concentration. After performing long-term toxicity tests with *Gammarus. Pulex*, Ashauer *et al*. (2007c) also concluded that the TWA concentration approach can be used to predict effects of repeated pulses of chlorpyrifos and pentachlorophenol.

Fate of azoxystrobin in the water column

The dissipation rate of azoxystrobin in the SAT_{33} treatment of the present study (18 days) is consistent with those of previous studies, which reported half-life values in the range of 15-25 d (Gustafsson *et al*. 2010) and 13 days (Jones and Lake, 2000). The dissipation is probably a result of photolysis, since the US-EPA (1997) reported a half-life of 11 to 17 days in aquatic environments for photolysis only. Also, the potential for accumulation in sediments is low (log K_{ow} =2.50) and azoxystrobin is a non-volatile compound (Henry's Law constant = 7.3 10^{-9} Pa x m^3/mol (Tomlin, 2011). The higher peak concentration in all treatments measured 4 h after application compared to the nominal concentrations may be attributed to non-homogeneity of azoxystrobin in the water layer as a result of dominant aquatic vegetation and therefore incomplete mixing of dosed solutions within the microcosm.

Biological effects of azoxystrobin

The PRC diagram of the zooplankton community indicated pronounced treatment dependent negative impacts of azoxystrobin (Fig. 3). The largest adverse effects were reported for nauplii, adult Calanoida and Cladocera, followed by adult Cyclopodia. For most taxa these changes persisted until the end of the study (Fig. 2 and 3). These results are consistent with the other model ecosystem studies available and suggest that some copepods are sensitive to azoxystrobin (Cole *et al*. 2000; Gustafsson *et al*. 2010). Furthermore, in the present study, naupliar stages of copepods were found to be more sensitive to azoxystrobin than adult cyclopoids, which is in agreement with observations reported by Gustafsson *et al*. (2010).

The decrease in numbers of calanoid copepods in all treatments just after the start of the treatment (Fig. 3B) is similar to other observations by Lauridsen *et al*. (2003). They performed a series of acute and sub-chronic toxicity tests with azoxystrobin on several different freshwater zooplankton and macroinvertebrate species and found that the calanoid copepod *Eudiaptomus graciloides* was the most sensitive among the tested taxa. In the present study, cyclopoid copepods

were vulnerable to all treatments except for CAT_{10} (Fig. 3C). This is not in line with the observations by Cole *et al*. (2000) who reported effects directly after a single application of 10 µg/L. This may be a result of differences between the species composition of the Copepoda community. The effects in this study agree with the laboratory tests performed by Lauridsen *et al*. (2003) with the cyclopoid copepod *Cyclops vicinus*, in which all individuals died within 48 h when exposed to 20 µg/L or higher, and a NOEC for reproduction of 10 µg/L was reported. Effects on Copepoda populations have also been reported longer than 3 weeks period on Copepoda populations after a single application of 3, 7.5, 15, and 60 µg/L (Gustafsson *et al*. 2010).

The PRC diagram and univariate analysis showed a negative impact of azoxystrobin on one taxon of Cladocera, i.e. *D.* gr. *longispina*, while other taxa like *Simocephalus vetulus*, *Alona* sp. and *Alonella* sp. experienced no effects (Fig. 2). This is in accordance with the study of Cole *et al*. (2000), who reported significant reductions of *Daphnia* spp. after single applications of 10 and 30 µg/L and also reported significant increase in numbers for *Chydorus* sp. It was shown by Lauridsen *et al*. (2003) that some cladocerans (*D. magna*, *Chydorus sphaericus* and C*eriodaphnia* sp.) were relatively tolerant to azoxystrobin while others (*D. galeata*) were much more sensitive. Their physiological experiments (e.g. pectoral limb, hind claw, mandible and heart activity) clearly demonstrated that azoxystrobin may affect zooplankton in different ways.

Treatment-related impacts on the macroinvertebrate community were not found. On the basis of information already known for azoxystrobin, it is reasonable to assume that invertebrates, in particular macroinvertebrate crustaceans and insects, are not highly sensitive to azoxystrobin, which is supported by the results of Cole *et al*. (2000). *C. obscuripes* was the only macroinvertebrate species which responded significantly to the FAT_{16} treatment (Fig. 4). The observed effect in the FAT_{16} treatment is probably not a direct effect since it did not show a clear treatment-response pattern. The effects observed are consistent with the microcosm study conducted by Cole *et al*. (2000) who reported no significant effects on any macroinvertebrates after single applications of 10 and 30 µg/L and observed some effects on Gammaridae and Mollusca at 100 µg/L. Lauridsen *et al*. (2003) reported no effects on *Chaoborus flavicans* up to azoxystrobin concentrations of 6000 µg/L and no significant effects were detected on Chaoboridae after a single application of 1000 µg azoxystrobin/L in the microcosm study performed by Cole *et al*. (2000). The observed effects might be a result of temporally decreased food availability in the form of *D.* gr. *longispina* (Fig. 3), while its fast recovery can be explained by the multivoltine life cycle of *C. obscuripes* in The Netherlands (Van Wijngaarden *et al*. 2006).

Further studies performed by Lauridsen *et al*. (2003) on three other macroinvertebrate species, i.e. *Chironomus plumosus*, *Cloeon dipterum* and *Hydropsyche angustipennis,* also revealed no effects at treatment levels of 525, 1500 and 3000 µg/L, respectively. Apparently, the sensitivity-tolerance spectrum for this chemical is very wide for different arthropod species.

Compared to the controls, the composition of the phytoplankton community was not altered by the azoxystrobin treatments, which is in agreement with Cole *et al*. (2000). On day 22, the abundance of *Tetraedron minimum* had increased in the control and the CAT_{33} treatments, and abundance was still elevated at day 42 in these treatments. It is difficult to explain the significantly lower numbers in the other treatments relative to the control, as a result of direct or indirect effects of azoxystrobin as effects were not observed in the treatment with the highest peak and TWA concentration (i.e. CAT_{33}). A significant increase of the macrophyte *Spirodela polyrhiza* coverage occurred in the CAT_{33}, SAT_{33} and FAT_{16} treatments in the range of % cover of < 1% and is, therefore, not considered important from ecological point of view. The absence of systematic effects on macrophytes in this study (Fig. 5) is in agreement with the high growth EC_{50} (3200 µg/L) determined for the macrophyte *Lemna gibba* in a laboratory toxicity study (Tomlin, 2011). As a result of this lack of effects on macrophytes, the water physico-chemical parameters were not greatly influenced by azoxystrobin treatments.

Of all the physico-chemical variables, pH values in treated systems were slightly (and sometimes statistically significantly) higher than in the controls (Fig. 6). This could be a result of increased algal biomass due to a reduced grazing pressure by *D.* gr. *longispina*, but this was, however, not reflected in significant increases in phytoplankton and periphytonic chlorophyll-a. The observations in the present study are in accordance with Gustafsson *et al*. (2010) who found marginally significant differences on one sampling day for chlorophyll-a concentration in water at azoxystrobin concentrations of 5 and 20 µg/L, although it should be noted that this experiment was performed in brackish water. The increase in pH values remained within one pH unit and is, therefore, considered to be of low ecological relevance. In line with this, Cole *et al*. (2000) also found significant differences in pH relative to the control, following a single application of 10 µg/L, but on individual sampling dates only. No effects were observed on decomposition in all treatment regimes which is consistent with Gustafsson *et al*. (2010) who reported that degradation of organic material in their microcosms was not affected by azoxystrobin treatment during the course of experiment.

According to the EU Guidance Document on Aquatic Ecotoxicology, the No Observed Ecologically Adverse Effect Concentrations (NOEAEC) is the concentration at or below which no long-lasting adverse effects were observed in the microcosm study (European Commission, 2002). If we

translate short-lasting effects to effect class 1 or 2 (based on effect classes; Brock *et al*. 2006), the NOEAEC of this study can be set at the CAT_{10} treatment, which is in accordance with the NOEAEC of a previous microcosm study (Cole *et al*. 2000) which was set at a single application of 10 µg/L.

Conclusions

The present study shows that under the tested exposure regimes and for the endpoints studied, the TWA is a more adequate predictor for long-term effects of azoxystrobin on the zooplankton community and species than the peak concentration. These results support the recommendation of the ELINK workshop that for long-term effects of pesticides, a risk assessment based on TWA concentrations may be more relevant than one based on peak concentrations. It should be noted however that this conclusion only applies to the zooplankton community and species, since effects on other endpoints were limited.

Acknowledgements

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Chapter 6

General discussion and concluding remarks

"It may not seem very important, I know, but it is, and that's why I'm bothering telling you so"

(Dr. Seuss)

"Look deep into Nature…….

……and then you will understand everything better"

(Albert Einstein)

The human population is still increasing exponentially and everyone in the world depends completely on the Earth's ecosystems and the services they provide, such as food, water, disease management, climate regulation, and aesthetic enjoyment. In the second half of the twentieth century, humans have changed the structure and functioning of the world's ecosystems more rapidly and extensively than at any time in human history, largely to meet rapidly, growing demands for food, fresh water, and fuel etc. Therefore, ecosystems are under escalating pressure and facing threats from human activities and a growing world population. This has resulted in substantial changes in marine and freshwater ecosystems, temperate grasslands, forests globally, and the depletion of fish stocks and an alarming and continuous loss in the biodiversity of life on Earth (all above cited from Millennium Ecosystem Assessment, 2005). Because of the increasing human population, agricultural activity needed to grow rapidly and an increased use of chemicals in terms of artificial fertilizer and plant protection products was required to satisfy and maintain the local and global demand for higher food production (Firbank *et al*. 2012). Hence, modern agriculture practises rely on the usage of synthetic pesticides (e.g. insecticides, herbicides and fungicides) in order to reduce crop losses due to pests and disease to achieve higher crop yields. The amount of pesticides used as part of industrial agriculture's methods significantly increased the chemical load on ecosystems (Timlan *et al*. 2001). Pesticides applied in crops may enter water bodies adjacent to agricultural fields via different entry routes such as spray drift, agricultural run-off, leaching and/or drainage (Brown *et al*. 2004; Dabrowski *et al*. 2002). These contaminations may have undesirable impacts on the ecology of fresh water ecosystems (Van den Brink, 2008; Liess *et al*. 2005; Holvoet *et al*. 2007). In order to prevent unacceptable, adverse effects on non-target aquatic communities of natural aquatic ecosystems, the evaluation of these effects is legally part of European Registration Procedures of pesticides (European Commission, 1991; 2009).

The present thesis aims to contribute to the issue of how to extrapolate effects characterized under relatively simple exposure regimes to the more complex exposure patterns occurring in the real world. Linking exposure and effects has been a challenge in ecotoxicology for many years (Boesten *et al*. 2007), especially because of the mismatch between the exposure profiles used in the experiments providing the pesticide effect data and the more complex and variable exposure profiles predicted to occur under natural conditions (Brock *et al*. 2010). In this thesis, I focus on evaluating the effects of different time-variable exposure regimes of pesticides on aquatic species and communities. For this purpose, I performed laboratory and semi-field experiments in order to compare the effects of different time variable exposure patterns (single, multiple and chronic exposure patterns), all of which are comparable in their time-weighted average (TWA)

concentration towards freshwater communities. In this synthesis, I will focus on the comparison of impacts of time-variable exposure regimes on aquatic communities and ecosystems, using not only the results from the experiments presented in this thesis **(Chapters 3, 4 and 5)** but also by performing a literature review on the effects of pesticides as observed in semi-field experiments **(Chapter 2).**

Dilemma and limitation of standard toxicity tests and risk assessment

Understanding and predicting the spatial and temporal impacts of pesticides on population and communities of organisms in aquatic ecosystems involves a combination of different disciplines, comprising environmental chemistry, toxicology and ecology (Van den Brink, 2008; Schmitt-Jansen *et al*. 2008; Solomon *et al*. 2008; Forbes *et al*. 2009). The myriad of effects induced by pesticides and other toxicants on flora and fauna have been the subject of an enormous number of studies in the past several decades. Standard toxicity tests provide the basic information about the potential toxicity of chemicals (Brock *et al*. 2010). The results of these toxicity experiments are evaluated by statistical models in order to get dose-response relationships, which give the magnitude of a certain response (e.g. mortality or mobility) over a range of exposure concentrations. From these dose-response relationships, an EC50, or LC50 i.e. the concentration that affects or, respectively, kills 50% of tested organisms and no observed effect concentrations (NOECs) may be derived. These toxicity parameters are used in the first tier of prospective risk assessment, intending to derive conservative "safe concentrations" (e.g. Brock and Van Wijngaarden, 2012). Standard toxicity tests are carried out at constant concentrations over a fixed duration of time and follow standardized guidelines (e.g. OECD, 1984). The higher tier risk assessment of pesticides for the aquatic environment may include the results of semi-field studies (i.e. cosm studies) (e.g. EU 2002). An overwhelming fraction of the cosm experiments published in the ecotoxicological literature, especially when focusing on invertebrates, involves an evaluation of a single application for assessing the impacts of pesticides. Fewer cosm studies evaluated multiple applications, and single and multiple applications are rarely compared. In realistic agricultural practises, however, often multiple applications are applied for the adequate protection of crops. Therefore, aquatic ecosystems surrounding agricultural fields are mainly subject to repeated pulses of pesticide inputs, which may cause direct and indirect effects on aquatic life (e.g. Schäfer *et al*. 2010).

Community level effects of time-variable exposure patterns

The issue of how to link complex fate scenarios with their caused effects was addressed in two recent EU workshops, called ELINK (Brock *et al*. 2010). One of the recommendations of these ELINK workshops is to investigate whether for the aquatic risk assessment of pesticides the peak concentration or the time-weighted average (TWA) concentration should be used when the predicted field exposure is variable in time. It is proposed in the ELINK document that further experimental work is required to scientifically investigate whether the TWA concentration approach is appropriate to be used for assessing long-term effects (Brock *et al*. 2010). In the scientific literature, some information on the effects of time-variable exposure to pesticides is available from laboratory studies (see Brock *et al*. 2010. for a small review), but results from semi-field experiments are mostly lacking. Therefore, the central focus of this thesis is whether the TWA is a more adequate predictor for long-term effects of pesticides as observed in semi-field experiments on communities and species than the peak concentration (Fig. 1).

According to Haber's law, different exposure patterns that have the same Area Under the Curve (AUC) concentration are assumed to have the same effect. The effect of pesticides may be similar when aquatic organisms are exposed to a high concentration for a short time or for a longer time to a low concentration. This phenomenon is called reciprocity. Haber's law states that toxicity is the product of concentration and time, and is the basis of the time-weighted average (TWA) approach where an exposure concentration is integrated over time (AUC) and then divided by a certain time period (Giesy and Graney, 1989). Theoretically, reciprocity should only apply where both uptake and elimination of a compound into the test organism (toxicokinetics, TK), and damage and repair processes (toxicodynamics, TD) have reached a steady state (Rozman and Doull, 2000). In general, however, it may be expected that the longer the duration of toxicity experiments, the higher the probability that TK and TD will approach a steady state during the study period. In long-term toxicity tests with *Gammarus pulex*, Ashauer *et al*. (2007c) demonstrated that the TWA concentration approach can be used to extrapolate results of a long-term test using pulsed exposure regimes to other long-term exposure profiles for both chlorpyrifos and pentachlorophenol. This and other observations invite the use of the TWA concentration approach in risk assessments when evaluating long-term exposure patterns.

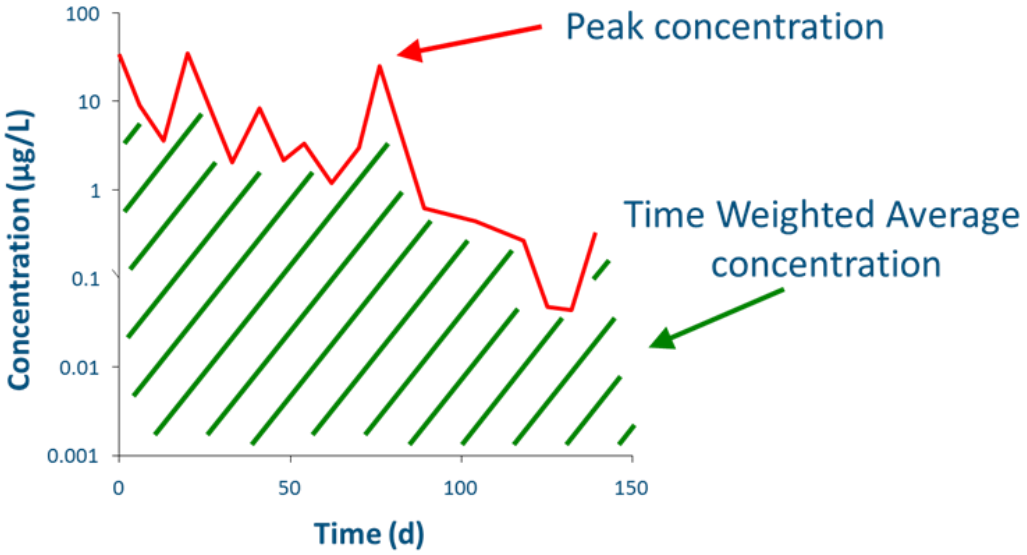

Figure 1: *Schematic representation of the central theme of the present thesis: Which type of concentration, the TWA or the peak, is more appropriate to assess the long-term risks of pesticides when the predicted or measured exposure profile is variable in time?*

Comparison of time-variable exposure regimes: peak versus TWA

In order to understand the effect pattern resulting from different time-varying exposure profiles, the scientific literature describing the effects of insecticides studied in cosm studies was reviewed. This to allow a comparison between the effects as observed in cosm experiments due to the peak concentrations of the exposure profiles evaluated in the studies and their TWA_{21d} concentrations. This comparison was performed using different sensitive endpoints (microcrustaceans, macrocrustaceans and insects) for separate chemicals and for groups of chemicals with the same toxicological mode of action **(Chapter 2)**. When groups of chemicals were analysed together, their concentrations were scaled to toxic units (TU) using the median laboratory sensitivity of arthropods towards the corresponding chemical. Figure 2 presents the comparison of effects for microcrustaceans and chlorpyrifos, when the concentration is expressed as the peak and the TWA_{21d} concentration. The results shown for microcrustaceans and chlorpyrifos show a clearer (distinct) dose-response relationship in case of TWA_{21d} exposures, when compared to peak exposure (Fig. 2A, B). It points out that for this type of insecticides (acetylcholinesterase inhibitor), the TWA_{21d} concentration can be used as a good predictor for long-term effects on sensitive endpoints. It is apparent from Figure 2 that when scaled on peak exposure concentrations, clear effects were generally observed for microcrustaceans at concentrations of 0.1 µg/L and higher, while when expressed as TWA_{21d} concentrations, they were reported at concentrations higher than 0.05 µg/L (Fig. 2; compare left (A) and right panel (B)). All effects observed at insecticide peak concentration

lower than 0.5 µg/L relate to chronic or multiple exposure studies. On the basis of this comparison between peak and TWA_{21d} concentrations, it was found that in the case of single applications, direct effects became apparent at TWA_{21d} exposure concentrations which were a factor of 5 lower than their peak exposure concentrations, while the factor is slightly lower for multiple applications and 1 in case of a chronic exposure regime. We therefore propose an extrapolation factor of 5 to scale peak exposure due to a single application of an acetylcholinesterase inhibiting insecticide to a TWA_{21d} concentration, which can be used in assessing the long-term effects feeding into the risk assessment of time-variable exposures **(Chapter 2).**

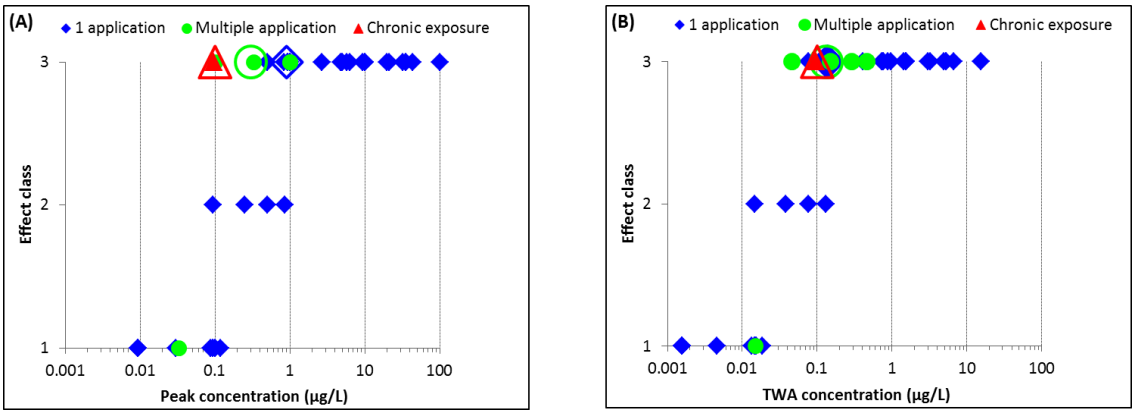

Figure 2: Comparison of effects of the organophosphate insecticide chlorpyrifos for the most sensitive endpoint "Microcrustacea" from **Chapter 3** of this thesis and literature values from freshwater model ecosystem studies as reviewed in **Chapter 2**. The figure comprises responses found in studies applying single applications, multiple applications and chronic exposure regimes. The effects are summarised according to magnitude: **1** = no effect, **2** = slight effect, **3** = clear effect. The x-axis displays the exposure concentration of chlorpyrifos evaluated in the cosm studies expressed as the peak **(left panel: A)** and TWA **(right panel: B)** concentrations. The responses of chlorpyrifos with different time-variable exposure regimes from **Chapter 3** are indicated by large unfilled symbols.

It is concluded that the TWA_{21d} concentration may provide the best correlation to the long-term ecotoxicological effects and can, thus, be considered as the Ecotoxicologically Relevant Concentration (ERC; Boesten *et al*. 2007) for acetylcholinesterase inhibiting insecticides **(Chapter 2, 3).** López-Mancisidor *et al*. (2008) studied the effects of multiple applications of the insecticide chlorpyrifos on plankton-dominated mesocosms under Mediterranean conditions and found that the threshold levels for long-term exposure (expressed as TWA_{7d} concentration) are up to a factor of 10 lower than the threshold levels observed in single application studies (expressed as peak concentrations). The difference between the factor proposed in this thesis and by López-Mancisidor *et al*. (2008) can be fully explained by the difference in time-span used to calculate the TWA. Several

groups have proposed to use the TWA concentration approach, instead of the peak concentration, for assessing effects of repeated exposures (ECOFRAM, 1999; Boxall *et al*. 2001; Reinert *et al*. 2002; Boesten *et al*. 2007; Brock *et al*. 2010), which is in line with the findings of **Chapter 2 and 3**.

Contrary to the acetylcholinesterase inhibitor insecticides, no clear dose-response relationship was found when classified effects were summarised for sodium channel modulator insecticides and ranked based on their Peak-TU and TWA_{21d}-TU concentrations (**Chapter 2**). For moulting inhibiting insecticides, when effects on microcrustaceans are scaled to their Peak-TU concentrations, the dose-response relationship of microcustaceans looks slightly better compared to when the scaling is based on the TWA_{21d}-TU concentrations, although the opposite was true for insects. Interestingly, for the insecticide diflubenzuron a clear dose-response relationship was found for both standardisations (Peak-TU and TWA-TU), with no preference for either of them (**Chapter 2**).

On the basis of the comparisons between peak and TWA concentrations we found that clear effects became apparent at TWA exposure concentrations that were a factor of 5 lower than when the same studies were evaluated on the peak concentrations (Fig. 2; left and right). The factor is, however, based on a range of studies that used different experimental set-ups, different ways of applying the compound, studied different endpoints and/or sampled the same endpoints in a different way, etc. Therefore, we tested this rule-of-thumb in a specifically designed experiment performed with chlorpyrifos. This experiment aimed at gathering empirical evidence for the use of either concentration as the ERC by comparing the effects of different time-variable exposure regimes with the same TWA_{21d} concentration but different peak concentrations towards freshwater communities **(Chapter 3).** As expected, chlorpyrifos exposure caused a decrease in densities of species belonging to the arthropod community, with the largest adverse effects reported for mayflies (*Cloeon dipterum*) and cladocerans (*Daphnia longispina*, *Alona* sp.*)*, followed by other insects (e.g. Phryganaidae and crustaceans (e.g. adult *Cyclopoids*, *nauplii* and *Gammarus pulex*). By the end of the experimental period, the multivariate principal response curve analysis showed the same effects magnitude for all treatment regimes, for both the zooplankton as the macroinvertebrate community (**Chapter 3**). This indicates that for long-term effects indeed the TWA_{21d} is more important than the peak concentration. This is, however, not true for one species, i.e. the mayfly *C. dipterum*, for whom the peak concentration seemed most important. The threshold values of this study are highlighted in large symbols in Figure 2. On the basis of comparing peak and TWA_{21d} concentrations from experimental evidences we found that our findings from **Chapter 3** are in accordance with the findings of literature values presented in **Chapter 2.**

A similar microcosm study was performed with another type of chemical, again in order to explore which type of concentration, the TWA or the peak, is a better predictor for long-term effects

of pesticides. This study compared the effects of four different exposure regimes (two chronic exposure profiles, one repeatedly pulsed and one single pulsed exposure regime) of the fungicide azoxystrobin. Out of them, two exposure regimes had similar peak but different TWA_{42d} concentrations, while the two pulsed regimes had different peak but similar TWA_{42d} concentrations. The TWA_{42d} concentrations of the two pulsed exposure regimes were intermediate relative to the two chronic regimes **(Chapter 5).** The findings of this experimental study showed that the zooplankton community exhibited significant alterations after the exposure, with the largest adverse effects reported for nauplii (copepod juveniles) and cladocerans (*D. longispina.)*, followed by Cyclopoidia and Calanoida. By the end of the experimental period, the principal response curve analysis showed the same effects magnitude for the pulsed treatment regimes, which are placed in between the chronic treatment regimes. This shows that for long-term effects of azoxystrobin, the TWA_{42d} correlates better with the effects observed on most zooplankton species than the peak concentration. Azoxystrobin only slightly affected some species of the macroinvertebrate, phytoplankton and macrophyte assemblages **(Chapter 5).** These findings are in accordance with observations reported by Gustafsson *et al*. (2010).

The findings of **Chapter 5** also support that the TWA_{42d} is the ERC for zooplankton species for another chemical than acetylcholinesterase inhibitors, while the findings as presented in **Chapter 2** suggest that this is not the case for pyrethroids and moulting inhibitors. It must be stated that the experiments presented in **Chapter 3 and 5** were specifically designed to answer the research question of this thesis while the experiments reviewed for **Chapter 2** were performed to answer different research questions (mostly to determine peak effect threshold concentrations). Therefore, in order to verify or falsify the results obtained in **Chapter 2**, experiments using mode of actions different from chlorpyrifos and azoxystrobin should be performed. Given the evidence up to date, this thesis supports that, whatever time varying exposure profile is evaluated, the TWA concentration might provide a better linkage between the exposure and long-term effects of insecticides and fungicides than the peak concentration (Reinert *et al*. 2002; Brock *et al*. 2010; Boesten *et al*. 2007; ECOFRAM, 1999; Boxall *et al*. 2001; **Chapter 2, 3, 5**). It must be stated that the evidence for fungicides is very scarce and it would be useful to perform a review as presented in **Chapter 2** for fungicides. For herbicides such a review would also be helpful, since only scarce information is available. However, Belgers *et al*. (2011) observed that TWA concentration can also be used as the ERC to assess the long-term effects of a herbicide to a macrophyte species. He studied the effects of the herbicide metsulfuron-methyl on the growth of the submerged macrophyte *Myriophyllum spicatum* under laboratory conditions using different exposure scenarios (the same TWA_{21d} concentrations but different peak exposure concentrations) and found that the TWA_{21d} or

TWA_{42d} concentrations were a better predictor of the treatment-related responses than the peak concentration (Belgers *et al*. 2011).

Species level effects of time-variable exposure patterns

The multivariate analysis of the community data sets **(Chapter 3),** shows that all time-variable exposure regimes of treatments have similar effect magnitudes at the end of the experimental period, indicating that the TWA concentration is more important than the peak concentration for assessing long-term risks of chlorpyrifos. This finding, however, does not hold true for all arthropod populations. The mayfly *C. dipterum* showed a different survival response to the different time-variable exposure regimes, compared to the water flea *D. longispina* (Fig. 3). *D. longispina* showed the response as extracted from the dominant community response, while *C. dipterum* only responded to the single application treatment. For the long-term effects of chlorpyrifos to *C. dipterum*, thus, the peak concentration is more relevant than the TWA_{21d} concentration. This difference in response could be related to a difference in toxicokinetics/toxicodynamics (TKTD) of chlorpyrifos in individuals of this species **(Chapter 4).** Intrinsic sensitivity is a product of the processes which include TK (uptake, biotransformation and elimination of the compound) and TD (internal damage, recovery and threshold) of a compound (Ashauer *et al*. 2006; Rubach *et al*. 2010a). Therefore, differences in field responses of species towards time variable exposure may relate to differences in the TKTD of chlorpyrifos in these species. The TK of chlorpyrifos in several freshwater arthropods has been investigated previously and a high variation in parameter values was observed between species (Rubach *et al*. 2010a). **Chapter 4** presents experimental and modelling results to determine parameter estimates for the TD parameters for a few species.

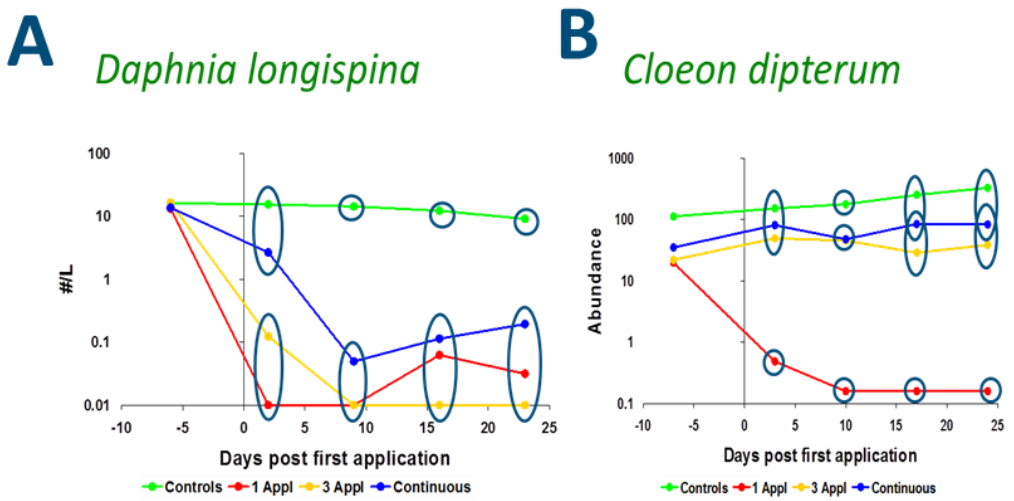

Figure 3: *Response of the water flea* (Daphnia longispina) ***(A)*** *and the mayfly* Cloeon dipterum ***(B)*** *to different chlorpyrifos exposure regimes having the same* TWA_{21d} *as observed in a microcosm experiment* ***(Chapter 3)****. Treatment time-points that share a circle do not differ statistically significantly from each other.*

Long-term survival experiments were performed in order to assess how these arthropod species respond to different time-varying exposures of chlorpyrifos in their survival and mobility. The results were used to estimate the TD parameters of a TKTD model (Threshold Damage Model (TDM), Ashauer *et al*. 2007a; b; c) for the insecticide chlorpyrifos and several aquatic macroinvertebrates and to compare the parameter estimates among species **(Chapter 4).** The experiment quantified mobility and survival of the four freshwater species *Chaoborus obscuripes, Cloeon dipterum, Plea minutissima* and *Daphnia magna* in relation to varying patterns of chlorpyrifos exposure. The killing rate constant, recovery rate constant, and the threshold for damage were estimated by fitting the TDM to the experimentally observed survival data. The species *C. obscuripes* and *D. magna* showed an immediate decrease in mobility and a delayed effect in survival whereas *C. dipterum* and *P. minutissima* responded immediately to the exposure in both endpoints. In general, the effect of successive pulses was smaller if the intervals between pulses were larger and thus allowed for elimination of the compound and potential recovery at the target site. Hence, the effects of a first exposure pulse may influence the biological response to a second pulse (Ashauer *et al*. 2007c). Using individual-level, such as TKTD models (Ashauer *et al*. 2007a) or time-to-event approaches (Newman and McCloskey, 1996), one can determine whether pulses are toxicologically dependent on each other, i.e. whether effects of sequential pulses are enhanced, reduced, or independent from each other. An important parameter to predict the toxicological independence of pulses is the t_{95}, which provides the duration of the time interval needed between repeated pulsed exposures for an

organism to eliminate 95% of the accumulated toxicant from its body when moved from contaminated to clean water.

After chlorpyrifos exposure, *C. obscuripes* showed a direct effect in mobility and a delayed effect in mortality, without individual recovery (i.e. move from an immobile to a mobile state). This delayed effect on survival and lack of individual recovery has also been observed by Rubach (2010), who hypothesised that low chlorpyrifos concentrations might act on the hydrostatic vesicles with which the animals are equipped. This results in floating animals, which is assumed to be an irreversible effect and acts as an additional stressor, eventually leading to the death of the animal. Our results support this hypothesis. When the interval between successive pulses was longer than 7 days, the effects of the second pulse were much lower compared to the first pulse. A hypothesis that can explain the observed difference is that the individuals with the highest threshold will survive the first pulse and thus the group of individuals that is left is less sensitive to a second pulse, especially when some time is available for depuration and repair. It can also be hypothesised that the individual tolerance is induced by earlier pulses by making the individuals stronger and more tolerant through acclimation and induction processes (Dauterman, 1994; **Chapter 4)**. On the other hand, cumulative effects on individuals occur when the first pulse weakens the organisms by making them less tolerant, consuming their energy and lowering the fitness of the organisms by carry-over damage (Dauterman, 1994) or when the interaction of substances with the receptors is irreversible (Van der Hoeven and Gerritsen, 1997; Verhaar *et al*. 1999). The experimental results shown here support the individual effective dose theory (Gaddum, 1953), which assumes a unique threshold for every individual so the individuals with the lower thresholds are killed by the first pulse, leaving the remaining individuals as able to withstand the second pulse better. This and the relatively high estimate for the threshold and recovery rate constant parameters explain the response observed for *Chaoborus* sp. in **Chapter 3**, where no response to the continuous application was observed. The individual effective dose theory is not included in the TDM model, which assumes stochastic death **(Chapter 4).**

A direct response in mobility and survival was observed for *C. dipterum* after the first chlorpyrifos pulse. *C. dipterum* showed direct effects on mobility, which was comparable to the response of *P. minutissima*, indicating acute inhibition of acetylcholinesterase AChE. The survival response after exposure was observed faster than for the other species. This can be explained by the higher uptake rate for *C. dipterum* and/or by potentially fast distribution to the target enzyme and fast binding kinetics, which would result in a high killing rate constant. This could be due to difference in the seasonality of sensitivity, highlighting the potential seasonal dependence of the TK parameter values, which themselves are a result of the species morphological, ecological and

physiological traits at a given point in time (Rubach *et al*. 2010a; in press). As intended and when the control mortality was taken into account, a somewhat smaller response was observed after the second pulse when the interval between the pulses increased. This suggests that when an animal is given the time to fully eliminate the chemical and to recover by reactivating the inhibited enzyme, no accrual in effect is observed, which might explain the response of this species as observed in **Chapter 3**. The decrease in effect size with increasing time intervals between pulses was, however, difficult to quantify since the mortality in the control increased substantially during the course of the experiment. Further experiments using lower concentrations should be performed with *C. dipterum* to further reveal its TKTD characteristics for chlorpyrifos. It may also be helpful to analyse the current data set with a model that includes the individual effective dose theory instead of stochastic death (Jager *et al*. 2011).

In the laboratory experiments of **Chapter 4**, direct effects on mobility and survival with some recovery after exposure were observed for *P. minutissima*. When time intervals between pulses were shorter, larger effects were observed, most likely a result of damage accrual. When enough time was provided between the pulses, the second pulse did not show much difference in mobility and survival after the second pulse. This indicates that most animals were able to eliminate chlorpyrifos from their body and thus had suffered less from the second pulse. However, individual tolerance could also have played a role **(Chapter 4)**. This species did not show significant treatment effects in the cosm experiment of **Chapter 3** due to its low intrinsic sensitivity (Rubach *et al*. 2011).

Daphnia magna showed direct immobility and a very prolonged effect over several days on mortality. In all cases, however, the second pulse had much lower effects compared to the first pulse, irrespective of the time-interval between the pulses (**Chapter 4**). When growing, animals may become potentially less sensitive and the chemical may be diluted by the increasing amount of body tissue, by moulting and by the release of offspring (Naddy *et al*. 2000). Another explanation could be again the individual tolerance principle. The delayed effect on survival could be explained by the definitions of the endpoint mortality, which is the lack of heartbeat and not total immobilization of the body as is often used in other studies **(Chapter 5).** Van der Hoeven and Gerritsen (1997) studied the effect of chlorpyrifos on *Daphnia pulex*, explaining that the agrochemical causes immobility in daphnids several days before death. Even when exposure was stopped, immobilized *D. pulex* died and gave the impression of irreversible effects. However, no delayed effects were recorded for daphnids that survived after initial exposure of chlorpyrifos in a study of Naddy and Klaine (2001), as long as adequate recovery time between exposures was allowed. Naddy and Klaine (2001) hypothesise that in the study of Van der Hoeven and Gerritsen (1997), a combination of higher exposure concentrations coupled with longer exposure periods (less recovery time), allowed the

daphnids to accumulate chlorpyrifos, exceeding their critical toxicity threshold. Klaine *et al*. (1997) also showed that *D. magna* survival after exposure to chlorpyrifos was age related, with older daphnids being more sensitive. When daphnids were exposed to two pulses, these authors found a higher mortality if the second pulse occurred later in their life cycle **(Chapter 3, 4)**. Hence, the time interval between pulses and the age of the test organisms at the start of the experiment are of importance for the effects of consecutive pulses. Normally, when a study is finished, latent, or delayed effects occur in the post-exposure period. This may be an important factor in the use of the results of toxicity studies in the ecological risk assessment for pesticides and prolong their population-level recovery after pesticide stress (Galic, 2012; Hurd *et al*. 1996). Galic (2010) showed that for some chemicals the latency of effects predicted by the TDM model beyond the duration of the acute standard toxicity tests might be considerable, indicating that using the dose-response model to estimate survival might underestimate the adverse effects of pesticides (Galic, 2010). On the other hand, many insecticides like pyrethroids, have a very low persistence and half-life (Van Wijngaarden *et al*. 2005) and their existence in the water column may be limited to a few hours (Laskowski, 2002). In such cases, the standard risk assessment also uses a 96 h dose–response model and the risks of their short-term exposure might be overestimated (Galic, 2010). This shows that occurrences of effects beyond the exposure period, but also toxicity dynamics that differ among the chemicals and organisms, are not captured by standard toxicity tests and TKTD approaches may help to overcome such shortcomings and improve the ecological risk assessment of pesticides (Ashauer *et al*. 2006; Ashauer *et al*. 2010, Rubach *et al*. 2010a; Rubach *et al*. 2011).

One of the key challenges in ERA of pesticides is how to deal with exposure regimes that vary in time and extrapolate effects observed after one peak exposure (e.g. laboratory) to multiple exposures in the field that occur due to e.g. spray drift, run-off and/or drainage. Techniques that can be applied to assess the effects of time-varying exposure include ecological modelling. The ELINK workshop provided some recommendations on this issue from a pesticides perspective (Brock *et al*. 2010). For a more mechanistic coupling of exposure and effects using internal concentrations, TKTD models can be used. As these models need extensive laboratory studies for model parameterisation, they are still scarcely parameterised compared to the wealth of species-chemical combinations available. Using species traits to explain the variation in TK parameters between species as has been done by Rubach *et al*. (in press) might provide a promising way to be able to construct models that are able to predict the TKTD parameters for unknown species-chemical combinations, as is outlined by Rubach *et al*. (2010c). For this, it is important that the different TD studies performed with chlorpyrifos as described in Rubach (2010) and **Chapter 4** of this thesis are brought together and analysed using trait-based approaches. To assess the ecological relevance of the individual-level

effects as predicted by TKTD models for populations, TKTD models can be linked to individual-based (meta-) population models (Galic, 2012). This integration of individual and population level models holds a big promise for the extrapolation of the effect of pesticides at the individual-level to the landscape-level.

Concluding remarks and outlook

Multivariate analysis of community data given in **Chapter 3 and 5** of this thesis revealed the same effect magnitude for all time-variable exposure regimes indicating that for risk assessment of long-term effects of the tested chemicals on aquatic communities the TWA concentration is more important than the peak concentration. This finding, however, does not always hold true at the species/population level. This was investigated by performing TD experiments with those species that showed relatively high estimates for the threshold and recovery rate constants compared to other species. Therefore, recommendations on whether to use the TWA or the peak exposure cannot be made at the species-level without experimental results. Although differences in response to time variable exposures are observed for some species, the TWA concentration can still be considered to be conservatively protective when chosen as threshold level for long-term effects. The use of time-weighted concentrations instead of the peak concentration in risk assessment should be investigated further for pyrethroids and other insecticides with a non-acetylcholinesterase inhibiting mode-of-action since there is no experimental evidence available in literature.

It was revealed in ecotoxicological studies performed with aquatic animals that the effects of pulsed exposure to pesticides may be similar to, smaller or larger than those observed at an equivalent TWA concentration but a lower, continuous exposure regime. The difference in effects depends, among others, on the following different factors: (1) the rate of pesticide accumulation in the organisms (uptake rate), (2) whether the threshold concentrations for lethal and sublethal effects are exceeded, (3) the ability and rate of elimination and/or detoxification of the accumulated pesticide in the organisms of concern (elimination and biotransformation) and (4) the rate of repair of the damage (individual recovery process) after exposure has ended (see Jager *et al*. 2006; Reinert *et al*. 2002; Ashauer *et al*. 2006; Hommen *et al*. 2010 and Rubach, 2010; and literature cited therein). This highlights the complexity of how species react to time-varying exposure patterns, the process of which can only be fully understood using TKTD models. The various existing TKTD models for survival were unified and incorporated into the “General Unified Threshold model for Survival” (GUTS). GUTS is a TKTD framework for ecotoxicology, from which a large range of existing models can be derived as special cases (Jager *et al*. 2011). It is considered that GUTS can help increase the application of

TKTD models in ecotoxicological research as well as environmental risk assessment of chemicals. It is recommended to re-analyse the data presented in **Chapter 4** using the GUTS model to explain patterns in the data by using various assumptions (e.g., stochastic death and individual tolerance) as compared to TDM, which only includes stochastic death.

Summary

Pesticides are broadly applied in current agriculture practices globally and may end up in interconnected water bodies i.e. ditches, ponds, and lakes via numerous routes such as spray drift, runoff and leaching. Given the fact that they are inherently designed to harm biota, pesticides may pose risks to a range of aquatic organisms. Non-target organisms may be exposed to fluctuating concentrations with successive pulses of pesticide contaminants. In general, pesticide risks are often assessed by performing laboratory experiments and/or semi-field experiments evaluating a continuous and single application, respectively, which does not necessarily correspond to the exposure pattern of realistic applications (e.g. time-varying exposure). This mismatch is one of the main challenges in contemporary ecological risk assessment. Evaluation of the potential adverse effects of multiple pulsed pesticide exposure on non-target aquatic organisms is therefore considered to be of importance and should also become a part of the standard European registration procedure.

This thesis aims to compare the effects of different time-variable exposure regimes, having the same Time Weighted Average (TWA) but different peak concentrations, of a pesticide on aquatic species and communities (**Chapter 1**). For the risk assessment of pesticides, an imperative question is addressed about which type of concentration, the TWA or the peak, is more appropriate to assess the longer-term risks of pesticides. In addition, this thesis also uses empirical approaches to establish rules-of-thumb to extrapolate from one type of exposure pattern to the other.

Chapter 2 addresses the issue whether peak or TWA_{21d} concentrations should be used in the aquatic risk assessment of insecticides when the predicted or measured exposure is variable in time. Therefore, in this chapter I aimed to compare the effects as observed in cosm experiments on the basis of the peak concentration of their exposure profile as well as their TWA_{21d} concentration using three sensitive endpoints, i.e. microcrustaceans, macrocrustaceans and insects. This comparison was performed for individual insecticides and also for groups of chemicals sharing the same toxicological mode-of-action. To achieve this aim, a review of the empirical PERPEST database was performed, which contains classified effects of insecticides on various endpoints as observed in freshwater model ecosystems that evaluate the effects of pesticides. The PERPEST (Predicting the Ecological Risks of PESTicides in freshwater ecosystems) model uses this database to predict the effects of a particular concentration of a pesticide on various community endpoints. Since the PERPEST data base only contains the peak concentrations of the exposure profiles evaluated in the cosm experiments, all cosm studies were re-reviewed in order to obtain the TWA_{21d}. In order to facilitate a comparison across insecticides, the exposure concentrations were expressed as toxic units (TU). On the basis of these TUs, threshold values were assumed to be equivalent for compounds with a similar mode-of-action. TUs were calculated by dividing the concentrations evaluated in the cosm

study by the Hazardous Concentration 50% (HC50), which was calculated as the geometric mean of all acute toxicity values of the insecticide for aquatic arthropods. For acetylcholinesterase inhibiting insecticides, we found that when comparing peak and TWA_{21d} concentrations, direct effects became apparent at TWA_{21d} concentrations that were a factor of 5 lower than their respective peak exposure concentrations. We therefore recommend an extrapolation factor of 5 to extrapolate safe peak concentrations to safe TWA concentrations, especially when the threshold value is based on a study evaluating a single application of an acetylcholinesterase inhibiting compound. For acetylcholinesterase inhibiting insecticides, TWA_{21d} concentrations can be used as good predictors for long-term effects on sensitive endpoint groups in the risk assessment process, since somewhat clearer dose-response relationships were obtained for all endpoints in case of TWA_{21d} exposures when compared to peak exposures. For pyrethroids, no clear dose-response relationship was found, neither when the comparison was scaled on peak concentrations, nor when scaled on TWA_{21d} exposures. For moulting inhibiting insecticides, the peak and TWA_{21d} concentrations may have equal importance in order to standardise the effects.

In **Chapter 3** I compared the effects of different time-variable exposure regimes having the same TWA concentration but different peak concentrations of the organophosphate insecticide chlorpyrifos on freshwater invertebrate communities. The experiment was performed in outdoor microcosms by introducing three different regimes: a single application of 0.9 µg a.i./L; three applications of 0.3 µg a.i./L with a time interval of 7 d; and continuous exposure to 0.1 µg a.i./L for 21 d. Our results indicated that the application of chlorpyrifos resulted in decreased abundances of species belonging to the arthropod community, with the largest adverse effects reported for the mayfly *Cloeon dipterum* and cladocerans *Daphnia gr. longispina* and *Alona* sp., while smaller effects were observed for other insects, copepods, and amphipods. At the population-level, most species showed the same effect magnitude at the end of the experimental period, indicating that the TWA concentration of chlorpyrifos is predictive for its long-term effects on arthropod species. The mayfly *C. dipterum*, however, only responded to the single-application treatment, which could be explained by the toxicokinetics of chlorpyrifos in this species. Intrinsic sensitivity is a product of the processes of toxicokinetics (TK: uptake, biotransformation and elimination of the compound) and toxicodynamics (TD: internal damage, individual recovery and threshold) of a compound (**Chapter 4**). Therefore, differences in field responses of species to time-variable exposure profiles may relate to differences in the TKTD of chlorpyrifos in these species. At the end of the experimental period the invertebrate community showed approximately the same effect magnitude for all time-variable exposure regimes of treatments. These results suggest that for this combination of concentrations and duration of the TWA, the TWA concentration is more important for most species than the peak

concentration for the assessment of long-term risks of chlorpyrifos. These results support the recommendations of the ELINK workshops, which suggest that for long-term effects the TWA concentration may be more relevant than the peak concentration.

In order to assess the effects of time-varying pulses of pesticides, the development of models that can describe the toxicokinetic (TK) and toxicodynamic (TD) of a chemical in individuals of a species is of major importance. This is because non-target organisms may be exposed to fluctuating concentrations or sequential pulses of pesticides in the environment**.** Furthermore, recovery of individuals after being exposed to pesticides, will occur as part of the TD processes, but is not routinely taken into account in risk assessment. The Threshold Damage Model (TDM) is a process-based model for predicting the acute effects of pulsed pesticide exposure on the survival of aquatic invertebrates and consists of a TK part in which uptake and elimination are described, and of a TD part accounting for processes such as damage, individual recovery, and internal thresholds.

Chapter 4 presents data from a series of laboratory experiments with the model substance chlorpyrifos, which were used to parameterize the TD part of the TDM model for four different species. The experiment quantified mobility and survival of the four freshwater species *Chaoborus obscuripes, Cloeon dipterum, Plea minutissima* and *Daphnia magna* after two subsequent 24 h pulses of chlorpyrifos with an intermediate time interval that either allowed for the elimination of the compound and potential individual recovery between successive pulses or not. The killing rate constant, recovery rate constant, and the threshold for damage were estimated by fitting the TDM to the experimentally observed survival data using estimates for the TK parameters for the same species from the literature. The species *C. obscuripes* and *D. magna* showed an immediate decrease in mobility and a delayed effect in survival whereas *C. dipterum and P. minutissima* responded immediately to the exposure with both endpoints. *C. obscuripes* was the only species showing no individual recovery. In general, the effect of the pulses was smaller if the intervals between pulses allowed for elimination and potential recovery. The experimental data were successfully fitted by the TDM model, however, not all parameters were estimated equally robustly. This expresses the need for further data collection and development of TKTD models for different species and compounds. Improved TKTD models could be combined with individual-based models to provide more accurate and detailed model predictions of direct effects of pesticides on immobility and mortality and how these direct effects propagate to population recovery in order to link the different levels of biological organisation.

Chapter 5 presents a study which aimed at evaluating the effects of different time-varying exposure patterns of the strobilurin fungicide azoxystrobin on freshwater microsocosm communities. These exposure patterns included two treatments with a similar peak but different TWA concentrations, and two treatments with similar TWA but different peak concentrations. The experiment was carried out in outdoor microcosms under four different exposure regimes; (1) a continuous application of 10 µg/L (CAT_{10}) for 42 days, (2) a continuous application of 33 µg/L (CAT_{33}) for 42 days, (3) a single application of 33 µg/L (SAT_{33}), and (4) a treatment with four applications with a time interval of 10 days of 16 µg/L (FAT_{16}). Multivariate analyses demonstrated significant changes in zooplankton community structure in all but the CAT_{10} treated microcosms relative to that of controls. The largest adverse effects were reported for zooplankton taxa belonging to Copepoda and Cladocera. By the end of the experimental period (day 42 after treatment), community effects were of similar magnitude for the pulsed treatment regimes, although the magnitude of the initial effect was larger in the SAT_{33} treatment. This indicates that for long-term effects the TWA is more important for most zooplankton species in the test system than the peak concentration. Azoxystrobin only slightly affected some species of the macroinvertebrate, phytoplankton and macrophyte assemblages. The overall No Observed Ecologically Adverse Effect Concentrations (NOEAEC) in this study was 10 µg/L.

Chapter 6 discusses the findings of this thesis. I aim to compare the effects of time-variable exposure regimes as observed in the cosm experiments described in this thesis as well as in the reviewed cosm studies published in the open literature in terms of peak and TWA concentrations on aquatic communities and ecosystems and draw conclusions from all the results presented in this thesis.

Samenvatting

Het gebruik van bestrijdingsmiddelen voor de productie van agrarische gewassen brengt het risico met zich mee dat deze in aangrenzende waterlichamen, zoals sloten, vijvers, meren en/of beken, terecht kunnen komen. Er zijn verschillende manieren waarop bestrijdingsmiddelen in deze waterlichamen terecht kunnen komen, bijvoorbeeld door bovengrondse afstroming op hellende agrarische velden, door overwaaiing wanneer de toepassing van bestrijdingsmiddelen dichtbij wateroppervlakten plaatsvindt of door uitspoeling van bestrijdingsmiddelen naar het oppervlakte- of grondwater. Omdat bestrijdingsmiddelen zijn ontwikkeld voor het aantasten van voor gewassen schadelijke biota, kunnen deze chemische gewasbeschermingsproducten ook schadelijk zijn voor verwante (aquatische) organismen. Mogelijke risico's van bestrijdingsmiddelen op aquatische organismen worden meestal getest met behulp van lab- en/of semi-veldexperimenten. Deze experimenten zijn gebaseerd op een eenmalige of continue toepassing van het bestrijdingsmiddel. Het resulterende blootstellingsregime komt niet altijd overeen met het blootstellingprofiel zoals voorspeld voor of gemeten in een veldsituatie, die vaak variabel in de tijd is. Een betere overeenkomst tussen de blootstellingspatronen van bestrijdingsmiddelen die gebruikt worden in experimenten waarop de risicobeoordeling gebaseerd is en die in het veld optreden kan de ecologische risicobeoordeling verbeteren. Dan kan rekening gehouden worden met in de tijd fluctuerende concentraties van bestrijdingsmiddelen die beter de werkelijke situatie nabootsen. Deze manier van risicoanalyse moet worden toegevoegd aan de reguliere Europese registratieprocedure van bestrijdingsmiddelen, niet alleen om de toelating van verschillende soorten bestrijdingsmiddelen te reguleren, maar ook om de toegestane hoeveelheid te bepalen.

Dit onderzoek vergelijkt de effecten van bestrijdingsmiddelen op individuele soorten en gemeenschappen van aquatische organismen. Om de effecten van tijdvariabele blootstelling te onderzoeken zijn verschillende, in tijd variërende blootstellingsregimes geëvalueerd, die dezelfde tijd-gewogen-gemiddelde concentratie van het bestrijdingsmiddel hebben, maar verschillende piekconcentraties **(hoofdstuk 1)**. Voor de risico-evaluatie van bestrijdingsmiddelen is het namelijk belangrijk om te bepalen welk type concentratie, de tijd-gewogen-gemiddelde of de piekconcentratie, het beste gebruikt kan worden voor het beoordelen van lange-termijn risico's. In dit proefschrift worden de resultaten van nieuwe en in het verleden uitgevoerde experimenten en empirische methoden gebruikt om vuistregels voor de extrapolatie van effecten van het ene naar het andere blootstellingsregime op te stellen.

Vervolgens wordt in **hoofdstuk 2** geanalyseerd welke concentraties van bestrijdingsmiddelen – de tijd-gewogen-gemiddelden of piekconcentraties van tijdvariabele blootstellingspatronen – gebruikt kunnen worden voor het evalueren van het risico van insecticiden op waterorganismen. Hiervoor worden de effecten van bestrijdingsmiddelen op organismen zoals geobserveerd semi-veldexperimenten die in het verleden uitgevoerd zijn, samengevat. In semi-veldexperimenten worden micro- en/of mesocosms (cosm) gebruikt als modelecosystemen, die kunnen dienen als replica voor de grotere en complexere aquatische ecosystemen in het veld. Voor alle geëvalueerde cosm-experimenten is de tijd-gewogen-gemiddelde concentratie over een periode van 21 dagen bepaald, alsmede de piekconcentraties. Tevens zijn de effecten op drie eindpunten, namelijk de sterfte van microcrustaceans, macrocrustaceans en insecten, geclassificeerd. Vervolgens zijn de effecten in de verschillende cosm experimenten vergeleken op basis van beide concentraties en is bekeken welk concentratietype het meest consistente beeld opleverde. Deze vergelijking is gemaakt voor individuele insecticiden alsmede voor insecticidegroepen met hetzelfde toxicologische werkingsmechanisme. De geclassificeerde effecten van de verschillende cosm experimenten werden verkregen vanuit een empirisch gegevensbestand. Dit bestand vormt de ruggengraat van het PERPEST (**P**redicting the **E**cological **R**isks of **PEST**icides) model dat de effecten van een specifieke concentratie van een pesticide op verschillende eindpunten van aquatische leefgemeenschappen in zoetwaterecosystemen kan voorspellen. Qua blootstelling, bevatte het PERPEST gegevensbestand slechts de piekconcentraties van de verschillende blootstellingsprofielen. Om de tijd-gewogen-gemiddelde waarden voor 21 dagen te verkrijgen, zijn alle cosm-studies opnieuw geëvalueerd. Om een vergelijking van de effecten van de verschillende soorten insecticiden mogelijk te maken, zijn alle blootstellingsconcentraties uitgedrukt in toxische eenheden (TU's: toxic units). Op basis van TU's wordt verondersteld dat de drempelwaarden (de waarden waarbij de insecticide een negatief effect veroorzaakt op aquatische organismen) voor verschillende insecticiden met hetzelfde toxicologisch werkingsmechanisme gelijk zijn. De TU's zijn berekend door de geëvalueerde concentraties, verkregen vanuit de cosm-studies, te delen door de concentratie die schadelijk is voor 50% van de arthropoda soorten (HC50: Hazardous Concentration 50%). De HC50 waarde is het geometrische gemiddelde van alle acute toxische waarden van de insecticiden voor aquatische arthropoda. Onze studie concludeerde dat voor acetylcholinesterase remmende insecticiden, de drempelconcentraties waarvoor directe effecten op aquatische organismen werden gevonden, een factor 5 lager waren op basis van tijd-gewogen-gemiddelde concentraties dan op basis van piekconcentraties. Daarom adviseert onze studie een extrapolatiefactor van '5', wanneer drempelwaarden op basis van piekconcentraties worden geëxtrapoleerd naar tijd-gewogen-gemiddelde concentraties, in het bijzonder wanneer de drempelwaarde is gebaseerd op een studie met slechts een enkele toepassing

van een acetylcholinesterase remmende insecticide. Tijd-gewogen-gemiddelde concentraties zijn goed te gebruiken in risico-evaluaties als indicatoren voor lange-termijn effecten op gevoelige eindpunten. Vergeleken met piekconcentraties, laten de tijd-gewogen-gemiddelde concentraties een betere dosis-response relatie zien met alle gevoelige eindpunten. Voor pyrethroïden, daarentegen, is er geen duidelijke dosis-respons relatie gevonden; noch voor tijd-gewogen-gemiddelde, noch voor piekconcentraties. Voor bestrijdingsmiddelen die de vervelling van organismen verstoren, lijken de tijd-gewogen-gemiddelde concentraties en piekconcentraties even belangrijk te zijn voor het voorspellen van effecten op aquatische organismen.

In **hoofdstuk 3** worden de effecten van verschillende concentratieprofielen van chloorpyrifos (een organofosfaat insecticide) op ongewervelde zoetwater gemeenschappen geëvalueerd. De verschillende tijdvariabele blootstellingsregimes hebben dezelfde tijd-gewogen-gemiddelde concentraties, maar verschillende piekconcentraties. Dit experiment werd uitgevoerd in openlucht microcosms. Deze microcosms werden blootgesteld aan drie verschillende blootstellingsregimes: (i) een enkele toediening van 0.9 µg a.i./L (a.i.: actieve ingrediënt), (ii) drie toedieningen van 0.3 µg a.i./L, met een tijdsinterval van 7 dagen en (iii) een continue blootstelling aan 0.1 µg a.i./L voor 21 dagen. De toediening van chloorpyrifos resulteerde in een afname in aantallen van soorten behorende tot de arthropoden gemeenschap. De eendagsvlieg *Cloen dipterum* en de watervlooien *Daphnia gr. Longispina* en *Alona sp.*, toonden de grootste afnames in aantallen als gevolg van de blootstelling aan chloorpyrifos. Geen negatieve effecten werden waargenomen voor andere evertebraten, zoals copepoda (roeipootkreeftjes) en amphipoda (vlokreeftjes). De meeste aangetaste soorten lieten eenzelfde effectgrootte zien tegen het einde van een experimentele periode van blootstelling aan chloorpyrifos. Dit geeft aan dat de tijd-gewogen-gemiddelde concentratie van chloorpyrifos een betere indicator is voor de lange-termijn effecten van chloorpyrifos op arthropoda soorten dan de piekconcentratie. Een uitzondering vormde de eendagsvlieg *C. dipterum*, die slechts aangetast werd door de behandeling met een eenmalige toediening van chloorpyrifos. Dit kan verklaard worden aan de hand van de toxicokinetiek (TK) van chloorpyrifos in deze arthropoda soort. De intrinsieke gevoeligheid van organismen voor mogelijke schadelijke bestrijdingsmiddelen wordt namelijk bepaald door toxicokinetische (TK: opname, biotransformatie en eliminatie van het a.i.) en toxicodynamieke (TD: interne schade voor het organisme, individueel herstel en drempelwaarden voor mogelijk effect) processen (**hoofdstuk 4**). Het verschil in respons voor de verschillende soorten, zoals gemeten in het semi-veldexperiment, is gerelateerd aan verschillen in de TKTD van het chloorpyrifos in deze soorten. Deze resultaten worden ook ondersteund door de aanbevelingen van de ELINK workshops. Tijdens deze workshops werd bediscussieerd hoe de effecten van bestrijdingsmiddelen gerelateerd kunnen worden aan de

blootstelling. In deze workshops werd ook benadrukt dat voor het evalueren van de lange-termijn effecten van bestrijdingsmiddelen op aquatische organismen, de tijd-gewogen-gemiddelde concentratie relevanter kan zijn dan de piekconcentratie.

Voor een mechanistische link tussen tijdvariabele blootstellingsregimes en de effecten op individuen kunnen modellen die de TK en TD beschrijven gebruikt worden. Het herstel van individuele organismen na blootstelling aan bestrijdingsmiddelen is een onderdeel van de TD processen. Dit herstel wordt echter niet altijd in beschouwing genomen bij de risico-evaluatie. Het Threshold Damage Model (TDM) is een model gebaseerd op TKTD processen. Dit model is bedoeld voor het voorspellen van de acute effecten van verschillende concentraties bestrijdingsmiddelen op aquatische organismen. Dit model voorspelt de mortaliteit van ongewervelde aquatische organismen als gevolg van een blootstelling aan een bepaald concentratieregime van een bestrijdingsmiddel. TDM bestaat uit een TK en TD gedeelte; het TK gedeelte beschrijft de opname van bestrijdingsmiddelen in organismen en de eliminatie van deze bestrijdingsmiddelen, het TD gedeelte beschrijft de processen van schade aan organismen door blootstelling, het individuele herstel en de interne drempelwaarden voor mortaliteit.

In **hoofdstuk 4** worden de gegevens van een serie laboratoriumonderzoeken, die uitgevoerd zijn met het insecticide chloorpyrifos, gepresenteerd. Deze experimenten werden gebruikt om het TD gedeelte van het TDM te parametriseren voor vier verschillende soorten aquatische organismen. De experimenten kwantificeerden de effecten op de mobiliteit en het overleven in de tijd van vier zoetwatersoorten, namelijk *Chaoborus obscuripes, C. dipterum, Plea minutissima* en *Daphnia magna*, als gevolg van verschillende tijdvariabele blootstellingspatronen van het insecticide chloorpyrifos. De verschillende patronen bestonden uit twee pulsen van 24 uur met verschillende tijdsintervallen tussen de pulsen voor de verschillende patronen. Het verschil in interval tussen de pulsen werd verondersteld óf te leiden tot de eliminatie van het bestrijdingsmiddel en het herstel van individuele organismen (lang interval), óf tot geen eliminatie van het bestrijdingsmiddel voordat de tweede puls werd toegediend (kort interval). De TD parameters (eliminatiesnelheid, de herstelsnelheid en de drempelwaarde voor schade) werden geschat door het TDM te fitten op de experimentele resultaten en gebruik te maken van de geschatte TK parameters voor dezelfde soorten gepubliceerd in de literatuur. De soorten *C. obscuripes* en *D. magma* toonden, na de blootstelling aan het chloorpyrifos, een onmiddellijke vermindering in mobiliteit, maar een vertraagd effect op het overleven van de individuen. De soorten *C. dipterum* en *P. minuttissima* lieten direct na de eerste puls een negatief effect zien voor beide eindpunten. *C. obscuripes* vertoonde als enige geen herstel van de individuen tijdens het interval tussen de pulsen. In het algemeen was het effect van de pulsen kleiner wanneer de intervallen tussen de pulsen langer was en dus eliminatie van het

bestrijdingsmiddel en het herstel van de individuen toestond. Hoewel de experimentele gegevens succesvol zijn gefit met het TDM, zijn niet alle parameters even robuust geschat. Dit geeft aan dat er meer experimentele data nodig zijn voor het verder parameteriseren van TKTD modellen voor verschillende soorten aquatische organismen en bestrijdingsmiddelen. Verbeterde TKTD modellen kunnen in de toekomst gecombineerd worden met individu-gebaseerde modellen. Deze combinatie kan zorgen voor accuratere en gedetailleerdere modelvoorspellingen van de effecten van bestrijdingsmiddelen op de verspreiding en het overleven van aquatische populaties en hersteltijden.

In **hoofdstuk 5** wordt een studie beschreven die de effecten van verschillende tijdvariabele blootstellingsregimes van het strobilurin fungicide azoxystrobin op zoetwaterleefgemeenschappen in microcosms evalueert. Deze blootstellingspatronen bestaan uit twee behandelingen met dezelfde tijd-gewogen-gemiddelde concentratie, maar verschillende piekconcentraties, en twee behandelingen met dezelfde piekconcentratie, maar verschillende tijd-gewogen-gemiddelde concentraties. Het experiment werd uitgevoerd in openlucht microcosms met vier verschillende blootstellingsregimes; (1) een continue toediening van 10 µg/L (CAT_{10}: continuous application treatment) voor 42 dagen, (2) een continue toediening van 33 µg/L (CAT_{33}) voor 42 dagen, (3) een enkele applicatie van 33 µg/L (SAT_{33}: single application treatment) en (4) een behandeling van vier applicaties met elk een tijdsinterval van 10 dagen (FAT_{16}: four application treatment). De resultaten van de multivariate analyses lieten, in vergelijking met de controle behandeling, significante veranderingen in de structuur van de zoöplankton leefgemeenschap zien in alle behandelingen, behalve de microcosms behandeld met CAT10. De grootste effecten werden gerapporteerd voor de zoöplankton taxa, behorende tot Copepoda (roeipootkreeftjes) en Cladocera (watervlooien). Aan het einde van de experimentele periode, namelijk 42 dagen na de behandeling, waren de effecten op de leefgemeenschappen van dezelfde omvang voor de behandeling met het hetzelfde tijd-gewogen-gemiddelde blootstelling. Echter, de omvang van de initiële effecten was groter voor de SAT_{33} behandeling. Dit geeft aan dat voor het bepalen van de lange termijn effecten, de tijd-gewogen-gemiddelde concentratie belangrijker is voor de meeste zoöplankton soorten in het testsysteem, terwijl voor korte-termijn effecten de piekconcentraties een betere voorspeller is. Buiten effecten op het zooplankton werden er nauwelijks effecten van azoxystrobin waargenomen op andere eindpunten (macro-evertebraten, phytoplankton en macrophyten). De veilige drempel concentratie (no observed ecologically adverse effect concentrations, NOEAEC) gebaseerd op de resultaten van deze studie was 10 µg/L.

Tot slot presenteert **hoofdstuk 6** een afsluitende en overkoepelende einddiscussie over de algemene resultaten van dit onderzoek. In dit hoofdstuk worden de empirische en experimentele data bij elkaar gebracht en met elkaar vergeleken. Hieruit blijkt dat over het algemeen de tijd-gewogen-gemiddelde concentratie een betere voorspeller is voor lange-termijn effecten dan de piekconcentraties.

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Abbreviations

a.i.	:	Active ingredient
%	:	Percent
≤	:	Equal or less than
Δt	:	Time interval between applications
≈	:	Almost equal to
≥	:	Equal or greater than
µ	:	Micro
µg	:	Micro gram
µg/L	:	Microgram per litre
µl	:	Micro litre
µm	:	Micro metre
µS/cm	:	Micro Siemens per centimetre
^{0}C	:	Degree Centigrade
100 X	:	100 times
25 X	:	25 times
AChE	:	Acetylcholinesterase
AM	:	Ante Meridiem
ANOVA	:	Analysis of Variance
AUC	:	Area Under the Curve
BCF	:	Bioconcentration factor
C_t	:	Concentration of pesticide in water at time t
C/L	:	Carbon per litre
CAS	:	Chemical Abstract Service
CAT_{10}	:	Continuous application treatment of 10 µg/L
CAT_{33}	:	Continuous application treatment of 33 µg/L
CAUC	:	Critical Area Under the Curve
CBR	:	Critical Body Residues
C_{int}	:	Internal concentration
Cm	:	Centimetre (s)
Conc.	:	Concentration
CTO	:	Critical Target Occupation
C_w	:	Concentration in water
d	:	Day
D	:	Damage
d^{-1}	:	Per day
DAM	:	Damage Assessment Model
DDT	:	Dichlorodiphenyltrichloroethane
DEBtox	:	Dynamic Energy Budget
DO	:	Dissolved Oxygen
DT_{50}	:	Dissipation time 50%
EC	:	European Commission
EC	:	Emulsifiable Concentrate
EC	:	Electrical Conductivity
EC_{50}	:	Median Effective Concentration
ECD	:	Electron Capture Detector
EEC	:	European Economic Community

EFSA	:	European Food Safety Authority
ELINK	:	Linking Aquatic Exposure and Effects
EPA	:	Environmental Protection Agency
ERA	:	Ecological Risk Assessment
ERC	:	Ecotoxicologically Relevant Concentration
EU	:	European Union
exp	:	Exponent
FAT_{16}	:	Four application treatment of 16 µg/L
Fig	:	Figure
FOCUS	:	FOrum for Coordination of pesticides fate models and their USe
g	:	Gram
GC-ECD	:	Gas Chromatography Electron Capture Detector
GUTS	:	General Unified Threshold model for Survival
h	:	Hour
H	:	Hazard
h_b	:	Background mortality
HC_5	:	Hazard concentration for 5% of species, predicted from SSD curve
HC_{50}	:	Hazard concentration for 50% of species, predicted from SSD curve
HCl	:	Hydrochloric acid
HP	:	Hewlett-Packard
HPLC	:	High Pressure Liquid Chromatography
IED	:	Individual Effective Dose
IT	:	Individual Tolerance
K	:	Dissipation rate constant
K_{in}	:	Uptake rate constant
K_k	:	Killing rate constant
K_{out}	:	Elimination rate constant
K_{ow}	:	Octanol-Water Partition Coefficient
K_r	:	Recovery rate constant
L	:	Litre
$l/Kg_{ww.d}$	:	Litre per Kilogram wet weight per day
LC_{30}	:	Lethal Concentration 30 %
LC_{50}	:	Median Lethal Concentration
ln	:	Natural logarithm
LOD	:	Limit Of Detection
LOEC	:	Low Observed Effect Concentration
Log	:	Logarithm
LOQ	:	Limit Of Quantification
m	:	Metre
m^3/mol	:	Cubic metre per mol
MASTEP	:	Metapopulation model for Assessing Spatial and Temporal Effects of Pesticides
meq/L	:	Miliequivalent per litre
mg	:	Milligram
mg/L	:	Milligram per litre
min	:	Minute

mL	:	Millilitre
mm	:	Millimetre
msg	:	Most sensitive group
n	:	Number of applications
N	:	Normal
N	:	Nitrogen
NH_4^+	:	Ammonium
nmol	:	Nanomol
NO_2^-	:	Nitrite
NO_3^-	:	Nitrate
NOEAEC	:	No Observed Ecologically Adverse Effect Concentration
NOEC	:	No Observed Effect Concentration
p	:	Probability level (level of significance)
Pa	:	Pascal
PEC	:	Predicted environmental concentration
PEC^1	:	Predicted Exposure Concentration from single application
PEC^n	:	Predicted Exposure Concentration from 'n' applications
PERPEST	:	Predicting the Ecological Risks of PESTicides in fresh water
POM	:	Particulate Organic Matter
PPR	:	Plant Protection Products and their Residues
PRC	:	Principle Responses Curves
rpm	:	Revolution per minute
RSS	:	Residual Sum of Squares
RT	:	Ralph Tollrian
S	:	Survival
s	:	Second
SAT_{33}	:	Single application treatment of 33 µg/L
SD	:	Standard Deviation
SD	:	Stochastic Death
SETAC	:	Society of Environmental Toxicology and Chemistry
SI	:	Supporting Information
sp	:	Species
t	:	Time
T	:	Temperature
T_1	:	Treatment one
T_2	:	Treatment two
T_3	:	Treatment three
t_{95}	:	Depuration time 95 %
TDM	:	Threshold Damage Model
TER	:	Toxicity to Exposure Ratio
THM	:	Threshold Hazard Model
thr	:	Threshold
TKTD	:	Toxicokinetic-Toxicodynamic
t_{TWA}	:	Length of period for time weighted average
TU	:	Toxic Unit
TWA	:	Time-Weighted Average

TWA_{21d}	:	Time-Weighted Average concentration for period of 21 days
U.S.	:	United State
UK	:	United Kingdom
USDA/NASS	:	United States Department of Agriculture/National Agricultural
WFD	:	Water Frame work Directive

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My doctorate studies have been a continuous learning experience, developing my academic as well as social and didactic skills. After a journey full of ups and downs, seeing this thesis still makes me feel incomplete because I cannot share this book with my Sweet Mother, who would finally understand what her son has actually been doing in The Netherlands. Through this note I remember the ones who have left me, giving me all the love and encouragement to complete the doctorate, and also express my acknowledge to those who supported me in developing my PhD thesis. Even though I have always missed my home back in Pakistan, having spent 5 years in Wageningen, this town feels like a second home to me now…..!!

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The days I spent at the Wageningen University and Alterra Research Center will always stay in my memories. I am proud of being a student of this prestigious university.

Mazhar Iqbal Zafar

Wageningen University

August 2012

Short biography

“Defeat is not when You Fall Down... It is when You REFUSE to get up.... So keep Getting Up Every Time You Fall, That’s the ATTITUDE of life”

(Anonymous)

"Life is like a coin…. Pleasure and pain are the two sides. Only one side is visible at a time. But remember, other side's also waiting for its turn"

(Anonymous)

Mazhar Iqbal Zafar (1981) was born in a small village nearby the city of Bahawalpur in Punjab, Pakistan. He is the youngest son of Faqir Muhammad (Late, 1987) and Shamim Begum (Late, 2012), who had their own farm, where he developed an enthusiasm for studying agricultural sciences.

Mazhar was the first one in his family who got the opportunity to enrol as a university student, due to his good grades and excellent performance at the secondary school. Mazhar obtained his bachelor degree in the Agricultural Sciences in 2005 from the University of Agriculture, Faisalabad. During his internship he worked as a trainee for FMC United (Pvt.) Ltd. (a pesticide company) in the Sahiwal District. Mazhar pursued his master's degree (Honours) with a specialization in Agricultural Entomology in 2007, also from the University of Agriculture, Faisalabad. During his master thesis research, he investigated together with his supervisor Prof. Dr. Sohail Ahmed, the mechanistic effect of plant chemicals on behaviour and gut enzymes of *Microtermes obesi*. This research determined the comparative efficacy of naturally occurring ant-termite compounds, extracted from locally available plants or trees in order to find a solution for the control of harmful termites. He post-graduated with a distinction, for which he received the Silver Medal.

When Mazhar post-graduated, he was awarded an overseas scholarship by the Higher Education Commission (HEC) in Pakistan, through Netherlands Organisation for international Cooperation in Higher Education (NUFFIC) to pursue his doctorate studies in The Netherlands (on my choice). In September 2007, Mazhar started his PhD at the Department of Aquatic Ecology and Water Quality Management group (AEW) at the Wageningen University, and the Environmental Risk Assessment of Pesticides team (ERA) which is connected to the Alterra Research Centre. His doctoral research was being supervised by Prof. Dr. Paul van den Brink and focused on the extrapolation of effects of pesticides on aquatic communities and ecosystems across different exposure patterns. Mazhar's interest in the environmental sciences and the possible adverse effects of pesticides on organisms came forth from his initial research on how insect pests, which are harmful to crops and other plants, can best be controlled from an entomology perspective. With his PhD research he wanted to bring his research interest a step further, namely to examine how insect control mechanisms may affect the environment.

During his PhD, Mazhar convened and chaired the session "Eco-toxicology and Chemical Stress Ecology" during the Netherlands Annual Ecology Meeting (NAEM, 8 -9 February 2011). He attended several SETAC meetings (Society of Environmental Toxicology and Chemistry) and other international conferences to present his PhD research. He was also involved in several extra-curricular activities at the Wageningen University. As such, he was an active member of Wimek/SENSE PhD council (SPC) (SENSE Research School for Socio-Economic and Natural Sciences of the Environment) and the Wageningen PhD council (WPC). He also organized social events such as the screening of "The PhD Comics Movie" and the "PhD Student Party" for PhD candidates of the Seven Graduate schools of Wageningen University.

When he is free, Mazhar likes to play badminton and football. After his dissertation, Mazhar plans to explore the possibilities for a project-based job in area of ecological risk assessment of chemicals, which he hopes will be refreshing after years of academic study and research.

Publications

Refereed Scientific Publications

Zafar M.I., R.P.A. van Wijngaarden, I. Roessink and P.J. van den Brink. 2011. Effects of time-variable exposure regimes of the insecticide chlorpyrifos on freshwater invertebrate communities in microcosms. *Environmental Toxicology and Chemistry* 30: 1383-1394.

Zafar M.I., J.D.M. Belgers, R.P.A. van Wijngaarden, A. Master and P.J. van den Brink. 2011. Ecological impacts of time-variable exposure regimes to the fungicide azoxystrobin on freshwater communities in outdoor microcosms. *Ecotoxicology* 21: 1024-1038.

To be submitted/ In preparation

Zafar MI, Van den Brink PJ. Explanatory power of peak and time-weighted average concentrations for effects of insecticides as observed in semi-field experiments.

Zafar MI, Diepens NJ, Rubach MN, Van den Brink PJ. Toxicodynamic experiment for different time-variable exposure regimes of the insecticide chlorpyrifos on freshwater arthropods.

Belgers, J.D.M., R.P.A. van Wijngaarden, M.C. Boerwinkel, A.M. Matser, **M.I. Zafar** & G.H.P. Arts**.** Population and community-level effects of chronic exposure regimes of the fungicide azoxystrobin in freshwater microcosms.

Book chapter

S. Ahmed, **M.I. Zafar**, A. Hussain, M.A. Riaz and M. Shahid. 2011. Evaluation of plant extracts on mortality and tunnelling activities of Subterranean Termites in Pakistan. In: Pesticides in the Modern World - Pests control and pesticides exposure and toxicity assessment. Stoytcheva, Margarita (ed.). InTech - Open Access Publisher. pp. 39-51.

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Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment

C E R T I F I C A T E

The Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment
(SENSE), declares that

Mazhar Iqbal Zafar

born on 10 March 1981 in Bahawalpur, Pakistan

has successfully fulfilled all requirements of the
Educational Programme of SENSE.

Wageningen, 3 October 2012

the Chairman of the SENSE board	the SENSE Director of Education
Prof. dr. Rik Leemans	Dr. Ad van Dommelen

The SENSE Research School has been accredited by the Royal Netherlands Academy of Arts and Sciences (KNAW)

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AKADEMIE VAN WETENSCHAPPEN

The SENSE Research School declares that **Mr. Mazhar Iqbal Zafar** has successfully fulfilled all requirements of the Educational PhD Programme of SENSE with a work load of 67 ECTS, including the following activities:

SENSE PhD Courses

- Environmental Research in Context
- Research Context Activity: Convening the session "Eco-toxicology and Chemical Stress Ecology" in Netherlands Annual Ecology Meeting (NAEM), 8 - 9 February, 2011
- Environmental Risk Assessment of Chemicals
- Basic & Advanced Statistics
- Toxicological Risk Assessment
- Special Topics in Ecotoxicology

Other PhD and General Courses

- Pollution in Europe: Sustainable management of polluted areas. The 13th Erasmus Intensive Program, Université de Pau et des Pays de l'Adour, France
- Ecological Risk Assessment and Management. SETAC Europe 22nd Annual Meeting, Berlin
- Current registration requirements for ecology risk assessment of crop protection products in the EU & India. SETAC Europe 21st Annual Meeting, Milan
- Statistical methods in ecotoxicology using R. SETAC Europe 20th Annual Meeting, Seville
- How to best conduct and report aquatic ecotoxicity tests according to the International Guidelines. SETAC Europe 19th Annual Meeting, Gothenburg
- Research Methodology: From topic to proposal
- Techniques for Writing and Presenting Scientific Papers
- Teaching and Supervising Thesis Students
- PhD Competence Assessment
- Scientific Publishing
- Advanced Course Guide to Scientific Artwork
- Dutch language course: Listening and speaking 1

Management and Didactic Skills Training

- Member of WIMEK/SENSE PhD Council
- Co-organizer of three social events for PhD students of Wageningen University and of SENSE
- ExPectations 2010 Career Day Event
- Teaching assistant for one MSc course at Wageningen University

Oral Presentations

- *Extrapolation of effects of pesticides on aquatic communities and ecosystems across different exposure patterns* . SENSE/EPCEM symposium "Looking ahead at emerging issues in the environmental sciences" in the session "The Cutting Edge of Freshwater Ecology", 10 October 2008, Wageningen and SETAC Europe 18th Annual Meeting, 20-25 May 2008, Warsaw, Poland
- *Effects of different time-variable exposure regimes of the insecticide chlorpyrifos on freshwater invertebrate communities in outdoor microcosms*, The Netherlands Annual Ecology Meeting (NAEM), 9 - 10 February 2010, Lunteren, SENSE symposium Land-Water-Atmosphere interactions, 9 March 2010, Wageningen and SETAC Europe 19th Annual Meeting, 31 May - 4 June 2009, Gothenburg, Sweden
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- *Toxicodynamic experiment for different time-variable exposure regimes of the insecticide chlorpyrifos on aquatic arthropods*, SETAC Europe 21st Annual Meeting, 15-19 May 2011, Milan, Italy
- *Ecological impacts of time-variable exposure regimes of pesticides on freshwater communities*. SETAC 6th World Congress & SETAC Europe 22nd Annual Meeting, 20-24 May 2012, Berlin, Germany

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Mr. Johan Feenstra