The Effects of mixtures of pesticides, in use in Thailand, on the aquatic macrophyte *Lemna minor*

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Abstract

The aim of the present study was to assess the effects of four herbicides commonly used in Thailand (atrazine, 2,4-D, alachlor, paraquat)on the aquatic plant *Lemna minor* under differing patterns of exposure (single-, mixture-, and sequential-exposure). The endpoint of interest was the growth rate of plants over time.

In the single-compound toxicity studies, paraquat was found to be the most toxic pesticide followed by alachlor, atrazine and 2,4-D. Mixture studies were then done on the pesticides to understand how they would interact. Comparison of data from toxicity tests on mixtures of the pesticides with modelling predictions indicated that atrazine and2,4-D interact antagonistically whereas alachlor and paraquat interact synergistically. These results are in agreement with other mixture studies with pesticides.

Studies were also done to understand the effects of the different pesticides when applied in sequence. Comparison of the experimental results with predictions from a simple model demonstrated that at low effect concentration herbicides, the model works well but at higher concentrations it falls down. To explore the reasons for this, a further study was done to assess the carry-over toxicity of the study compounds.

This work demonstrates approaches to understand the effects of pesticides under more realistic exposure conditions. It demonstrates that while modelling approaches are available for estimating impacts under more realistic exposures, the accuracy of the predictions is likely to be highly dependent on the mode of action and concentration of the pesticide and the duration of the exposure.

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CHAPTER 1

1. Introduction

Background and significance of the problems

Thailand is known as an agricultural country due to its geographic conditions which are suitable for plant growth and which allow a wide variety of crops to be grown which are of high quality(Panuwet et al., 2012a). The agricultural sector is the main source of income for a large proportion of the Thai population(Plianbangchang *et al*., 2009). Major agricultural activities in Thailand include the cultivation of field crops, rice, orchards and tree plantations. All of these agricultural activities require extensive use of pesticides to control pests and weeds. In recent years, the total amount of imported pesticides has dramatically increased with a 3 fold increase seen from 1994 to 2005 with the amount used reaching more than 80,000 tonnes in 2004 (Department of Pollution Control, 2005; Iwai *et al*., 2007; Department of Agriculture, 2010; Iwai *et al*., 2011).

As a result of the increasing use of pesticides, there is an increased likelihood that pesticides may contaminate the Thai environment. For example, the contamination of drainage water from crops and paddy fields has been one of the major non-point sources of pollution in aquatic ecosystems in Thailand (Sanchez *et al*., 2006; Iwai *et al.*, 2007). Around 95% of freshwater in Thailand is used to irrigate more than 5 million hectares of agricultural land (Iwai *et al*., 2007) and waste water from this activity can result in significant contamination of aquatic ecosystems (Iwai *et al*.,

2011b). This contamination can lead to a range of adverse effects on the environment from cellular effects in organisms to effects at the level of the whole ecosystem (USGS, 2000; Iwai *et al*., 2007). Furthermore, the contamination might affect wildlife species either by direct exposure or through bioaccumulation through the food web causing a loss of biodiversity and malfunctions in the aquatic ecosystem (Fairchild *et al*., 1999; Hanazato, 2001; Iwai *et al*., 2007).

Agriculture and pesticide use in Thailand

Thailand is predominantly an agricultural country and has a long history of exporting agricultural products due to its climate which is suitable for the growth of a wide variety of crops and also high quality strains of agricultural products(Semathong *et al*., 2008). Agricultural activities such as field crops and rice cultivation, orchards and tree plantations require extensive use of pesticides to control pests and weeds (Semathong *et al*., 2008). There are variations in pesticide use in different regions of Thailand. For example in the Northern regions of Thailand, where a large variety of crops are grown (including rice fields, orchards and tree plantations), the most used pesticides are glyphosate, paraquat, chlorpyrifos, mancozeb and methomyl (Chalermphol and Shivakoti, 2009, Semathong et al., 2008, Panuwet et al., 2008b, Thapinta and Hudak, 1998).The imported quantity of herbicide was 15,536 tonnes in year 2000 and has continued to rise over the last decade. In addition, The Office of Agricultures Economics (OAE) and the Office of Agriculture Regulation (OAR) showed that the quantity of herbicides used has also continued to increase over the last decade (Figure 1; (Panuwet et al., 2012a)). In terms of active ingredients, the most imported herbicide was glyphosate, followed by 2,4-D (Table 1-1).

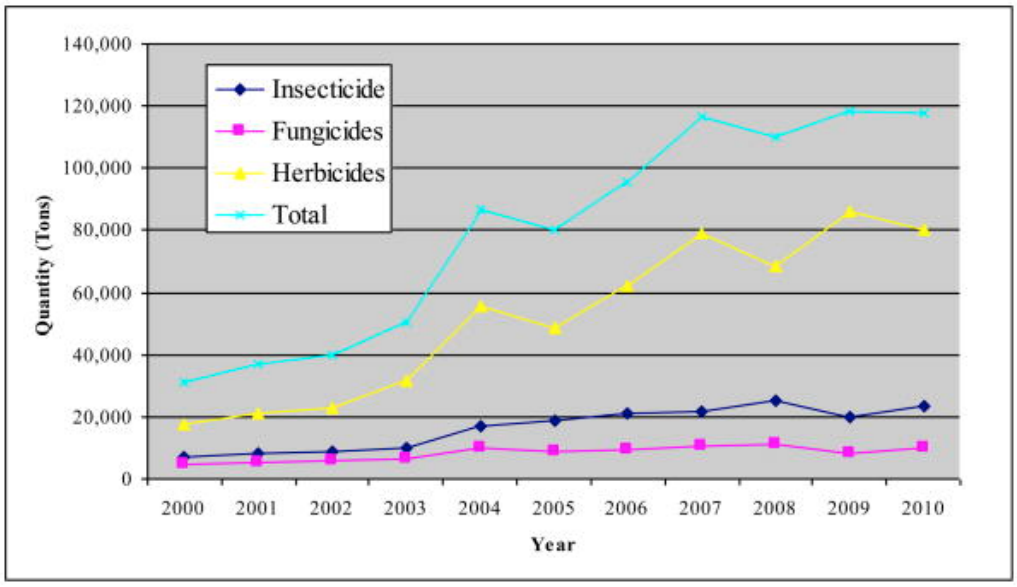


Figure 1-1: Summary of imported pesticides between 2000 to 2010 (Panuwet et al., 2012a).

Table 1-1:The most imported pesticide active ingredients in Thailand in the year 2000 (Sematong *et al.*, 2008). Amounts are provided for the product and the active ingredient.

Pesticide	Quantity of product (tonnes)	Active ingredient (tonnes)
glyphosate	15,536	7,787
2,4-D	2,356	1,965
methamidophos	2,778	1,941
atrazine	1,568	1,227
mancozeb	1,540	1,225
parathion methyl	1,257	1,041
endosulfan	1,066	994
paraquat	2,160	982
sulfur	1,121	8
copper oxychloride	848	25

Previous studies have indicated that, in order to save labour costs associated with spraying chemicals in fields, farmers in Thailand will usually mix two or more pesticides in one application(Panuwet et al., 2008b, Chalermphol and Shivakoti, 2009). As a result of the extensive usage of pesticides and these practices, large amounts of mixtures of pesticides are applied to agricultural systems where they can contaminate and degrade water bodies. As a result, in rural areas, nearly 70% of the populations are facing problems related to water quality due to chemical contamination in both surface and groundwater sources (Tirado *et al*., 2008).Furthermore, the Pollution Control Department reported that more than 40% of surface waters were of poor or very poor quality. From 1993 to 1997 the main rivers in Thailand were monitored for the presence of pesticide residues to determine whether they are at concentrations above advisable limits (PCD, 2001). Organochlorine pesticides were detected in 40.62% of the samples at concentrations ranging from 0.01 to 1.21 µg /L and organophosphate pesticides were detected in 20.62% of samples at concentrations ranging from 0.01 to 5.74 µg/L. These concentrations were compared to the safe limits for pesticides in drinking water established by the European Union of 0.1µg /L for single pesticides and 0.5 µg /L for the sum of all pesticides detected(Chulintorn, 2002). Other pesticides have been detected in streams and rivers, including carbamate pesticides (detected in 12.39% of the samples in concentrations ranging from 0.01 to 13.67 µg /L), atrazine (detected in 20% of the samples in concentrations ranging from 0.01 to 6.64 µg /L) and paraquat (detected in 21.63% of the samples in concentrations ranging from 0.14 to 87.0 µg /L)(Chulintorn, 2002, Tirado et al., 2008).

There are several reports of intensive usage of pesticides in Northern Thailand in the Chiang Mai province (Chalermphol and Shivakoti, 2009, Panuwet et al., 2008b). The

province consists of mountainous regions and the climate is quite cold all year round compared to other parts of Thailand, with an average temperature of 19.8 ^{0}C (Panuwet et al., 2008b). The amount of rainfall and the availability of water supplies makes the region suitable for crop cultivation. This province produces large amounts of agricultural products such as tangerines, cut flowers, temperate vegetables and fruits. Chemicals have been used intensively, especially pesticides. There are reports that farmers in Chiang Mai province spend more money on pesticides than farmers in any of the other provinces in Northern Thailand (The 1st Office of Agricultural Economics, 2007; Chiang Mai Office of Agriculture Economics, 2007; Panuwet *et al*., 2008). The identities of the most widely used pesticides in Chiang Mai are provided in Table 1-2 (Panuwet et al., 2008b, Chalermphol and Shivakoti, 2009). The next section provides an overview of the fate and the behaviour of pesticides in aquatic environments.

Table 1-2: The most used pesticides in rural areas in Chiang Mai,Thailand (Panuwet *et al*., 2008).

Order of usage	Pesticide
1	Glyphosate
2	Mancozeb
3	Paraquat
4	Methomyl
5	2,4-D
6	atrazine
7	chlorpyrifos

Pesticidesin aquatic environment

Fate and behaviour of pesticides in aquatic environments.

Once a pesticide is introduced into the aquatic environment through application to crops, disposal or spillages, the behaviour of the pesticide will be influenced by many factors, in particular the persistence and mobility of the pesticide (Fishel, 1991, Kerle et al., 2007). The fate of pesticides is influenced by many factors including the properties of the soil, the properties of the pesticides, hydraulic loading on the soil and crop management practices (Fishel, 1991, Kerle et al., 2007). The behaviour of a pesticide is somewhat predictable based on the information on the properties of the compound. Some of the most important properties of a pesticide that can be used to predict its environmental fate are the degradation half-life, soil sorption coefficient, water solubility, vapour pressure and Henry's Law constant (Tiryaki and Temur, 2010). The main factors and processes affecting the level of contamination of pesticides in the environment are described below.

1) Release of the pesticide into the environment

The extent that a pesticide is released into the natural environment will be very important in determining the levels of contamination. The amount of pesticide released into the environment will be determined by the characteristics of the formulation, method and rate of application as well as topography, amount and type of vegetation and groundcover and the weather conditions (Fishel, 1991, Kerle et al., 2007, Tiryaki and Temur, 2010).

2) Persistence

Persistence reflects the potential for a pesticide to break down into other compounds (degradation products) that have different chemical structures and properties. Ultimately pesticides may be completely broken down into CO_2 and H_2O. The persistence of a pesticide is determined by the chemical structure of the pesticide as well as the activity and nature of microbes found in the soil, soil and water properties (such as pH, soil moisture content) and the level of sunlight (Das *et al*., 1995). Mulla (1996) stated that the longer a pesticide persists before it breaks down, the greater chance it has for contaminating surface waters and groundwaters. Pesticide degradation occurs mainly in the biologically active zone of soils where plant roots are abundant. It is important to keep pesticides from leaching out of the rooting zone because pesticides break down more slowly in the deeper soils and sediments (Mullar, 1996, Kerle et al., 2007, Beard, 2009).

Photogradation is an important degradation process and involves the breakdown of pesticides by sunlight. The intensity and spectrum of sunlight, length of exposure and properties of the pesticide affect the rate of photodegradation or photolysis (Kerle et al., 2007, Beard, 2009).

Pesticide persistence is often described in terms of half-life. This is a constant for a given compound and a given environmental degradation process that occurs under specific conditions (Connell *et al*., 1999). The half-life is the length of time required for one half of the original quantity of a pesticide to break down. The half-life can be used to classify substances in terms of the general persistence properties (e.g. Table 1-3; Mackay *et al*., 1997; Kerle *et al*., 2007).

Table 1-3: Pesticide persistence classification based upon degradation half-lives (Kerle *et al.*, 2007).

Non persistent (half-life less than 30 days)	**Moderately persistent** (half-life greater than 30 days, less than 100days)	**Persistent** (half-life greater than 100 days)
aldicarb (Temik)	atrazine (AAtrex)	bromacil (Hyvar)
alachlor (Lasso)	carbofuran (Furadan)	DBCP (Nemagon)
butylate (Sutan)	DCPA (Dacthal)	dieldrin (Alvit)
dicamba (Banvel)	glyphosate (Roundup)	diuron (Karmex)
metalaxyl (Apron)	metribuzin (Sencor)	picloram (Tordon)
	pronamide (Kerb)	
	simazine (Princep)	
	terbacil (Sinbar)	
	triallate (Fargo), trifluralin (Treflan)	

3) Mobility

Pesticide mobility reflects the potential for a pesticide to move off site. The pesticide mobility is affected by the sorption behaviour of the compound in soil, volatilization and water solubility (Kerle et al., 2007, Beard, 2009). Each of these is discussed below.

3.1) Adsorption

Adsorption is the process by which a chemical bonds to colloidal materials, such as soil organic matter, clay particles or other surfaces(Kerle *et al*., 2007). Adsorption is an extremely important process affecting pesticide fate. Strongly adsorbed pesticides will be less mobile when applied to soil than weakly adsorbed pesticides(Connell *et al*., 1999). Pesticide adsorption is controlled by environmental factors such as pH, temperature, and water content of the soil and the amount and

type of organic matter present. In general, pesticide adsorption relates inversely to pesticide solubility in water. Highly soluble pesticides are typically more weakly adsorbed in a given soil than are sparingly soluble pesticides. Thus, highly soluble pesticides pose a greater threat for contamination of groundwater(Fishel, 1991, Kerle et al., 2007).

3.2)Water solubility

Water solubility describes the amount of pesticide that will dissolve in water at saturation (Kerle *et al.*, 2007). The solubility of pesticides that are weak acids or bases is influenced by pH. Kerle *et al*., (1996) stated that highly soluble pesticides are more likely to move within the site or off site by runoff or leaching. In addition, the degree of plant uptake is determined by the pesticide's water solubility.

3.3). Volatilization

Volatilization from moist soil is described by the Henry's Law constant (K_h). K_h is defined as the concentration of the pesticide in the air divided by the concentration in water at equilibrium. This value can be calculated from the pesticide vapour pressure and solubility (Kerle *et al*., 2007). K_h can be used to determine the likelihood of a pesticide moving between air and the soil water. The higher the K_h, the more likely that a pesticide will volatilize from moist soil (Connell et al., 1999, Kerle et al., 2007).

4) Site conditions

Areas with high rates of rainfall or irrigation may have large amounts of water moving through the soil and this increases the risk of pesticides contaminating ground water and surface water. Runoff is the movement of water over a sloping

surface and can carry both dissolved pesticides as well as those adsorbed to eroding soil(Taylor *et al*., 1991).

5) Patterns of pesticide applications

Pesticides are frequently applied on a fixed schedule of sequential applications irrespective of the occurrence or the level of pest infestation (Matthews, 1979). Besides this, the exposure to pesticide released into the environment often occurs in pulses and involves runoff after the rain or spray drift (Rosenkrantz *et al*., 2013). The duration of a pulse sequential pesticide application can vary from a few hours and up to 1-2 days, and the concentration of the pesticide's pulse is dependent on the type of the pesticide and the recipient's characteristics (Cedergreen et al., 2005, Rosenkrantz et al., 2013).

An ideal pesticide is one that should elicit an effect on a target organism but should also be degraded immediately to non-toxic chemical constituents(Calow, 1998). Pimentel and Edwards (1982) stated that the environmental quality and function of ecosystems may be reduced by pesticides. The effects of pesticide on the ecosystem can occur via a number of pathways, including modifications in species diversity, modifications of the food chain structure, which change the patterns of energy flow and nutrient cycling as well as modifications in the quality of soil, water and air. Some of these impacts of pesticides are described in the next section.

The impacts of pesticides in aquatic environment

Pesticides are generally chosen based on their efficacy or cost rather than on their impact on the environment (Kovach *et al*., 1992). Therefore, many of the pesticides get through to water bodies *via* the many routes described above. Many of these

pesticides are not easily degradable. They persist in the aquatic environment and, depending on their chemical properties, can enter aquatic organisms either directly through ingestion or absorption of contaminated water or indirectly by feeding on previously contaminated organisms (Williams *et al*., 1996). At the cellular level, pesticides can inhibit cell division, photosynthesis, and growth, alter membrane permeability, change metabolic pathways and inhibit the action of enzymes (Reese *et al*., 1972). In addition, the storage of pesticide residue in the bodies of aquatic organisms may affect the vitality of the developing growth stage (Reese *et al*., 1972). Furthermore, the impact of a pesticide may result in acute poisoning which leads to immediate flora or fauna kill. Chronic effects, which occur when the degree of exposure of an organism to a pesticide exceeds the capacity of the organism to detoxify and eliminate the pesticide residue, may cause structural imbalance in the aquatic community.

The impact of pesticides on aquatic organisms and ecotoxicological assessment of pesticides on aquatic plant.

Once a pesticide enters the aquatic environment, aquatic organisms may be exposed to it in several ways including direct entries of pesticides into their habitats and the movement of organisms into areas previously contaminated by retaining pesticides (Reese *et al*., 1972). As a consequence of this, aquatic organisms are potentially at risk from pesticides (Wilson and Koch, 2013).

Aquatic ecosystems support an enormous diversity of fauna and flora around the world (Lydeard and Mayden, 1995). Freshwater ecosystems comprise diverse communities of species and provide food and water for mammals and birds (Ricciardi and Rasmussen, 1999).

As a key component of the ecosystem, aquatic macrophytes are routinely used in the assessment of the risks of chemicals to the aquatic environment. The aquatic vascular plants of the genus *Lemna* (duckweed), especially *Lemna minor* and *Lemna gibba*, have been widely used as a model organism for phytotoxicity testing (Wang, 1991, Zezulka et al., 2013). The advantages of *Lemna* for ecotoxicity include its small size, ease of handling and culturing in the laboratory, rapid growth rate, and sensitivity to a wide range of pollutants (Zezulka *et al*., 2013). *L. minor* and *L. gibba* (also known as duckweed) belong to the family Lemnaceae and are widespread in Europe and also in Thailand (Dudley *et al*., 1981).

Ecotoxicological assessment of chemicals: Phytotoxicity testing

Phytotoxicity is the capacity of a compound such as a plant protection to cause temporary or long lasting damage to plants (European and Mediterranean Plant Protection, 1997). European and Mediterranean Plant Protection (1997) explained that there are several ways to observe the symptoms of phytotoxicity and these are described in Table 1-4 below.

Table 1-4: The symptoms of phytoxicity in plants(European and Mediterranean Plant Protection, 1997).

Type of phytotoxicity	Symptoms
Modification in the development cycle	- Delays in flowering, fruiting and ripening. - Non-appearance of certain organs (i.e. leaves, flowers and fruits.)
Thinning	Loss of whole plants by failure to emerge or to grow after transplanting or by disappearance of the plants after emergence.
Modification in colour	- Chlorosis, browning, and reddening. - Discoloration may be localised such as internal or external spots.
Necrosis	Local death of tissue or organ, generally appearing first as discolorations or necrotic spots on leaves.
Inhibition or stimulation	Numbers of individual organs, height, shoot length, diameter or area.
Deformation	The abnormality of plant morphology such as curling, rolling, stunting or elongation, changes in size or volume and the effects on quantity and quality of the yield of plant.

Phytotoxicity experimental tests are frequently used as part of the ecotoxicological assessment of chemicals. Many reports have assessed the phytotoxicity of herbicides using a variety of aquatic plants such as algae and aquatic macrophytes (Wang and

Williams, 1990, Wang, 1991). A list of some of the ecotoxicity tests that have been done on the pesticides studied in this thesis using aquatic plants is provided in Table 1-5. The data in the Table indicate that, of the pesticides tested, paraquat and alachlor have an effect at low concentrations whereas, 2,4-D is less toxic to aquatic organisms.

Table 1-5: List of ecotoxicity tests with aquatic organisms for the study compounds investigated in this thesis.

Pesticide	Mode of action of pesticide	Species test	Symptoms	EC50 (µg l^{-1})	Duration test	Reference
atrazine	*Photosystem II inhibitor*	*L. minor*	Dwarf frond	48-70	48-h	Kirby *et al*., 1994
		L. minor	-Loss of chlorophyll a	122	3-d	Teodorovic *et al*., 2011
		Myriophyllum aquaticum	-Loss of chlorophyll a	94	3-d	Teodorovic *et al*., 2011
		S. capricornutum	N.D	69.7	24-h	Turbak *et al*., 1986
			N.D	9.5	7-d	Robers *et al*., 1990
2,4-D	Auxin mimic	*L. minor*	Non-toxic	>100000	4-day	Fairchild *et al*., 1997
			N.D	6500(1000-8600)	24-h	Sander, 1970
			N.D	5900(3100-11000)	48-h	Sander, 1970
			N.D	1400(1100-1800)	24-h	Sander, 1970

		Myriophyllum spicatum	N.D	0.9 (n.c.)	7-d	Mohr *et al*., 2013
Paraquat	Photosystem I inhibitor	*L. minor*	N.D	62	6-d	Fairchild *et al*., 1997
			N.D	51 (25-77)	4-d	Kuster *et al*., 2007
			Death and bleaching effect at high concentration (100,100ppb)	31	28-d	Mohammad and Itoh, 2007

Pesticide	**Mode of action of pesticide**	**Species test**	**Symptoms**	**EC50 (µg l^{-1})**	**Duration test**	**Reference**
alachlor	Shoot inhibitor	*P. subcapitata*	Loss of biomass	12	3-d	Pavlic *et al*. 2006
		L. minor	Dwaft frond	482	4-d	Fairchild *et al*., 1994
		L. minor	N.D	198	4-d	Fairchild *et al*., 1997
		L. minor	N.D	482	4-d	Fairchild *et al*., 1998

Aquatic macrophyte for risk assessment for pesticide

Primary producers play critical roles in the aquatic system, providing a food source for birds and fish and shelter and protection for aquatic animals. *Lemna minor* is one such aquatic plant and has been used extensively in phytotoxicity tests (Kirby and Sheahan, 1994)as a representative of higher aquatic plants. It has a small size and rapid reproductive rate with a doubling time of 1-4 days (Lewis, 1994). As a consequence, numerous aquatic ecotoxicological studies have been done to assess the effects of herbicides on this macrophyte(Geoffroy et al., 2004). The *Lemna* growth inhibition test is widely used in ecotoxicology. There are many standard test protocols including the OECD guidelines for the testing chemical, *Lemna* sp. growth inhibition test 221 and the ISO/FDIS 20079 which are used for determining the toxic effect of water constituents and waste water on the plant(Maltby et al., 2010).

In terms of the risk assessment for herbicide by the European Union (EU), the risk of herbicides on aquatic plants and algae are initially evaluated by calculating toxicity exposure ratio (TERs) between toxicity endpoints (EC50 values) derived from standard laboratory work with algae or *Lemna* species and the predicted environmental concentration (PECs). The resulting TER is compared with a trigger of 10. TER value exceeding 10 indicate that the compound can be considered to pose an acceptable risk to aquatic plants, whereas TER value that falls below 10 indicate a potentially unacceptable risk and a need for a higher-tier risk assessment (Maltby et al., 2010).

Pesticide mixtures in aquatic ecosystems and chemical interactions

In the aquatic ecosystem, it is not uncommon to find a combination of several pesticides in agricultural areas (Daam *et al.*, 2009). The type of pesticide that will be present within the mixture is dependent on the dominant crops in that area(Deneer, 2000). The presence of mixtures of pesticides may lead to a lower or higher toxic effect than would be expected from exposure to single compounds (Larsen *et al*., 2003). Therefore, it is important that the effects of pesticide mixtures are assessed on particular systems. Chemicals can interact with each other during uptake and metabolism to produce a greater effect (synergism) or smaller effect (antagonism) than expected (Firpo, 2011). In order to assess the toxicity of pesticide combinations, it is necessary to have information on the composition of the mixture and the mechanism of action of the compounds in the mixture (Reffstrup *et al*, 2010). The next section reviews the different types of toxic interactions that can occur.

Mixture toxicity theory

Type of combined actions

In order to understand how mixtures of pesticides affect an environmental system, it is necessary to understand the combined action of compounds in a mixture. A number of combination effects are possible including: no interaction in the form of simple similar action (dose addition), simple dissimilar action (response addition) or interaction of a combined effect (antagonism or synergism) (Teuschler, 2009; Reffstrup *et al.,* 2010).

1) No-interaction

There are two models of no-interaction of chemical combinations. Firstly, simple similar action or concentration addition (CA) is the model that assumes that the chemical compounds in a mixture act on the same biological site by the same mechanism or mode of action (Alexander *et al*., 2008; Reffstrup *et al*., 2010). In contrast, simple dissimilar action or independent action (IA) is the model that assumes that the compounds in the mixture do not interfere with each other and also do not act by the same mode of action. Even though the mechanisms of the chemicals are always different, the presence of one chemical will not affect the toxicity of the other chemical (Alexander *et al*., 1998).

For compounds that interact *via* simple similar action and simple dissimilar action, the combined doses of a mixture may lead to a toxic response even if the individual compounds are at levels below the effect threshold (no-effect level) (Alexander *et al*. 2008). Particularly if "no-effect" is defined as a statistical NOEC, which is often in the range of EC10-EC30 dependent on the design and variance in the system.

2) Interactions

Interactions are defined as combined actions that may result in either a weaker (antagonistic) or stronger (synergistic) combined effect than the additive affect. The interactions can be divided into direct chemical-chemical interactions, or interactions on toxicokinetic or toxicodynamic processes(ATSDR, 2001; Alexander *et al*., 2008).

Antagonism is defined as the situation where the combined effect of two chemicals is less than sum of the effects of each chemical and typically occurs when interaction takes place at the same receptor site. Synergism is defined as a situation where the

combined effect of two chemicals is greater than the sum of the effects of each chemical given alone (Table 1-6).

Table 1-6: Classification of combined toxic actions of two compounds in a mixture (The Danish Veterinary and Food Administration, 2003 modified after Placket and Hewlett, 1952).

Interaction	**Combined action**	
	Similar action	**Dissimilar action**
Absent (No interaction)	Simple similar action (Dose addition)	Simple dissimilar action (Independent action, Response multiplication)
Present (Interaction)	Complex similar action, (Antagonism or Synergism)	Complex dissimilar action (Antagonism or synergism)

Experimental methods to assess pesticide mixture interactions

To understand the impacts of a pesticide mixture, it is important to appreciate that the toxicological characteristic of a mixture is dependent upon the identity of the chemical components, the concentration of the mixture and the concentration ratio of the components in the mixture (Borgert *et al*., 2001).

The concept for calculating predictions with isobolographic methods

Investigating the predictive power of concentration addition and independent action

There are several methods for estimating effects from concentration addition or independent action the approaches most frequently used to justified the nature of mixture interaction. The isobologram approach is one of the experimental methods

that has been widely used to understand the combined effects of a mixture (Altenburger et al., 1996, Cedergreen et al., 2013).

Isobole

An isobole is contour line that is constructed from the equi-effective quantities of two agents (Loewe and Muischnek, 1926; Larsen, 2001). The theoretical line of additivity is the straight line that connects the individual doses or concentration of each of the single compounds. In the case of an antagonistic interaction the equi-effective concentrations in the mixtures represent a convex line (displaced to the top right). In contrast, synergistic interaction would produce a concave line (displaced to the bottom left) (Figure 1-2).

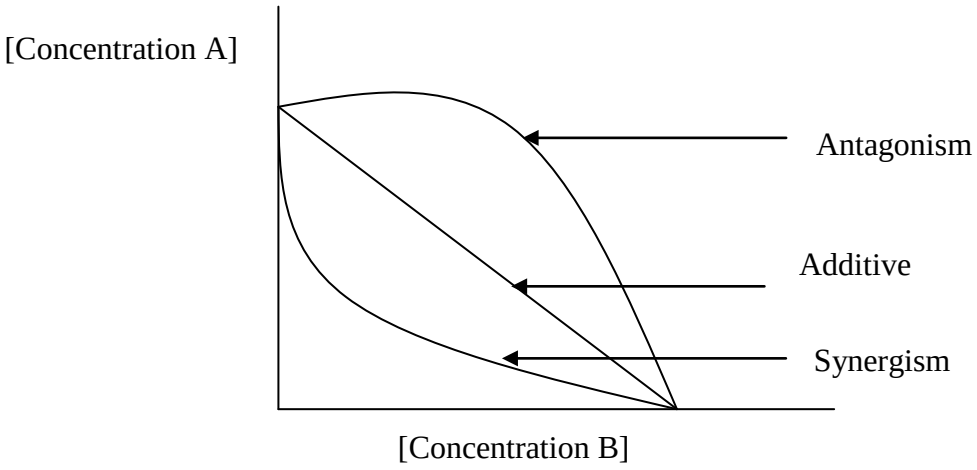


Figure1-2:Isobologram showing antagonism, additive and synergism lines

The results can be compared with the predictions by the concentration addition (CA) or independent action (IA) models which are described below. The comparison can be done either statistically or graphically, for example, a graphical comparison is the isobologram (Cedergreen et al., 2013). The response surface modeling is created by mixture ratio ray design between two chemicals. The ratios are usually chosen to

cover the response surface evenly based on a certain proportion of the effect concentration from the compound A, while the residual effect concentration comes from compound B. Also, the effect ratio can be set in variety which depends on the experimental set up, for example, it can be set up for 3 ratios: 25:75%, 50:50% and 75:25% or 7 ratios: 17:83%, 63:37%, 50:50%, 37:63% and 83:17% (Sorensen et al., 2007, Cedergreen et al., 2013).

Models to predict pesticide mixtures

There are two different models to predict mixture toxicity called Concentration Addition (CA) and Independent Action (IA), which are used to predict the combined effects of chemicals with similar and dissimilar modes of action (MOA). Both models allow the prediction of the effects of a mixture based on the knowledge of the toxicity of single chemicals (Mikkelsen, 2012). These two models are the most commonly used to predict joint effects of chemicals (Cedergreen et al., 2008). The concept of CA assumes that the toxicants with the same mode of action will act upon the same target in the organism (Rider and LeBlanc, 2005, Cedergreen et al., 2007c, Cedergreen et al., 2007b, Ferreira et al., 2008, Pavlaki et al., 2011). The theoretical assumption for IA is that the chemicals in a mixture do not interact physically, chemically or biologically due to the fact that they act independently of each other (Cedergreen et al., 2008, Ferreira et al., 2008). When a stressor's mode of action is unknown, experimental data can be generated for the mixtures and both models are then used - the one that best fits the data is then chosen for further use (Pavlaki *et al*., 2011)

1). Concentration addition

The concept of concentration addition assumes that the mixtures have a similar action and it was originally outlined for binary mixtures (Loewe and Muischnek, 1926) and is generally defined by the formula

$$\sum_{i=1}^{n} \frac{c_i^*}{ECx_i} = 1$$

Where c_i^* are the individual concentrations of the substance 1 to *n* and ECx_i denote the equivalent effect concentration of the single substances (e.g. $EC50_i$).

2). Independent action (IA)

The concept of independent action assumes a dissimilar action of mixture component (Bliss, 1939). The theoretical basis of this model is that the toxicant will interact with different molecular sites and that they have different modes of action. IA is commonly defined for a binary mixture by the equation

$$E(c_{mix}) = E(c_1) + E(c_2) - E(c_1) \times E(c_2)$$

Which can be extended to any number of mixture components using the following equation

$$E(c_{mix}) = 1 - \prod_{i=1}^{n} (1 - E(c_i))$$

Where C_i are the actual concentrations of the individual substance 1 to *n* in the mixture. $E(c_i)$ are the fractional effects (x%) caused by the individual substances and $E(c_{mix})$ is the total expected effect of the mixture. IA assumes the response is binary.

Pesticide mixtures in aquatic ecosystems and previous studies into ecotoxicological interactions of pesticide mixtures

In aquatic ecosystems, it is not uncommon to find a combination of several pesticides present in surface waters in agricultural areas (Daam *et al.*, 2009). The type of pesticide used is dependent on the dominant crops in that area(Deneer, 2000). The presence of mixtures of pesticides may lead to lower or higher toxic effects than would be expected from exposure to single compounds (Larsen *et al*., 2003). Therefore, it is important that the effects of pesticide mixtures are assessed on particular systems.

Over the past few years, a number of studies have attempted to begin to understand the toxic interactions of chemical mixtures in order to determine whether or not components of mixtures could interact to produce increased toxicity in target or non-target organisms compared with individual chemical exposures (Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment,2002). The findings of some of these studies are reviewed below and are summarised in Table 1-6.

Cedergreen *et al*. (2005) explored the development of the shape of dose-response relationships for four different recommended endpoints of *Lemna minor* (surface area, frond number, and fresh weight-specific and dry weight-specific relative growth rate) in mixture toxicity experiments with metsulfuron-methyl and terbuthylazine and used two models to predict the toxicity: independent action (IA) and concentration action (CA) models. The result showed that after a test time of 6 days, predictions of IA based on RGR_A and RGR_{FW} showed antagonism and RGR_{DW} showed synergism. When the CA model was applied different conclusions

were reached depending on the endpoint. Cedergreen (2005b) also studied the combination effects of ten herbicides on *Lemna minor* and *Pseudokirchneriella subcapitata* to predict factors and hazards in the aquatic environment. The result showed that the two mixtures of herbicide with the same mode of action produced a joint effect that was additive. In the studies with eight mixtures of herbicides with different modes of action, two of the mixtures were antagonistic. Furthermore, Junghans *et al*. (2005) explored the application and validation of approaches for predictive hazard assessment of realistic pesticide mixtures using two models: concentration addition in the case of similarly acting substances and independent action when substances were dissimilarly acting. *Scenedesmus vacuolatus*was used to test the effects of pesticide mixtures with the same and different modes of actionin run-off water. The results indicated that the concentration addition model can provide a better prediction of the toxicity of pesticide mixtures even though those pesticides do not share the same mode of action. However, concentration addition models cannot predict contaminant interactions in complex mixtures under realistic exposure scenarios.

Faust *et al*. (1994) observed the toxicity of pesticides in binary combinations on the freshwater algae *Chlorella fusca*. In order to predict the toxicity, the experimental data were assessed using a concentration addition model and estimated concentration function response using probit transformation of data and weighted linear regression analysis. The results showed that only four mixtures of compounds were more toxic than expected and that the combination of anilazine and tri-allate acted synergistically to the algae. In addition, Folt *et al*. (1999) explored synergism and antagonism among multiple environmental stressors using three models such as additive, multiplicative and simple comparative effects model, and the models were

used to compare the data from a test in the laboratory into the effects on cladoceran zooplankton. Thermal stress, toxin exposure, reproduction and survival were observed during the test. Synergism occurred when combined toxins were tested using low food concentrations and a test temperature of 30 ^{0}C.

Belden *et al*. (2000) studied the acute toxicity of atrazine and four organophosphate insecticides, chlorpyrifos, methyl parathion, diazion and malathion on *Chironomus tentana*. The toxicity tests were performed on both single compounds and mixtures of substances. When tested individually, atrazine was not toxic even at high concentrations (1000 µg/l).However, when combined with chlorpyrifos, methyl parathion and diazinon, the toxicity increased. Similary, Lydy and Linck (2003) observed the impact of chlorpyrifos when mixed with three triazine herbicides, atrazine, cyanazine and simazine, on *Eisenia fetida*(an earthworm species). The acute toxicity tests were done on both individual and combinations of compounds. The results showed that atrazine and cyanazine were more toxic at low concentrations than chlorpyrifos. However, chlorpyrifos was more toxic when combined with atrazine and cyanazine (7.9-fold and 2.2-fold increase in toxicity respectively). In addition, Lydy and Austin (2004) examined the toxicity on *Chironomus tentans* of nine pesticides that had been detected in the Sacramento-San Joaquin Delta. The toxicity tests were done both on single and binary mixtures and the results indicated that chlorpyrifos and diazinon when tested individually were more toxic than when tested in combination. Deneer (2000) reviewed the literature data between 1972 and 1998 that describe the toxicity of pesticide mixtures in aqueous environments. Concentration addition was generally found to be the best model for describing the joint effect of mixtures of pesticides with similar modes of action rather than different mode of action. Key *et al*.(2006) studied the toxicity of

three pesticides, atrazine, fipronil and imidacloprid, on both individual and combinations of substances using *Palaemones tespugio*. The result showed that fipronil was the most toxic in shrimp larvae with an LC50 of 0.68 µg/Lover 96-hour and atrazine was not shown to be toxic to the shrimp at concentrations up to 10,000 µg/L. However, when atrazine was combined with the two herbicides, the toxicity was found to be greater than the additive effect. In addition, Belden et al (2007) reviewed publications on the toxicity of pesticide mixtures to aquatic organism which had used different types of models including concentration addition (CA), independent action (IA) and simple interaction (SI), to model the experiments. They found that CA model was often used to evaluate the toxicity of mixtures followed by SI and IA and 90% of all mixtures was described well with CA with experimental observations being within a factor of two of the prediction. Therefore, CA was generally found to be the best performing approach and the IA was found to generally under-predict toxicity. On the other hand, IA was more accurate than CA for mixtures comprising compounds with different mode of actions. Furthermore, Cedergreen (2014) reviewed the scientific literature on three main groups of environmentally relevant chemical toxicants including pesticides, metal ions and antifouling compounds. She found that synergy occurred in 7%, 3% and 26% of the binary pesticide, metal and antifoulants mixture respectively.

In summary, the effects of pesticide mixture are likely to be additive if the mixture comprises chemicals from the same mode of action, particularly pesticides that inhibit photosynthesis (Table 1-6). However, if pesticide mixtures contain compounds with different modes of action, the interaction is likely to be antagonistic.

Table 1-6: Summary of pesticide mixture toxicity studies on aquatic organisms.

Pesticides	Chemical group	Mode of action		Species test	Interaction	References
Atrazine+metribuzine	triazine	Inhibit photosystem II	Similar mode of action	*Chlorella fusca*	additivity	Altenburger *et al* 1990
Diuron+atrazine	Triazine	Inhibit photosystem II	Similar mode of action	Diatoms	addtivity	Legrand et al 2006
Diuron+hexazinone	triazine	Photosynthesis efficiency	Similar mode of action	Lemna sp	synergism	Kumar and Han 2011
Atrazine+hexazinone	triazine	Photosynthesis efficiency	Similar mode of action	Lemna sp	synergism	Kumar and Han 2011
Atrazine+simazine	triazine	Inhibit photosystem II	Similar mode of action	*Chlorella fusca*	additivity	Faust et al 1993
Bentazone+simazine	-Benzothiadiazines -triazine	- inhibit photosystemII - inhibit photosystemII	Similar mode of action	*Chlorella fusca*	additivity	Faust et al 1993
Bentazone+2,4-D	-Benzothiadiazines -phenoxy acid	-inhibit photosystemII -hormone….	Dissimilar mode of action	*Chlorella fusca*	additivity	Faust et al 1993
Bentazone+metazachlor	-Benzothiadiazines -Chloroacetamide	-inhibit photosystemII -inhibit lipid synthesis	Dissimilar mode of action	*Chlorella fusca*	antagonistic	Faust et al 1993
Chlorotoluron+2,4-D	- Phenylureas - phenoxy acid	-inhibit photosystemII -hormone	Dissimilar mode of action	*Chlorella fusca*	additivity	Faust et al 1993
Metazachlor+2,4-D	-Chloroacetamide - phenoxy acid	-inhibit lipid synthesis -hormone	Dissimilar mode of action	*Chlorella fusca*	additivity	Faust et al 1993
Matazachlor + Methabenzthiazuron	- Chloroacetamide - Benzoylthiazolylureas	-inhibit lipid synthesis - inhibit photosystemII	Dissimilar mode of action	*Chlorella fusca*	synergistic	Faust et al 1993

Mecoprop+terbuthylazine	- Aryloyalkanoic acid - 1,3,5-Triazine	- Synthetic auxin - inhibit photosystemII	Dissimilar mode of action	*Lemna minor*	Antagonistic	Cedergreen et al 2007
Acifluorfen+diquat	-Diphenylether -Bipyridyliums	-Cell membrane disrupter -inhibit photosystemI	Dissimilar mode of action	*Lemna minor*	Antagonistic	Sorensen et al2007

Chronic and pulsed exposure of aquatic organisms to pesticides

Chronic exposure refers to repeated, continuous exposure to pesticides over an extended period or long-term (Arcury et al., 2010). In order to understand the chronic toxicity of a compound, ecotoxicity tests need to be performed over longer time periods of weeks to months. However, in the real environment, continuous exposure rarely occurs (Cedergreen, 2014). Instead, chemicals typically occur in pulses due to the irregular nature of most anthropogenic discharges and the variable hydrology of receiving waters (Hogan et al., 2012). There are several reports that have compared chronic and pulsed exposures in term of ecotoxicity. For example, Stoughton et al (2008) investigated the acute and chronic toxicity of imidacloprid to the aquatic invertebrate *Chironomus tentans* and *Hyalella Azteca* under contant- and pulsed exposures and the results showed that Chironomid was more sensitive to acute and chronic imidacloprid exposure, but less sensitive to a single pulse, than *H. Azteca.* In addition, the two organisms were able to recover four days after a short – term pulse exposure.

Test durations are usually defined within protocols that have been developed for the test species, and are rarely adjusted to reflect environmental exposure durations (Diamond et al 2006, Zhao & Newman 2006, Erikson 2007). Boxall et al (2013) studied the effects of repeated pulses of four herbicides on *L. minor* and the results showed that there were different response depending on the herbicide, which may be explained by compound-specific uptake and degradation or dissipation rates in the plant.

Not only does the pattern of exposure matter but also the duration of time of the toxicity test(Drost, 2011). There are many reports that have evaluated the

relationship between the toxicity and duration of contaminant exposure(Oflaz et al., 2004, Ashauer et al., 2007a, Vallotton et al., 2008a, Ashauer et al., 2011b). However, more limited numbers of studies have examined the length of pulse exposure (Ashauer et al., 2006, Dennis et al., 2012). The length of pulse exposure has different effects on organisms which are explained by the toxicokinetic and toxicodynamic of a compound (Ashauer et al., 2011a, Kretschmann et al., 2012). Toxicokinetic (TK) refers to rates of absorption, distribution, storage, biotransformation and elimination. Toxicodynamic (TD) deals with the mechanism by which the toxicant interacts with the site of action within an individual organism (Ashauer and Brown, 2008). In addition, the effect of pulse exposure on plants is expected to depend on the rate of herbicide accumulation in the plant and ability of plant to recover after herbicide treatment. Cedergreen et al (2005) explained that herbicides that accumulate quickly will have a prolonged target-site exposure in contrast to slowly accumulating herbicides, which might not reach equilibrium between the plant and the environment, before the herbicides pulse concentration is declining. Therefore, the rate of herbicide accumulation mainly consists of three processes including the rate of uptake, the rate of inactivation of herbicide and the rate of herbicide release. The uptake and release rate depend on the physico-chemical properties of aherbicide such as the lipophilicity and charge of the herbicide. A lipophlic compound will diffuse more quickly into plant cells than hydrophilic compound and negatively charged ions will diffuse more slowly than neutral and positively charged ions (Cedergreen et al., 2005).

Modes of action / site of action of herbicides and type of damage on plants

According to the Herbicide Resistance Action Committee and Weed Science Society of America (HRAC and WSSA: http://wssa.net/wp-content/uploads/HerbicideMOAClassification.pdf), herbicides can be classified by their modes of action into growth regulators, seedling growth inhibitors, photosynthetic inhibitors and cell membrane disruptors. Each of these modes of action will result in different effects on a plant. Table 1-7 provides information on the sites of action/modes of action of herbicides and the resulting injury symptoms that will occur to plants (Gunsolus and Curran, 2002).

Table 1-7: Herbicide sites of action and injury symptoms to plant

Herbicide mode of action	Function	Injury symptoms and plant recovery	Herbicides
Growth regulators	- Growth regulator herbicides include synthetic auxin and auxin transport inhibitor compounds which are used to control broadleaf weed and are more effective on perennial broadleaf weed and brush control. -This group of herbicides are translocated through roots and foliage via xylem and phloem	-The growth and reproduction are abnormal, especially on new growth. Epinasty and leaf malformations are found in the forms of parallel venation, crinkling, leaf strapping and cupping	**Synthetic Auxin** - Phenoxy: 2,4-D, 2,4-DB, 2,4-DP, MCPA and MCPP - Benzoic acid : dicamba - Pyridinecarboxylic :picloram, clopyraid, fluroxypyr, triclopyr and aminopyralid - Quinoline: quinclorac Auxin Transport Inhibitor - Semicarbazone: Diflufenzopyr
-Seedling growth inhibitor	-Work during germination and emergence and include seedling shoot inhibitor, seedling shoot and root inhibitors and microtubule assembly inhibitor -Used for preemergence or with shallow soil incorporation to control annual grasses -Herbicides in this group are not readily translocated in the plant so herbicide placement and availability are important	- New shoots fail to emerge from coleoptile and whorl of the shoot of grass species -Susceptible germinating grasses fail to emerge from the soil	**Seedling shoot inhibitor** - Carbamothioate: EPTC **Seedling shoot and root inhibitors** - Acetamine : alachlor, S-metalachlor, metoloachlor, acetochlor, flufenacet, dimethenamid-P **Microtubule assembly inhibitor** - Dinitroaniline: trifluralin, pendimethalin, ethalfluralin, benefin

Photosynthetic Inhibitor	Control broadleaf and some grass weeds. All of these herbicides work by disrupting photosynthesis at different binding site. -Inhibit photosynthesis by binding to the Q_B-binding niche on the D_1 protein of the photosystemII complex in chloroplast thylakoid membrane -These herbicides are absorbed by both shoots and roots but are translocated only in the xylem	- Susceptible broadleaf plants will exhibit interveinal chlorosis and necrosis beginning around the leaf margins	**Photosystem II** - Triazine : atrazine, simazine, ametryn, prometon - Triazinone: metribuzin, hexazinone - Uracil : terbacil, bromacil **Photosystem II site B** - Phenylurea: linuron, diuron, tebuthiuron Photosystem II site C - Benzothiadiazole: bentazon - Nitrile: bromoxynil
Cell membrane disrupters	-This group are primarily nontranslocated herbicides which are used to control all existing vegetation as preharvest crop	-Quick damage on plants. The injury symptoms can occur within a few hours. -The symptoms will occur more quickly under high temperature and sunny conditions of application	**Protoporphyrinogen oxydase (PPO) inhibitor** - Diphenylether: aciflourfen, lactofen, fomesafen, pyraflufen - Aryl triazolinone: sulfentrazone, carfentrazone, flumioxazin - N-Phenylphthalimide: flumiclorac, fluthiacet - Pyrimidinedione: Saflufenacil Photosystem I electron diverters - Bipyridilium: paraquat, diquat

Rationale for this study

So far, limited works have been done on herbicides in use in Thailand due to the limited access to usage data and a lack of research in this area. Most work to date in the country has focused on monitoring pesticide poisoning in crops and the impacts on the human health. As a consequence of this, there are few studies that monitor environmental or ecological toxicity (Hudak and Thapinta, 2005, Iwai et al., 2007, Jaipieam et al., 2009, Iwai et al., 2011a). To our knowledge, no study has yet evaluated the combination of toxicity and interaction of pesticides in the aquatic system in Thailand. Aquatic plants are usually exposed to complex mixtures of many contaminants, mostly with different modes of action. Therefore, it is worthwhile investigating the toxicity of single herbicides with different modes of action as well as the effects of their mixtures under continuous and pulsed exposure scenarios.

Aims and Objectives:

The overall aim of this thesis is to address the ecotoxicological effects of four herbicides that are widely used in agriculture in Thailand, namely, atrazine, 2,4-D, alachlor and paraquat, on the aquatic organism *Lemna minor* which is treated as the surrogate toxicity test species.

The results from this study will further the understanding of the toxicity of pesticide mixtures on non-target aquatic organisms. In order to enable the incorporation of mixture's toxicity in future risk assessments, the study's aims were achieved through the following objectives:

1. To assess the usage amounts and the types of pesticides that farmers use in rice fields in Chiang Mai as well as the application practices that are employed.

2. To assess the effects of single pesticides and mixtures of pesticides that are widely used in Thailand on the aquatic macrophyte *Lemna minor*.

3. To assess the effects on aquatic duckweed (*L. minor*) of sequential exposure to different pesticides with different modes of action.

4. To establish the recovery patterns of *L. minor* after exposures to herbicides with different modes of action.

5. To use the data to determine implications for risk assessments of pesticide mixtures in Thailand.

Test chemicals and test organism

Test chemicals

The study chemicals were atrazine, 2,4-D, paraquat and alachlor. Information on their properties is summarised in Table 1-7.These compounds have been selected because they represent some of the most used pesticides in Thailand.

Table 1-8: Physicochemicalproperties of atrazine, 2,4-D, paraquat and alachlor (according Tomlin,2006).

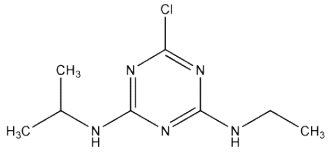
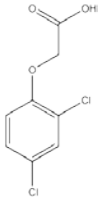
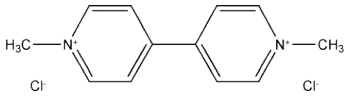
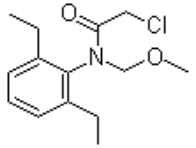
	atrazine	2,4-D	paraquat	alachlor
Molecular structure				
IUPAC name	6-chloro-N-ethyl-*N*'-isopropyl-1,3,5-triazine-2,4-diamine	(2,4-dichlorophenoxy) acetic acid	1,1'-dimethyl-4,4'-bipyridimedium dichloride	2-Chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide
CAS number	1912-24-9	94-75-7	1910-42-5	15972-60-8

Table 1-8: (Cont.) Physicochemicalproperties of atrazine, 2,4-D, paraquat and alachlor (according Tomlin,2006).

	atrazine	**2,4-D**	**paraquat**	**alachlor**
Molecular weight	215.69	221.04	257.2	269.8
Water solubility	33 mg/L (22^0C)	Very soluble in water	620 g/l ($20\ ^0$C)	0.14 g/L ($23\ ^0$C)
pH	5.04 ($25\ ^0$C)	N/A	N/A	N/A
Vapor pressure	0.039 mPa ($25\ ^0$C)	0.0187 mPa ($25\ ^0$C)	<1 x 10^{-2}mPa (25^0C)	Negligible
Henry's Law	1.5 x 10^{-4} Pa m^3mol^{-1}	1.3 x 10^{-5} Pa m^3mol^{-1}	4 x 10^{-9} Pa m^3mol^{-1}	3.2 x 10^{-3} Pa m^3mol^{-1}
Log K_{ow}	2.5 ($25\ ^0$C)	2.81	-4.5($20\ ^0$C)	3.53

$LogK_{oc}$	1.73-3.17	0.7-2.33	4.19 – 4.71 (non-mobile)	2.08 to 2.28; medium to high mobility in soil

The study pesticides

Pesticides are classified based on their target pest and include herbicides, insecticides, nematicides, rodenticides, acaracides, algicides, bactericides, fungicides, grain preservatives as well as wood preservatives(Baird, 1999, Connell et al., 1999). This study will focus on atrazine, 2,4-D, alachlor and paraquat.

Atrazine

Atrazine is an herbicide that was first registered for use in 1958 (Tomlin, 1997) and is also one of the most widely used pesticides around the world (Solomon, 1996). Atrazine is used to control broadleaf and grassy weeds in corn, pineapple, sorghum and other crops and also used as a non-selective herbicide on non-cropped industrial lands and on follow lands. Atrazine works by inhibiting the Hill reaction and its associated noncyclic photophosphorylation in electron transport chains in the photosynthesis system and also readily penetrates the chloroplasts of resistance as well as susceptible plants and seems to accumulate there until the equilibrium concentration is reached(Shimabukuro, 1969).

2,4-D

2,4-D is an herbicide that was first registered in the USA and is used for the control of broadleaf weeds or plant growth in agriculture, and for the control of woody plants along roadsides, railways and utilities rights of way. In addition, it has been mainly used on crops such as wheat and corn (Technical factsheet on: 2,4-D). 2,4-D is a selective systemic herbicide and works as growth inhibitor in plants. In the translocation system, the accumulation of 2,4-D occurs in the meristematic region of

shoots and roots. Due to the fact the compound contains salt, it is readily absorbed by roots, whereas foliage can absorb ester compounds as well (Lloyd et al., 1980, Lloyd, 1987, Tu et al., 2001).

Paraquat dichloride

Paraquat dichloride is an herbicide which was first registered for use in 1964(Tomlin, 1997). It has broad-spectrum control of broadleaf weeds and grasses in fruits and is also used for general weed control on non-crop land (Tomlin, 1997; Rely chemical Ltd, 2010). Paraquat works by damaging plant cell membranes as well as the cytoplasm as a result of superoxide generation in the photosynthesis system. Paraquat is a non-selective contact herbicide; it can be absorbed by the foliage with some translocation in the xylem.

Alachlor

Alachlor is an herbicide and endocrine disruptor, which was registered in 1969 as a selective herbicide and used as a pre-emergent, early post-emergent pesticide for control of broadleaf weeds and grasses (Herbicide Handbook, 1989; Schwab *et al*., 2006). It has been classified as a carcinogen of the B2 group by USEPA (US EPA, 1998; Hai-yan *et al*., 2006). Alachlor works by inhibiting biosynthesis of fatty acids, lipids, proteins, isoprenoids, flavonoids and gibberellins (US EPA, 1998).

Test organisms

The aquatic macrophyte *Lemna minor* (also known as common duckweed) was selected as the surrogate species since it is recommended for ecotoxicity testing (OECD221: Lemna sp. Growth Inhibition test).

L. minor is a monocotyledonous free-floating vascular plant in the Lamnaeae family. The morphology of this species is the lack of stems or leaves. It has a round, slightly oval-shaped body called the frond and a small root-like structure known as the rootlet (Wang, 1990). Fronds are small, often not exceeding 5 mm in length and two or three fronds typically make up one colony (Figure 1-3).

Figure 1-3: Aquatic plant *Lemna minor* (common duckweed).

Source:http://www.biopix.com/common-duckweed-lemna-minor_photo-47907.asp

Kingdom: Plantae

Division: Magnoliophyta

Class: Liliopsida

Order: Arales

Family: Lemnaceae

Genus: Lemna

Species: *Lemna minor*

Common name: common duckweed

L. minor as a model species test

L. minor represents a model species for Thai aquatic macrophytes due to the fact that it is widely distributed and is a dominant lentic species in the Thai aquatic environment. *L. minor* is also used for human consumption in some parts of Thailand (Leng, 1999). For the risk assessment of aquatic macrophytes from herbicides, guidelines are available in many countries such as the ASTM guideline, a draft OECD guideline and the EPA guideline. In this study, we follow the OECD 221 *Lemna* toxicity test guideline that has been modified from temperate countries. The environmental conditions such as temperature and light are different between temperate and tropical zones. However, there is limited information available on tests of aquatic macrophyte species done in Thailand or South East Asia, both of which have hot and sunny tropical conditions. There are a few publications that used zooplankton and algae but none that concerns aquatic plants (Iwai et al., 2011a). There are reviews on the sensitivity of *Lemna* species, *Myriophyllum* species and standard algal test species to herbicides in relation to other macrophytes (Fairchild et al., 1997, Teodorovic et al., 2012). The reports showed that no single species consistently represents the most sensitive macrophyte. However, *Lemna* is still a good choice for a species test model in Thailand as described above.

With the protection of aquatic macrophytes being a relevant assessment endpoint, the sustainability of the population of non-target organisms can then be systematically ensured and aquatic macrophytes protected on both the local and global bases.

Environmental risk assessment of pesticides in Thailand

Environmental risk assessments for pesticides vary from country to country (Kagaku, 2008). Differences can be observed in the EU, the United States and Japan. Unfortunately, risk assessments have not been carried out in Thailand. Instead, the data requirements for aquatic ecotoxicological tests in an Asian country like Japan are provided below.

Table 1-9: Data requirement and aquatic ecotoxicological risk assessment in Japanese pesticide registration

	Lower Tier	**Higher Tier**
Effect	**Acute/Short-term LC50 or EC50** Fish: Carp or Medaka, 96 h Invertebrate: Daphnia magna, 48h Aquatic plant: Green alga, 72h **Chronic/Long-term NOEC** Invertebrate: Daphnia magna, 21d	Additional species test (2-6 species) Bioavailability in natural water LC50 orEC50 at TOC1.5mg/L Life stage (adult/neonate)sensitivity Geometric mean L(E)C50
Exposure	**Tier I Simulation PEC** Input parameter: Use pattern	**Tier 2/3 Simulation PEC** Input parameter: Use pattern, chemical properties (e.g. measured concentration), Scenarios (e.g. water flow)
Risk assessment	**Comparison of AEC and PEC** AEC= fish LC50/10, Daphnia EC50/10, algal EC50/1	Comparison of AEC and PEC AEC = Lowest L(C)50/(2-4), L(E)C50 at TOC1.5mg/L, Geometric mean L(E)C50

Europe (European Union)

The data requirements and the risk assessment method for the EU are given in table 1-10

Table 1-10: Data requirement and aquatic ecotoxicological risk assessment in Europe pesticide registration

	Lower Tier	**Higher Tier**
Effect	Acute/Short-term LC50 or EC50 Fish: Rainbow trout: 1 fish, 96h Invertebrate: Daphnia magna, 48h: Midge, 48 h Aquatic plant:Green alga or Diatom 72h, Duckweed, 7d Chronic/Long-term NOEC Fish: prolong or ELS or FLC test Invertebrate:D. magna, 21d, Midge, 28d	Microcosm/Mesocosm Modified exposure test Indoor multi-species test Outdoor multi-species test Species Sensitivity analysis Additional species tests Probabilistic approach
Exposure	FOCUS STEP 1 or 2 Simulation PEC Input parameter: Use pattern, Chemical properties (e.g. Koc)	FOCUS STEP 3 or 4 simulation PEC Input parameter: Use pattern, chemical properties (e.g. Koc), Scenario (e.g. meteorological)
Risk Assessment	TER evaluation TERst= L(E)C50/PEC>100 TERlt=NOEC(plantEC50)/PEC>10	Case by case

The environmental risk assessments of the impact of pesticides on aquatic systems in Thailand are limited because studies have tended to focus on the effects of pesticide exposures on human health (Praneetvatakull and Waibel, 2006, Panuwet et al., 2012b). In addition, most of the data from ecotoxicity tests rely on guideline tests which were developed for temperate zones(Iwai et al., 2011b) that may give different result due to different climate factors such as temperature, rainfall and agricultural practices (Daam and Van den Brink, 2010).

To assess the risk of pesticides in the surface water of rice fields, several risk indices have been proposed (Kovach et al., 1992, Sangchan et al., 2014). The ratio of predicted environmental concentration (PEC) to predicted no-effect concentration (PNEC) has frequently been applied (Sangchan et al., 2014). In the risk assessment of surface water in rice fields, a Tier I model is typically used for estimating pesticide concentrations (Daam et al., 2013). The Tier I rice model is the screening level model which is based on the Interim Rice Model used in EFED to estimate pesticide concentrations in rice fields (USEPA, 1997). The formula of the Tier I Rice Model v1.0 is described below:

$Cw = (m_{ai'})/(0.00105 + 0.00013k_d)$ Equation

Where

K_d = 0.01Koc

C_w= water concentration (µg/L)

K_d= water-sediment partitioning coefficient (L/kg)

K_{oc}=organic carbon partitioning coefficient (L/kg)

mai' = mass applied per unit area (kg/ha)

This model has been applied in numerous areas. Daam et al. (2013) investigated the preliminary aquatic risk assessment of imidacloprid application in an experimental rice plot in Portugal. MED-rice model and Tier I rice model (USEPA) have been used to evaluate the risk of pesticides. The results showed that the application of imidacloprid at the recommended dose affects various species in the rice plot. In addition, models evaluating imidacloprid indicate clear long-lasting effects at the same concentrations as measured in the present study.

Structure of the Thesis

This thesis presents a study into the effects of herbicide mixtures under continuous and pulsed exposures on the non-target aquatic macrophyte, *Lemna minor.*

Chapter 1

Chapter 1 provides an introduction based on the background and the significance of the problem in Thailand of pesticides and environmental contaminants in the aquatic ecosystem. The aims and the objectives of the thesis are presented.

Chapter 2

This chapter presents data gathered through a questionnaire survey of farmers who work on paddy fields in Chiang Mai, Thailand, regarding their pesticide usage. This data were used to guide the mixture experiments that are reported in Chapter 3.

Chapter 3

This chapter presents work to understand the effects of herbicide mixtures on the aquatic plant *Lemna minor*. The herbicides tested are atrazine, 2,4-D, alachlor and paraquat, which were identified based on the data from pesticide survey in Chapter 2

as the compounds farmers frequently used and mixed before application on rice fields. Experimental observations from the mixture toxicity tests of the four compounds were compared to predictions from independent action (IA) and Concentration addition (CA) mixture modelsto determine the nature of their interactions.

Chapter 4

This chapter presents the results from short-term and long-term sequential pulsed exposure studies using *L. minor* and different concentrations of herbicides for different exposure durations. Four commonly used herbicides from different family groups were tested, namely atrazine, 2,4-D, alachlor and paraquat. A model has been developed to predict the effects of short-term and long-term exposures.

Chapter 5

This chapter presents data on the recovery of aquatic macrophyte *L. minor* after prolonged exposure to the four herbicides with different modes of action following either short-term or long-term exposures. Observations were made of how quickly the plants recovered and how enhanced the impacts on the plants were.

Chapter 6

This chapter presents a general discussion of the research within the context of the original aims and objectives, and suggests a future direction for the study of aquatic ecotoxicity of pesticide mixtures and risk assessment of aquatic macrophyte in Thai aquatic environment.

CHAPTERII

2. Survey of Pesticides Used in Chiang Mai, Thailand

Introduction

Rice is the staple food for more than half of the world's population and its production is the most important source of employment and income for rural Asians (Tirado *et al*., 2008). Thailand has a long history of exporting rice (Tirado *et al*., 2008). In 2008, Thailand was the world's sixth largest producer of rice and the world's largest exporter, selling around 10 million tonnes(Babel *et al*., 2011). Since 1970, economic plans have promoted the use of agrochemicals such as fertilizers and pesticides to help boost agricultural growth. Since then, imported pesticide volumes have dramatically increased annually (Pimentel et al., 1992, Tirado et al., 2008). Dechachete and Nuthall(2002) indicated that since 1992, most of the imported pesticides in the agricultural sector are for rice crops, followed by fruits and trees. A large number of chemicals have been used extensively to maintain high agricultural yields. Patterns of pesticide use are significantly different between countries and crops. In terms of the global pesticide consumption, herbicides accounted for 36% of the total usage, insecticides 25%, fungicides 10%, and others (nematicide, rodenticides, etc.)29% (College of Agriculture, University of Arizona, 2011)http://ag.arizona.edu/crops/vegetables/advisories/more/ weed49.html.In Portugal, Daam *et al*. (2009) assessed the risk on aquatic systems from pesticide applications on rice fields. They found that insecticides were the most widely used

pesticide group in order to control aphids, and that the recommended dose of 100g a.i./ha was affecting the aquatic organisms. However, based on data from the OAR of the Department of Agriculture (DOA) about 70,000 tonnes of herbicides and 9000 tonnes of insecticides have been imported annually in the past decade (Maneepitak and Cochard, 2014). Maneepitak and Cochard (2014) stated that the use of herbicides has been boosted in Thailand, which leads to contamination of water systems as well as effects on non-target organisms.

Chiang Mai province, known as the capital city of Northern Thailand, covers an area of approximately 20,107 km^2 with a population of 1,670,317 (Department of Provincial Administration, 2008). The province has a tropical wet and dry climate. The temperature throughout the year varies between 14 ^{0}C - 30 ^{0}C with the yearly average temperature being 19.8 ^{0}C (Guo *et al.*, 2012). The main economic crops of Chiang Mai are rice, longan, garlic, soy bean, potato, and onion (Dechachete and Nuthall, 2002). Chiang Mai's geographical location and climate encourage good harvests. Accordingly, this province has produced large amounts of agricultural products such as tangerines, cut flowers, temperate vegetables and fruits. In addition, there has been a report that this province is one of Thailand's main rice producers (Reunglertpanyakul, 2001).Furthermore, there are reports that farmers in the province spend more money on pesticides than those in any of the other northern Thailand provinces(Panuwet et al., 2008a).

Several studies have investigated the use of pesticides and exposure of farmers in Chiang Mai and other provinces in Thailand. Panuwet (2008) performed a pilot survey of pesticide specific urinary metabolites among farmers in Chiang Mai highland agriculture areas. A total of 40 urine samples from Hmong farmers were analysed for 19 specific pesticide metabolites. The farmers were classified into

groups according to the type of plantation or crop. The results showed that there was no significant difference among all the analytes detected in the farmers despite different crop types. Para-nitrophenol (PNP, a specific metabolite of methyl parathion and parathion) was the dominant analyte with the highest detection rates in all urine samples tested. Semathong (2008) studied pesticide use and farmers' knowledge and awareness in the Thong Pha Phum region, Kanchanaburi province, using a questionnaire with closed and open-ended questions to interview 100 farmers during the period 2006-2007. The results showed that the most widely used pesticides were glyphosate, paraquat dichloride, methomyl, chlorpyrifos and methyl parathion. In addition, the heaviest use of herbicides occurred in May or in the beginning of the rainy season, while the heaviest use of insecticides occurred in April in attempts to control the outbreak of aphids.

The aim of this research

So far, most of the studies undertaken by researchers have focused on the effects of pesticide exposure on human health in other areas of Thailand (Semathong et al., 2008, Plianbangchang et al., 2009, Iwai et al., 2011b, Sangchan et al., 2012, Schreinemachers and Tipraqsa, 2012). However, there are little or no data available on pesticides' impact on aquatic environment in Thailand. Therefore, this study aims to identify the pesticides that are the most commonly used in the paddy fields in Chiang Mai province; to explore the patterns of use of these pesticides; and, using these data, to estimate the likely levels of pesticides in rice field in Thailand.

Methodology

A survey to establish the use of pesticide in Chiang Mai was carried out by interviewing 30 farmers from three different districts in Chiang Mai during the period of 10 December 2011 to 4 January 2012. The study area is given in Figure 2-1. All of the three districts are important rice crop production areas in Chiang Mai province (Wiboonpongse and Chaovanapoonphol, 2001).

Figure 2-1:A map showing Mae Taeng, Mae Rim and San Patong districts which are major rice producing areas in Chiang Mai province, Thailand.

Study areas

Chiang Mai province is located in the North of Thailand and covers an area of 20,107 km^2, making it the second largest province in Thailand (Panuwet et al., 2008b). This province is one of the most important for agricultural production (Chiang Mai Office of Agriculture Economics, 2007). The three districts that were

studied were Mae Taeng (19°7′19″N, 98°56′37″E),Mae Rim (18°54′50″N, 98°56′42″E) and San Patong (18°37′43″N, 98°53′44″E), all of which produce a large quantity of rice.

Field sampling and data collection

The interviewing was done in two stages. A pre-test of a draft questionnaire in English was done through face-to-face interviews with four farmers. Based on the experience from the pre-tests, the questionnaire was adapted and then translated into the Thai language for use in the full survey. The questionnaire contained both closed and open-ended questions about types, quantities and patterns of pesticide use, as well as questions about awareness of the impacts of pesticides on aquatic systems (Appendix A).For the full survey, the interviews were conducted in the farmer's rice field. In total, the interviews of approximately 10 rice farmers from each district were conducted (30 farmers in total).Following completion, the questionnaires were gathered and the data were compiled and analysed with Microsoft Excel.

Assessment of aquatic exposure to pesticides in rice fields in Thailand

The results from the survey were used to explore the level of exposure of herbicides, in use in rice fields in Chiang Mai, in surface waters. To perform the exposure assessment, the Rice screening level model developed by the US EPA was used to generate exposure concentrations (US EPA, 2012). The tier I Rice Model relies on an equilibrium-partitioning concept to provide conservative estimate for the environmental concentrations resulting from application of pesticides to rice fields. The equation of the Tier I Rice Model v1.0 is as follows:

$$Cw = (m_{ai'})/(0.00105 + 0.00013k_d)$$ Equation 1

Where

C_w= concentration in water (µg/L)

K_d= water-sediment partitioning coefficient (L/kg) – which is 0.01 x the Koc

K_{oc}=organic carbon partitioning coefficient (L/kg)

mai' = mass active ingredient applied per unit area (kg/ha)

Results

General information

Thirty small-scale farmers were randomly surveyed in this study. The majority were males at83.3% (25 of 30) and the rest females at 6.67% (5 of 30). The respondents were between 32-71 years of age with an average age of 58 and a standard deviation of 8.5 years. The majority of the farmers were aged between 50-70 years old (73.3%). As the United Nations Development Programme in Thailand (UNDP, 2013) pointed out, the average Thai farmer's age is 55 years old, which is in agreement with Bryant and Gray (2005) who stated that the age of a typical Thai farmer is over 50 years old according to the data in year 2013. The age of rice farmers in this survey is given in figure 2-2.

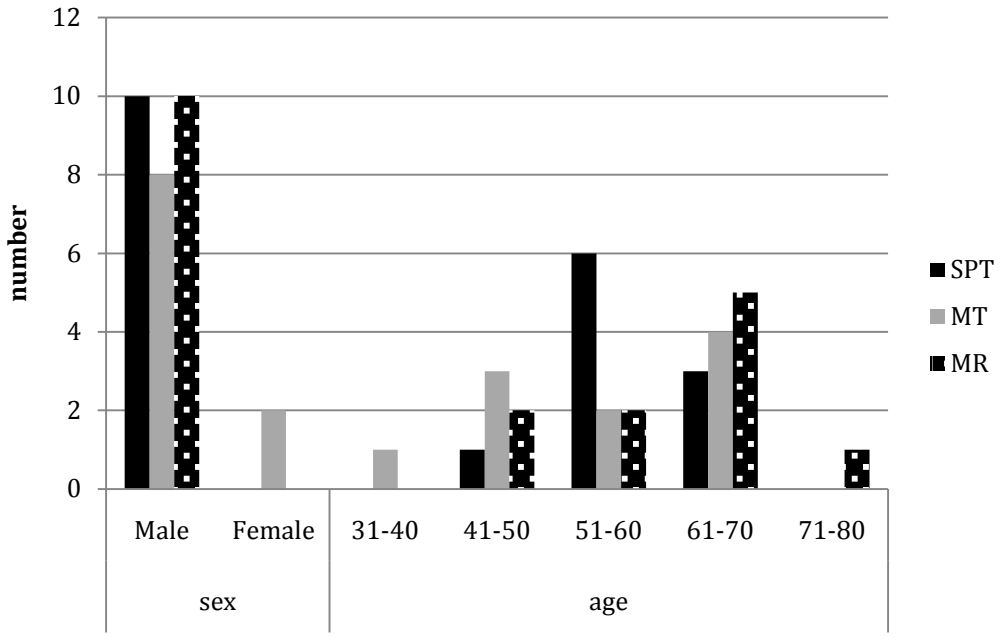


Figure 2-2: General information of the farmers from Mae Taeng (MT), Mae Rim (MR) and San Patong (SPT) districts, Chiang Mai province, Thailand during the period December 2011-Januray 2012.

Rice farming season

All the farmers reported growing crops using a rotation system. In the wet season (June to October), rice was the major crop, while in the dry season (November to May), corn, watermelon and soybean were grown instead of rice(Sangchan *et al*., 2012).

Pesticides used

Approximately 80% of the farmers surveyed used pesticides in their paddy fields. The most common pesticide products used by the farmers in rice paddy fields were Grammoxone (15%) followed by Lannate/methomyl, Hecdonan95 and 2,4-D80 (13%), Glyphosate48 (10%), Lannate, Furandan (7%), Paraquat and Lasso (5%) Tamaron, Dimethoate40, SanturnD and Round up (3%). In terms of the use of

mixtures, only four farmers in this survey mixed pesticides and used them at the same time. The mixtures were Lannate with Tamaron, Furadan with SanturnD and Lasso 180cc+gramoxone. The data from this survey are provided in Table 2-1.

Table 2-1: Pesticides used in Chiang Mai as recorded from a survey undertaken during the period December 2011-January 2012.

Pesticide product	Group of pesticide	Active ingredient	Number of famers using the pesticide	% farmers using
Gramoxone	Herbicide/Bipyridilium	Paraquat27.6%	6	15%
Lannate L (liquid)	Insecticide/Carbamate	Methomyl18%	5	13%
2,4-D80	Herbicide/chlorophenoxy acid or ester	2,4-D sodium salt80%	5	13%
Hecdonan95	Herbicide/chlorophenoxy acid or ester	2,4-D sodium salt95%	5	13%
Glyphosate48	Herbicide/Phosphanoglycine	Glyphosate48%	4	10%
Lannate	Insecticide/Carbamate	Methomyl18%	3	7%
Furadan	Insecticide/Carbamate	Carbofuran3%	3	7%
Paraquat	Herbicide/Bipyridilium	Paraquat27.6%	2	5%
Lasso	Herbicide/Chloroacetanilide	Alachlor48%	2	5%
Tamaron	Insecticide/Organophosphate	Metamidophos58%	1	3%
Dimethoate40	Insecticide/Organophosphate	Dimethoate40%	1	3%
Santurn-D	Herbicide/mixture	Thiobencarb5%,2,4-D2%	1	3%
Round up	Herbicide/ Phosphanoglycine	Glyphosate48%	1.	3%

Annual Quantity of Active Ingredients

The farmers used 13 commercial pesticides in the rice field. 2,4-D80 and Hecdonan were the most commonly applied on paddy fields at 130 and 70 litres/year followed by Furadan at 75 litres/year, respectively. Eight of the twelve products used were herbicides and five were insecticides (Table 2-3). In terms of pesticide active ingredients, 2,4-D was the most frequently used with the total of170.5 litres of active ingredient being applied followed by glyphosate which had 10.56 litres of active ingredient applied(Table 2-2).

Rate of pesticide application

According to the results, the farmers frequently used a higher than recommended concentration for almost one-half of the total pesticides used (6 of 13 products) (Table 2-2, 2-3). The was particularly the case for 2,4-D80 which was found to be applied to rice fields at over 10-fold the recommended rate. Hecdonan (2,4-D) was applied at around 8-fold the recommended concentration.

Table 2-2: Ranking of pesticide products in terms of annual quantity of active ingredient used in the three districts studied in Chiang Mai.

Pesticide product	Quantity (kg or L/year)	Active ingredient (kg/kg or kg/L)	Area (hectare)	Rate of application (kg or L/ha)	Recommended rate of application (kg or L/ha)	Active ingredients applied (kg or L of active ingredient)	Active ingredients applied per hectare (kg or L of a.i./ha)
2,4-D80	130	0.8	15.2	8.55	0.78	104	6.8
Hecdonan	70	0.95	11.36	6.16	0.78	66.5	5.8
Furadan	75	0.03	7.36	10.19	NA	2.3	0.31
Lannate L	17	0.18	9.6	1.77	NA	3.1	0.32
Santurn-D	15	0.02	4.48	3.35	31	0.3	0.07
Paraquat	10	0.276	5.44	1.84	2.18	2.8	0.51
Gramoxone	13	0.276	4.64	2.8	2.5	3.3	0.71
Glyphosate48	12	0.48	16.48	0.73	2.18	5.8	0.35
Lasso	4	0.48	1.6	2.50	3.1	1.9	1.2
Lannate	0.15	0.4	4.96	0.03	0.15	0.1	0.01
Dimethoate	2	0.4	1.6	1.25	0.12	0.8	0.5
Tamaron	1	0.56	0.64	1.56	0.25	0.6	0.88
Round up	10	0.48	1.92	5.21	2.18	4.8	2.5

Table 2-3: Ranking of pesticides used based on active ingredient on paddy fields in the Chiang Mai farms that were surveyed.

Rank	Active ingredient	Total applied (kg or L.)	Active Ingredients applied (kg or L.)	Area (hectare)	Active ingredients apply per hectare (kg or L of a.i./ha)
1	2,4-D	215	170.5	31.04	5.49
2	carbofuran	75	2.3	7.36	0.31
3	paraquat	23	6.1	10.08	0.61
4	methomyl	17.5	3.2	14.56	0.21
5	glyphosate	22	10.56	18.4	0.57
6	alachlor	4	1.9	0.48	3.95
7	demethoate	2	0.8	0.4	2
8	methamidophos	1	0.6	0.56	1.07

Frequency of pesticide application

All farmers use knapsack sprayers for pesticide application. The majority of them (47%) apply pesticides on their paddy fields during the period of crop growth to prevent pest invasion. Preventive spraying is usually done less than once a year during crop seasons. Some farmers sprayed pesticide in response to pest manifestation (Table 4). Only 13.3% (4 of 30) of the farmers used pesticide mixtures and only mixed these before applying to the crop. Farmers usually apply pesticides in June (39%) followed by May (22%) and 2,4-D is the most heavily used pesticide. Data on patterns of pesticide use reveal that the heaviest use period for herbicides and insecticides from May to June (Figure 2-3).

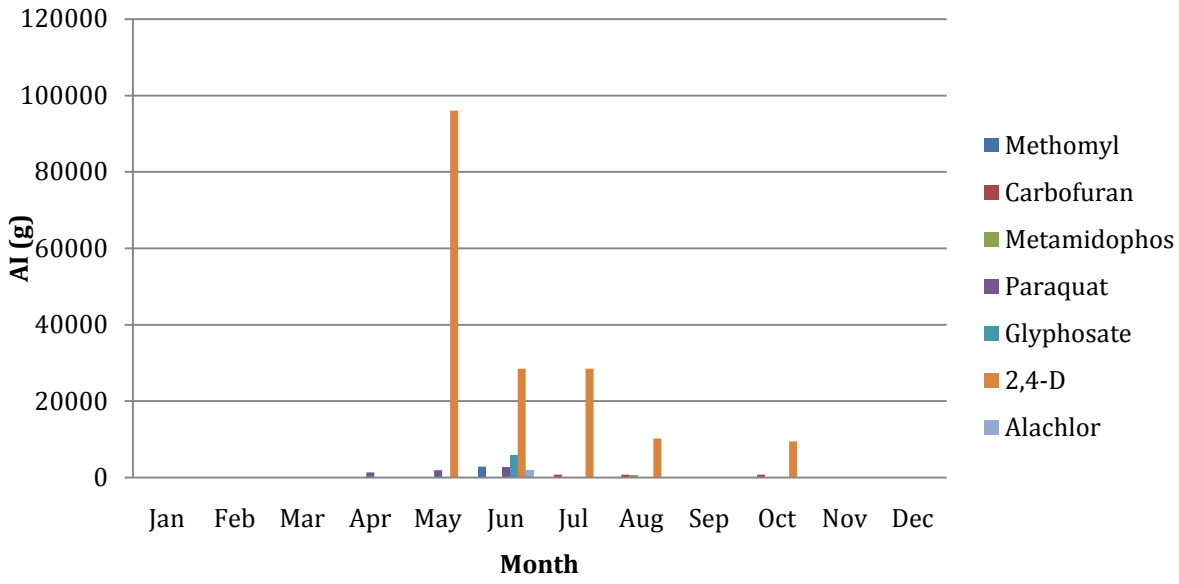


Figure 2-3: Amount of active ingredient (A.I.) in pesticide use at the three sites studied from paddy fields in Chiang Mai.

Table 2-4: Frequency of pesticide application of small-scale farmers in Chiang Mai, Thailand during December 2011.

Frequency of pesticide application	Number (%)
Less than once a month (1-2 times/season)	14 (47)
More than two times/ season	8 (26.7)
Depends on the pest manifestation	1 (3.3)
No use pesticide	7 (23)
Use pesticide mixture (only in same time)	
- Lannate with Tamaron	4 (13.3)
- Furadan with SanturnD	
- Lasso 180cc+Gramoxone	

Farmer perceptions

The open question at the end of the questionnaire raised a question about the awareness of pesticides' negative impact on the rivers or aquatic systems in the region. 17 out of 30 farmers said they avoided using a higher dose of pesticides and they try to avoid applying pesticides before heavy rain since they might be washed away and seep into the river which might affect aquatic organisms such as fish and ducks. In addition, they were also concerned about pesticide residue in soil and water on their fields.

Exposure assessment for pesticides in rice fields in Thailand

Calculated environmental exposure concentrations in rice fields, obtained using the TIER I rice model, are shown in Table 2-5 (Table 2-5). 2,4-D had the highest exposure concentrations followed by alachlor then paraquat.

Table 2-5: Input values used for the first-tier PEC (predicted environmental concentration) calculations in accordance with US-EPA (2007)

Input scenario data	**Herbicides**			
	atrazine	**2,4-D**	**alachlor**	**paraquat**
Application dose (m_{ai}; kg/ha)	-	5.49	3.95	0.61
K_{oc}	122	20	131	15473-51856
K_d	1.22	0.2	1.31	154.73-518.56
Concentration (µg/l)	-	5102	3237	8.92

Discussion

The general information from Thai farmers in this survey showed that the majority of farmers are male aged between 50-70 years old, which is the age range of the majority of farmers in Thailand after retirement of over 50 years old (Bryant and Gray, 2005). The average age of rice farmers in Thailand was 44 years old ranging from 23 to 63 years (Kongtip *et al*., 2009).

Pesticide used

In terms of the pesticide in use in Thailand from this survey, the results indicate that a wide variety of pesticides is used by farmers in the areas. The most used pesticides were 2,4-D, paraquat and glyphosate, which are herbicides. The results are in agreement with previous studies. For example, Panuwet (2012) pointed out that herbicides are used in the largest proportion, followed by insecticides, fungicides, and plant growth regulators. Similarly, Jungbluth (1997) stated that the majority of pesticides used in Thailand include glyphosate, 2,4-D, atrazine and paraquat. In addition, Primentel (1992) indicated that the most common pesticides found in groundwater are the insecticide aldicarb and the herbicides alachlor and atrazine. Plianbangchang (2009) stated that paraquat is one of the most popular herbicides throughout Thailand. In Vietnam, it has been reported that the most used pesticide class in the Mekong Delta was insecticides (394 grams a.i. per ha), followed by herbicides (323 grams a.i per ha) and fungicides (300 grams a.i. per ha) (Hosamani, 2009). In the Philippines, the most used herbicides were Butachlor and 2,4-D to control weed and these were applied once throughout the growing cycle (Fabro and Varca, 2012).

In terms of the amount of pesticides use per area, the most used herbicide from this study was 2,4-D, which was applied at a rate of 4.5 kg a.i./ha. The Philippines use approximately 0.8 kg a.i./ha of 2,4-D in rice fields (Fabro and Varca, 2012). Thai farmers usually apply pesticides once or twice per season and at higher doses than the recommended concentration. This frequency is similar to the pesticide usage by farmers in Pagsanjan-Lumban catchment of Lanuna de Bay in the Philippines who applied pesticides one to three times per season in rice fields.

Use of pesticide mixtures

In this survey, the farmers were usually using pesticide mixtures in the rice paddy fields to enhance the spectrum of the control when multiple pests were attacking simultaneously. Farmers usually mixed pesticide themselves by tank-mixing the products (Jungbluth, 1996). The pesticides in the mixture, with different modes of action, are mixed on the assumption that they would complement the action of each other for killing the target pests. From the questionnaire, the reasons for farmers using mixtures of pesticides are that they give the best control of a multitude of pests.Pests that are resistant to one or more pesticides may be susceptible to a combination of toxicants (Abd El-Mageed and Shalaby, 2011). Furthermore, in order to make them as effective as possible, the pesticides were applied at double the concentrations recommended by the manufacturers. Similarly, in Ghana, Ntom *et al*., (2006) found that farmers usually spray combinations of pesticides out of their desire to have rapid knockdown of pests. In South and South-East Asia, Gupta (2012) found that farmers usually mix pesticides with their bare hands before applying.

The gathered information was comprehensive but should in the future be complemented with further interviews of farmers about their awareness and perception of pesticides and aquatic systems. The farmers were aware of the negative impact pesticides can have on the environment. During one interview, a farmer indicated that he considers the pesticides to have effects on species that are consumed such as watercress, spirogyra (filamentous green algae) and duckweed. The pesticides directly drain into the rivers in many cases. Our study was limited to only survey questionnaire since December and January are dry season. There are two main growing seasons for rice: the wet season and the dry season. The first crops (or wet season crop) is cultivated from June to August, and harvested during October to

January (Wiboonpongse and Chaovanapoonphol, 2001). The second crop (or dry season crop) is cultivated from February to April and harvested during April to June. Wiboonpongse and Chaovannapoonphol (2001) stated that the production in the wet season accounts for more than in the dry season, approximately 18 million tonnes and 4 million tonnes, respectively. Since the rising production leads to increased use of pesticides (Praneetvataku *et al*., 2013), future research could alternatively collect data during both the wet and the dry seasons to compare pesticide use in rice fields.

Modelled herbicide concentrations in rice field

In the exposure assessment of herbicides in rice field, PEC estimated for surface water was made using Tier I rice model. The highest modelled PEC surface water was 2,4-D 5102 µg/L followed by alachlor 3237 µg/L and paraquat 8.91 µg/L. This is not surprising due to the fact that the farmer heavily use herbicides through application by direct overspray. The resulting predicted concentrations can be used to calculate risks to non-target aquatic macrophyte ecotoxicology – this is done in Chapter 6.However, there are a limited data about the level of herbicide-measured concentration in rice field. Therefore, it is difficult to compare the concentrations that are predicted with experimental measurements.

In the next chapter, a selection of the most commonly used pesticides in Chiang Mai and in Thailand more generally is studied to understand the effects of pesticide mixtures on aquatic macrophytes.

In the next chapter, a selection of the most commonly used pesticides in Chiang Mai and in Thailand more generally is studied to understand the effects of pesticide mixtures on aquatic macrophytes.

CHAPTER III

3. The Effects of Mixtures of Herbicides on *Lemna minor*

Introduction

The survey described in Chapter 2 indicated that in some instances herbicides are applied as a mixture and that a number of compounds might be applied at a similar time of year. It is therefore likely that aquatic systems in Thailand will be exposed to mixtures of herbicides rather than to single substances. In this Chapter, experiments to explore the combined effects of herbicides are therefore presented. The work described in the Chapter focused on mixtures of atrazine with 2,4-D and alachlor with paraquat as these are combinations that some of the farmers used in reality.

The U. S. Environmental Protection Agency (EPA) recently estimated that more than 540 million kilograms of pesticides are applied to crops around the world and the most used pesticide class is the herbicides (Ecobichon, 2001, Thapinta and Hudak, 2003). The use of herbicides has been continuously increasing year on year. In addition, several reports have highlighted the problems associated with pesticide overuse and misuse due to a lack of knowledge about safe and correct use(Ecobichon, 2001, Grovermann et al., 2013). Pesticides can be released into aquatic systems via spray drift, runoff and leaching from soil (Laetz et al., 2009, Boxall et al., 2013). Once released into aquatic system they may then cause unintended adverse health impacts on humans and non-target organisms(Laetz *et al*., 2009).

Herbicides will not occur in the natural environment alone but will likely occur alongside other herbicides and other chemicals used in agriculture (Sorensen et al., 2010, Larras et al., 2013). A range of interactions are possible from these mixtures of contaminants including greater than additive toxicity, less than additive toxicity and additive toxicity(Belden and Lydy, 2000). Greater than additive (sometime referred to as synergistic) interactions are of the greatest concern in environmental risk assessments as they result in larger impacts than expected based on the toxicity of individual components of a mixture(Hertzberg and MacDonell, 2002). To better understand the impacts of pesticides on aquatic environment, it is therefore important to establish the mixture interactions of pesticides.

Two models have been used to assess the ecotoxicological impacts of chemical mixtures: concentration addition (CA) and independent action (IA)(Cedergreen et al., 2007a, Cedergreen et al., 2007b, Cedergreen et al., 2007c, Munkegaard et al., 2008, Syberg et al., 2008). The model of concentration addition (CA) introduced by Loewe and Muischnek (1926) assumes that the components of mixture have the same molecular site of action and can be regarded as dilutions of one another(Cedergreen *et al*., 2007c). Independent action sometimes referred to as response addition, which was introduced by Bliss (1939), is based on the concept of dissimilar modes of action of compounds in a mixture where the individual components interact with different molecular target sites (Cleuvers, 2003). A number of studies have been examined the effects of chemical mixture on a wide range of aquatic organisms (nontarget species) including aquatic plants, bacteria, fish and macroinvertebrate (Belden and Lydy, 2000, Hertzberg and MacDonell, 2002, Cedergreen et al., 2007a, Cedergreen et al., 2007b, Cedergreen et al., 2007c, Syberg et al., 2008). The data from these studies show that mixtures of chemicals tend to not

enhance each other's action and that synergistic interactions are rare. The majority of chemical mixture interaction is more likely to be additive(Altenburger et al., 1996, Deneer, 2000, Junghans et al., 2006, Belden et al., 2007, Syberg et al., 2009, Zhang et al., 2010, Rodney et al., 2013). For example, the two largest studies of pesticide mixture interactions on aquatic organisms were performed by Faust *et al* (1994) and Altenburger *et al* (1996). Faust *et al* (1994) examined the effects of pesticides with different mode of action on algae and found that 60% of the exposures showed additive effects. Similarly with Alterburger *et al* (1996) investigated 137 pesticide mixtures and found that the majority of mixtures had additive acute and chronic effects.

Synergism and antagonism have been reported in some instances (Cedergreen *et al*., 2006, Cedergreen *et al*., 2007c, Sorensen *et al*., 2007). For example, Belz *et al* (2008) examined the effects of pesticide mixture acifluorfen with mesotrione and acifluorfen with terbuthylazine on aquatic macrophyte *L. minor*, it was showed that acifluorfen with mesotrione was antagonistic effects. Cedergreen *et al* (2007) tested the toxicity of six binary herbicide mixtures on chlorophyll content and plant growth by concentration addition model (CA) and independent action model (IA), both model showed acifluorfen combined with diquat were antagonistic. Besides that, the synergistic has been observed by Cedergreen *et al* (2006), they studied the effect of prochloraz, imidazole combined with diquat, azoxystrobin, acifluorfen, dimethoate, chlorfenvinphos and pirimicarb on four aquatic organism including bacteria, daphnia, algae and duckweed. The result showed the combination between prochloraz with azoxystrobin and diquat with esfenvalerat resulted in a synergistic effect on Dapnia and that diquat with prochloraz interacted synergistically in algal studies.

In this study we explore the effects of mixture interactions of four commonly used herbicides, atrazine, 2,4-D, alachlor and paraquat which are the widely use in single and combination in Thailand (Chapter2).The aim of present study was to examine the interactions of the herbicide mixtures on *Lemna minor* the monocotyledonous free floating, rooted aquatic macrophyte*(Muller et al., 2010). L. minor* is widely used as a test organism in the environmental risk assessment (Kiss et al., 2003, Cedergreen et al., 2007c, Bisewska et al., 2012, Dalton et al., 2013) and currently recommended as a regulatory phytotoxicity test to support the registration of pesticides(Organisation for Economic Co-operation and Development., 2006). We provide knowledge that will allow a better understanding of the mixture toxicity of some of the most widely used herbicide as combinations that are likely to occur in surface water. The objectives of this research were (1) to measure the toxicity of four commonly used herbicides as single compounds and binary mixtures; and (2) to use the results to determine whether the study compounds interacted in an additive, synergistic or antagonistic manner. As the bulk of the literatures suggest that pesticides interact antagonistically, the underlying hypothesis of this study was that herbicides with different modes of action, which are in use in Thailand, will interact antagonistically.

Materials and Methods

Chemicals

Atrazine (98.5%purity), 2,4-D (99%purity), alachlor (98% purity), paraquat dichloride (99% purity) and analytical grade solvents (methanol and acetone) were

obtained from Sigma Aldrich (Poole, Dorset, UK). The characteristics and sites of action of the four herbicides are summarized in Table 3-1.

Table 3-1:Chemical characteristics and sites of action of the four herbicides used in the present study (Tomlin, 2006)

Hebicide	CAS RN[a]	MW[b]	Family group	Site of action
Atrazine	1912-24-9	215.68	Triazine	Inhibitors of photosynthetic electron transport
2,4-D	94-75-7	221	Phenoxyacetic acid	Disruption of the hormonal equilibrium of the auxin-cytokinin system and inhibits root and shoot growth for both broad-leaved plants and grasses.
Alachlor	15972-60-8	269.77	Chloroacetanilide	Interfere with biosynthesis of lipid, protein and flavonoids.
Paraquat dichloride	1910-42-5	257.16	Bipyridilum	Affected on photosynthesis electron transport by redox catalyst at photosystem I

Test species and test conditions

Lemna minor is an aquatic macrophyte that grows on surface water in lentic ecosystems. *L. minor* is fast growing and widely distributed. They are easy to culture and test. In addition, this species has been recommended as a standard test species (Wang, 1990). The endpoints of the tests are addressed in OECD221: *Lemna* sp. Growth inhibition test (2006). It is recommended that the estimated toxicity be based on the average specific growth rate of the frond number but it is preferable to use the measurements of biomass such as the total frond area, dry weight or fresh weight because some substance may affect the frond size without affecting the frond number. Therefore, the guideline (OECD221, 2006) stated that the total frond area is often preferred for being the most sensitive to change. In addition, some researchers

discussed the advantage of establishing the growth rate via the frond area (Drost, 2011; Eberius *et al*., 2012). They stated that with this approach standard deviations are minimized, confidence intervals are reduced, and the test sensitivity is enhanced because of individual measurements of the plant area at the beginning of the experiment. While the number of fronds may be identical, the frond area may vary and growth depends more on the photosynthetically active area. Furthermore, EC-values based on the frond number are similar to the EC-values based on the frond area (Drost, 2011). Additionally, in the case of alachlor, the compound had an impact on frond size by causing dwarfish fronds and broken colonies. Therefore, the frond number was not the appropriate endpoint to establish the alachlor-caused growth inhibition. For this reason, the growth rates of this study were based on the total frond area.

***Lemna minor* culture**

L. minor were cultured in Swedish media (Syberg *et al*., 2009). Cultures were maintained in a Sanyo Environmental test chamber (model MLR-351H) at 20 ^{0}C under continuous illumination at 125 $\mu E^{-2}S^{-1}$. *L. minor* was kept in the logarithmic growth phase by sub-culturing the stocks every 7 days. Prior to use in the ecotoxicity studies, the pH of the growth media was adjusted to 6.5 with either 0.1 M HCL or NaOH.

Single compound ecotoxicity tests.

The tests were conducted in accordance with the OEDC 221: Lemna sp. Growth inhibition test guidelines for 7-d static tests. Total frond area was used as endpoint. Three replicates of a range of pesticide concentrations were prepared from stock

solutions of each study pesticide in acetone. Atrazine concentrations ranged from 0.05 to 0.8 mg/L, 2,4-D ranged from 5 to 100 mg/L, and for alachlor and paraquat the range was 5 to 80 µg/L. The final acetone concentration in each test was kept less than 0.05% v/v to avoid phytotoxicity effects of the organic solvent (Dewez *et al*., 2003). Associated control and solvent-control solutions were also prepared in triplicate.

L. minor were exposed in triplicate to the individual pesticide solutions or controls. For atrazine and 2,4-D, borosilicate glass petri dishes were used in the exposures (Duran®; height = 22mm; diameter =60mm) whereas for alachlor and paraquat plastic petri dishes were used (Sterilin® Ltd; diameter = 60 mm) to avoid pesticides adsorption onto the glassware(Yeo, 1967).One *L. minor* colonies, comprising three fronds, were added to each petridish with 10-mL of medium. Digital photographs (Cannon ixus210) were then taken of the *L. minor* from above. The areas of the *L. minor* colonies were then determined using image J (Boxall *et al*., 2013). Each petri dish was transferred into a Sanyo Environmental test chamber (model MLR-351H) for 7 days test period. The test chamber incubation was set at a temperature of 20 ^{0}C under continuous illumination at 10,000 Lux. The dishes were then removed and photographed as detailed above and the areas of the *L. minor* colonies determined using image J. At the end of the test period, water samples were kept at 4^{0}C until analysis with high performance liquid chromatography (HPLC), and pH was measured using a Thermo Orion pH meter (Benchtop pH/ISE meter).

Mixture ecotoxicity tests

The interactions of two herbicide combinations were explored: atrazine with 2,4-D and alachlor with paraquat. The mixture experiments were conducted following a fixed ratio design (Greco *et al*., 1995)on the basis of the EC50s from the single

compound experiment, exchange ratios were initially determined(Sorensen *et al*., 2007).The herbicides were then mixed at perceived effective concentration ratios of100:0%, 83:17%, 63:37%, 50:50%, 37:63%, 17:83%, 0:100% effect concentrations(Cedergreen et al., 2005, Munkegaard et al., 2008, Norgaard and Cedergreen, 2010)and from these seven chemical dilutions, three replicates and 12 controls were developed.

Calculation of specific growth rate

The growth rates of *L. minor* were calculated from the results of the image analysis of *L. minor* frond area in each treatment. The growth rates were calculated according to equation1 and, in order to calculate the percentage of growth inhibition, equation 2 was used.

$$ASGR = \frac{\ln(Nj) - \ln(Ni)}{tj - ti} \quad \text{Equation 1}$$

Where ASGR is the specific growth rate, N_i is the frond area at day i and N_j is the frond area at day j.

$$Ii = \frac{(Ac - At)}{Ac} \times 100 \qquad \text{Equation 2}$$

Where Ii is the inhibition of measured endpoint for concentration, A_c is the growth rate of total frond area in the control and A_t is the growth rate of total frond area in the tested sample concentration.

Based on the inhibition of chemicals on *L. minor* from day 0 to day 7, calculation of the effective concentrations resulting in 50% growth inhibition (EC50) was

determined using nonlinear curve fitting based on a sigmoid model four-parameter logistic function (equation 3) namely upper lower limit at 100 and 0%, EC50 and Hillslope (Cleuvers, 2003, Cedergreen et al., 2007a, Belgers et al., 2009).

$$y = \min + \frac{(\max - \min)}{1 + \left(\frac{x}{EC50}\right)^{-Hillslope}} \qquad \text{Equation 3}$$

Where min is the bottom of curve, max is the top of curve while EC50 is the concentration giving a response of 50% and Hillslope characterizes the slope of the curve at its midpoint (Sigmaplot, UK).

Mixture modeling

There are various models used to predict the mixture toxicity. In order to predict the joint effect of herbicides, two models have been suggested for use: independent action (IA) and concentration addition (CA). The EC25 and EC50 data for the individual toxicants were therefore used in the CA and IA model (Equation 4,5) to estimate the effects of the different pesticide combinations tested at different effective concentration in the mixture studies described below.

Concentration addition (CA)

The CA-reference model is typically interpreted as compounds of a mixture with sharing mode of action (Cedergreen et al., 2013). The equation can be expressed as

$$\sum_{i=1}^{n} \frac{c_i}{EC_{xi}} = 1 \qquad \text{Equation 4}$$

Where c_i gives the concentration of the *i*th component in an *n*-component mixture that provoke x% effect.

Independent action (IA)

The IA-reference model was selected as the model to use in this study since the mixtures are made from toxicants with dissimilar modes of action (Syberg et al., 2008, Phyu et al., 2011, Hadrup et al., 2013).

$$E(c_{mix}) = E(c_1) + E(c_2) - E(c_1)E(c_2) \qquad \text{Equation 5}$$

Where $E(c_1)$ and $E(c_2)$ represent the fractional effects (ranging from 0 to 1) caused by the individual toxicants 1 and 2 in the mixture. This usually requires that the concentration-response curves of the individual chemicals (Backhaus and Faust, 2012). $E(c_{mix})$ is the total effect of the mixture.

Isobologram

The isobologram approach is a commonly used and powerful graphical approach for exploring the joint action of chemical mixtures (Tallarida, 2006, Chen, 2009).By comparing the isoboles based on the IA predictions and experimental mixture data, conclusions can be drawn on the type(s) of interaction occurring. When an experimental point falls below the model lines, this indicates that synergism is occurring whereas if an experimental point falls above a modelled point, this indicates that antagonism occurs (Machado and Robinson, 1994). Isoboles were therefore constructed from the results of the IA modelling and the experimental mixture toxicity data in order to draw conclusions on the mixture interactions of the study compounds for two effect levels: EC50 and EC25.

High performance liquid chromatography analysis

The concentration of atrazine and 2,4-D were confirmed using a PerkinElmer Flexar HPLC equipped with a Supelco 516 C18-db 5µm x 15 cm x 4.6 mm column. For

atrazine a methanol:water (60:40, v/v) mobile phase was used, the flow rate was 1 ml/min and the temperature was set at to 40 ^{0}C. The detection wavelength was 220 nm and the injection volume was 15 µl(Fu, 2008). The calibrations were done using atrazine standard covering a concentration range with high correlation (r^2= 0.998) and retention times were 6-7 minutes. The limit of detection was 0.02 mg/L and the limit of qualification was 0.04 mg/L. For 2,4-D, a methanol:water with 0.1% formic acid (70:30, v/v) mobile phase was used. The temperature was set to 30 ^{0}C and the detection wavelength was 236 nm (Connick *et al.*, 1982)and calibration was by external standards (Chandra *et al.*, 2001) (r^2= 0.999), with retention times between 3-4 minutes. The limit of detection was 0.02 mg/L and the limit of qualification was 0.08 mg/L.

Enzyme linked immunosorbent assay (ELISA)

Alachlor ELISA test kit was purchased from Abraxiskits® (PA, USA). For alachlor analysis, water samples were removed from the refrigerator and allowed to attain room temperature. Afterward, 25 µl of standard, control and water sample were added into the 96 well flat-bottomed polystyrene ELISA plate. An enzyme conjugate (50 µl) alachlor antibody solution was then added to each well. Wells were then covered with parafilm to prevent contamination and evaporation and incubated at room temperature for 60 minutes. The plate was washed three times with the diluted wash buffer, and then150 µl of color solution was then added to each well and the plates then incubated for a further 20 minutes. Finally 100 µl of stopping solution was added to each well. The absorbance was read at 450 nm within 15 minutes after addition of the stopping solution.

Paraquat analysis, ELISA test kits were purchased from US Biocontract® (San Diego, USA). 96-wells microplate coated with anti-paraquat antibody was used.

Firstly, add 25 µl of standard and samples of each well, and then 100 µl of Paraquat-Horseradish Peroxidase Conjugate (PRQ-HRP) were added in each well and incubate at room temperature for 30 minutes. After incubation, the plate was washed three times with wash buffer, and then 100 µl TMB substrate was added. Plates were then left at room temperature for 15 minutes after which 100 µl of stopping solution was added to each well and the plate was then read using an absorbance at 450 nm.

Statistical analyses

In order to determine the differences of pH and chemical analysis at the beginning and the end of test, a student t-test was performed by sigma plot 12 software (Systat, Chicago, IL). A Shapiro-Wilk's test was chosen to check the normal distribution of data, if failed the Man-Whitney U test was performed instead(Mohr *et al.*, 2013).

Results

Chemical analysis

The pH of the exposure media for all the treatments increased slightly over the study period but this increase was less than one pH unit (Figure 3-1,3-2). From the atrazine and 2,4-D mixture, the pH slightly increased due to the effects of the chemical property of 2,4-D which is acid (2,4-Dichlorophenoxyacetic acid) (Figure 3-1). During the seven-day test, the concentrations of the study compounds in the single and binary mixture solutions at the end of the study were determined to be within ±20% of the starting concentration. The HPLC analysis of test solutions of atrazine, 2,4-D , alachlor and paraquat showed that the test substance concentration was

maintained during 7 days ($p>0.05$) (Figure 3-3). The raw data for the chemical analysis are provided in Appendix B.

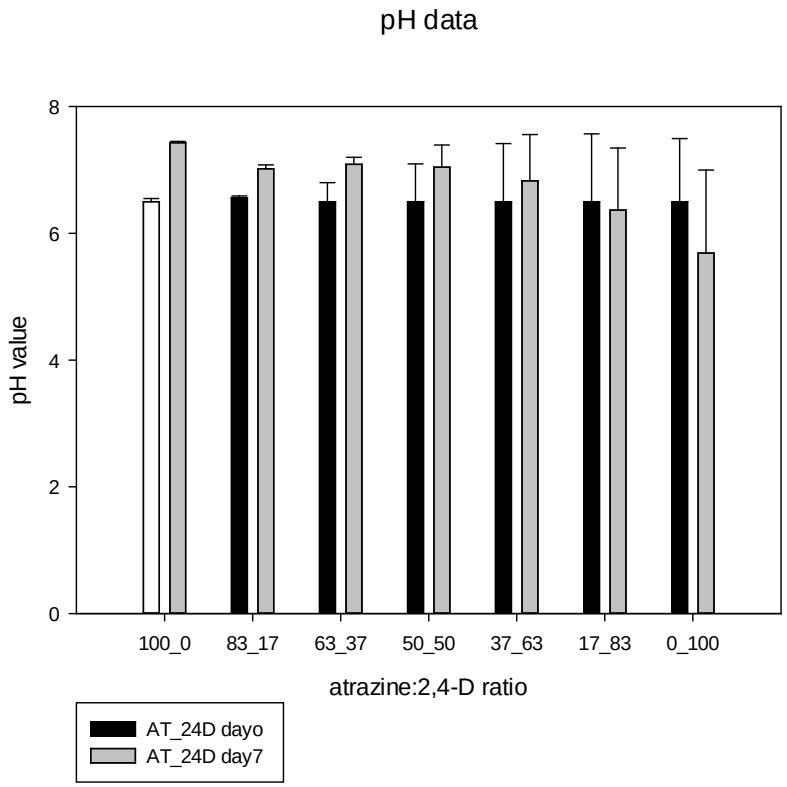


Figure 3-1: pH value including mean and standard deviation (SD) *(n=3)* at day 0 and day7 atrazine and 2,4-D mixture during the experiment.

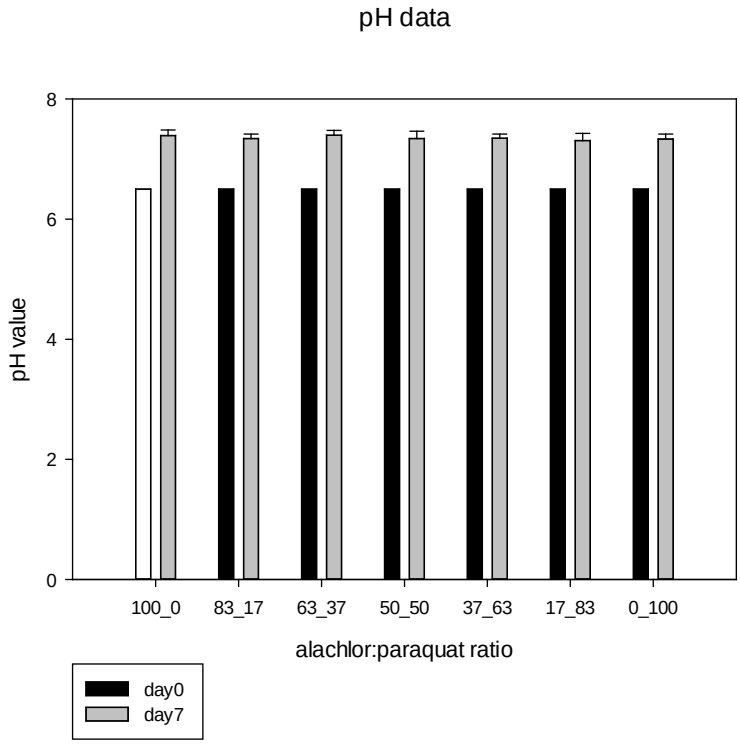


Figure 3-2:pH value including mean and standard deviation (SD) *(n=3)* at day 0 and day7 alachlor and paraquat mixture during the experiment.

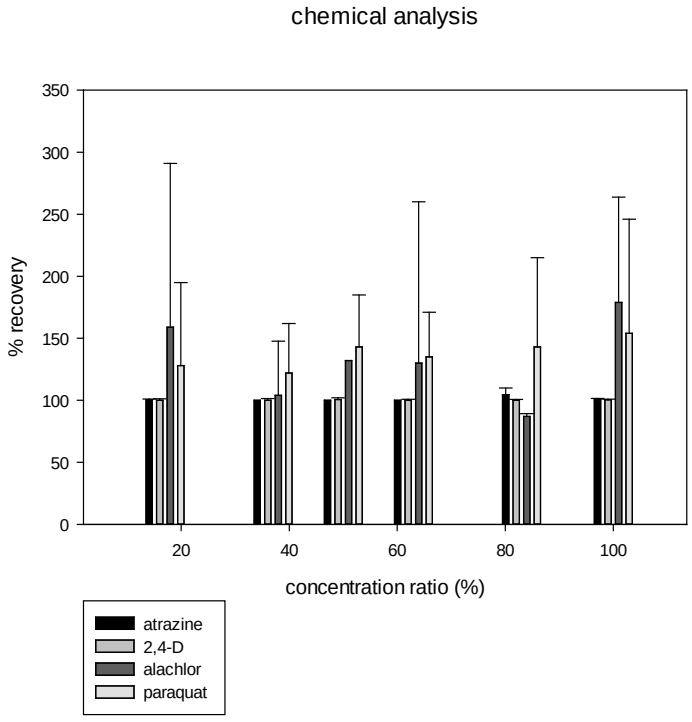


Figure 3-3: the percentage of recovery chemical analysis including mean and standard deviation (SD) (*n=3*) of four herbicides.

Single compound toxicity tests

There were no significant differences in growth rates between the controls and the solvent-controls ($p>0.05$). This indicated that the solvent did not affect the growth rates of *L. minor*. The single toxicity test showed that paraquat was the most toxic of the four study compounds to *L. minor* followed by alachlor, atrazine and 2,4-D. The EC50s for the single compound toxicity tests were 13 µg/L, 16 µg/L, 170 µg/L and 42.0 mg/L, for paraquat, alachlor, atrazine and 2,4-D respectively (Table 3-3). Dose responses curved for determined effective concentration 50 (EC50) are provided in Figures 3-4 -3-7.

Mixture toxicity tests

Isoboles were developed for the EC25 and EC50 levels using the experimental data and predictions using the CA and IA models. At both levels, the observed toxicity for mixtures of 2,4-D and atrazine was found to be lower than estimated by both models indicating that these compounds interacted antagonistically (Figures 3-8 and 3-9). However, for alachlor and paraquat, at both effect levels, the observed toxicity was greater than predicted by the CA and IA models, indicating that these substances interact synergistically (Figures 3-10 and 3-11).

Dose response model with equation

Equation: Standard Curves, Four Parameter Logistic Curves

f1 = min + (max-min)/(1 + (x/EC50)^(-Hillslope))

f = if(x<=0, if(Hillslope>0,min,max), f1)

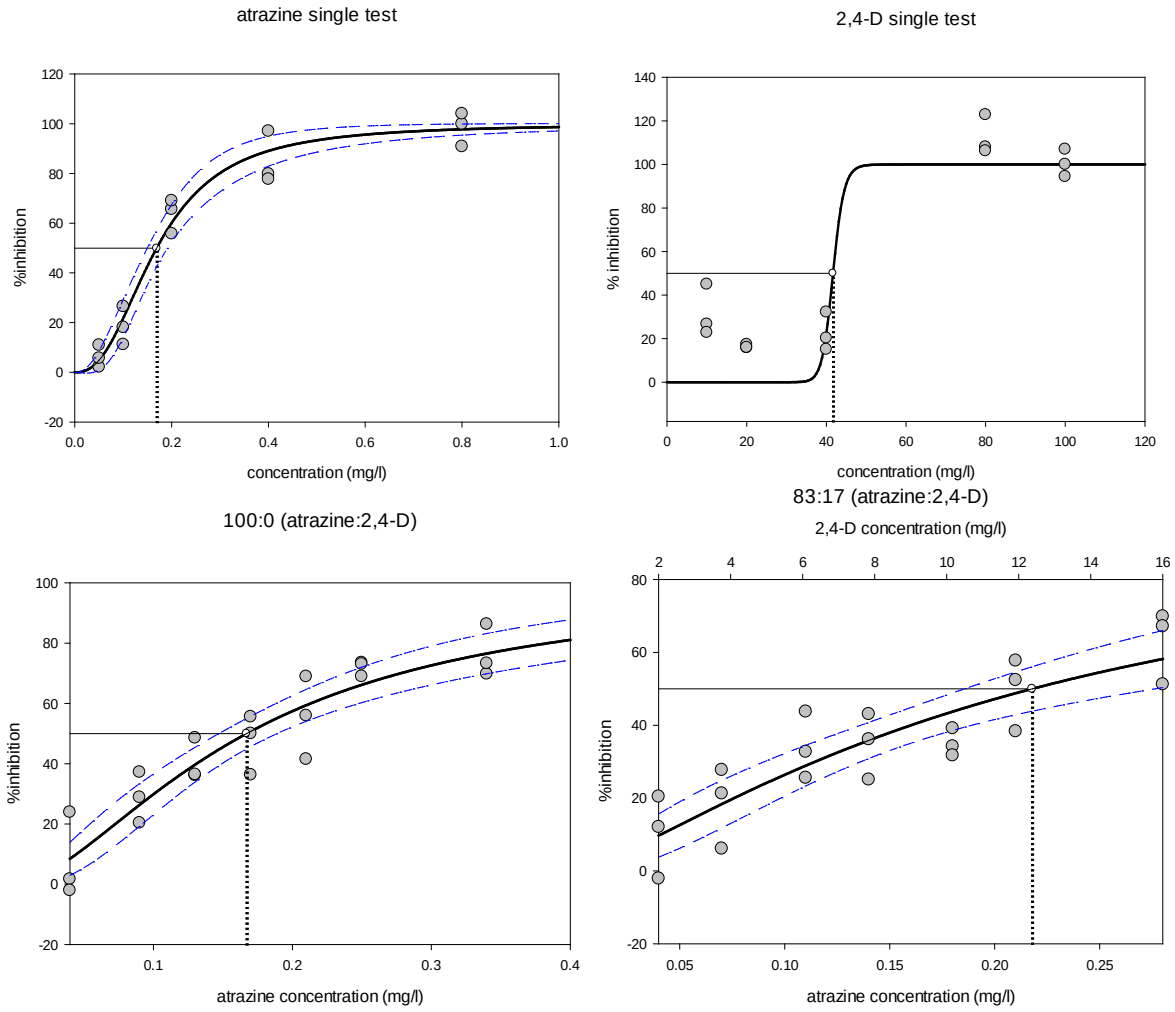


Figure 3-4: Dose response curve of atrazine and 2,4-D in single and mixture in each ratio; atrazine in single test (3-4A), 2,4-D in single test (3-4B), atrazine:2,4-D 100:0 (3-4C) and atrazine:2,4-D 83:17 (3-4D)

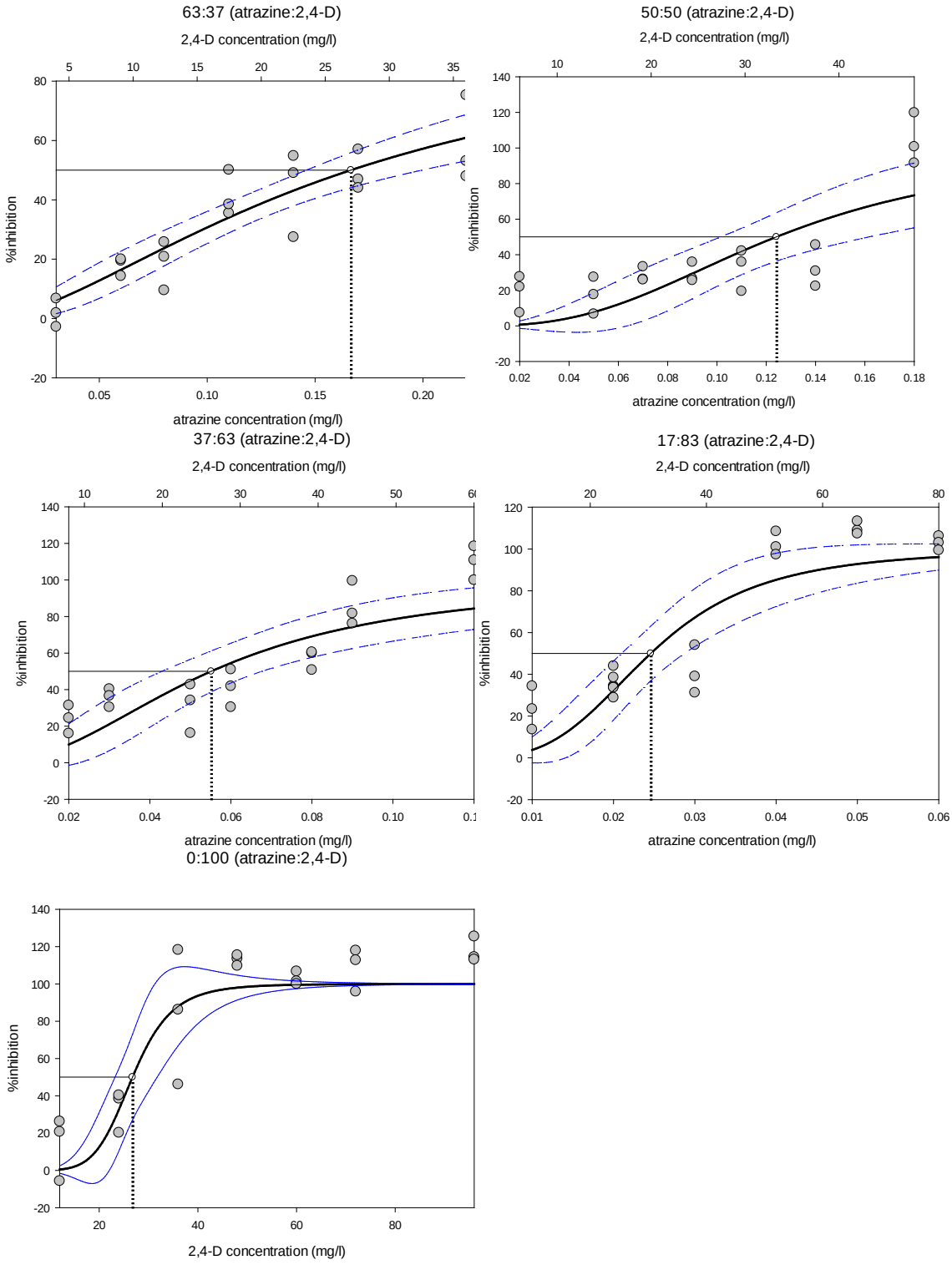


Figure 3-5: Dose response curve of atrazine and 2,4-D mixture each ratio; atrazine:2,4-D 63:37 (3-5A) and atrazine:2,4-D 50:50 (3-4B), atrazine:2,4-D 37:63 (3-5C), atrazine:2,4-D 17:83 (3-5D), atrazine:2,4-D 0:100 (3-5E)

Alachlor and paraquat

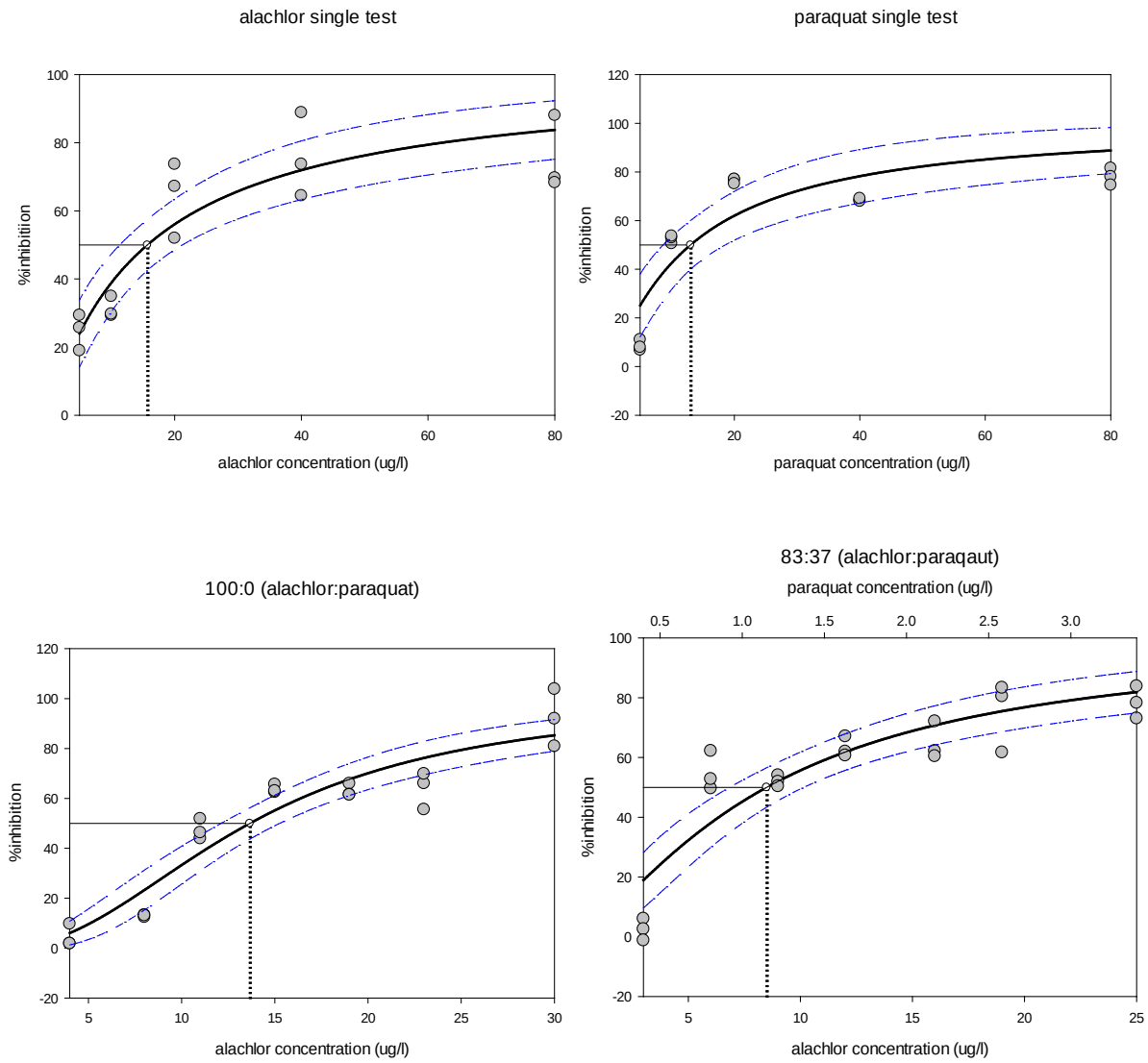


Figure 3-6: Dose response curve of alachlor and paraquat in single and mixture each ratio; alachlor in single test (3-6A), paraquat in single test (3-6B), alachlor:paraquat 100:0 (3-6C) and alachlor:paraquat 83:17 (3-4D).

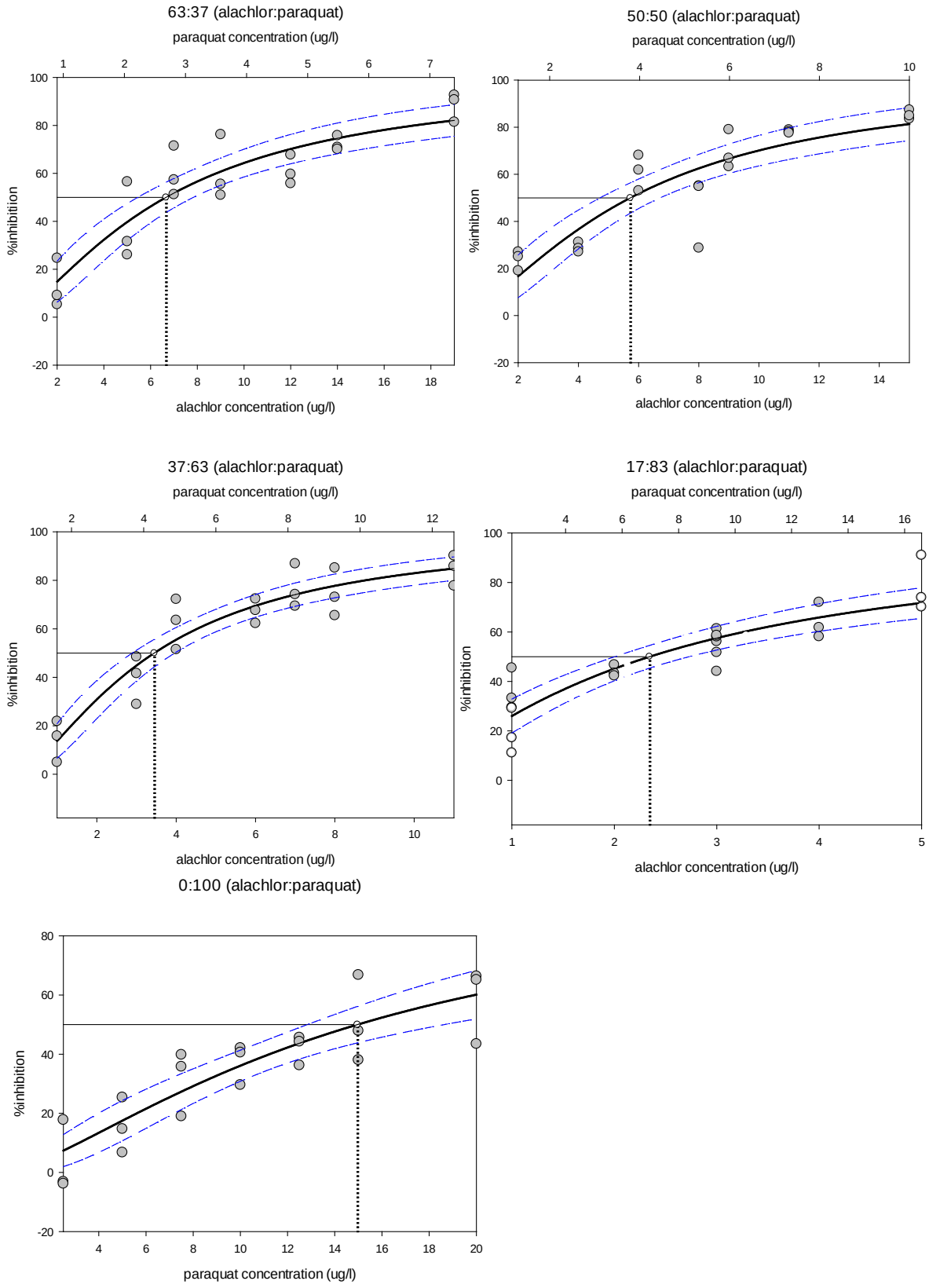


Figure3-7: Dose response curve of alachlor and paraquat mixture each ratio; alachlor:paraquat 63:37 (3-7A) and alachlor:paraquat 50:50 (3-7B), alachlor:paraquat 37:63 (3-7C), alachlor:paraquat 17:83 (3-7D), alachlor:paraquat 0:100 (3-7E)

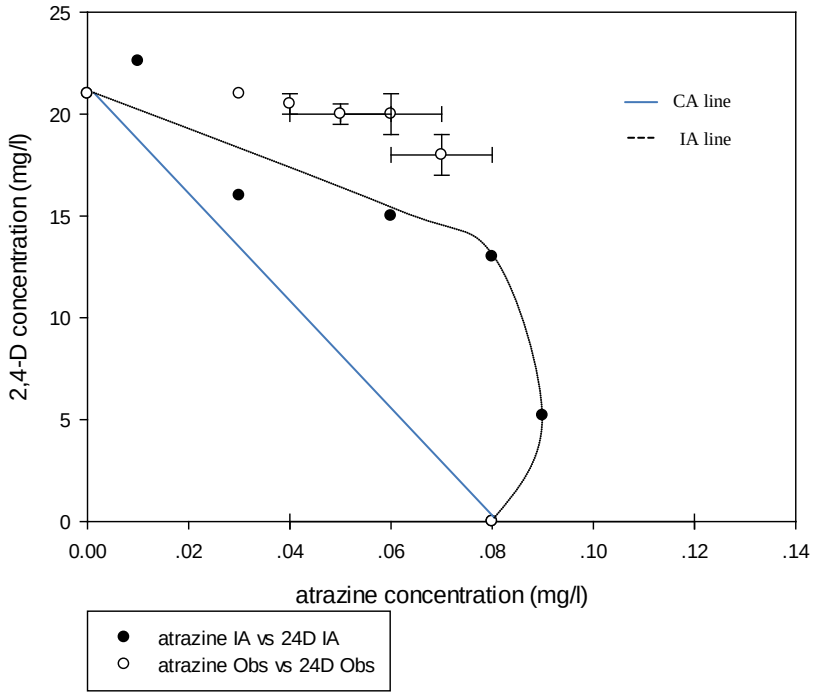


Figure 3-8: Isobole at the EC_{25} level for the seven mixtures of atrazine and 2,4-D. Points represent concentration where 25% reduction in growth was observed and error bar represent the associated 95% CIs.

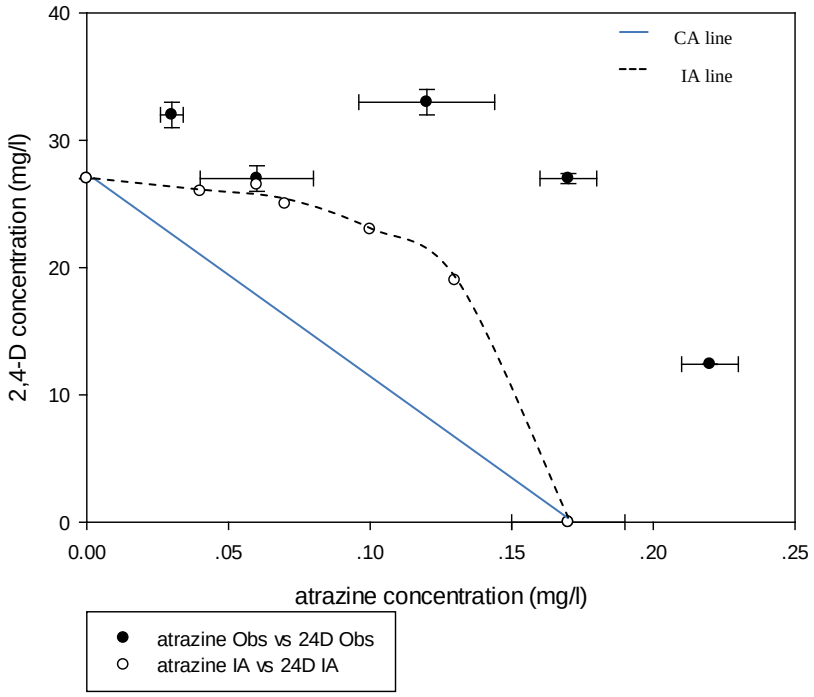


Figure 3-9: Isobole at the EC_{50} level for the seven mixtures of atrazine and 2,4-D. Points represent concentration where 50% reduction in growth was observed and error bar represent the associated 95% CIs.

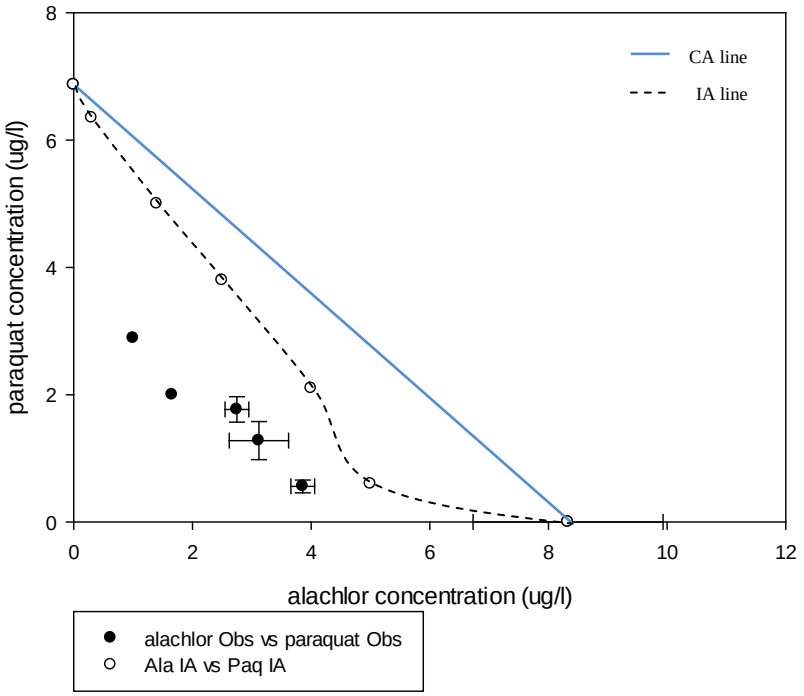


Figure 3-10: Isobole at the EC_{25} level for the seven mixtures of alachlor and paraquat. Points represent concentration where 25% reduction in growth was observed and error bar represent the associated 95% CIs.

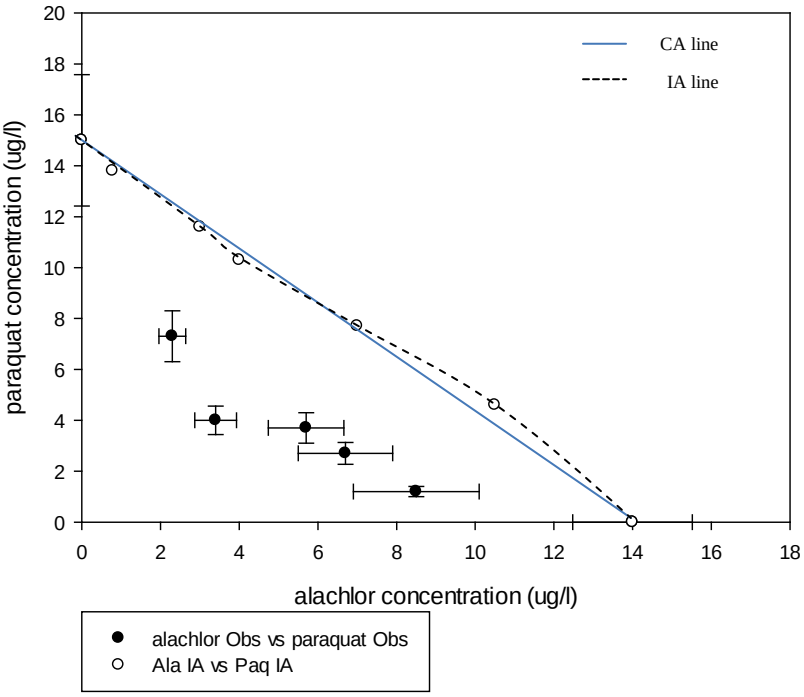


Figure 3-11: Isobole at the EC_{50} level for the seven mixtures of alachlor and paraquat. Points represent concentration where 50% reduction in growth was observed and error bar represent the associated 95% CIs**.**

Discussion

Single toxicity

The results are similar to previous studies on the toxicity of the study compounds to *L. minor* and related macrophytes. For example, Mohammad *et al* (2010) reported that paraquat is more toxic than alachlor and atrazine to duckweed. Previously reported EC50s for the compound to *L. minor* are: 51µg/L for paraquat, 198 µg/L for alachlor, 153 µg/L for atrazine and >100,000 µg/L for 2,4-D (Fairchild et al., 1997). *L. minor* responds differently to different herbicides, which reflect differences in the physicochemical properties of the study compounds, the degree of translocation into the plant, metabolic degradation and the presence or absence of molecular target sites(Michel et al., 2004). The high toxicity of paraquat is explained by the fact that it is a bipyridylium herbicide that can damage the plant tissue very quickly(Brian, 1976). Under sunny conditions leaf discoloration can occur within an hour of applying paraquat to plants. Colour changes were visible on the *Lemna* fronds in the paraquat treatment. Alachlor is a chloroacetamide or amide pesticide and affects root elongation, RNA, protein synthesis, amylase and proteinase activity (Ashton and Bayer, 1976). In our study exposure to the compound resulted in dwarfish fronds. This observation is in agreement with other studies that have shown that alachlor has an impact on frond size due to a disruption of cell division processes (Drost et al., 2007, Vallotton et al., 2008b).Atrazine was moderately toxic in this experiment. Atrazine belongs to the triazine group which is characterised by the photosynthesis inhibition in photosystem II by blocking electron transport (Holzmann *et al*., 1999), leading to a reduction in photosynthetic oxygen production and finally reducing the relative growth rate. Britton *et al*. (1989) examined the membrane of chloroplasts

that were damaged by this chemical(Belden and Lydy, 2000). Exposure to 2,4-D showed limited effects on the plants compared to the other compounds (paraquat, alachlor and atrazine). There are many published studies on the toxicity of 2,4-D on aquatic macrophytes. All of these studies indicate that duckweed are insensitive to or experience moderate toxicity from 2,4-D. Their EC50 values range from 500 to >6000 µg/L (Belgers *et al*., 2009) and from this present study the EC50 was >2700 µg/L. Others have reported that 2,4-D's toxicity is enhanced specifically in dicotyledonous plants rather than monocotyledons because of their differences in morphology and physiology of the two plant groups.

Mixture toxicity

The results indicate that the interaction between atrazine and 2,4-D at EC25 and EC50 levels was antagonistic (Figure 3-4 – 3-5) based on the IA and CA model predictions. There are no literature data on atrazine and 2,4-D mixture toxicity to organisms but there are ecotoxicity data for closely related chemicals and organisms. For example, Bisewska *et al* (2012) examined the toxic interactions of two herbicides, MCPA (2-methyl-4-chlorophenoxyacetic acid) and chloridazone, to the green microalgae and duckweed *L. minor*. Like 2,4-D, MCPA is a chlorophenoxy herbicide. Like atrazine, chloridazone inhibits photosynthesis system II by blocking the electron transport from quinone b(Qb) to plastoquinone (PQ) in the PSII reaction center (Bisewska *et al*., 2012). The two compounds were found to interact antagonistically in studies with *Lemna.* Nielsen and Dahllof (2007) examined the toxicity of mixtures of MCPA and bentazone (PSII inhibitor) to eelgrass *Zostera marina* and found that a synergistic interaction occurs at the low concentrations of the pesticide mixture but at high concentration, an antagonistic effect occurred.

For this work, the results of this experiment agree with those previously reported by other researchers that the antagonistic interaction is the most common form of herbicide mixture interaction. For example, Belden and Lydy (1999) stated that the variety of joint actions produced by atrazine mixed with other compounds indicates that the effect of atrazine on an organism is dependent on the species, co-contaminant, and levels of atrazine used. In addition, the key factors which lead to decreased or increased antagonism on plants include the herbicide rates, mode of action, plant species, formulation, adjuvants, timing, stage of growth and the environment(Green, 1989). Antagonism has been found to occur frequently in other studies using mixtures of herbicides belonging to different chemical groups and monocot species (Zhang et al., 1995, Damalas, 2004). Furthermore, the most common antagonism is when post emergence grass herbicides are mixed with post emergence broadleaf herbicides (Minton *et al*., 1989). In terms of the biochemistry when exposing plants to two herbicides, atrazine has been reported to affect oxidative phosphorylation and decrease net photosynthesis by CO_2 uptake. The phenoxy herbicide 2,4-D also decreases net photosynthesis of plants but higher concentrations are needed (Van Oorschot, 1976).Also, there have been many reports of antagonism occurring with mixtures of herbicides belonging to different chemical groups and monocot species (Zhang et al., 1995, Phyu et al., 2011, He et al., 2013).

Alachlor and paraquat showed greater than additive toxicity (synergism) when experimental observations were compared to predictions based on the IA and CA model. Alachlor is a seedling growth inhibitor and is active at two main sites of the developing shoot and roots(Tomlin, 1997).This herbicide inhibits the dividing of plant cells, which interrupts shoot elongation and lateral root formation(Minton et

al., 1989, Tomlin, 1997). There is evidence to suggest that these herbicides can affect multiple sites within a plant. Similarly, paraquat dichloride is activated by exposure to sunlight to form oxygen compounds such as hydrogen peroxide (Van Oorschot, 1976).These oxygen compounds destroy plant tissues by rupturing plant cell membranes (Van Oorschot, 1976, Tomlin, 1997). Among the reports on pesticide mixture toxicity, they found little evidence of synergism. However, according to the earlier reviews, there is evidence that synergistic interaction occur with mixtures of pesticided with low doses(Cedergreen, 2014, Dennis et al., 2012). In this study the concentration of alachlor and paraquat were tested in low concentration. Regarding the synergy interaction of the pesticide mixture, many studies have attempted to identify the mechanism behind the synergistic interactions, but the mechanisms are not well understood. Therefore, Cedergreen (2014) described that the mechanism causing synergistic interaction can basically affect six processes leading to enhanced toxicity to organisms including effects on bioavailability, uptake, internal transportation, metabolization, binding at the target site and excretion.

It has been suggested that the success of the reference model either IA or CA in predicting effects of mixtures depends on many factors including the effect level under consideration, the number of mixture components, the concentration ratio, the steepness of individual concentration response curves and the regression models (Faust *et al*., 2001).

From the results of this study of atrazine and 2,4-D mixture, the observed effect concentrations were slightly higher than predicted by IA an CA model over a wide range of exposure concentrations, which means that the IA model is likely to overestimate effects of the study herbicides on *Lemna*. Alachlor and paraquat mixtures showed a synergistic interaction based on IA and CA model. Therefore, the

risk assessment of atrazine and 2,4-D mixtures using the IA, CA model would provide an environmentally conservative assessment of the toxicity of these mixtures. However, in terms of alachlor and paraquat it would be beneficial to identify what the mechanism is behind the synergistic effects in order to develop alternative approaches for risk assessment of combinations of these compounds.

Conclusion

Toxicity tests on both the single compounds and binary mixtures of the four herbicides frequently used in Thailand on *L. minor*, which represent one of the non-target aquatic organism of the country, showed that paraquat was the most toxic, followed by alachlor, atrazine and 2,4-D, respectively. For the mixtures, we explored the toxicity of herbicide mixtures that farmers frequently apply on their farms according to the data.

This Chapter explored the effects of herbicide mixtures applied at the same time. However, it is likely that macrophytes will be exposed to different compounds over time due to multiple applications of pesticides to fields or differences in fate characteristics which will mean that different substances may enter aquatic systems at different times. In the next Chapter, work to understand the effects of mixtures of pesticides in time (i.e. pulsed exposure studies) is described.

CHAPTER IV

4. The Effects of Sequential Exposures to Multiple Herbicides on the Aquatic Macrophyte *Lemna minor*

Introduction

Work in the previous Chapter explored the effects of pesticide combinations on *Lemna minor*. In reality, pesticides are not only applied in combinations, but are also applied through other methods such as in sequences, rotations and mosaics (Tabashnik, 1989). Sequential application, which involves pesticides with multiple modes of action, is one of the frequently employed methods in agriculture (Matthews, 1979) and will likely result in aquatic organisms being exposed to different pesticides over time. This therefore adds a temporal dimension to the mixture issue.

After a pesticide is applied to the field, it may undergo a variety of fate processes (Harold, 1990). Some may be lost to the atmosphere through volatilization, leaching into surface water by runoff and erosion, broken down in the sunlight by photolysis, broken down with microorganism by degradation or remaining stable in the environment (Harold, 1990). The process may take from hours to years, depending on environmental conditions and chemical characteristic of pesticides. As a consequence of this, it there are often more than one pesticide present on cropland from sequential exposure to non-target organism in the environment.

Despite this, few studies have observed the evidence showing that aquatic non-target organisms are being exposed to fluctuating concentrations and sequential pulses of different pesticides due to these types of application (Ashauer et al., 2011b). There have been reports that the effects of mixture of pulses of pesticides depend on the order of the exposures, while the duration of sequential application of different groups of pesticides matters as to whether the toxicity increases or decreases (Drost, 2011). In order to determine the risk from pesticide sequential applications, questions have therefore been raised as to whether laboratory data into the effects of single substances on organisms can be used to make predictions for real environmental conditions or, at least, to appraise potential hazards. In addition, such data would provide more realistic scenarios to study the impact of different compounds across modes of action, in different concentrations and orders of application (Dennis *et al*., 2012). Studies of duration and sequence of chemical applications may help refine the risk assessment of aquatic organisms and identify pulse sequences that may be more or less harmful to the environment (Drost et al., 2007, Drost, 2011).

However, there seems to be a lack of studies dealing with fluctuating and sequential long-term exposures (Ashauer et al., 2007a, Dennis et al., 2012). While there are numerous studies that have examined the effects of pesticide exposures, these have focused mostly on single pulses of substance(Angel et al., 2010, Alonso and Camargo, 2009, Bearr et al., 2006, Diamond et al., 2006, Milne et al., 2000, Hosmer et al., 1998). In real practice, single chemical exposure rarely occurs in aquatic systems (Dennis *et al*., 2012). Limited data are available on the effects of mixed pulses of pesticides and reports on repeated or fluctuating pulse are even rarer (Boxall et al., 2013, Dennis et al., 2012). Nonetheless, there have been recognitions

of the lack of a systemic approach and the need to perform tests in more realistic testing regimes.

This Chapter therefore describes a study to assess the effects of pulse exposures to mixtures of pesticides on *L. minor*. The hypothesis of this study was that it is possible to estimate the effects of sequential exposures of macrophytes to different herbicides using data from single-compound ecotoxicity studies. Therefore, the objectives were to (1) evaluate whether the data from single compound toxicity studies using short-term plant ecotoxicity tests are predictive of short-term and long-term effects; (2) evaluate the effects of the order of pesticide exposure on toxicity to *L. minor*; and (3) explore the effects of pulse exposure of herbicides that have different modes of action.

Materials and methods

Chemicals

Atrazine (98.5% purity), 2,4-D (99% purity), alachlor (98% purity), paraquat dichloride (99% purity) and analytical grade solvents (methanol and acetone) were obtained from Sigma Aldrich, Poole, Dorset, UK.

Lemna minor cultures

L. minor was cultured in Swedish media (Organisation for Economic Co-operation and Development., 2006). Cultures were maintained in a Sanyo Environmental test chamber (model MLR-351H) at 20 ^{0}C under continuous illumination at 10,000 Lux. *L. minor* was kept in the logarithmic growth phase by sub-culturing the stocks every 7 days. Prior to use, the pH of the growth media was adjusted to 6.5 with either 0.1 M HCL or NaOH (OECD221:Lemna, 2006).

Sequential exposure studies

The effects of sequential exposure combinations of two sets of two pesticides were assessed. The test combinations were: atrazine then 2,4-D; 2,4-D then atrazine; alachlor then paraquat; paraquat then alachlor. Control treatments included solvent control treatments and single pesticide treatments where plants were exposed sequentially to the same pesticide. There were two separate experimental sets with different exposure times, namely short-term 7-day tests and long-term 14-day tests. Each experiment was separated into different orders of application and concentrations, which are described below.

Short-term exposure

The two exposure scenarios were assessed over a 7-day test period. In the first experiment, a pre-exposure corresponding to a set of varying effective concentrations was used, followed by an exposure to varying concentrations of a second substance. In the second experiment, a pre-exposure corresponding to the 50% effective concentration was used, followed by exposure to varying concentrations of a second herbicide.

The varying concentrations were selected based on the single compound concentration-response data generated previously in Chapter3,and were selected to give either a 10, 25, 50, 75 or 90% reduction in the growth of *L. minor* (concentrations are given in Table 4-1). A simple study design was adopted where plants were exposed to the first herbicide for 3.5 days (50% of the study duration) and then removed and exposed to the second pesticide for the remainder of the study. Three replicate glass petri dishes were set up for each concentration and exposure scenario, and further three petri dishes were also set up to act as controls. Each petri

dish was transferred to a Sanyo Environmental test chamber (model MLR-351H) for 3.5 days for the first exposure and continued to the second exposure for 3.5 days afterwards. The photographs were taken at day 3.5 and day 7.

Long-term sequential exposure

This experiment assessed effects over a 14-day test period. A pre-exposure, corresponding to the 10, 25, 50, 75 and 90% effect concentrations, was used for 10.5 days for the first pesticide, followed by an exposure to varying concentrations of the second substance for 3.5 days. The varying concentrations were selected based on the single compound concentration-response data generated previously and were selected to give 10, 25, 50, 75 and 90% reduction in the growth of *L. minor* (concentrations are given in Table 4-1).

Three replicate glass petri dishes were set up for each concentration and exposure scenario, and further three petri dishes were also set up as controls. One colony of *L. minor* with three fronds was then added to each petri dish and digital photographs of the *L. minor* were taken using Cannon ixus210from above. The areas of the *L. minor* colonies were then determined using Image J (Boxall *et al*., 2013). Each petri dish was transferred to a Sanyo Environmental test chamber (model MLR-351H) for 10.5 days for the first exposure and renewed with fresh substances every 3.5 days before continuing to the second exposure for 3.5 days afterwards. Control test solutions were changed at the same frequency as the semi static (3.5 days) until day 10.5 and exposed to substance for 3.5 days afterwards. The photographs were taken at day 10.5 and day 14.

Test conditions and observation of sequential toxicity

The test chamber incubation was set at a temperature of 20 ^{0}C under continuous illumination at 10,000 Lux. the dishes were removed and *L. minor* were photographed using Cannon ixus210 from above. Areas of *L. minor* colonies were then determined using Image J (Boxall *et al*., 2013). At the end of the test periods, samples of the exposure media were taken for chemical analysis, and pH was measured using a Thermo Orion pH meter (Benchtop pH/ISE meter). Samples of stock solution of test media (2 mL) were taken for analysis of pesticide concentrations.

Control test solution and control media were changed at the same frequency (every 3.5 days) as the semi static exposure test solutions.

Table 4-1: Dosage of solvents and pesticide concentrations in different sequential exposure studies.

Dosage (µg/l) / Pesticide	Solvent	10%	25%	50%	75%	90%
Atrazine	<0.05% acetone	70	110	170	270	420
2,4-D	-	19000	22000	28000	32000	37000
Paraquat	-	1.9	5	13	34	89
Alachlor	<0.05% acetone	1.9	5	16	46	100

Table 4-2: Experiment plan for short-term and long-term exposure to pesticides

Experiment	**Day**	**AT/2,4-D**	**2,4-D/AT**	**control/AT**	**control/2,4-D**
Short-term	0-3.5	Atrazine	2,4-D	media	media
	3.5-7	2,4-D	Atrazine	Atrazine	2,4-D
	Day	**Ala/Paq**	**Paq/Ala**	**Control/Ala**	**Control/Paq**
Short-term	0-3.5	Alachlor	Paraquat	media	media
	3.5-7	Paraquat	Alachlor	Alachlor	Paraquat
Experiment	**Day**	**AT/2,4-D**	**2,4-D/AT**	**control/AT**	**control/2,4-D**
Long-term	0-10.5	Atrazine	2,4-D	media	media
	10.5-14	2,4-D	Atrazine	Atrazine	2,4-D
	Day	**Ala/Paq**	**Paq/Ala**	**Control/Ala**	**Control/Paq**
Long-term	0-10.5	Alachlor	Paraquat	media	media
	10.5-14	Paraquat	Alachlor	Alachlor	Paraquat

Calculation of the measured and predicted growth rates

The model for calculating measured and predicted growth rates was adopted from OECD221 (Organisation for Economic Co-operation and Development., 2006). The predicted growth rate was calculated using average specific growth rate (ASGR) from herbicide control, while the measured rate was collected from the experiment itself. Details of the equations used for calculating average specific growth rates and predicted frond areas are given below.

The growth rates of duckweed were calculated via image analyses of *L. minor*'s frond area in each treatment. The growth rates were determinedusingEquation1.

$$ASGR = \frac{\ln(Nj) - \ln(Ni)}{tj - ti} \qquad \text{Equation 1}$$

Where ASGR is the specific growth rate, N_i is the frond area at day_i and N_j is the frond area at day_j.

In order to calculate the frond area to evaluate predicted and measured endpoint of *L. minor*, Equation 2 was used.

$$\ln X_7 = 3.5GR_A + 3.5GR_B + \ln X_0 \qquad \text{Equation 2}$$

In Equation 2, GR_A denotes the growth rate of *L. minor* in chemical A and GR_B is the growth rate of *L. minor* in chemical B, whereas X_0 is the frond area at $day_{0.}$

For the second scenario, the growth rates from the control chemicals at day 3.5 to day 7 were used to calculate the area at day 7 (X_7) of *L. minor* by following Equation 3.

$$\ln X_7 = 3.5GR_A + \ln X_{3.5} \qquad \text{Equation 3}$$

For long-term sequential exposure, in order to derive the predicted endpoint, the growth rates from the control chemicals were used to calculate the frond areaof *L. minor* at day 14 (X_{14}) following Equation 4

$$\ln X_{14} = 3.5GR_{A,B10.5d} + \ln X_{10.5A,B} \qquad \text{Equation 4}$$

In Equation 4, $GR_{A,B}$ denotes the growth rate of *L. minor* in chemical A or B, whereas $X_{10.5A,B}$ is the frond area in chemical A or B starting from $day_{10.5.}$

Analytical methods

Concentrations of atrazine in water samples were determined by high performance liquid chromatography (HPLC) using an Agilent 1100 HPLC system. The mobile phase (methanol: water; 55%: 45%) was set at a flow rate of 1 ml/min. The column was a C18 Supelco Discovery (15 cm x 4.6 mm x 5µm). The oven temperature was adjusted to 40 ^{0}C and the detection wavelength was 220 nm (Fu, 2008). The injection volume was 15 µl. The calibrations were done using pesticide standard with a concentration range with high correlation (r^2= 0.999) and the retention time was between 6-7 minutes. The limit of detection was 0.02 mg/l and the limit of qualification was 0.06 mg/l.

Concentrations of 2,4-D were also determined by HPLC. The mobile phase (methanol:water; 70%: 30%, 0.1% HCOOH) was set at a flow rate of 1 ml/min and the volume injection set to 15 µl. The column was a C18 Supelco Discovery (15 cm x 4.6 mm x 5µm). The oven temperature was adjusted to 30 ^{0}C with the detection wavelength of 236 nm (Connick *et al*., 1982). An analytical set consists of five analytical standards of various concentrations, covering the range of concentrations tested, and will be used to perform the calibration graph (Chandra *et al.*, 2001) (r^2= 0.999).The retention time was between 3-4 minutes. The limit of detection was 0.12 mg/l and the limit of qualification was 0.39 mg/l.

Enzyme Linked Immunosorbent Assay (ELISA) test kits were used to determine concentrations of alachlor and paraquat. The alachlor ELISA test kit was purchased from Biosense (Biosense, Norway) and the paraquat ELISA test kit from EnviroLogix (Portland, USA). For both alachlor and paraquat, semi-log or 4-parameter curve fit is used to interpret the results.

Statistics

The differences between predicted and measured toxicity at different effective concentrations were determined in two-way analyses of variance (ANOVA) using SigmaPlot 12. Where the test of normality failed, a Scheirer-Ray-Hare test in non-parametric was executed. The test was performed with $\alpha = 0.05$ using SPSS Software, version 18 (SPSS Inc., Chicago, IL, USA).

Results

Comparison of mean measured concentrations in test solutions with nominal concentrations for the different sequential exposures indicated that actual concentrations of atrazine and 2,4-D were generally within ±5% of the nominal concentrations (Table 4-3). The actual concentrations of paraquat and alachlor were generally within ±20% and ±40%, respectively. Chemical analysis of the four compounds indicated that the chemical concentrations in each treatment remained relatively stable throughout the test period. The pH of the exposure media for all treatments increased slightly over the study period but this increase was less than 1 unit (± <1) during the experiment (see Appendix C). The results of the chemical analyses agreed with previous research and indicated that four herbicides atrazine, 2,4-D, alachlor and paraquat are stable in the aquatic environment (Larson et al., 1997, Solomon et al., 2013).The pH values can be found in Appendix C.

Table 4-3: Analytical results for pulsed exposure studies and standard deviation.

Nominal exposure concentration (µg/l)	Measured concentration (µg/l)		
	Short-time exposure		Long-time exposure
	Sequential exposure I	Sequential exposure II	
Atrazine			
70	68(±4)	62(±10)	80(±0)
110	108(±2.3)	111(±18)	124(±0)
170	165(±3.2)	184(±18.7)	177(±5.7)
270	265(±5)	269(±26.7)	283(±5.7)
420	413(±9.1)	406(±18.3)	426(±5.7)
2,4-D			
19000	18920(±0.35)	18450(±6.7)	1696(±0.77)
22000	22125(±0.66)	22543(±5.7)	1928(±1.21)
28000	27825(±1.03)	28427(±5.7)	2864(±1.53)
32000	31775(±1.66)	32770(±4.8)	3444(±2.76)
37000	36065(±1.55)	37760(±2.3)	3731(±1.05)
Alachlor			
1.9	2.21(±0.15)	2.32(±0.05)	1.85(±0.8)
5	7.2(±0.88)	6(±1.4)	4.92(±1.87)
16	21.2(±2.3)	19(±1.1)	15.98(±3.2)
46	42.5(±3.4)	46.5(±3)	44(±10.7)
100	146(±12)	117(±10.2)	102.2(±7)
Paraquat			
1.9	1.46(±0.2)	1.15(±0.3)	1.76 (±0.09)
5	4.4(±0.5)	3.4(±0.8)	5.6(±0.44)
13	11.4(±1.5)	18(±6.2)	12.58(±0.79)
34	29.3(±5.2)	20(±11)	32.97(±1.7)
89	91(±12)	73(±1)	84.25(±4.38)

Toxicity of herbicides on *Lemna minor* based on the frond area

Short-term exposure

In the first scenario with atrazine and 2,4-D, the toxicity of atrazine and 2,4-D in the experiment that started with exposure of *L. minor* to atrazine and then to 2,4-D, showed less toxicity than predicted ($p>0.05$) (Figure4-1). However, when the plants were exposed to 2,4-D followed by atrazine, the toxicity was greater than predicted with significant differences between the experimental observations and predictions seen at the low concentration of two herbicides ($p<0.05$) (Figure4-2). The experiments that started with the pre-exposure of alachlor and the second exposure of paraquat and, in the second scenario, with the pre-exposure of paraquat followed by alachlor, showed no significant differences($p>0.05$) between the predicted and measured frond areas of *L. minor* in each treatment (Figure4-3; 4-4).

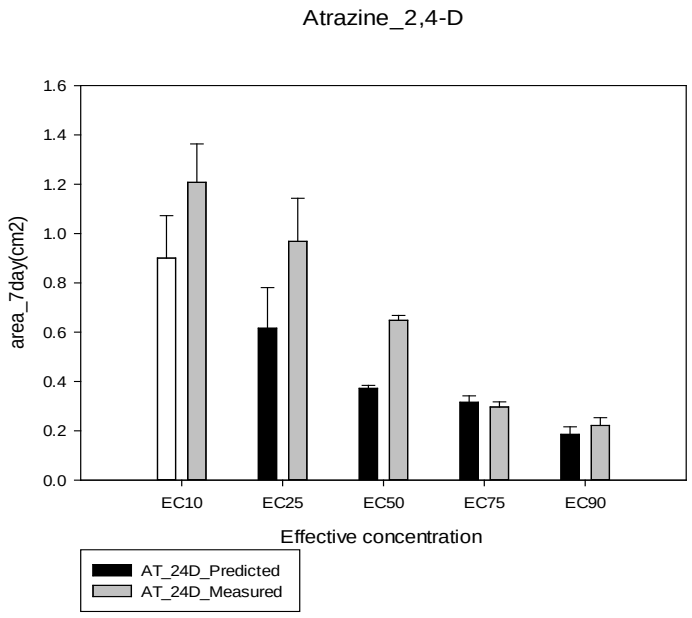


Figure 4-1: Mean frond area at 7-d (cm^2) (± standard deviation) of *L. minor (n=3)* in atrazine/2,4-D at different effective concentrations (EC10, EC25, EC50, EC75 and EC90; x-axis) where the graph describes either the predicted area (■) derived from calculation of the growth rate of chemical control itself or the measurement obtained from the experiment (■). Asterisk (*) indicates that there was a significant difference between the predicted and measured areas ($p < 0.05$).

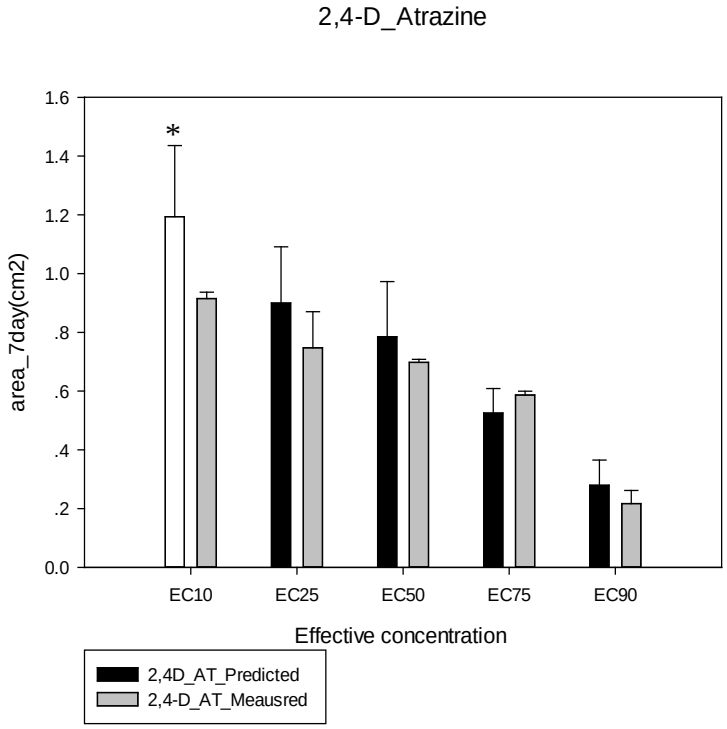


Figure 4-2: Mean frond area at 7-d (cm^2) (± standard deviation) of *L. minor (n=3)* in 2,4-D/atrazine at different effective concentrations (EC10, EC25, EC50, EC75 and EC90; x-axis) where the graph describes either the predicted area (■) derived from calculation of the growth rate of chemical control itself or the measurement obtained from the experiment (■). Asterisk (*) indicates that there was a significant difference between the predicted and measured areas ($p < 0.05$).

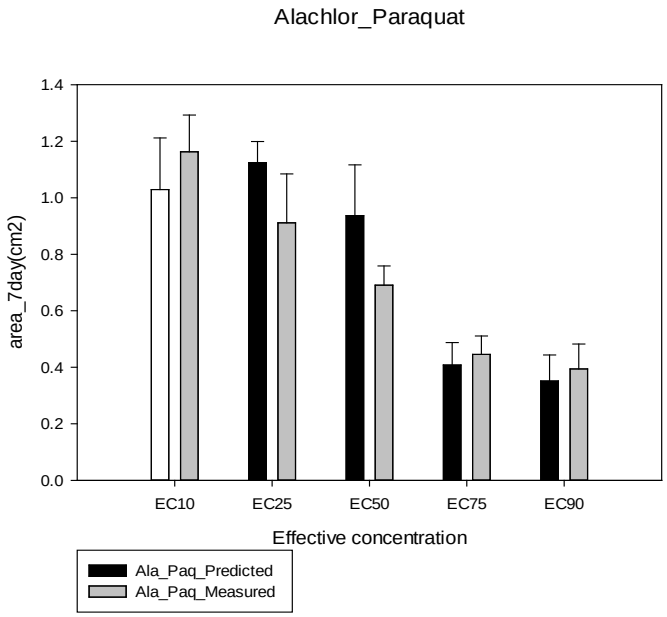


Figure 4-3: Mean frond area at 7-d (cm^2) (± standard deviation) of *L. minor (n=3)* in alachlor/paraquat (c), and at different effective concentrations (EC10, EC25, EC50, EC75 and EC90; x-axis) where the graph describes either the predicted area (■) derived from calculation of the growth rate of chemical control itself or the measurement obtained from the experiment (■). Asterisk (*) indicates that there was a significant difference between the predicted and measured areas ($p < 0.05$).

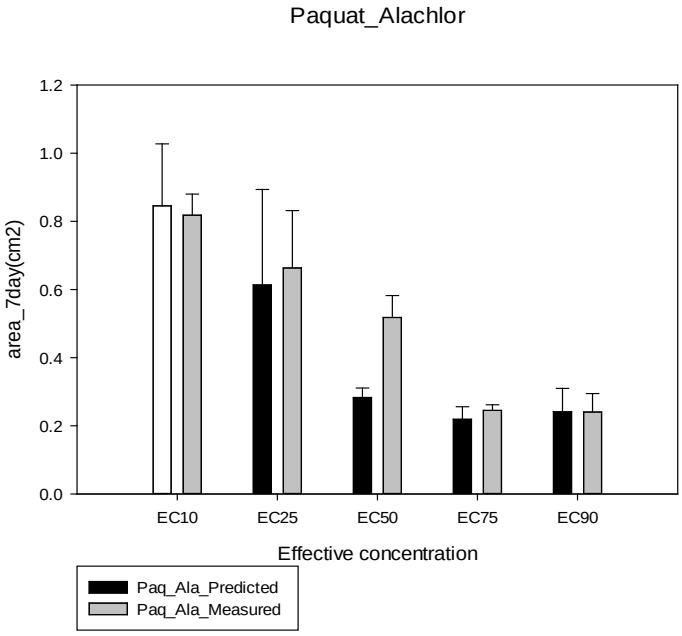


Figure 4-4: Mean frond area at 7-d (cm^2) (± standard deviation) of *L. minor (n=3)* in paraquat/alachlor at different effective concentrations (EC10, EC25, EC50, EC75 and EC90; x-axis) where the graph describes either the predicted area (■) derived from calculation of the growth rate of chemical control itself or the measurement obtained from the experiment (■). Asterisk (*) indicates that there was a significant difference between the predicted and measured areas ($p < 0.05$).

For the second scenario, in order to interpret the concentration response between the pre-exposure and the second substance, the first substance was fixed at one level at the 50% effective concentration (EC50) and then combined with varying concentrations of the second substance. This meant that the plants were pre-treated in the same manner.

The estimated areas after 7days of atrazine or 2,4-D with a second exposure to atrazine or 2,4-D at varying concentrations were determined. The same result was found with both atrazine as pre-exposure followed by 2,4-D, and 2,4-D as pre-exposure followed by atrazine. They showed no differences between predicted and measured areas (p>0.05) (Figure4-5; 4-6).Estimated areas after 7 days of alachlor and paraquat with pre-exposure of alachlor followed by paraquat also showed no significant differences between predicted and measured frond areas (p>0.05) (Figure4-7; 4-8).

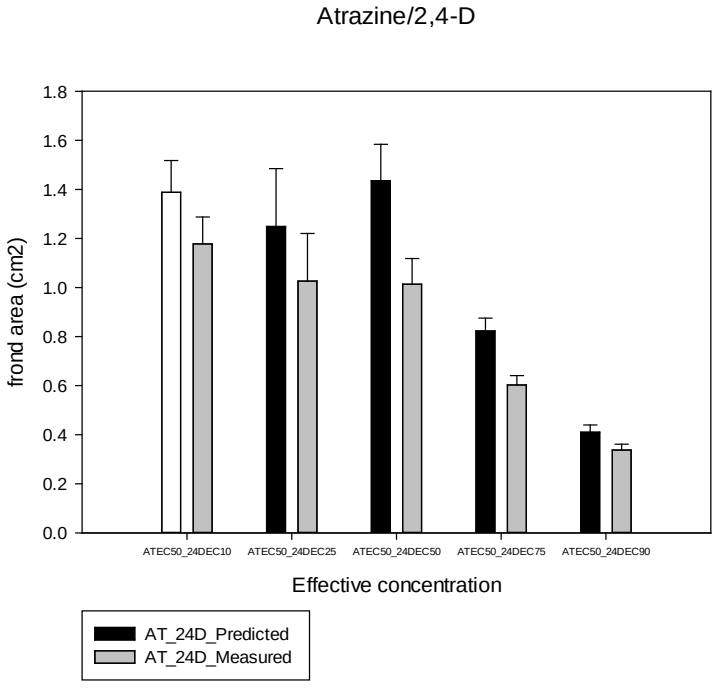


Figure 4-5: Mean frond area of *L. minor* at 7-d (± standard deviation) (n=3) in atrazine/2,4-D at different effective concentrations where the graph described either the predicted area (■) derived from the calculation of the growth rate of chemical control itself or the measurement obtained from the experiment (■). Asterisk (*) indicates that there was a significant difference between the predicted and measured toxicity *(p < 0.05)*.

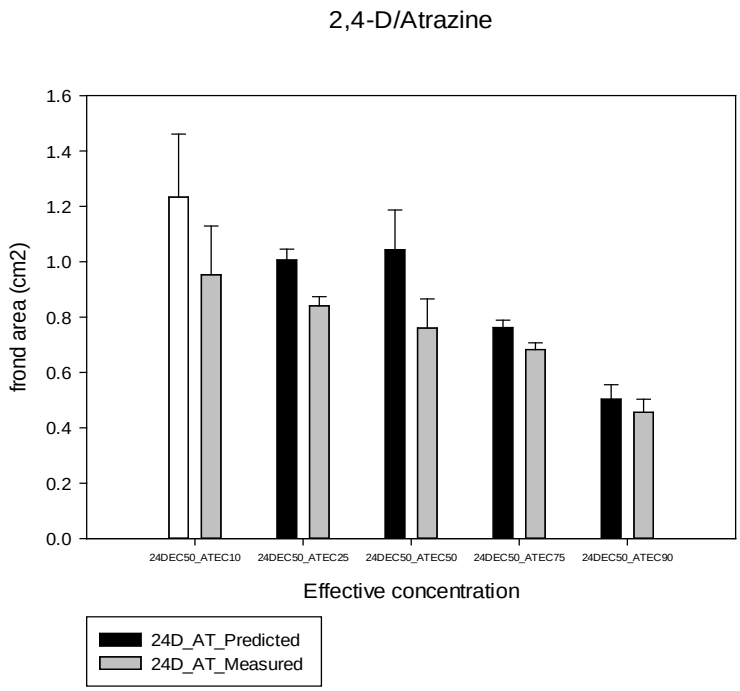


Figure 4-6: Mean frond area of *L. minor* at 7-d (± standard deviation) (n=3) in 2,4-D/atrazine at different effective concentrations where the graph described either the predicted area (■) derived from the calculation of the growth rate of chemical control itself or the measurement obtained from the experiment (■). Asterisk (*) indicates that there was a significant difference between the predicted and measured toxicity *(p < 0.05)*.

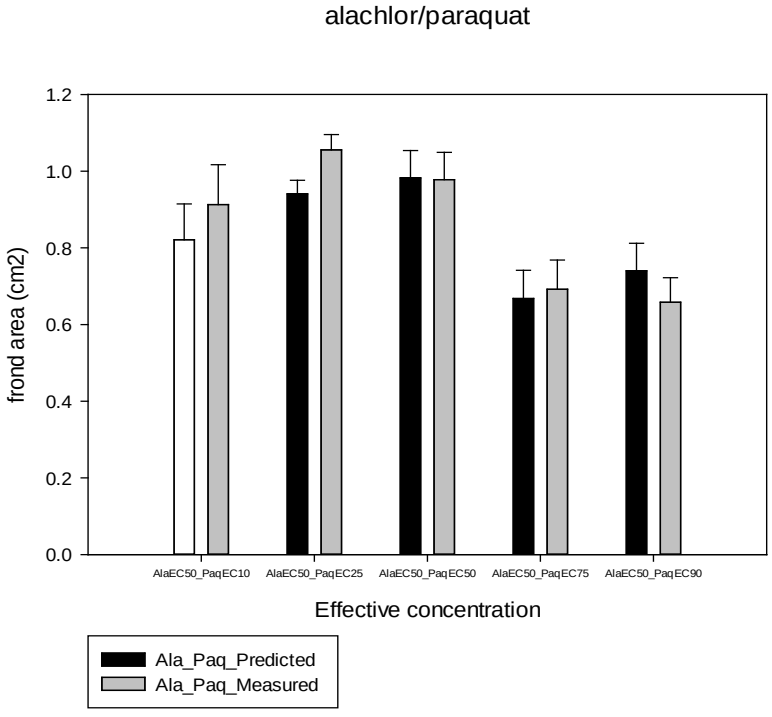


Figure 4-7: Mean frond area of *L. minor* at 7-d (± standard deviation) (n=3) in alachlor/paraquat at different effective concentrations where the graph described either the predicted area (■) derived from the calculation of the growth rate of chemical control itself or the measurement obtained from the experiment (■). Asterisk (*) indicates that there was a significant difference between the predicted and measured toxicity *(p < 0.05)*.

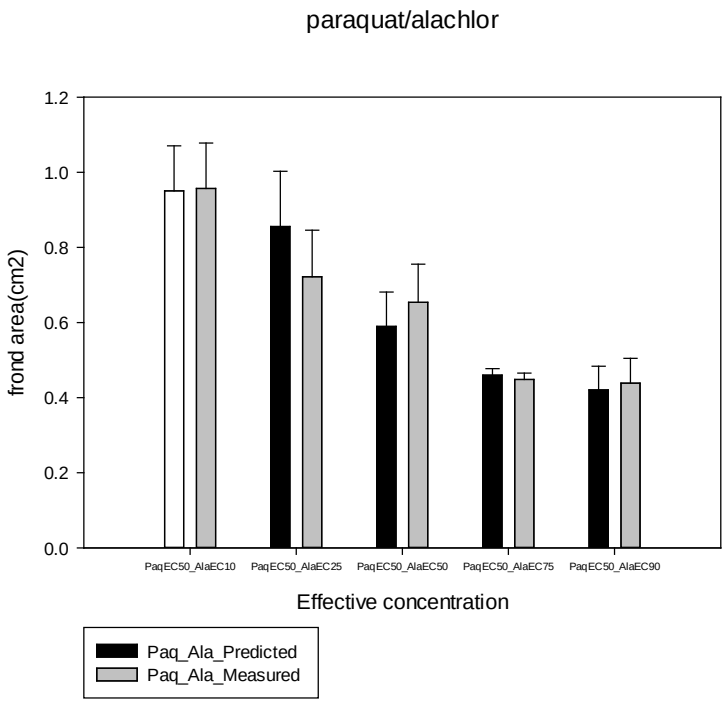


Figure 4-8: Mean frond area of *L. minor* at 7-d (± standard deviation) (n=3) in paraquat/alachlor at different effective concentrations where the graph described either the predicted area (■) derived from the calculation of the growth rate of chemical control itself or the measurement obtained from the experiment (■). Asterisk (*) indicates that there was a significant difference between the predicted and measured toxicity *(p < 0.05)*.

Longer sequential exposure

To test if the model can be used to predict the influence of a 10.5-day pre-exposure to one herbicide at a fixed concentration level (based on the single toxicity test data from previous experiment) followed by an exposure to varying concentration of a second herbicide exposure for 3.5 days, *L. minor* were pre-treated in the same manner and the plants were subsequently exposed to a second substance (Drost, 2012).

In the experiment where *L. minor* were pre-exposed to atrazine followed by 2,4-D, the model showed that the predictions were overestimates of the measurements ($p<0.05$) (4-11, 4-12 and 4-13) and the observation of the predicted and measured areas are shown in Table 4-4. This means that the measured area sizes were smaller

than predicted. However, for *L. minor* pre-treated with atrazine at the effective concentration of 10 and 25 followed by 2,4-D, the measured result was as predicted (Fig.4-9 and 4-10).

1. Atrazine/2,4-D

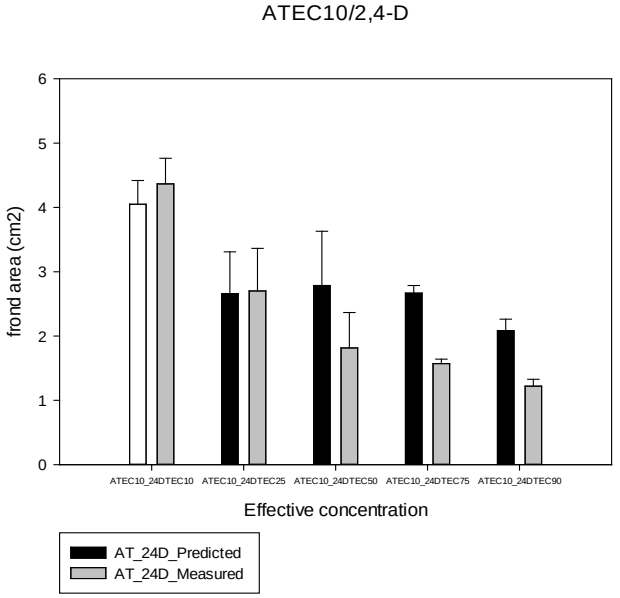


Figure 4-9: Predicted (■) and measured (■) mean frond area (cm^2) ± standard deviation of *L. minor* (*n=3*). The plants were pre-treated with atrazine for 10.5 days at effective concentration of 10 (EC10). Subsequently, the plants were transferred to various concentrations of 2,4-D and were exposed for 3.5 days. Asterisk (*) indicates that there was significant difference between the predicted and the measured areas (*p<0.05*).

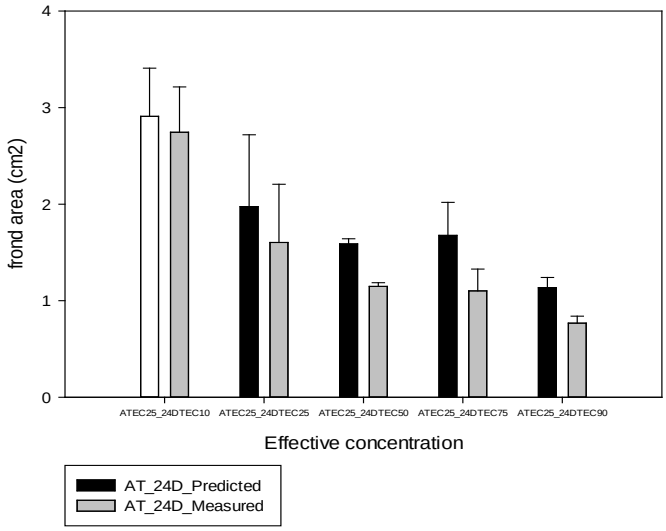


Figure 4-10: Predicted (■) and measured (■) mean frond area (cm^2) ± standard deviation of *L. minor* (*n=3*). The plants were pre-treated with atrazine for 10.5 days at effective concentration of 25 (EC25). Subsequently, the plants were transferred to various concentrations of 2,4-D and were exposed for 3.5 days. Asterisk (*) indicates that there was significant difference between the predicted and the measured areas (*p<0.05*).

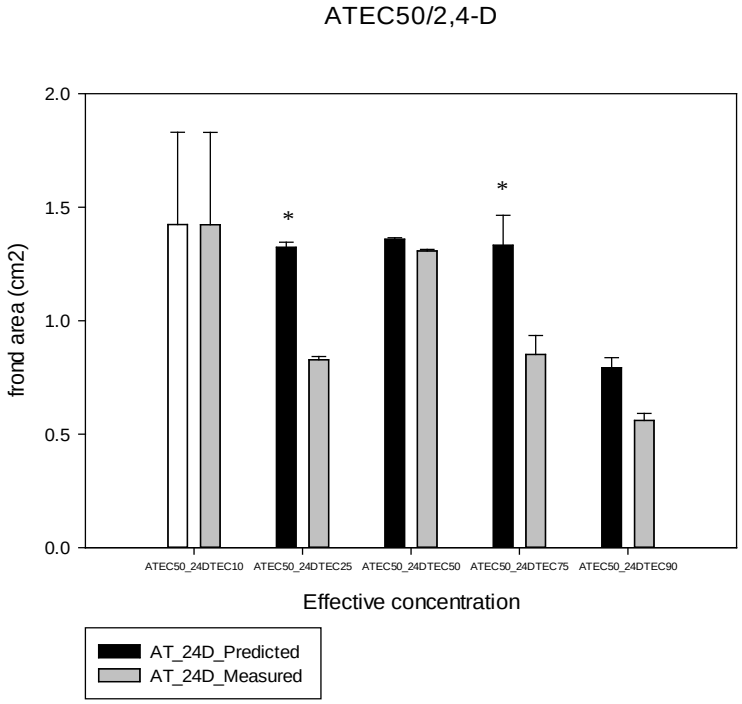


Figure 4-11: Predicted (■) and measured (■) mean frond area (cm^2) ± standard deviation of *L. minor* (*n=3*). The plants were pre-treated with atrazine for 10.5 days at effective concentration of 50 (EC50). Subsequently, the plants were transferred to various concentrations of 2,4-D and were exposed for 3.5 days. Asterisk (*) indicates that there was significant difference between the predicted and the measured areas (*p<0.05*).

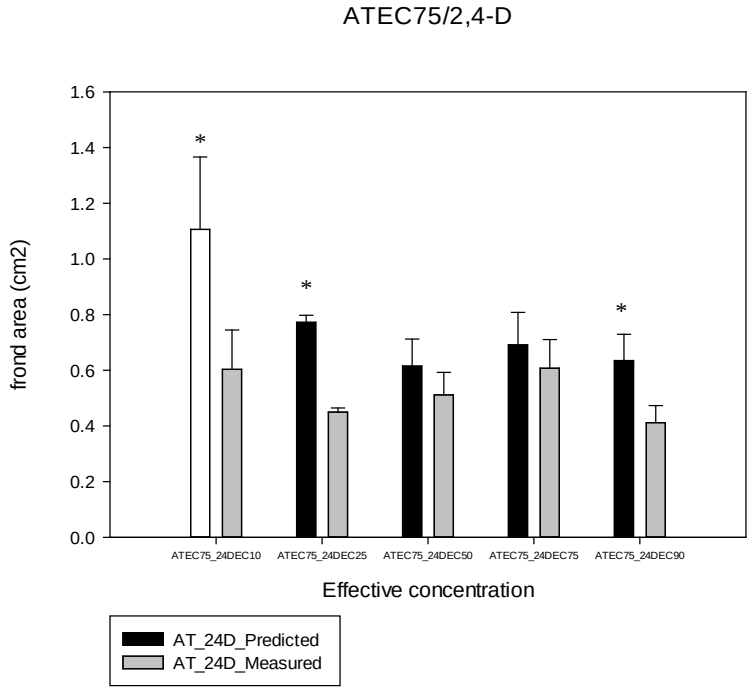


Figure 4-12:Predicted (■) and measured (■) mean frond area (cm^2) ± standard deviation of *L. minor* (*n=3*). The plants were pre-treated with atrazine for 10.5 days at effective concentration of 75 (EC75). Subsequently, the plants were transferred to various concentrations of 2,4-D and were exposed for 3.5 days. Asterisk (*) indicates that there was significant difference between the predicted and the measured areas (*p<0.05*).

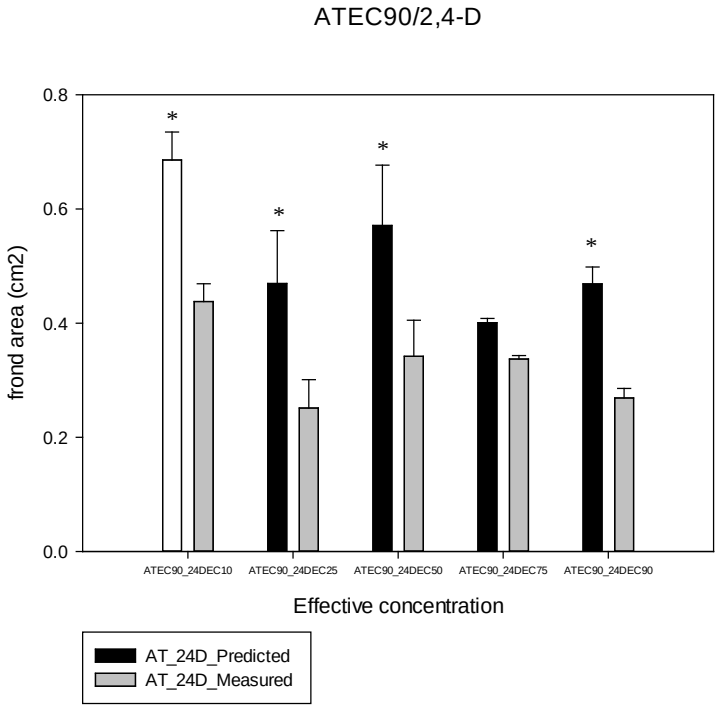


Figure 4-13:Predicted ( ) and measured ( ) mean frond area (cm^2) ± standard deviation of *L. minor* (*n=3*). The plants were pre-treated with atrazine for 10.5 days at effective concentration of 90 (EC90). Subsequently, the plants were transferred to various concentrations of 2,4-D and were exposed for 3.5 days. Asterisk (*) indicates that there was significant difference between the predicted and the measured areas (*p<0.05*).

2. 2,4-D/atrazine

For the experiment with pre-exposure to 2,4-D followed by atrazine, the predicted models were higher than the measurements, but the differences were not significant ($p>0.05$) among the lower effective concentrations (Figs. 4-14, 4-15, 4-16 and 4-17). This shows that the models can be used to make predictions of the toxicity of sequential exposure to two herbicides, 2,4-D and atrazine, at lower concentrations, but not for the higher effective concentrations of pre-treatment (Fig. 4-18).

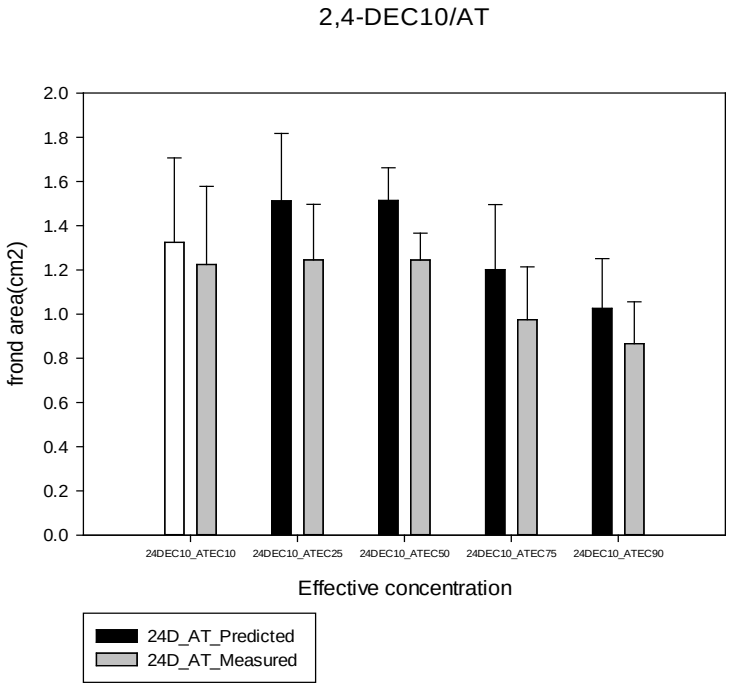


Figure 4-14: **Predicted (■) and measured (■) mean frond area (cm^2) ± standard deviation of *L. minor* (*n=3*). The plants were pre-treated with 2,4-D for 10.5 days with the effect concentration of 10 (EC10). Subsequently, the plants were transferred to various concentrations of atrazine and were exposed for 3.5 days. Asterisk (*) indicates that there was significant difference between the predicted and the measured areas (*p<0.05*).**

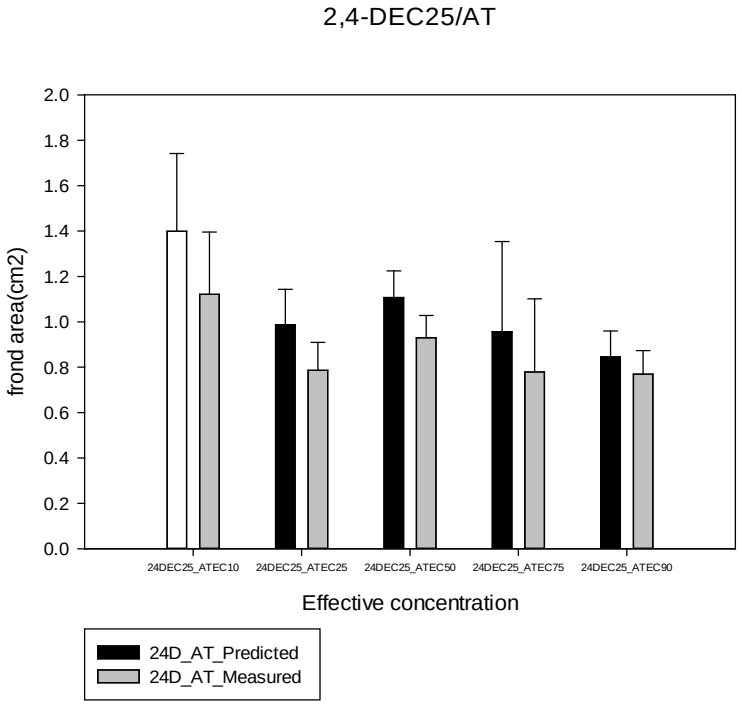


Figure 4-15: Predicted (■) and measured (■) mean frond area (cm^2) ± standard deviation of *L. minor* (*n=3*). The plants were pre-treated with 2,4-D for 10.5 days with the effect concentration of 25 (EC25). Subsequently, the plants were transferred to various concentrations of atrazine and were exposed for 3.5 days. Asterisk (*) indicates that there was significant difference between the predicted and the measured areas (*p<0.05*).

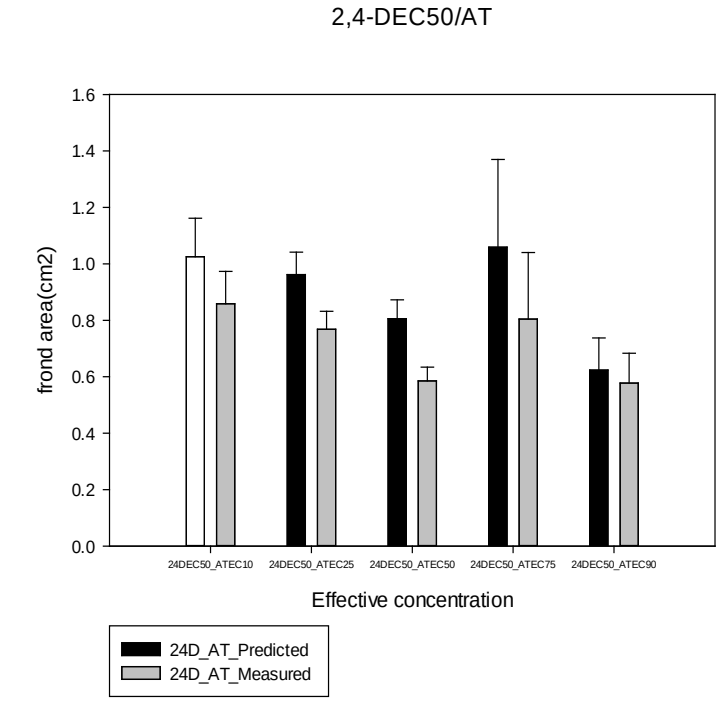


Figure 4-16: Predicted (■) and measured (■) mean frond area (cm^2) ± standard deviation of *L. minor* (*n=3*). The plants were pre-treated with 2,4-D for 10.5 days with the effect concentration of 50 (EC50). Subsequently, the plants were transferred to various concentrations of atrazine and were exposed for 3.5 days. Asterisk (*) indicates that there was significant difference between the predicted and the measured areas (*p<0.05*).

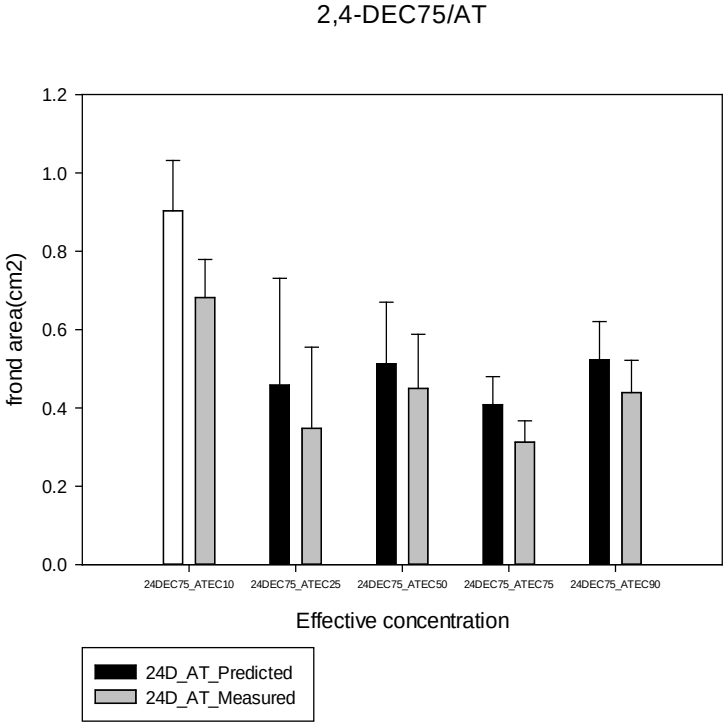


Figure 4-17: Predicted (■) and measured (■) mean frond area (cm^2) ± standard deviation of *L. minor* (*n=3*). The plants were pre-treated with 2,4-D for 10.5 days with the effect concentration of 75 (EC75). Subsequently, the plants were transferred to various concentrations of atrazine and were exposed for 3.5 days. Asterisk (*) indicates that there was significant difference between the predicted and the measured areas (*p<0.05*).

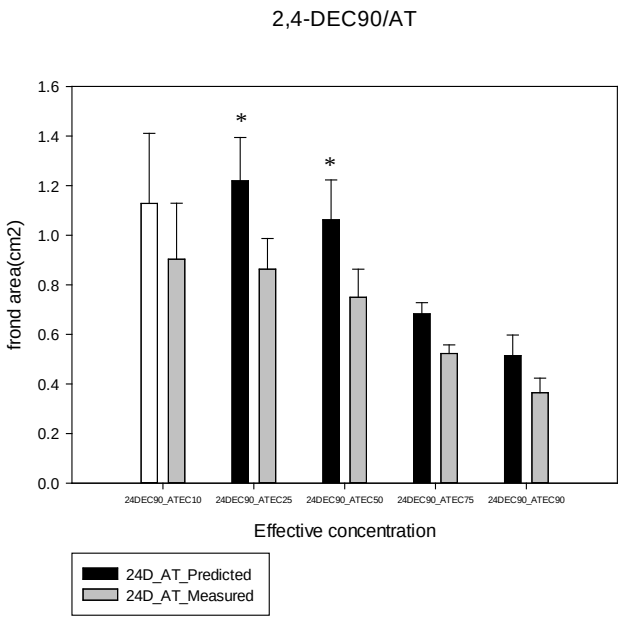


Figure 4-18: Predicted (■) and measured (■) mean frond area (cm^2) ± standard deviation of *L. minor* (*n=3*). The plants were pre-treated with 2,4-D for 10.5 days with the effect concentration of 90 (EC90). Subsequently, the plants were transferred to various concentrations of atrazine and were exposed for 3.5 days. Asterisk (*) indicates that there was significant difference between the predicted and the measured areas ($p<0.05$).

3.Alachlor/paraquat

In the experiment with alachlor as pre-treatment followed by paraquat, the model predictions were higher than measured ($p<0.05$) or overestimated effects at the higher effective concentrations (Fig. 4-22 and 4-23), but for the lower effective concentrations of pre-treatment the model predictions were not significantly different from measurements ($p>0.05$) (Fig. 4-19, 4-20 and 4-21). It can be concluded that the models of the effect of these two herbicides as sequential mixture can be used to make predictions.

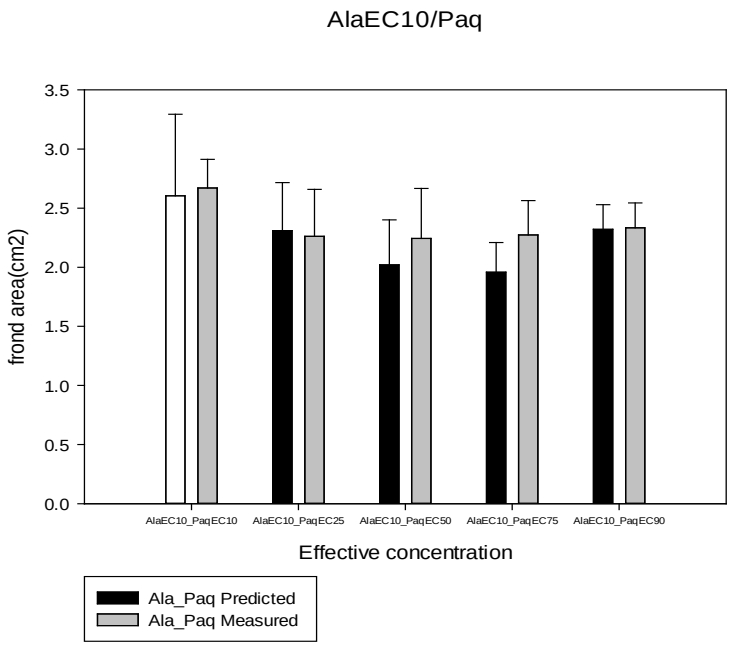


Figure 4-19: **Predicted (■) and measured (■) mean frond area (cm^2) ± standard deviation of *L. minor* (n=3). The plants were pre-treated with alachlor for 10.5 days with the effective concentration of 10. Subsequently, the plants were transferred to various concentrations of paraquat and were exposed for 3.5 days. Asterisk (*) indicates that there was significant difference between the predicted and the measured areas (*p<0.05*).**

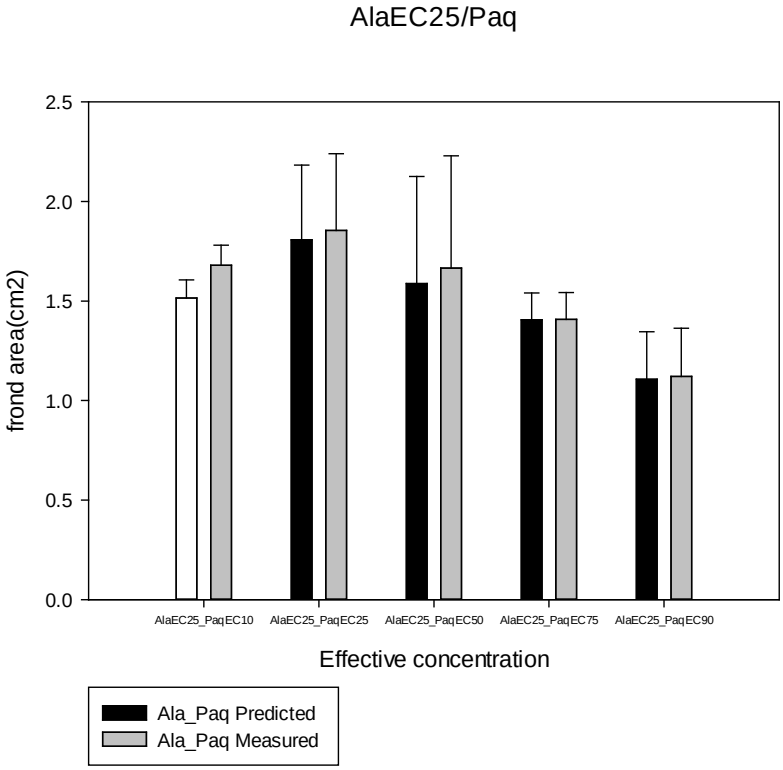


Figure 4-20: Predicted (■) and measured (■) mean frond area (cm^2) ± standard deviation of *L. minor* (n=3). The plants were pre-treated with alachlor for 10.5 days with the effective concentration of 25 (EC25). Subsequently, the plants were transferred to various concentrations of paraquat and were exposed for 3.5 days. Asterisk (*) indicates that there was significant difference between the predicted and the measured areas (*p<0.05*).

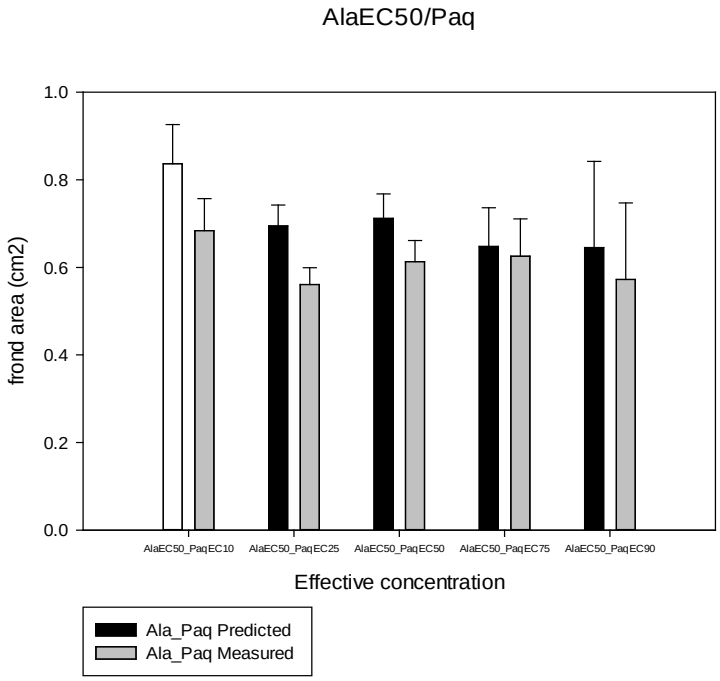


Figure 4-21: Predicted (■) and measured (■) mean frond area (cm^2) ± standard deviation of *L. minor* (n=3). The plants were pre-treated with alachlor for 10.5 days with the effective concentration of 50 (EC50). Subsequently, the plants were transferred to various concentrations of paraquat and were exposed for 3.5 days. Asterisk (*) indicates that there was significant difference between the predicted and the measured areas (*p<0.05*).

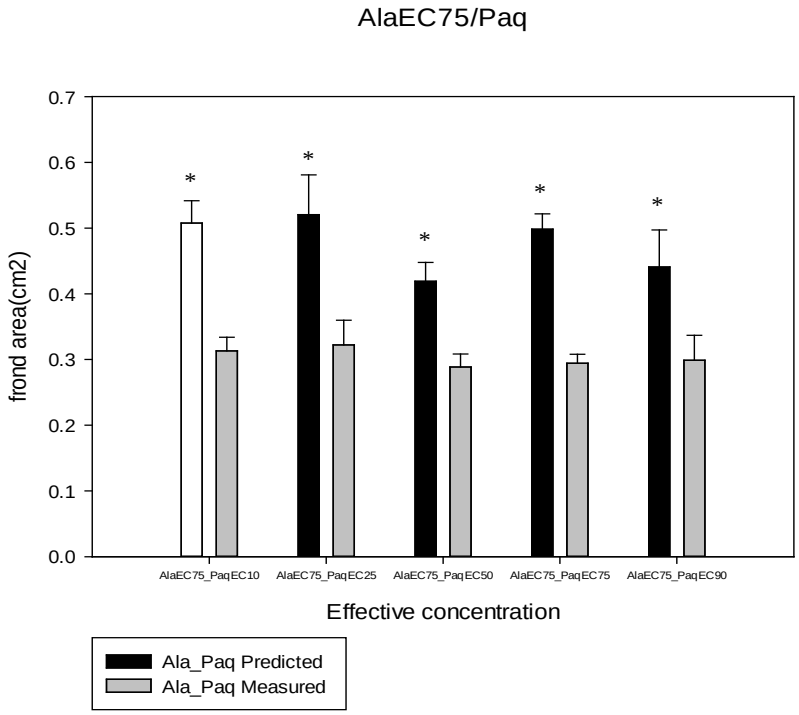


Figure 4-22: Predicted (■) and measured (■) mean frond area (cm^2) ± standard deviation of *L. minor* (n=3). The plants were pre-treated with alachlor for 10.5 days with the effective concentration of 75 (EC75). Subsequently, the plants were transferred to various concentrations of paraquat and were exposed for 3.5 days. Asterisk (*) indicates that there was significant difference between the predicted and the measured areas (*p<0.05*).

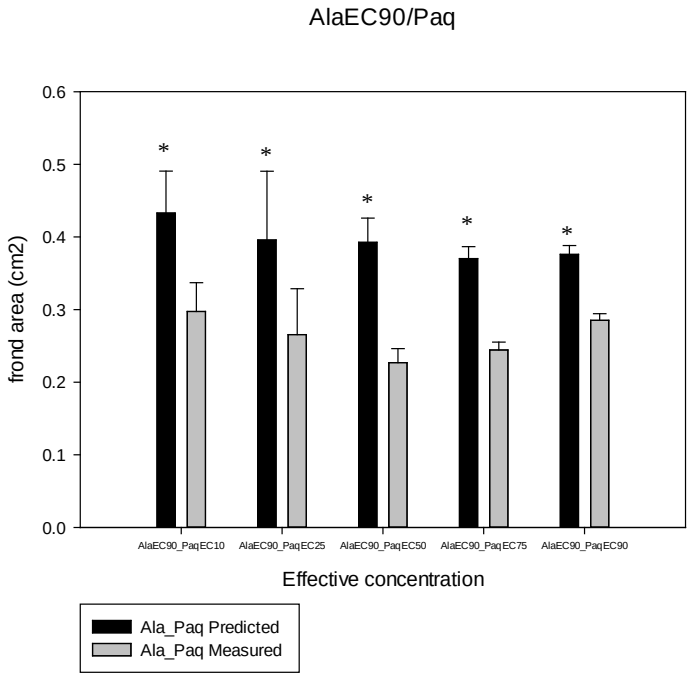


Figure 4-23: Predicted (■) and measured (■) mean frond area (cm^2) ± standard deviation of *L. minor* (n=3). The plants were pre-treated with alachlor for 10.5 days with the effective concentration of 90 (EC90). Subsequently, the plants were transferred to various concentrations of paraquat and were exposed for 3.5 days. Asterisk (*) indicates that there was significant difference between the predicted and the measured areas ($p<0.05$).

4. Paraquat/alachlor

The experiment that started with exposures to paraquat followed by alachlor showed that there were significant differences at concentrations of paraquat at EC75 and EC90 ($p<0.05$) (Figs. 4-27, 4-28). The predicted models overestimated effects at the high effective concentrations for the EC75 level. However, at the lower effective concentrations, the effect of sequential application of these herbicides was predictable (Fig 4-24, 4-25 and 4-26).

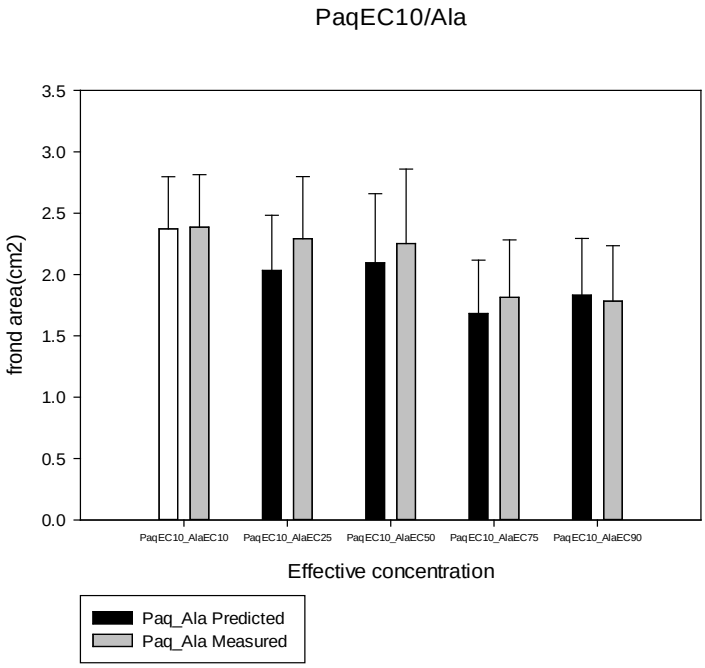


Figure 4-24: Predicted (■) and measured (■) mean frond area (cm^2) ± standard deviation of *L. minor* (n=3). The plants were pre-treated with paraquat for 10.5 days with the effect concentrations of 10 (EC10). Subsequently, the plants were transferred to various concentrations of alachlor and were exposed for 3.5 days. Asterisk (*) indicates that there was significant difference between the predicted and the measured areas (*p<0.05*).

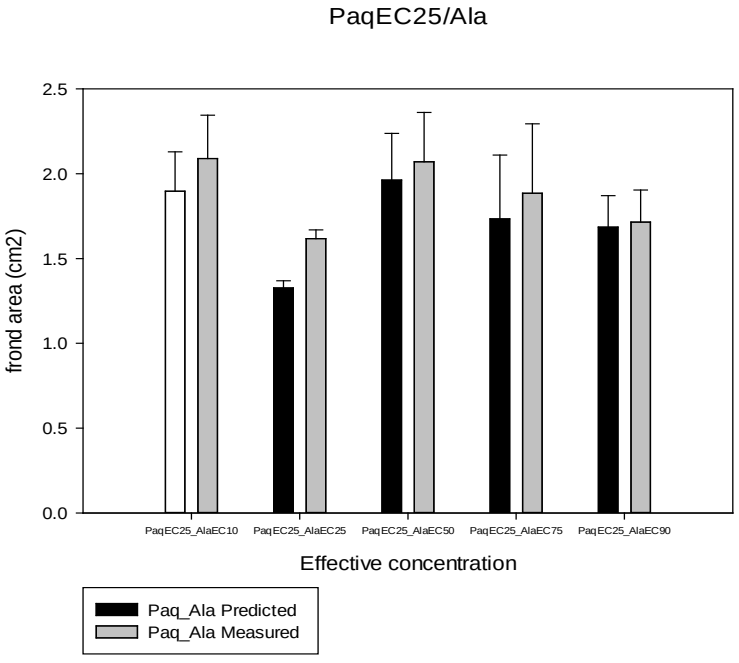


Figure 4-25: Predicted (■) and measured (■) mean frond area (cm^2) ± standard deviation of *L. minor* (n=3). The plants were pre-treated with paraquat for 10.5 days with the effect concentrations of 25 (EC25). Subsequently, the plants were transferred to various concentrations of alachlor and were exposed for 3.5 days. Asterisk (*) indicates that there was significant difference between the predicted and the measured areas (*p<0.05*).

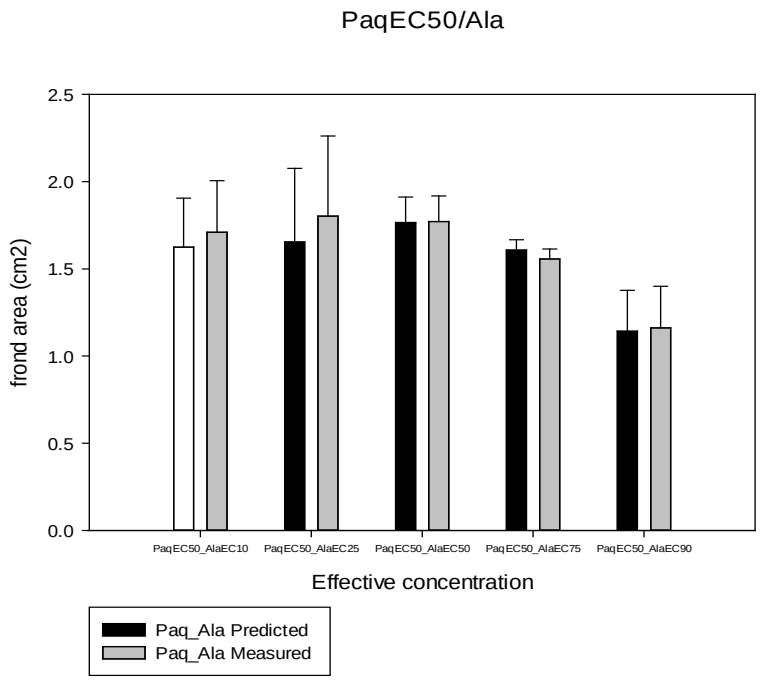


Figure 4-26: Predicted ( ) and measured ( ) mean frond area (cm^2) ± standard deviation of *L. minor* (n=3). The plants were pre-treated with paraquat for 10.5 days with the effect concentrations of 50 (EC50). Subsequently, the plants were transferred to various concentrations of alachlor and were exposed for 3.5 days. Asterisk (*) indicates that there was significant difference between the predicted and the measured areas ($p<0.05$).

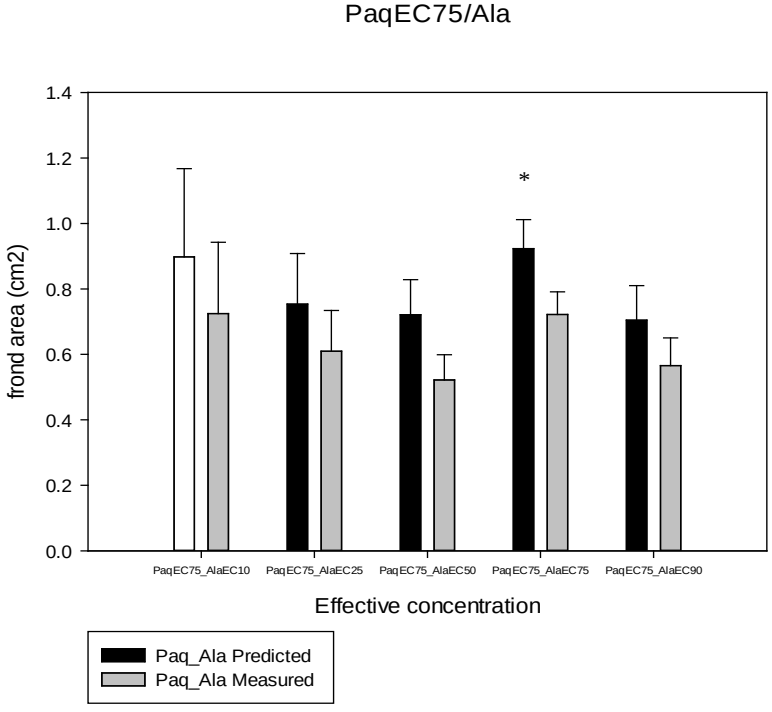


Figure 4-27: Predicted ( ) and measured ( ) mean frond area (cm^2) ± standard deviation of *L. minor* (n=3). The plants were pre-treated with paraquat for 10.5 days with the effect concentrations of 75 (EC75). Subsequently, the plants were transferred to various concentrations of alachlor and were exposed for 3.5 days. Asterisk (*) indicates that there was significant difference between the predicted and the measured areas ($p<0.05$).

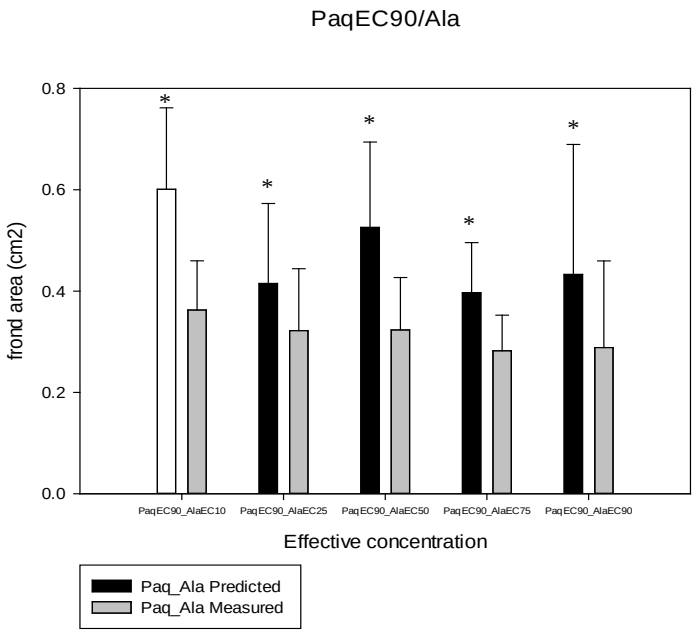


Figure 4-28: Predicted (■) and measured (■) mean frond area (cm^2) ± standard deviation of *L. minor* (n=3). The plants were pre-treated with paraquat for 10.5 days with the effect concentrations of 90 (EC90). Subsequently, the plants were transferred to various concentrations of alachlor and were exposed for 3.5 days. Asterisk (*) indicates that there was significant difference between the predicted and the measured areas ($p<0.05$).

Dose response model for measured and predicted data

The dose response relationships were used to explore how far off the predicted effects were from the measured data. The results showed that in short-term sequential exposures, at low concentrations of the first chemical, the lines of measured and predicted effects are close with the narrow 95% Cis(Figure 4-29 and 4-30). However, the similarities are less clear in the long-term sequential exposure, (Figure 4-31-4-34). It could be said that in high concentration, the dose response model cannot use to predict the effects.

Sequential exposureI

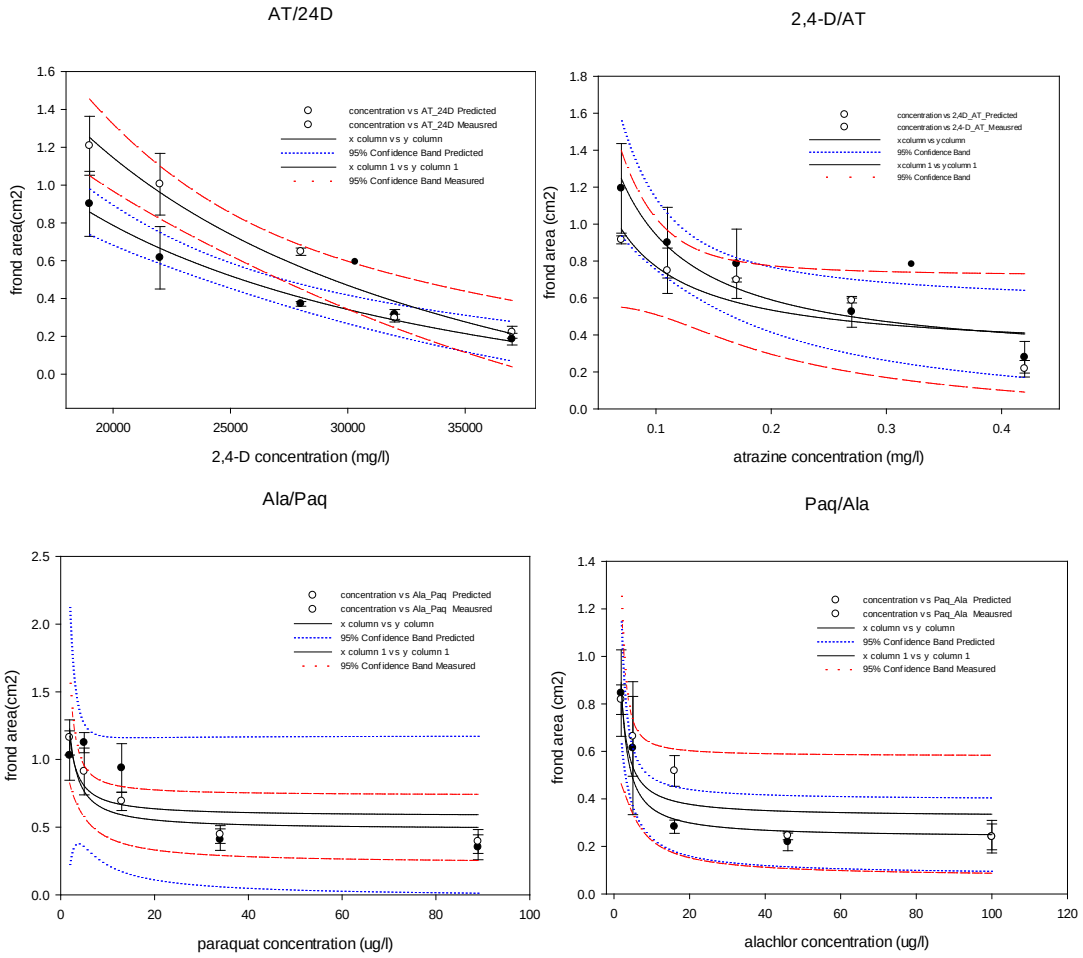


Figure 4-29: Dose response model for measured and predicted data of sequential exposureI

Sequential ExposureII

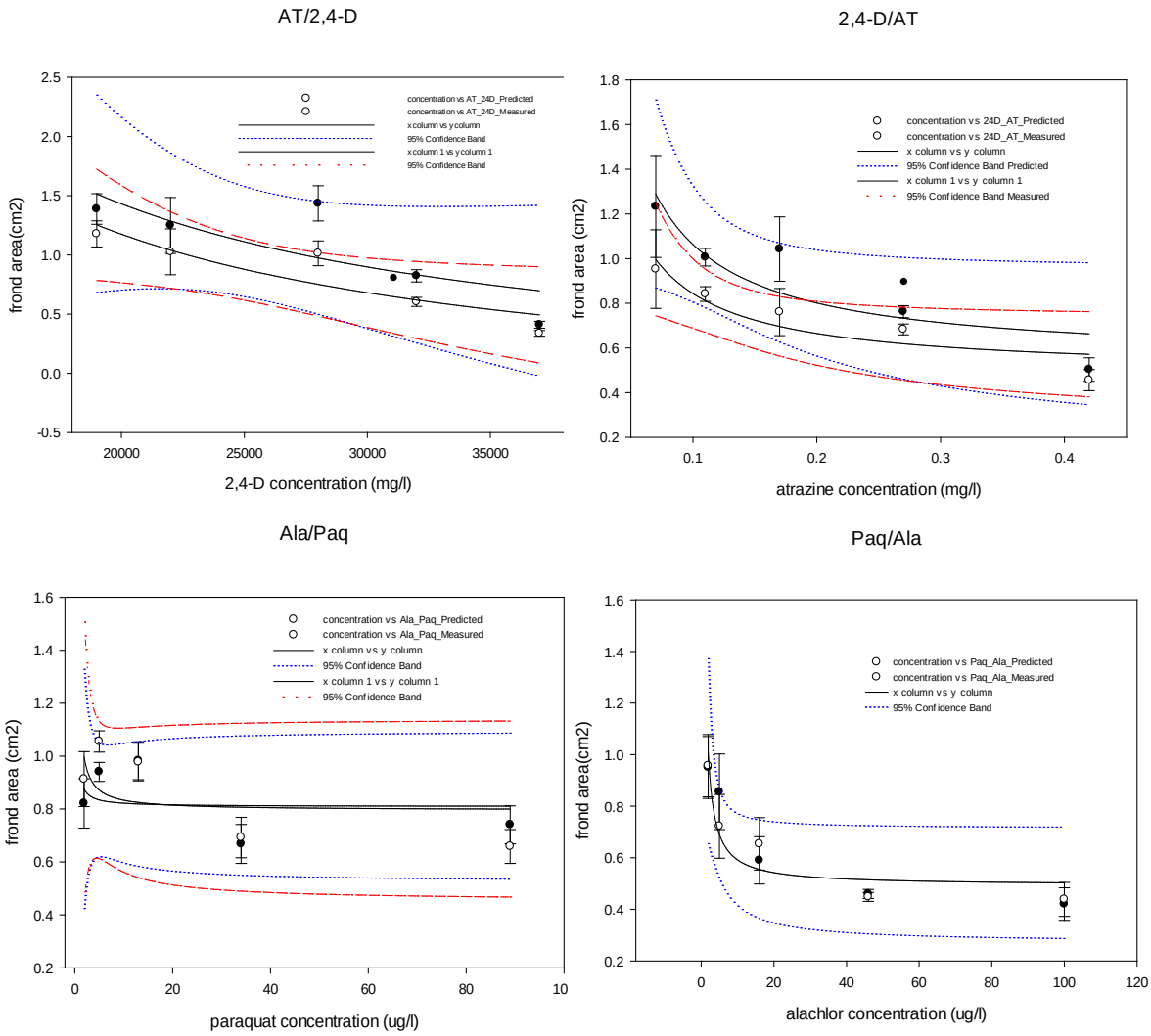


Figure 4-30: Dose response model for measured and predicted data of sequential exposureII

Long-term sequential exposure

Atrazine/2,4-D

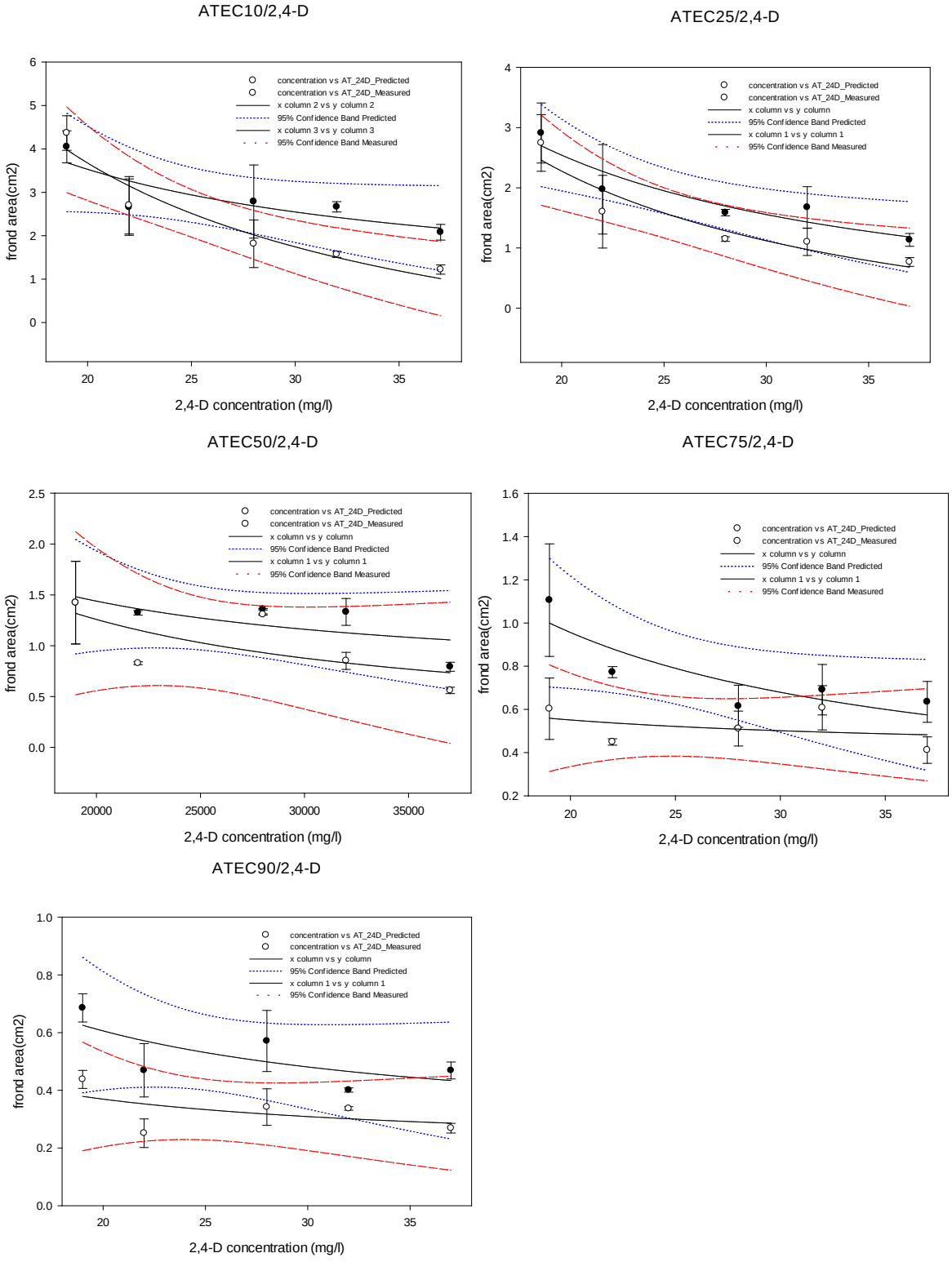


Figure 4-31: Dose response model for measured and predicted data of long-term sequential exposure

2,4-D/atrazine

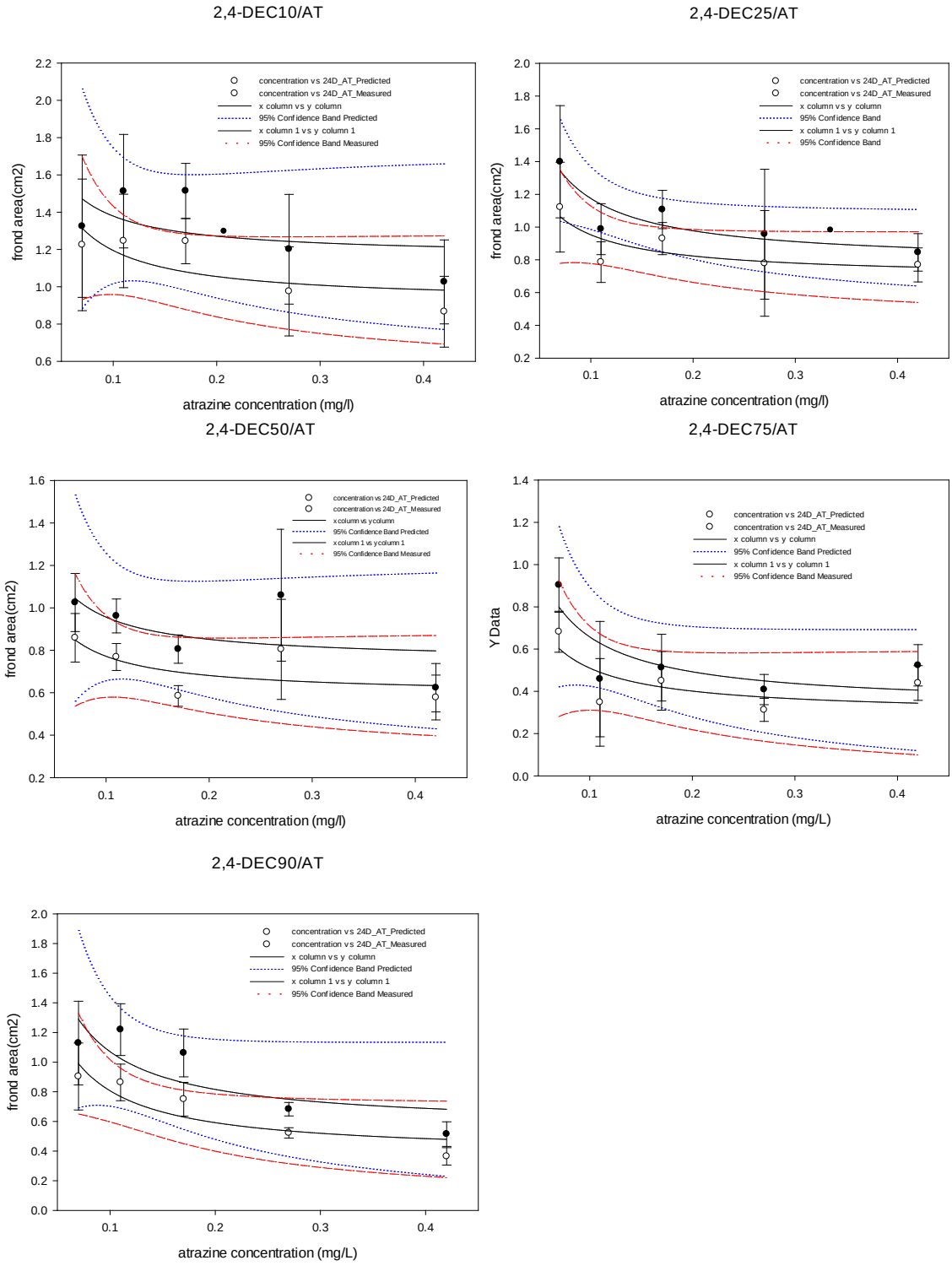


Figure 4-32: Dose response model for measured and predicted data of long-term sequential exposure

Alachlor/Paraquat

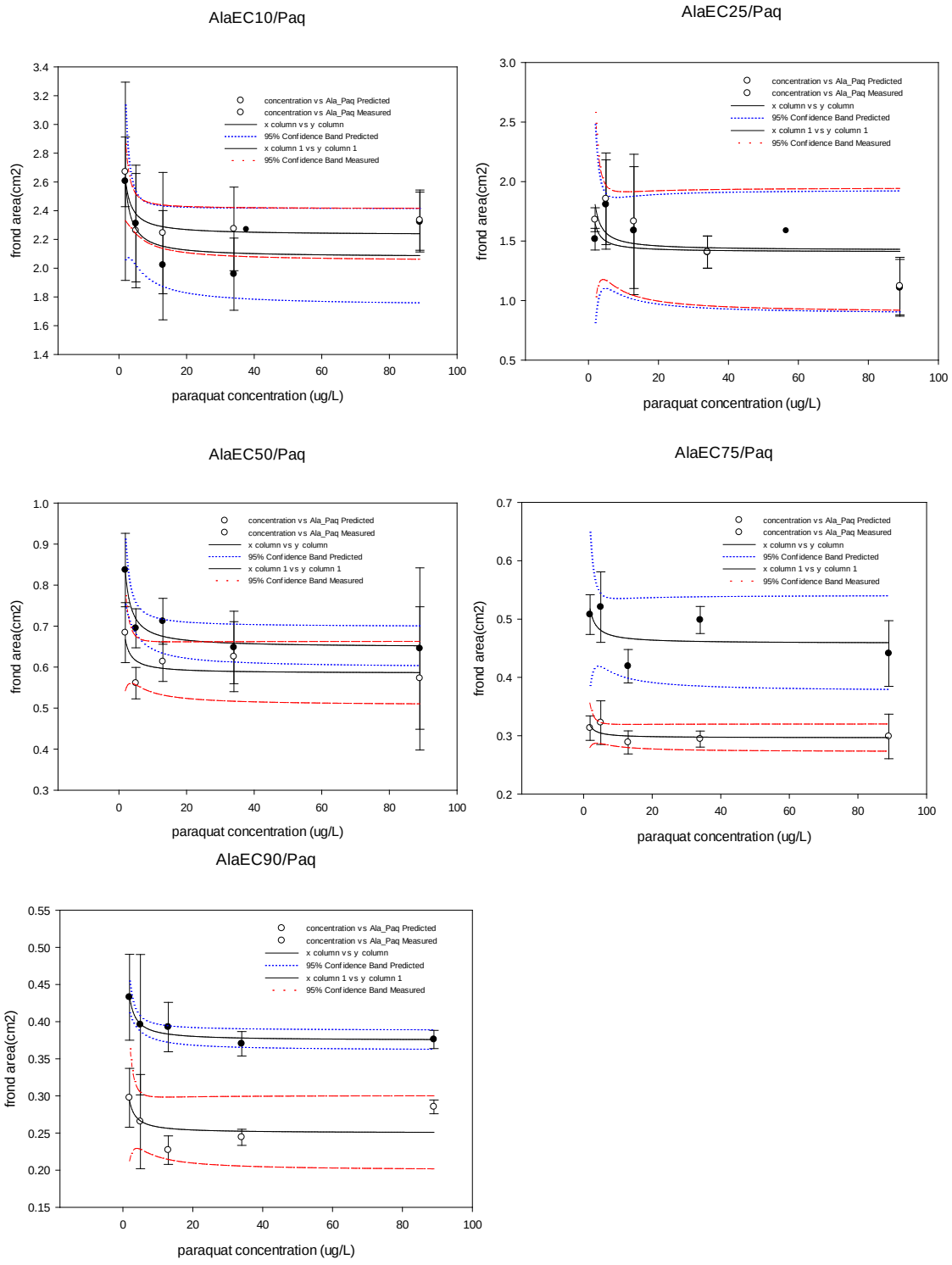


Figure 4-33: Dose response model for measured and predicted data of long-term sequential exposure

Paraquat/alachlor

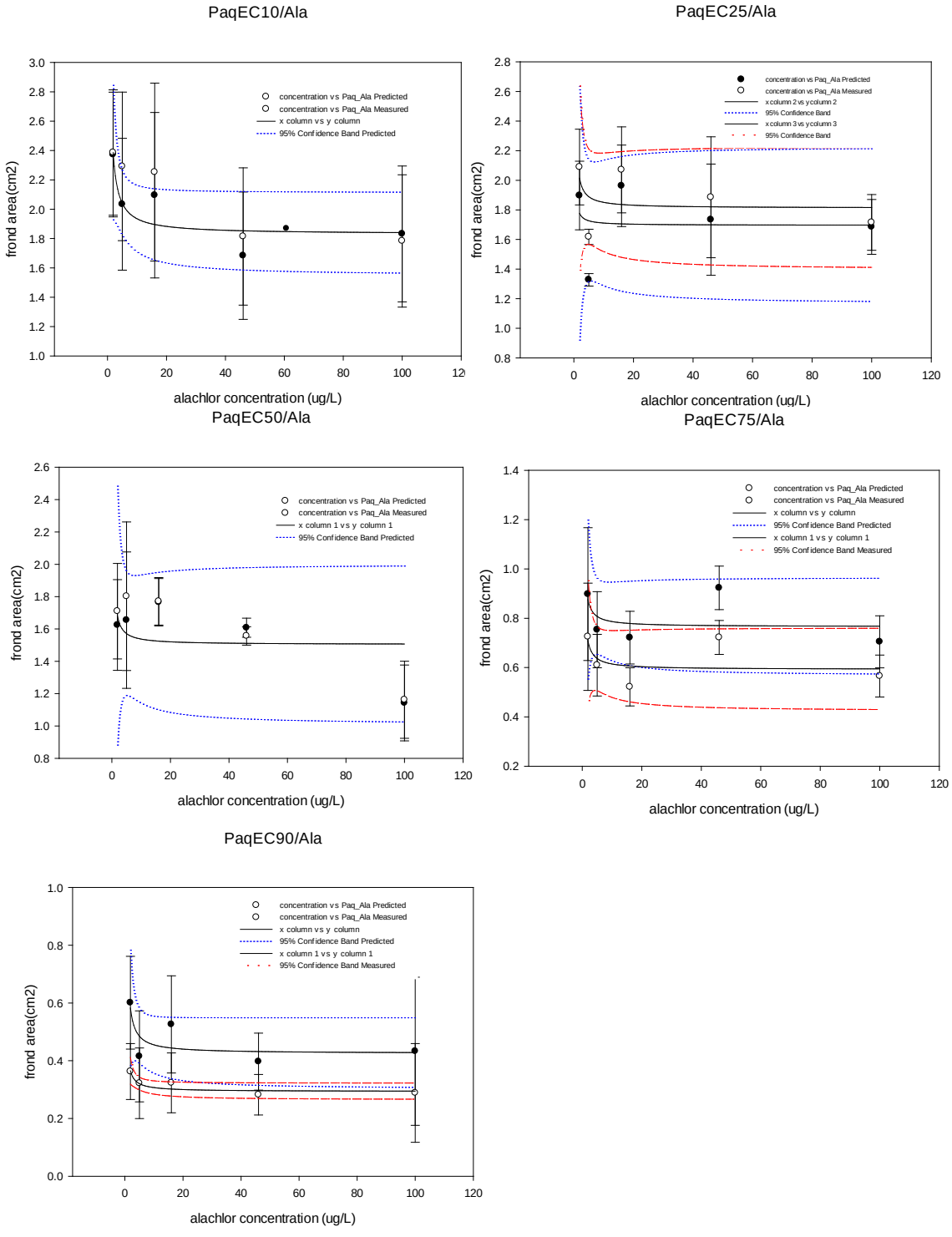


Figure 4-34: Dose response model for measured and predicted data of long-term sequential exposure

Table 4-4: The results of predicted models and actual observations in short-term and long-term sequential exposure

Short-term sequential exposure

Sequential exposure I		Sequential exposure II	
Treatment	Effects	Treatment	Effects
Atrazine/2,4-D	predictable	Atrazine/2,4-D	Predictable
2,4-D/Atrazine	Unpredictable	2,4-D/Atrazine	Predictable
Alachlor/paraquat	Predictable	Alachlor/paraquat	Predictable
Paraquat/Alachlor	Predictable	Paraquat/Alachlor	Predictable

Long-term sequential exposure

Treatment	**Effects**	**Treatment**	**Effects**
ATEC10/2,4-D	predictable	2,4-DEC10/AT	Predictable
ATEC25/2,4-D	Predictable	2,4-DEC25/AT	Predictable
ATEC50/2,4-D	Unpredictable	2,4-DEC50/AT	Predictable
ATEC75/2,4-D	Unpredictable	2,4-DEC75/AT	Predictable
ATEC90/2,4-D	Unpredictable	2,4-DEC90/AT	Unpredictable
Treatment	**Effects**	**Treatment**	**Effects**
AlaEC10/Paq	Predictable	PaqEC10/Ala	Predictable
AlaEC25/Paq	Predictable	PaqEC25/Ala	Predictable
AlaEC50/Paq	Predictable	PaqEC50/Ala	Predictable
AlaEC75/Paq	Unpredictable	PaqEC75/Ala	Unpredictable
AlaEC90/Paq	Unpredictable	PaqEC90/Ala	Unpredictable

The concentrations of herbicide rely on the single toxicity test that can be used to predict the toxicity of sequential exposure in long-term experiment.

Discussion

This study explored the effects of two sets of two different herbicides when applied to aquatic macrophytes at fluctuating concentrations and in sequential pulses over short-term and long-term exposures. Experimental observations were compared against a simple growth-effect model.

For the short-term exposure, apart from the experiment of pre-exposure to 2,4-D at low concentrations followed by atrazine, the model was able to predict the toxicity in all of the orders of exposure and levels of concentration. However, in the long-term exposure, the model was unable to predict the toxicity at high concentrations of pre-exposure to herbicides.

There are several reports exploring how herbicide concentrations influence the toxic effect on the frond area of *L. minor* (Drost et al., 2003, Mohammad et al., 2008, Mohammad et al., 2010, Brain et al., 2012a). A number of authors (e.g. Ashauer *et al*.; 2006, 2007a; Raymond, 2008;Dennis *et al*., 2012) described that exposure to high concentrations may affect the physiochemical setups of organisms such as the functioning of voltage-gated sodium-channels and may stress organisms leading to lethargic responses during exposure. In addition, it was argued that the production of detoxifying enzyme might be connected to the exposure duration and concentrations. High concentrations might decrease the enzyme used in the detoxification process (Drost et al., 2003, Chesworth et al., 2004, Ashauer et al., 2006, Ashauer et al., 2007b, Boxall et al., 2013). Cedergreen *et al.* (2005) stated that herbicide pulse can reach concentrations that would affect aquatic plants if applied over a long period of time. It can be said that the *L. minor* that were pre-exposed to a high concentration of herbicide followed by either a low or high concentration of a second herbicide

showed more damaging effect compared to those exposed to a low concentration of pre-exposure herbicide.

Among the reports on exposure to pesticide mixtures, it was argued that the concentration dependent thresholds may be responsible for the variation in toxicity and order of exposure. The available reports articulated that the effects of pulse exposure on aquatic organisms might rest on the physical and chemical properties of the toxicants (Vallotton, 2008, Vallotton et al., 2008a, Cedergreen et al., 2008, Brain et al., 2012b, Boxall et al., 2013).Reymond (2008) found that the toxicity of three insecticides varied widely as a result of differences in the molecular structure of their compounds that helps to explain the toxicity via route of uptake, metabolic pathways and target sites. A high concentration of pretreatment resulted in significantly reduced frond area ($p<0.05$) from the predicted model; therefore, the model is unable to predict the effects. It can be said that the recovery potential from the first substance may be influenced by the second substance by decreasing the recovery rate and increasing the sensitivity of plants, which is in agreement with the findings of Drost (2012). In addition, there is evidence to show that the sensitivity of species is due in part to difference in toxicokinetics which consists of several processes, including absorption, distribution, metabolism and excretion (Escher *et al.*, 2011). As described by Drost (2012), plants pretreated with alachlor showed a slight increase of sensitivity toward a second substance, especially in the high effect concentrations. Since the plants were exposed to the herbicide for a long time, they potentially absorbed more toxic than in the short-term exposure. In addition, the effects of pulse exposure depend largely on many reasons such as compound specific uptake, degradation, dissipation rates and recovery potential of plants, carry-over

effects and depuration rate (Cedergreen et al., 2005, Ashauer et al., 2010, Boxall et al., 2013).

Ashauer *et al.* (2010) point out that the carry-over toxicity occurs when organisms exposed to an environmental toxicant survive but carry some damage resulting in reduced fitness of organism. Because of the impact from first exposure, stronger effects are possible if the organisms have not yet recovered. In addition, carry-over may cause increased toxic effects after the second pulse compared to organisms which were not prestressed due to incomplete organism recovery. They stated that the incomplete recovery may be caused either by incomplete elimination or by mechanisms of toxicity with slow or incomplete reversibility.

According to Ashauer *et al.*(2007), the order in which the toxicants are applied has a bearing on the toxicity of organisms, especially if there is additional stress on the species such as pH change during the experiment. In addition, the effect of carry-over toxicity might result in reduced fitness. Ashauer *et al*. (2010) explored the carry-over toxicity of *Gammarus pulex* to repeated pulses of diazinon at varying intervals and the results indicate that the organisms need more time to recovery from long-term damage due to possible carry-over toxicity.

The accumulative effect from the herbicide pretreatment leads to injury or damage on organisms that cannot fully recover. The toxicant will have an effect if the internal concentration of the toxicant in the organism exceed sits specific threshold. Therefore, the different modes of action and sequential exposure being investigated may lead to different toxicity duration.

Furthermore, the damage on plants does not only depend on the level of concentration, but also on the duration of herbicide exposure. Cedergreen *et al*.

(2005) explained that short-term exposure has minor effect and allows for rapid recovery compared to long-term exposure to low concentration of herbicides.

In terms of the mode of action, after the pre-exposure the *L. minor* were transferred to an atrazine test solution for 3.5 days. The fronds still showed chlorosis. Further, the fronds were affected with necrosis and chlorosis symptoms at the lowest effective concentration of 25. When atrazine was pulsed first on *L. minor* for 3.5 days up to a concentration of 0.17 mg/l (EC50), there were no chlorosis or necrosis symptoms but the colonies broke up during the test period. With the second exposure to 2,4-D, the fronds showed chlorosis and completely died at the concentration of 32 mg/l (EC75). Corbett (1984) stated that the symptoms of plants when treated with phenoxyacteic herbicide include leaf chlorosis, altered stomatal function, and abnormal stem tissue and apical growth.When applied at higher concentrations these herbicides affect cell walls and nucleic acid metabolism and inhibit cell division and growth, leading to the plant's death. This is evident in this study with the fronds of *L. minor* being damaged from 2,4-D by a bleaching effect at the effective concentration of 25. Under such herbicide, the plant is unable to photosynthesize or grow well (Zimdahl, 1999). Cedergreen (2005) found that the effect of the s-triazine group on the growth of *L. minor* is easily reversible due to the binding of s-triazine to PSII via non-covalent hydrogen bonds.

The test of alachlor and paraquat with fixed concentrations of pre-exposure followed by a second exposure at varying concentrations showed higher toxicity than predicted but there was no significant difference between the measurements and the predicted models ($p<0.05$). It can be said that the single toxicity data can be used to predict the toxicity of sequentially applied herbicides when the pre-treatment has low concentration.

Conclusion

Overall, this study shows that the model adopted from the OECD221 guideline can be used in combination with single toxicity data to predict the effect of short-term sequential pulse exposures to herbicides from different groups as well as the effect of low concentrations of pre-treated herbicide in long-term scenarios. The model can predict the effects of sequential pulse in short-term and long-term exposures if the pre-treatment involves low concentrations of herbicide, but cannot be used to predict the effects of high concentrations of pre-treatment in a long-term exposure.

The order of exposure matters in terms of the interactions that occur and these interactions may be affected by concentrations and time of exposure. Vollotton (2009) stated that greater effects during sequential exposures can be expected since the effects of the first pulse might influence the response to the second pulse. However, the interactions may be more complex when the modes of action of the pesticides in the mixture are different. Therefore, it is very important to take into account the impact of chemicals across different modes of action, species traits in the test system, and different environmental features as well as the effect of the exposure period on the test chemical. In addition, aquatic organisms when exposed to hazardous substance may recover depending on the quality and quantity of the damage and their detoxification capability(Drost et al., 2007) .

This study identifies the time factor and the effective concentrations that are harmful for the plant *L. minor*. As demonstrated in this study, the simple model can be used to predict the detrimental effects on plants of intermittent releases of toxicants or sequential pulse exposure to herbicides in the aquatic system and help form better practices of herbicide use for the ecosystem. Nevertheless, this model seems to fall

down in predicting the effects of pulse exposures to high concentrations of herbicides. In order to explain such scenarios where there may be carry-over toxicity, the next chapter's studies are done to understand the speed of recovery of *L. minor* following exposure to the study pesticides.

CHAPTER V

5. The recovery potential pattern after short and prolonged exposure of *Lemna minor* to herbicides

Introduction

The recovery of herbicide-injured plants depends on many factors such as the amount of herbicides that the plants have been exposed to, the type of herbicide used, their persistence in the environment, the growing condition after contact, and the sensitivity of the plants (Davies et al., 2003, Wilson and Koch, 2013). The mechanism of plant recovery from herbicides has been investigated by a number of researchers (Mohammad et al., 2010, Brain et al., 2012b). A good understanding of a plants' recovery mechanisms as long been recognised in Weed Science as important inmaking a selection of which herbicide to use (Pinto de Carvalho *et al.*, 2009).Each herbicide activates different metabolic pathways and interacts with different sites of action in the plant (Pinto de Carvalho *et al.*, 2009). Therefore, the detoxification or the recovery of plants from herbicides also depends on the herbicide's metabolism in plants, which can be caused by the natural metabolic process of plant detoxification(Drost et al., 2007).

A few publications have found evidence that the toxic effects of chemicals on aquatic organisms still remained even after the chemical was removed (Ashauer *et*

al., 2010). This phenomenon may cause a carry-over effect from the first exposure. Carry-over toxicity occurs when a chemical that is used to treat an organism is still effecting the organism after the chemical exposure has been removed (Ashauer *et al*, 2010). This incomplete recovery may be caused either by slow or incomplete elimination (toxicokinetics; TK) or by mechanisms of toxicity with slow or incomplete reversibility (toxicodynamics; TD) (Vale, 1998, Ashauer et al., 2013). Therefore, toxicokinetics and toxicodynamics play important roles in the recovery and can be used to explain the time-course of the processes of toxicity, including processes that cause carry-over toxicity or delayed effects (Ashauer *et al*., 2012). Toxicokinetics deals with the time-course of the toxicant's concentration at the site of the toxic action as well as processes such as absorption (i.e. how toxicants enter the organism); distribution (i.e.how toxicants travel within the organism); storage (i.e. how some tissues preferentially harbor a toxicant); biotransformation (i.e. how toxicants are altered or detoxified by chemical changes in the organism); and elimination (i.e. how toxicants are removed from the organism). On the other hand, toxicodynamics deals with the mechanisms by which toxicant's action at the target site affects individual organisms (Ashauer et al., 2011a).

Several studies have employed toxicokinetic and toxicodynamic models to quantify the time-course of the internal concentration that is defined by uptake, elimination and biotransformation, and the processes that lead to toxic effects (Nyman et al., 2012).

A few publications have focused on the phytotoxicity of herbicides, explaining that when a plant is exposed to herbicides, the physiological and metabolic distresses are revealed as irreversible injuries or chronic symptoms (Larcher, 2000). When a herbicide reaches the target site of action, the plant may express phytotoxic

symptoms that can be divided into structural damage such as chlorosis, necrosis, albinism, wilt, epinasty, leaf shriveling and rolling, or physiological damage such as cycle reduction and growth rate reduction. There are a few specific reports that have focused on these effects on plants and the phytostatic and phytocidal concentrations. Phytostatic concentration is defined as the concentration that allows no net growth of the population of the test organisms only during the exposure, while phytocidal concentration is defined as the lowest concentration tested which allows no net increase in population density during both the exposure and the recovery period, meaning that the organism does not recover when transferred to a fresh medium (Hughe *et al.*, 1933).

In this study, we focus on four herbicides namely, atrazine, 2,4-D, alachlor and paraquat that we used in earlier chapters.

In recent years, the risk assessment for aquatic macrophytes has received increasing scientific attention (Marvier, 2002). The *Lemna* species is commonly used in phytotoxicity tests as part of risk assessments (USEPA, 1996; OECD221, 2006). Many researchers have determined the toxicity levels of herbicides, such as EC50, in order to determine the potential impact. However, only a few studies have explored the recovery of plants after exposures to herbicides (Mohammad et al., 2006, Mohammad et al., 2010, Mohammad et al., 2008, Teodorovic et al., 2011, Brain et al., 2012b). For example, Mohammad *et al.* (2010) investigated the potential recovery of *L. gibba* after exposures to four herbicides, including atrazine, alachlor and paraquat, with different exposure periods of 7, 14, 21 and 28 days followed by a 7-day recovery. The results showed that paraquat is more toxic than alachlor, while atrazine produced no phytostatic effect400µg/L of alachlor caused phytostatic effect on day 14, and 200 ppb on day 21.1600 µg/L of triazine produced phytostatic effect

on day 14 and 800 µg/Lon day 28. This study suggests that the recovery depends on the concentration of herbicides. Similarly, Brain *et al*. (2011) evaluated the recovery of *L. gibba* after exposures to atrazine for the varying durations of 1, 3, 5, 7, 9 and 14 days with herbicide concentrations starting from 5 to 160 µg/L, followed by either a 7 or 14-day recovery in fresh medium. The results showed no phytocidal effect on chlorosis or necrosis and complete recovery was achieved by day 7. These results are in agreement with Teodorovic *et al*. (2011) who explored the recovery potential of *L. minor* after exposure to atrazine during 3- and 7-day tests. *L. minor* recovered after 6 days in the recovery phase.

The work in the previous chapter indicated carry-over toxicity of some of the study herbicides under certain conditions. Therefore, in the experiments reported in this chapter, work was done to understand the rates of recovery of *Lemna* following short and long-term exposures to the study herbicides.

Material and method

Plants and culturing

Lemna minor were cultured in Erlenmeyer flasks 250-ml in Swedish media (Organisation for Economic Co-operation and Development., 2006). The cultures were maintained under continuous light in the Sanyo Environmental test chamber (model MLR-351H) at 1,000 LUX and 20 ^{0}C. *L. minor* were kept in logarithmic growth phase by sub-culturing the stocks every 7 days. Prior to use, the pH of the growth media was adjusted to 6.5 with either 0.1M HCl or NaOH.

Chemicals

Herbicides were chosen to represent a range of compound widely used across a range of general classes and modes of action. Atrazine (98.5% purity), 2,4-D (99% purity), alachlor (98% purity), paraquat dichloride (99% purity) and analytical grade solvents (methanol and acetone) were obtained from Sigma Aldrich, Poole, Dorset, UK.

Experimental method

The recovery of *Lemna minor* from the four herbicides from different families and with different modes of action—namely, atrazine, 2,4-D, alachlor and paraquat—was observed by monitoring growth rates following exposure to different concentrations of the test compounds. Visible symptoms of herbicide damage were also considered during the experiments. The experiments were divided into two scenarios, short-term exposures and long-term exposures, in order to study the recovery rates of *L. minor* after exposures to the herbicides. The effects of each compound were assessed separately.

The tests were performed using six control treatments (three media-only controls and three solvent controls). Nine concentrations of study chemicals, each with three replicates, were selected to give 10, 20, 25, 30, 50, 60, 75, 80 and 90 percent of growth reduction of *L. minor* based on the single compound standard toxicity test reported in Chapter 3 (the concentrations are given in Table 5-1). The final acetone concentration in each test was kept to less than 0.05% v/v. Glass petri dishes of 60 mm diameter (Duran®) were used for atrazine and 2,4-D, but for alachlor and paraquat plastic petri dishes were employed to avoid adsorption of the herbicides onto glassware.

Table 5-1: The effective concentrations tested in the experiment of four herbicides (µg/L)

Effective concentration	Concentration (µg/L)			
	atrazine	2,4-D	alachlor	paraquat
EC10	70	19000	1.9	1.9
EC20	90	21000	4	4
EC25	110	22000	5	5
EC30	130	24000	9	8
EC50	170	28000	16	13
EC60	200	29000	23	19
EC75	270	31000	46	34
EC80	300	32000	62	44
EC90	420	37000	100	89

The *L. minor* tests were performed according to OECD 221: *Lemna* sp. Growth Inhibition test (Organisation for Economic Co-operation and Development., 2006). Eachcolony consisting of three fronds was transferred to a petri dish that contained 10 ml of the test solution and kept in the Sanyo Environmental test chamber for 14 days (3.5 days of exposure followed by a 10.5-day recovery phase in Swedish media). The test solutions were renewed every 3.5 days, as were the controls. For the recovery test of *L. minor* after the short-term exposure, the plants were exposed for 3.5 days. The fronds were then rinsed and transferred to clean media for 10.5 days. For the recovery test following the long-term exposure, *L. minor* were exposed to herbicide for 10.5 days then transferred to fresh media for 17.5 days. *L. minor* were kept under the same conditions as above. In the long-term exposure, weekly

subsampling of *L. minor* were essential due to the doubling time of the growth of untreated plants. Therefore, plants were subsampled at the end of each week (every 7-day) (Boxall *etal*., 2013). At the beginning and the end of the test period, water samples were taken for analysis. Measurements of pH (Thermo orion; Benchtop pH/ISE meter) were conducted at the start, then at day 3.5, day 10.5 and day 14. The experiment plan is illustrated in Table 5-2.

Table 5-2: Experiment plan and exposure durations of *L. minor* to four herbicides

Herbicide	Short term duration (14 days)		Long term duration (28 days)	
	Exposure period	**Recovery period**	**Exposure period**	**Recovery period**
atrazine	3.5 days	10.5 days	10.5 days	17.5 days
2,4-D	3.5 days	10.5 days	10.5 days	17.5 days
alachlor	3.5 days	10.5 days	10.5 days	17.5 days
paraquat	3.5 days	10.5 days	10.5 days	17.5 days

The total area of the fronds was determined daily with image analysis. Digital photographs were taken using Cannon ixus210.

During the test period, the frond's areas and symptoms of toxicity were recorded. Symptomatic fronds were identified based on a distinguishable pattern of chlorosis and necrosis (Wilson and Koch, 2013). The phytostatic and phytocidal concentrations of the test chemicals for *L. minor* were determined according to the definition described by Hughes *et al*. (1988).

Statistical analysis

Frond area as the function of time fitting the growth rates with to a linear regression with a log-transformed area of *L. minor*. Initially, exponential modeling based on relative growth rate was performed but it was found that variation increased, therefore, prior to all analysis the area of Lemna was log transformed (base e).

The overall average growth rate and the daily (time-point) growth rates of *L. minor* after their exposure to each effective concentration were used to determine the differences from the controls'. Overall average growth rate refers to the average of *L. minor*'s growth rates from day 3.5 to day 14 for the short-term exposures, and from day 10.5 to day 28 for the long-term, while the time-point growth rates are measured each day for comparison with the controls'.

All data analyses used SPSS (Flores *et al*., 2013). Mean and standard deviations (SD) were calculated for specific growth rate. One-way analysis of variance (ANOVA) with Tukey's as a post-hoc test was performed to compare the treatments and controls day by day after exposure. Normality was evaluated using Shapiro-Wilk test and the equal variance was evaluated using Levene's test(Teodorovic *et al.*, 2011). If false, non-parametric Kruskal-Wallis was used instead.

Calculations of the average specific growth rate

The response variable was calculated based on the basis of changes in the logarithms of the frond area overtime as expressed each day in the controls and the treatments. The ASGR was calculated using the following Equation 1:

$$ASGR = \frac{\ln(Nj) - \ln(Ni)}{tj - ti} \quad \text{Equation 1}$$

Where ASGR is the average specific growth rate, N_i is the frond area at day_i, N_j is the frond area at day_j and t is the time period from i to j. To determine the time to recovery following exposure to different concentrations of the study herbicides, ASGR values were expressed as natural logarithms (ln) and compared to ln ASGRs of the control treatments.

Chemical analysis

High performance liquid chromatography analysis

The concentrations of atrazine and 2,4-D were confirmed withhigh performance liquid chromatography (PerkinElmer Flexar HPLC) equipped with Supelco 516 C18-db 5µm x 15 cm x 4.6 mm. The mobile phase for atrazine was prepared with methanol: water (60: 40 v/v), flow rate 1 ml/min, and the temperature was adjusted to 40 ^{0}C. The detection wavelength was 220 nm. The injection volume was 15 µl (Fu, 2008). The calibrations were done using atrazine standard. Retention was 5.4 mins and r^2 = 0.999. For 2,4-D, methanol: water with 0.1% formic acid (70: 30 v/v) was prepared as the mobile phase. The temperature was set to 30 ^{0}C and the detection wavelength was 236 nm (Connick *et al.,* 1982). Retention time was 4.5 mins and r^2 is 0.999.

Enzyme linked immunosorbent assay (ELISA)

For alachlor, the water samples were removed from the refrigerator and allowed to attain room temperature. Afterward, 25 µl of standard, control and water sample were added into a96-well flat-bottomed polystyrene ELISA plate. 50 µl of enzyme conjugate and alachlor antibody solution followed into each well. The wells were

covered with parafilm to prevent contamination and evaporation. After incubation at room temperature for 60 minutes, the plate was washed three times with diluted wash buffer. Then, 150 µl of colour solution was added into each well and left to incubate for 20 minutes. Finally, 100 µl/well of stopping solution was added. The absorbance was read at 450 nm within 15 minutes after adding the stopping solution.

For the paraquat analysis, the ELISA test kit was purchased from US Biocontract, USA. 96-well microplate coated with anti-paraquat antibody was used. Firstly, 25 µl of standard and sample were put into each well, followed by 100 µl of Paraquat-Horseradish Peroxidase Conjugate (PRQ-HRP), before leaving it to incubate at room temperature for 30 minutes. After the incubation, the plate was washed three times with wash buffer, and then TMB substrate 100 µl was added and left at room temperature for 15 minutes. 100 µl of stopping solution was added to each well and the plate was read under absorbance at 450 nm.

Results

Chemical analyses

Table 5-3 shows the mean concentrations and standard deviations for the four herbicides in water samples, which are measured and calculated after the experiment. The results of the chemical analyses indicate that the four herbicides were stable during the period of the test (Table 5-3). Atrazine concentrations ranged from98-106% of the nominal concentration. 2,4-D concentrations were ranged95-101% of the nominal concentration and paraquat and alachlor were ranged from 80-110% and 60-176%, respectively. The pH values of the exposure media during the experiment increased slightly and were around 6.5 ± 1 unit (see in Appendix D1 and D2).

Table 5-3: The mean concentrations and standard deviations of the four herbicides in water samples

	Short term exposure		**Long term exposure**	
Exposure concentration (µg/l)	**Measured (µg/l)**	**%recovery**	**Measured (µg/l)**	**%recovery**
Atrazine				
70	70(±0)	106(3.5)	70(±0)	100(13.2)
90	95(±0)	106(2.2)	93(±0)	103(1.6)
110	109(±0)	99(1.9)	110(±0)	101(0)
130	132(±0)	102(4.2)	133(±0)	102(3.2)
170	171(±0)	101(3)	167(±0)	98(2)
200	203(±0)	102(2.1)	201(±0)	101(2.4)
270	276(±0)	102(1.3)	270(±0)	100(2.6)
300	303(±0)	101(1.3)	300(±0)	100(2.2)
420	423(±0)	101(1.3)	419(±0)	100(1)
2,4-D				
19000	18620 (±211)	98(±1.1)	18720(±476)	99(±2.5)
21000	20720(±811)	99(±4.7)	21240(±420)	101(±2)
22000	21620(±302)	98(±1.4)	20980(±270)	95(±1.5)
24000	23900(±695)	100(±2.8)	24060(±365)	100(±1.5)
28000	27820(±716)	99(±2.5)	27460(±210)	98(±0.75)
29000	27720(±476)	99(±1.6)	27740(±517)	96(±1.8)
31000	29940(±317)	97(±1)	30460(±173)	98(±0.55)
32000	30400(±750)	96(±1.2)	3120(±480)	98(±3.2)
37000	37060(±0.8)	100(±0.9)	37440(±159)	101(±0.4)

	Short term exposure		Long term exposure	
Exposure concentration (µg/l)	**Measured (µg/l)**	**%recovery**	**Measured (µg/l)**	**%recovery**
Alachlor				
1.9	1.27(±0.1)	60(±8.1)	1.38(±0.4)	66(±16)
4	7.1(±1.2)	176(±29)	6.3(±1.4)	159(±37.2)
5	7.3(±0.8)	146(±16)	7.9(±1)	158(±21.03)
9	9(±1)	101(±11.1)	9.5(±11)	105(±13.1)
16	12.3(±2.9)	77(±18.1)	16.6(±2.5)	104(±16)
23	21.9(±7.6)	95(±33)	21.2(±5)	93(±21.3)
46	32.9(±3.7)	72(±8.2)	35.4(±7.1)	77(±15.3)
62	82.4(±6.4)	133(±10.3)	79.5(±11)	128(±18)
100	115.6(±22.8)	116(±22)	120.5(±28)	121(±28)
Paraquat				
1.9	ND	ND	ND	ND
4	ND	ND	ND	ND
5	ND	ND	ND	ND
8	8.8(±1.3)	109(±16.6)	8.7(±0.3)	109(±0.4)
13	13.8(±1)	107(±8.3)	11(±3)	85(±23)
19	16.6(±2.2)	87(±11.4)	15.7(±1.7)	84(±7.5)
34	35(±3)	103(±8.7)	35.6(±3.6)	110(±10.7)
44	41.3(±7.4)	94(±17)	35.2(±6)	80(±14)
89	75.9(±9)	85(±10)	77.1(±9.1)	87(±10.2)

Symptoms of herbicide toxicity (visible observe)

Damage to *L. minor* was manifested by different symptoms depending on the type of the herbicide. With atrazine, after 10.5 days of exposure, the *Lemna* showed a reduced growth rate and the fronds were smaller at high effective concentrations than the control plants, but the colour of the fronds was still green (Fig.5-1b). With regards to 2,4-D,the plants that were exposed to a high concentration of the compound showed disintegrated colonies with necrosis also recorded at high concentrations (Fig.5-1c). For alachlor, *Lemna* remained a normal green colour but developed dwarfish daughter fronds and malformed colonies (Fig.5-1d).The morphological features of *Lemna* changed when exposed to high concentrations of paraquat with loss of pigment, chlorosis and necrosis, leading to pale green or white fronds (Fig.5-1e).

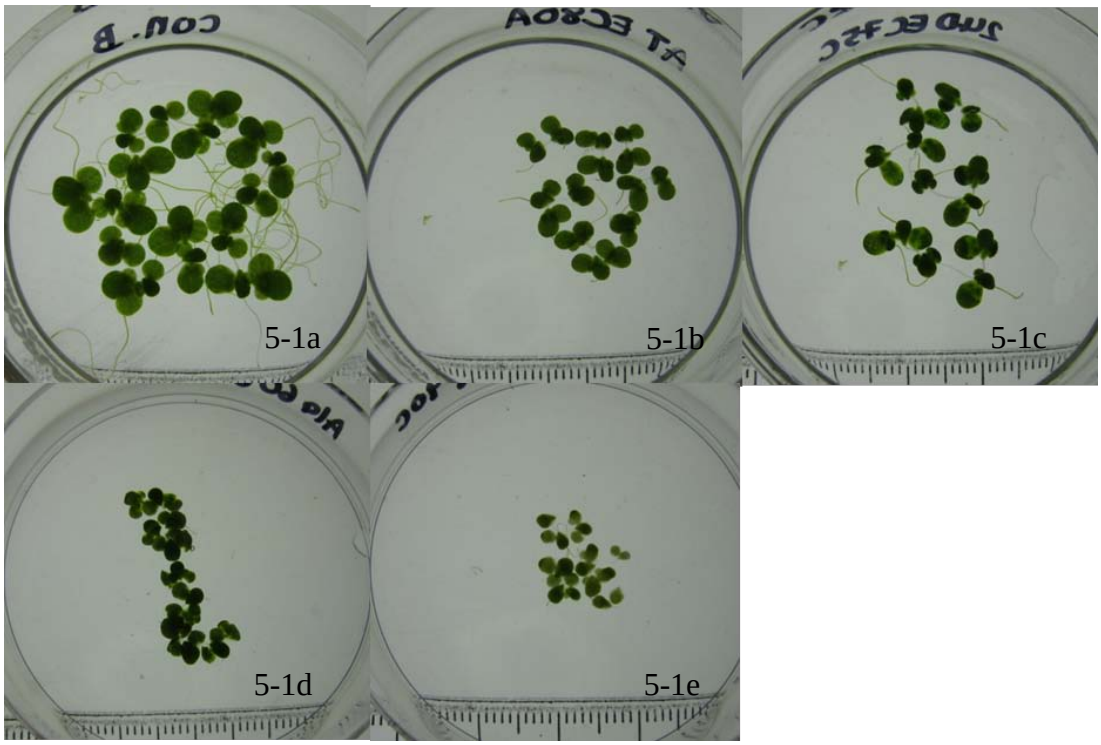


Figure 5-1: (a-e): The photographs of *L.minor* in different herbicides exposure were taken with a light box. (5-1a) - *L. minor* in fresh media. (5-1b) - *L. minor* exposed to atrazine. (5-1c) - *L. minor* exposed to 2,4-D. (5-1d) - *L. minor* exposed to alachlor. (5-1e) - *L. minor* exposed to paraquat.

Short-term and long-term recovery patterns

The results showed that the growth rate of *L. minor* was most affected by paraquat, then alachlor, atrazine and 2,4-D, respectively. The growth rate in long-term recovery of plants exposed to paraquat ranged between 0.21 to 0 cm^2day^{-1}, to alachlor from 0.20to 0.04cm^2day^{-1}, to2,4-D from 0.14 to 0.11cm^2day^{-1}, and to atrazine from 0.23 to 0.16cm^2day^{-1}. The data showing the growth rates of short-term and long-term exposure recovery can be found in Table 5-4.The results from the short-term and long-term recovery following damage from four herbicides are presented in terms of linear regression (r^2) and rate of recovery expressed in term of slope of the linear regression line were shown in appendix E.

Table 5-4: Mean and standard deviations of growth rate at the end of test period

	Effective concentration	**Short exposure** (cm^2day^{-1})	**Long exposure** (cm^2day^{-1})
	control	0.29 (±0.13)	0.28(±0.11)
atrazine	EC10	0.26 (±0.13)	0.23(±0.08)
	EC20	0.26 (±0.11)	0.22(±0.07)
	EC25	0.26 (±0.11)	0.22(±0.07)
	EC30	0.26(±0.09)	0.22(±0.07)
	EC50	0.27(±0.08)	0.21(±0.08)
	EC60	0.26(±0.08)	0.20(±0.08)
	EC75	0.25(±0.1)	0.20(±0.08)
	EC80	0.25(±0.07)	0.19(±0.09)
	EC90	0.22(±0.11)	0.16(±0.14)
2,4-D	EC10	0.24(±0.23)	0.14(±0.09)
	EC20	0.22(±0.12)	0.15(±0.12)
	EC25	0.22(±0.22)	0.14(±0.07)
	EC30	0.19(±0.18)	0.11(±0.07)
	EC50	0.20(±0.14)	0.12(±0.09)
	EC60	0.21(±0.11)	0.11(±0.08)

	Effective concentration	**Short exposure** (cm^2day^{-1})	**Long exposure** (cm^2day^{-1})
2.4-D	EC75	0.20(±0.17)	0.11(±0.08)
	EC80	0.20(±0.13)	0.11(±0.08)
	EC90	0.19(±0.1)	0.15(±0.19)
	control	0.28(±0.12)	0.28(±0.11)
alachlor	EC10	0.25(±0.13)	0.20(±0.16)
	EC20	0.24(±0.1)	0.20(±0.05)
	EC25	0.25(±0.08)	0.20(±0.07)
	EC30	0.25(±0.12)	0.20(±0.08)
	EC50	0.22(±0.09)	0.16(±0.17)
	EC60	0.24(±0.09)	0.12(±0.08)
	EC75	0.18(±0.14)	0.08(±0.09)
	EC80	0.21(±0.11)	0.05(±0.1)
	EC90	0.20(±0.09)	0.04(±0.23)
paraquat	EC10	0.25(±0.12)	0.21(±0.1)
	EC20	0.19(±0.37)	0.21(±0.08)
	EC25	0.19(±0.11)	0.21(±0.08)
	EC30	0.24(±0.24)	0.20(±0.10)
	EC50	0.24(±0.10)	0.19(±0.07)
	EC60	0.24(±0.09)	0.18(±0.07)
	EC75	0.20(±0.27)	0.04(±0.52)
	EC80	0.14(±0.16)	0(±0.17)
	EC90	0.19(±0.43)	0(±0.13)

Atrazine recovery pattern

After *L. minor* were exposed to atrazine in the short-term test for 3.5 days, their average growth rates from day 3.5 to day 14 for all concentrations of the herbicide showed no significant differences when compared with the growth rate of the control treatment ($p>0.05$) (Figure 5-2). The recovery of the exposed plants could be seen to match the control growth rate within 6 days($p<0.05$), while plants exposed to low concentrations of atrazine showed no significant differences from the controls. For the long-term exposures, the growth rates of *L. minor* slightly decreased during the exposure period of 10.5 days. After the transfer of *L. minor* to fresh media, the growth rates rapidly increased, matching the rate of the controls after 8 days for the long-term exposures (Figure 5-3). In addition, there are significant differences among the effective concentrations during the test period ($p<0.05$) in the high effective concentration of 80 and 90. The results from the short-term and long-term exposures of *L. minor* to atrazine indicate that the long-term exposures had more impact than the short-term exposures.

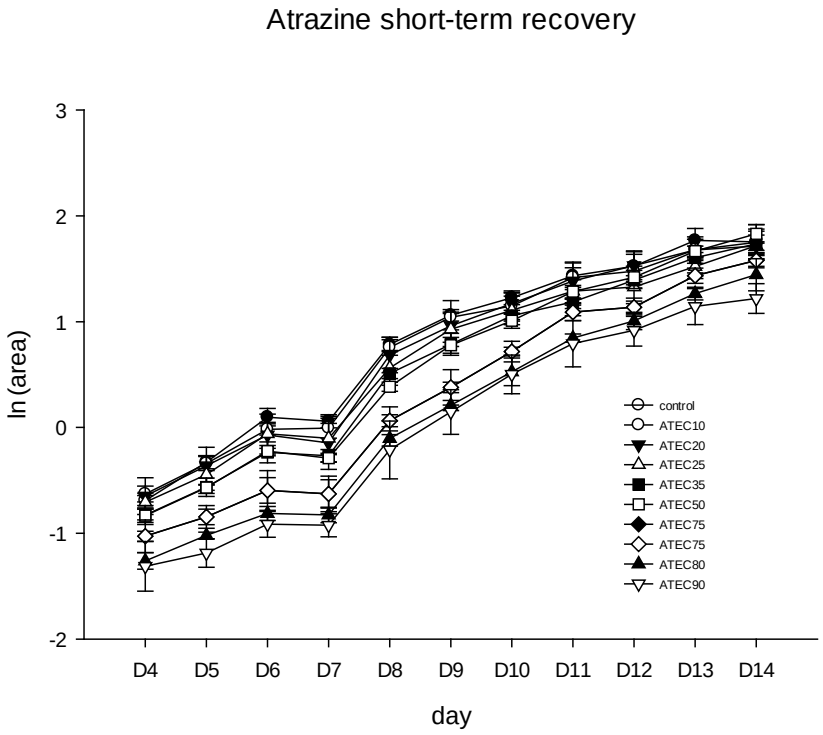


Figure 5-2: The day-to-day growth rates (day^{-1}) based on the frond area of *L. minor* after being exposed to atrazine for 3.5 days followed by a recovery phase from day 3.5 to day 14.

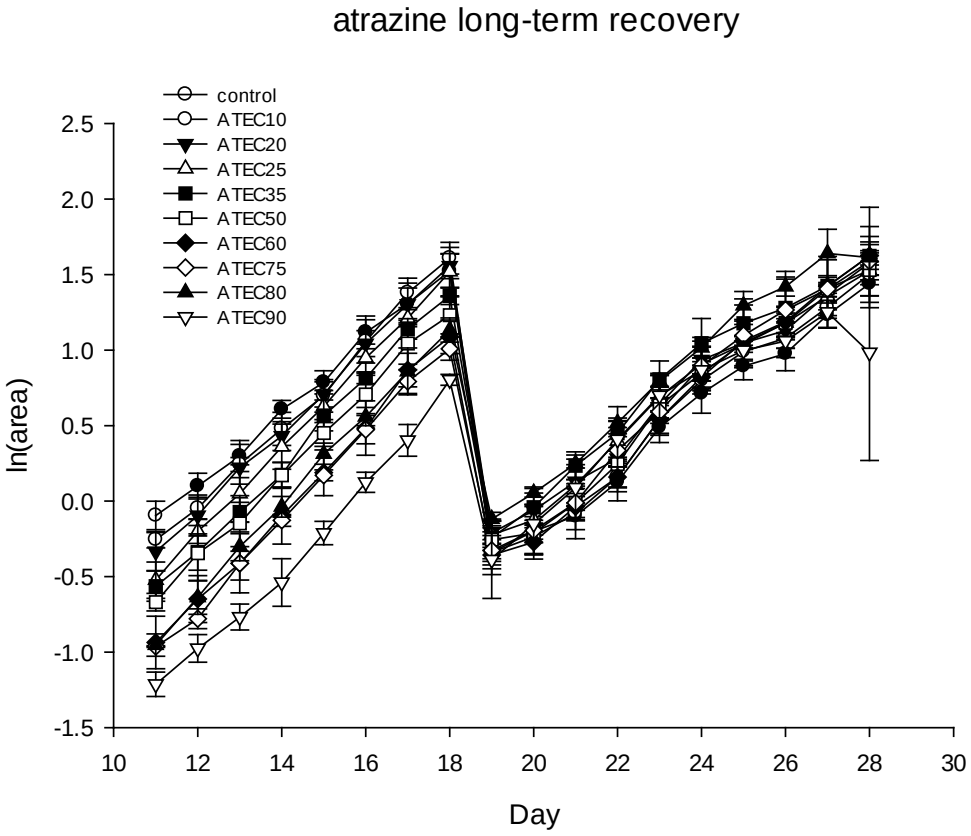


Figure 5-3:The day-to-day growth rates (day^{-1}) based on the frond area of *L. minor* after being exposed to atrazine for 10.5 days followed by a recovery phase from day 10.5 to day 28.

2,4-D recovery

During the recovery after short-term exposures to varying concentrations of 2,4-D, the average growth rates of *L. minor* slightly decreased then modestly recovered, but still with significant differences ($p<0.05$), particularly at high effective concentration. However, for the overall average there were no significant differences ($p>0.05$) (Figure 5-4). For the long-term exposures (Figure 5-5), the growth rates were significantly different between the control treatment and each effective concentration ($p<0.05$) during the recovery (day 10.5- day 28). The growth rates matched the controls' within a couple of weeks after the exposure phase.

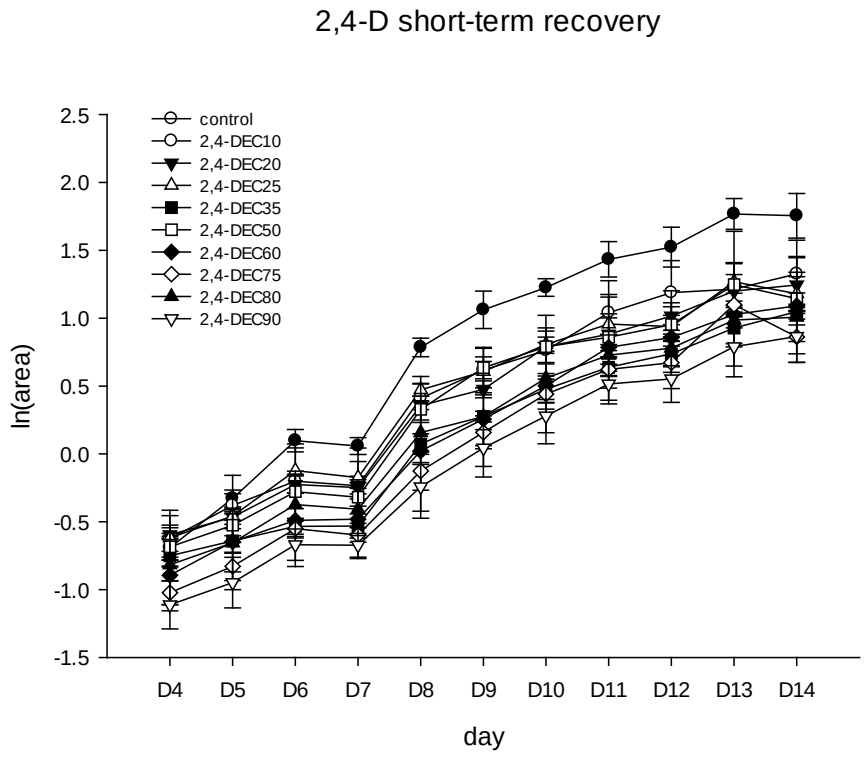


Figure 5-4: The day-to-day growth rates (day^{-1}) based on the frond area of *L. minor* which were exposed to 2,4-D for 3.5 days followed by a recovery phase from day 3.5 to day 14.

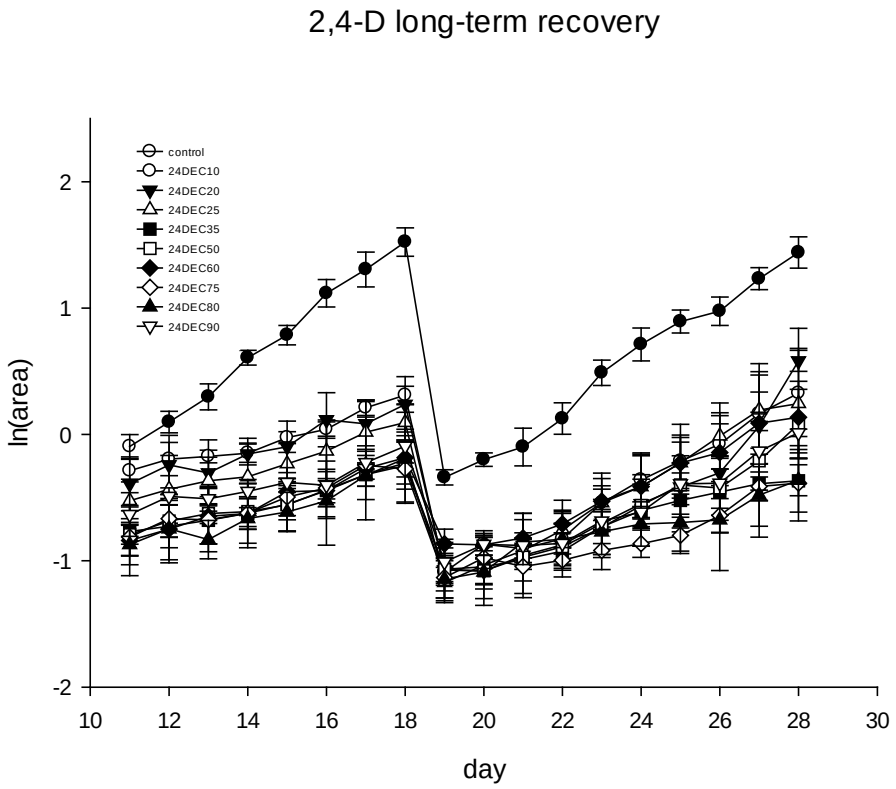


Figure 5-5: The day-to-day growth rates (day^{-1}) based on the frond area of *L. minor* which were exposed to 2,4-D for 10.5 days followed by a recovery phase from day 10.5 to day 28.

Alachlor recovery

For the short-term exposures, the average growth rates (day 3.5 – day 14) of *L. minor* during the recovery for all effective concentrations were not significantly different from the control treatment ($p>0.05$) (Figure 5-6). When looking at the time-point in the recovery for the short-term test, the growth rates matched the controls' rate within 9 days ($p<0.05$).

For the long-term exposures, the average growth rates (day 10.5 – day 28) showed significant differences from the controls' for every effective concentration except EC10 and 20 ($p<0.05$) (Figure 5-7). In terms of the time-point in the recovery, the growth rates for the high concentrations (EC80 and EC90) could not recovered during the test ($p<0.05$).

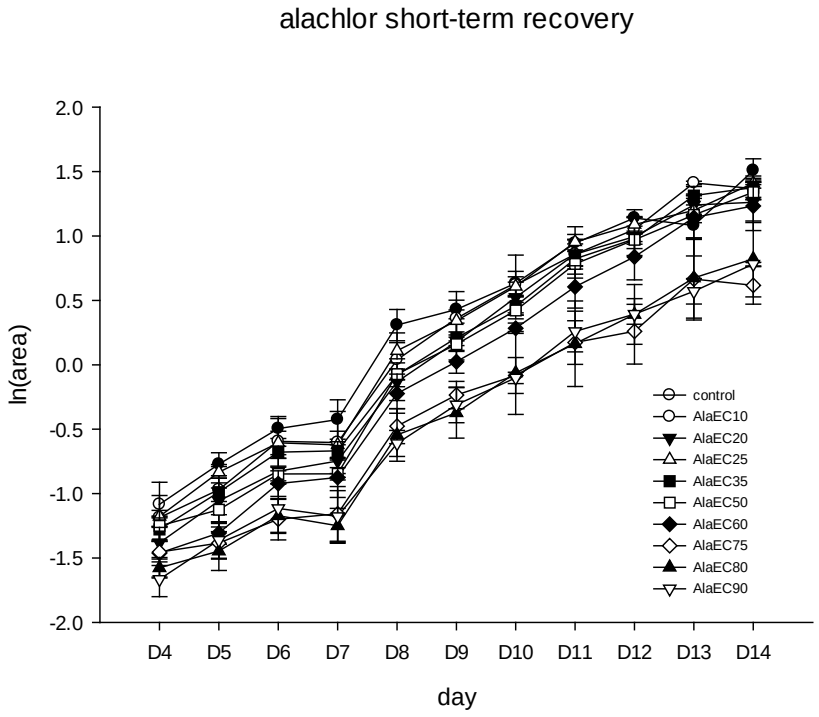


Figure 5-6: The day-to-day growth rates (day^{-1}) based on the frond area of *L. minor* which were exposed to alachlor for 3.5 days followed by a recovery phase from day 3.5 to day 14.

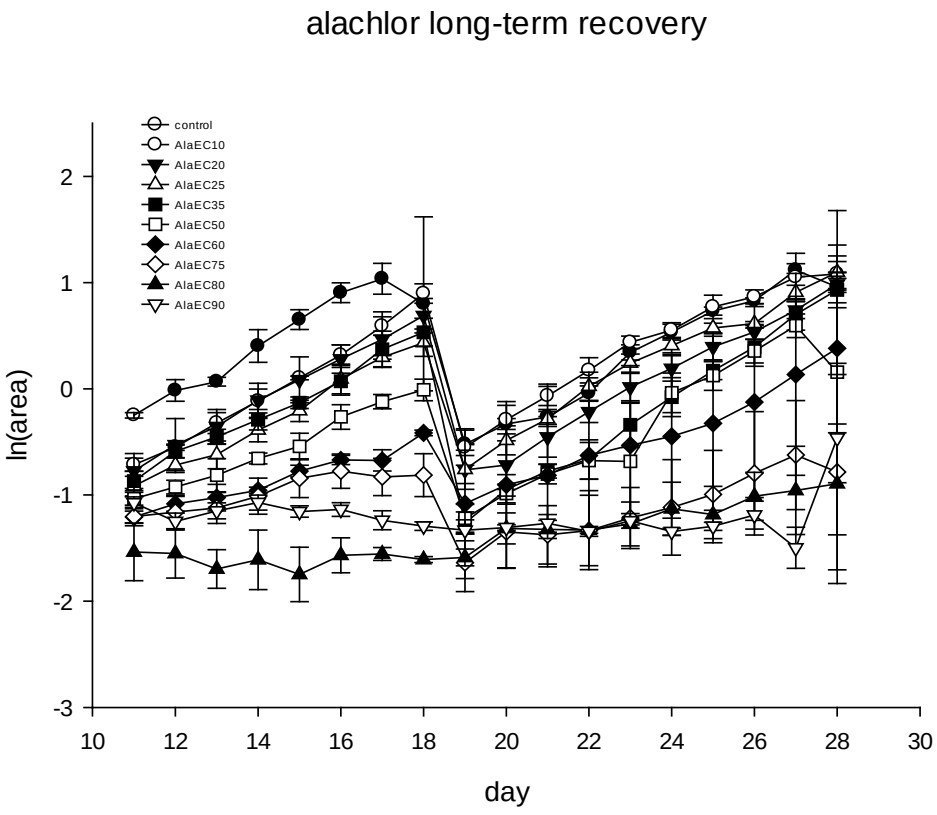


Figure 5-7: The day-to-day growth rates (day^{-1}) based on the frond area of *L. minor* which were exposed to alachlor for 10.5 days followed by a recovery phase from day 10.5 to day 28.

Paraquat recovery

In the short-term exposures to paraquat, the average growth rates during the recovery period (day 3.5 – day 14) of *L. minor* were significantly different from the control treatment's ($p>0.05$) at high effective concentration 80 (Figure 5-8), and for the long-term exposures, the growth rates of *L. minor* during the recovery phase were significantly different from the controls' ($p<0.05$) at high effective concentration 75, 80 and 90 (Figure 5-9). Plant could not recovery at high effective concentration 80 and 90 during the test.

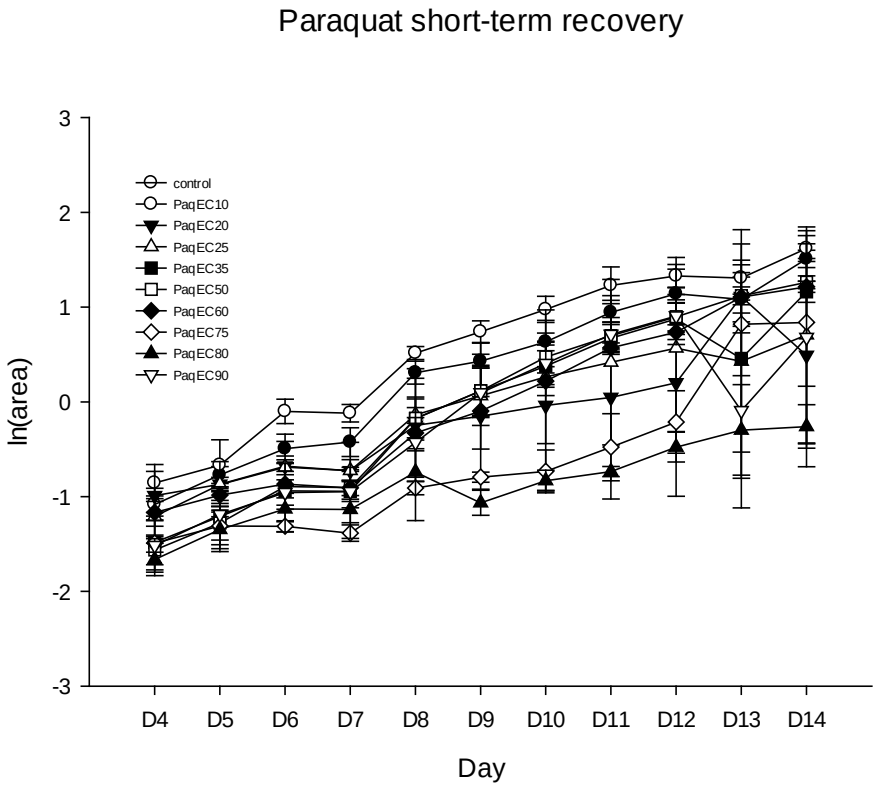


Figure 5-8: The day-to-day growth rates (day^{-1}) based on the frond area of *L. minor* which were exposed to paraquat for 3.5 days followed by a recovery phase from day 3.5 to day 14.

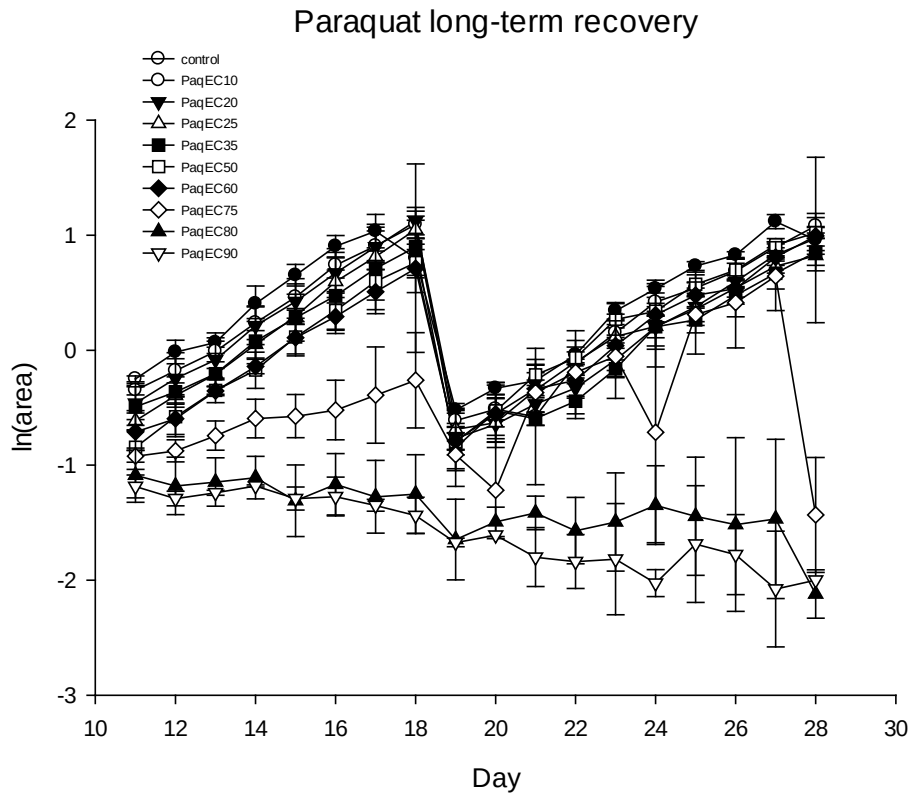


Figure 5-9: The day-to-day growth rates (day^{-1}) based on the frond area of *L. minor* which were exposed to paraquat for 10.5 days followed by a recovery phase from day 10.5 to day 28.

Phytocidal and phytostatic concentrations

Phytostatic concentrations are the concentrations that allow no net growth of *L. minor* during the exposure, but the plants can still recover when transferred to a fresh medium. A phytocidal concentration is defined as the lowest concentration tested that allows no net increase in population density during both the exposure and the recovery period. This means that the organism does not recover even when transferred to a fresh medium. This symptom can generally be observed in high concentration tests. However, for the highly toxic paraquat and alachlor the phytocidal concentrations started from at the EC80 and EC90. The phytocidal and phytostatic concentrations are given in Table 5-5.

Table 5-5: Phytostatic and phytocidal concentrations of atrazine, 2,4-D, alachlor and paraquat on *L. minor* in different exposure periods

Chemical	Short-term exposure and recovery		Long-term exposure and recovery	
	Phytostatic concentration (µg/L)	Phytocidal concentration (µg/L)	Phytostatic concentration (µg/L)	Phytocidal concentration (µg/L)
Atrazine	EC80,90	ND	EC90	ND
2,4-D	ND	ND	ND	ND
Alachlor	EC60,75	EC80	EC75	EC80
Paraquat	EC75	EC80	EC75	EC80

Discussion

Chemical analysis showed that the concentrations of the four herbicides were stable during the test period. However, the analytical concentrations of paraquat and alachlor in ELISA showed greatly fluctuating concentrations.

Many studies that employed the ELISA method have found that the false results can be due to a variety of factors such as inconsistencies during the preparation and the experiment, which can lead to up to 20% result variance. False positives results occur regularly with higher lab detection limits and high selectivity when applied to multi-residue. Another possible cause is that alachlor and paraquat water samples had to be diluted to get the concentrations within the linear dynamic range of ELISA

(Fisher and Michel, 1997). To get accurate results, alachlor's concentration had to fall in the middle of the ELISA kit's range.

In terms of the observable symptoms, the four herbicides are from different family groups and, therefore, their effects on *L. minor* exhibited different types and degrees of damage depending on the herbicides' mode of action, the duration of exposure and the concentration of the herbicide (Mohammad et al., 2011, Drost, 2011). The observable symptoms noted in this study are the same as those previously observed by other researchers (Kirby et al., 1994; Mohammad and Itoh, 2007; Teodorovic *et al*., 2011). For example, the colonies appeared broken up and the fronds were dwarfish from exposures to alachlor and atrazine (Kirby et al., 1994; Drost, 2011). Chlorosis and bleaching were caused by atrazine and paraquat (Mohnammad and Itoh, 2007). For phytostatic and phytocidal concentration in this experiment, the results showed unclear this observation due to the growth rate of *L. minor* fluctuate which slightly falling and rising during the recovery phase. Regarding the control plants, these seemed to stop growing exponentially, perhaps due to the fact that in this experiment, small petri-dishes were used and plants were growing very fast. To counteract this, plants were sub-cultured every week during the experiment to in an attempt to keep them in the exponential growth stage.

The recovery following the short-term exposures to the four herbicides was quicker than following the long-term exposures. It can be said that the effects of these herbicides are heavily dependent on the duration of the exposure. These results are in agreement with Mohammad *et al*. (2010) who stated that the growth of duckweed was more significantly affected in long-term exposures than in short-term exposures. The toxicological response varied after different exposure durations and concentrations of the compound. Brain *et al*. (2012) found that the time to recovery

was largely dependent on the duration of exposure except with the highest concentration of exposure. According to the results, it seems that carry-over toxicity plays a major role in the plants' recovery. Carry-over occurs when organisms are exposed to an environmental toxicant and survive but carry with it some damages resulting in reduced fitness (Chen *et al*. 2011; Ashauer *et al,* 2010). In addition, toxicokinetics (TK) and toxicodynamics (TD) are the concepts that can be used to explain the patterns of toxic effects on organisms overtime by simulating the underlying processes (Ashauer *et al*, 2011). There are several publications that pointed out that the time to recovery depends on the mechanisms of TK and TD. For example, Nyman *et al.* (2012) stated that the recovery of organisms can be driven either by TK (i.e. elimination) or TD (i.e. damage recovery). They found that TD generally dominated an organisms' recovery. Similarly, Ashauer *et al*. (2010) reached the same conclusion when they exposed *Gammarus pulex* to diazinon. Therefore, TK and TD play an important role in the recovery of organisms. In addition, they found that slow recovery of organisms is due to the possibility of carry-over toxicity by slow toxicodynamic and toxicokinetic processes. Ashauer *et al*. (2010) pointed out that whether a reversible or irreversible cellular injury occurs will depend on the duration of the exposure as well as the specific toxicokinetic properties of that toxicant.

However, it is not only the duration of exposure but also the type of the herbicide that affects the toxicity and degree of damage, with the concentration of the herbicide also playing a role. According to the growth rates from the results, out of the four herbicides, paraquat has proven to be the most toxic, followed by alachlor, atrazine and 2,4-D, respectively. It is clear from this experiment that the chemicals

with different physico-chemical properties and different modes of action display varied toxicity effects on *L. minor* as discussed below.

For atrazine, the results indicated that the growth rates of the controls and all the effective concentrations in the short-term exposures were not significantly different. However, when looking at the day-to-day recovery, the growth rates showed a rapid increase within 3 days into the recovery. In the long-term exposures, the growth rates were significantly affected by the differing effective concentrations and the recovery reached the same level as the controls' within 7 days. The results are in agreement with many other researches (Wilson and Koch, 2013, Mohammad et al., 2010). As for the recovery after the long-term exposures, the results showed that the growth of *Lemna* rapidly recovered within two or three days for the low concentrations. In the high concentrations, the fronds disintegrated but recovered within seven days. There are many studies indicating that the effects of photosystem II inhibitors are reversible (Trebst, 2008, Brain et al., 2012b). Cedergreen *et al*. (2005) observed that when *L. gibba* were exposed to triazine herbicide, they recovered within five days. This is because the triazine herbicide acts by reducing the site of photosystem II, followed by lipid peroxidation. In the case of the PSII inhibitors, the unchanged toxicity over time indicates that the bindings to PSII are weak H-bonds which is quickly reversible (Drost *et al*., 2007). Drost *et al*. (2010) mentioned that the recovery experiments conducted with the PSII inhibitors indicate that the effects of PSII inhibition quickly subside even for the high effective concentrations.

2,4-D is a phenoxy acid herbicide, which acts at multiple sites to disrupt hormonal balance and protein synthesis and cause a variety of plant's growth abnormalities (Tomlin, 1997). Its mode of action is to selectively kill broadleaf weeds by translocation via both the xylem and phloem. As a result, this herbicide has minor

impacts on monocots as they do not possess any vessels (Song, 2014, Fairchild et al., 1997). However, this experiment exposed the plants to 2,4-D at fairly high concentrations ranging between 19-37 mg/L. Therefore, the effects on the growth rates subsided within 4 days for the short-term test and within a couple of weeks in the long-term exposures. In terms of the injury symptoms to *Lemna*, leaf malformations such as cupping, crinkling, parallel veins and leaf strapping, were found in the experiment.

Alachlor interferes with plants' metabolism and inhibits the synthesis of fatty acids (Mohammad *et al.*, 2010). This herbicide is moderately toxic to *L. minor* (Fairchild et al., 1997, Drost et al., 2007). There are several studies that have explored the effects of alachlor on *L. minor*. In the short-term exposures, the growth rates recovered within 7 days but in the long-term exposures, the growth rate recovered within 15 days for the high concentrations. Mohammad *et al*. (2010) has mentioned that short exposures to higher concentrations caused longer lag periods for the initiation of growth in recovery, while a longer exposure period caused a slower growth rate without the lag period. In terms of the injury symptoms, the leaf tissue will be chlorotic or necrotic and leaves can be easily separated from the plant (Tomlin, 1997).

In terms of paraquat's toxicity, from the previous results indicate that paraquat is the most toxic herbicide toward *L. minor*. However, the result from this experiment was not clear enough to see the recovery from paraquat damage.The growth can be divided into two groups based on the impact of paraquat on the growth rate, which are the low impact group (EC10, EC20, EC25, EC30, EC50 and EC60) and the high impact group (EC75, EC80 and EC90). This herbicide disrupts photosynthetic electron transfer by accepting electrons from PSI and produces highly destructive

superoxide radicals (Tomlin, 1997; Mohammad *et al*., 2010). Therefore, plants, which are photosynthetic organisms, are deeply affected by exposure to paraquat and often die (Mohammad *et al*., 2010). Huges (1975) mentioned that the extent of the damage can also play a role as smaller damages may be more easily negated.

To sum up, as we can see from the results above, the four herbicides with different modes of action as well as different physico-chemical properties had different impacts on the plants. It can be said that the reversibility of cellular injuries in duckweed depends on different toxicokinetic mechanisms that eliminate the toxicant. In addition, the highly concentrated exposures cause higher bioaccumulation and thus more severe toxic effects than the lower constant exposures (Nyman *et al.*, 2012). By varying the concentrations of the herbicides, it became apparent that the high effective concentrations resulted in greater recovery time. As Liu *et al.* (2011) pointed out, the concentration level influences the bioaccumulation. In addition, the delay in the recovery after the exposures to paraquat and alachlor in comparison to the atrazine exposures is in agreement with a previous study by Cedergreen *et al.* (2005) which showed that the effects following exposures to photosynthesis inhibitors were readily reversible, while exposure to herbicides that impaired cell division induced delayed recovery of the fronds (Vallotton et al., 2008b). Therefore, it can be concluded that the mode of action of pesticides, the reversibility of their binding at the target site and the degree of damage during the exposure can have an influence on the potential recovery following exposures (Vallotton et al., 2008b).

Conclusion

The results of the present study demonstrated that a longer period of exposure caused more serious effects on *Lemna minor* and the toxicological responses of the plants

varied after different exposure durations and concentrations of the herbicide. In addition, other factors that may cause differences in the growth of duckweed are the route of exposure and the lag phase (Teodorovic *et al.*, 2012).

Ecologically relevant information for aquatic risk assessment of aquatic plant recovery potential and patterns should be obtained via laboratory tests by incorporating a recovery phase after the exposure. Additionally, the durations of both the exposure and the recovery should be taken into consideration. It would be worthwhile to investigate into the toxicokinetics and the toxicodynamics of the exposures, which would help to explain how the toxicant interacts with living organisms.

CHAPTER VI

6. General Discussion

The overall aim of the present study was to investigate the toxicity of the four herbicides atrazine, 2,4-D, alachlor and paraquat in single, mixture and sequential pulse exposures using the aquatic macrophyte *Lemna minor* as the test organism. In order to achieve this, the study pesticides were chosen based on the frequency of their use in actual rice fields in Thailand using a questionnaire survey that was performed in December 2011 in Chiang Mai, Thailand. The experiments investigated the effects of the four herbicides in mixtures and sequential pulse exposures, and the potential recovery of the plants after exposures to the herbicides. This investigation into the effects of herbicides toxicity was conducted with aquatic plants in an effort to fill a gap in the Thai ecotoxicological data. In addition, the present study aimed to use models to predict the effects on aquatic organisms of herbicides that are applied in mixture or in sequential pulse exposures.

This chapter provides a synthesis of the results and the conclusions of this research, which include the patterns of pesticide use, the comparative toxicity of pesticides in single and mixture exposures, and the model predictions of the toxicity of pesticides. Some suggestions regarding future research directions and limitations will also be discussed.

Synthesis of the data from the three experimental chapters

The observations from the three experimental investigations using mixture, short-term and long-term sequential exposure, and recovery studies with the herbicides were generally in agreement with findings obtained from other researchers. The mixture studies of atrazine and 2,4-D show that the interactions were antagonistic or the toxicity levels were over-predicted by the CA and IA model but the mixture of alachlor with paraquat showed synergistic interaction based on CA and IA as reference models. In comparison, the results of the short-term and long-term sequential exposures show that the growth rate model overestimates the toxicity and, the model can be used to make predictions for low concentrations of the short-term and the long-term tests. In contrast, at high concentrations of pretreated exposure, the model was unable to predict the toxicity.

As a result of this, the last experiments were performed in order to determine if the effects might be caused by carry-over toxicity. The speeds of recovery in different concentrations of the four herbicides were observed in this experiment. The results showed varying effects depending on the concentrations and type of herbicides. The highest concentrations of the four herbicides lead to more than a couple of weeks of recovery time for *L. minor* (Table 6.1). In particular, with, paraquat and alachlor at high concentrations, the growth rates were very low and the plants showed necrosis symptoms. Therefore, it can be said that the model is poor at predicting at high concentrations of pre-treated exposure in the sequential exposure due to carry-over toxicity.

Table 6-1: Summary of the results from the studies of mixtures, short-term and long-term sequential exposures, and recovery.

Experiment	**Second chemical** / **First chemical**	**Low concentration**	**Medium concentration**	**High concentration**
Mixture	Atrazine: 2,4-D	NA	Antagonism	Antagonism
	Alachlor: Paraquat	NA	Synergism	Synergism
Sequential exposure I	Low concentration	The model able to predict toxicity	The model able to predict toxicity	The model able to predict toxicity
	Medium concentration	The model able to predict toxicity	The model able to predict toxicity	The model able to predict toxicity
	High concentration	The model able to predict toxicity	The model able to predict toxicity	The model able to predict toxicity
Sequential exposure II	Medium concentration	The model able to predict toxicity	The model able to predict toxicity	The model able to predict toxicity
Long-term sequential exposure	Low concentration	The model able to predict toxicity	The model able to predict toxicity	The model able to predict toxicity

Table 6.1: (cont.) Summary of the results from the studies of mixtures, short-term and long-term sequential exposures, and recovery.

Experiment	**Second chemical** / **First chemical**	**Low concentration**	**Medium concentration**	**High concentration**
Long-term sequential exposure	Medium concentration	The model able to predict toxicity	The model able and unable to predict toxicity	The model unable to predict toxicity
	High concentration	The model unable to predict toxicity	The model unable to predict toxicity	The model unable to predict toxicity
Experiment	**Range of concentrations**	**Speed of recovery**		
Recovery (short-term)	Low concentration	Fast recovery within a couple of days:		
	Medium concentration	Fast recovery within a couple of days		
	High concentration	Fast recovery within a week		
	Overall	Atrazine showed fast recovery followed by 2,4-D, alachlor and paraquat, respectively.		
Recovery	Low concentration	Fast recovery within a week		

	Medium concentration	Fast recovery within a week
	High concentration	Fast recovery within a couple of weeks
	Overall	Atrazine showed fast recovery followed by 2,4-D, alachlor and paraquat, respectively.

Risk of herbicide exposure in rice fields to the aquatic macrophyte *Lemna minor*

The aquatic macrophyte *Lemna minor* is a dominant species in Thai aquatic systems. It plays a vital role in the aquatic system as a primary producer. However, in an agricultural area, there is a high risk of pesticides being released into the environment, which could damage non-aquatic organisms. Therefore, it is necessary to determine the risk of herbicide exposure for non-target organisms such as *Lemna minor*.

In Thailand, the use of herbicides in rice fields commonly involves farmers applying pesticides in mixtures to kill unwanted plants. In this section, an attempt has been made to establish the level of risk of herbicides in use in Thailand for aquatic macrophytes. The assessment is based on the risk assessment procedures described under EU Directive 91/414/EEC and AMEG, a new SETAC advisory group on aquatic macrophyte ecotoxicology (Arts et al., 2010) that provides scientifically based guidance for chemical risk assessments for aquatic macrophyte testing. According to Fenner et al. (2002) and Arts et al. (2010), in the lower-tier risk assessments, acute toxicity data (i.e. IC or EC50) are divided by the predicted exposure concentration (PEC) value to generate an acute toxicity exposure ratio or risk quotient ($RQ_{short\text{-}term}$). The $RQ_{short\text{-}term}$ should be less than 1 for sensitive plant

species. The equation for lower-tier risk assessment of aquatic macrophyte is given below

$$\text{Risk quotient} = \frac{PEC}{PNEC} \qquad \text{----------Equation 1}$$

If the $RQ_{short\text{-}term}$ value is found to be less than 1, this means that the pesticide passes Tier 1 and no further testing is necessary. If the $RQ_{short\text{-}term}$ value is more than 1, it means the higher tier assessment should be performed such as multispecies tests or micro/mesocosm studies.

Risks based on single pesticides

To assess the risks of the pesticides studied in this project for *Lemna*, the exposure concentrations estimated in Chapter 2 were taken and used alongside the ecotoxicity data to establish the level of risk (Table 6.2).

Table 6-2: input values used for risk quotient of aquatic macrophyte *Lemna minor*

Herbicides	PEC (µg/L) (from rice model)	EC50 (µg/L)	PNEC(µg/L)	RQ
2,4-D	5102	2800	280	18.2
Alachlor	3237	16	1.6	2023
paraquat	8.91	13	1.3	6.8

The results show that RQs of the three compounds are greater than one. For alachlor the value was 2023, whereas paraquat and 2,4-D had values of 18.2 and 6.8, respectively (Table 6.2). According to these results, it appears that, for the studied herbicides, there is a high potential risk for the aquatic plant *L. minor* in rice fields.

Therefore, these herbicides are considered to be candidates for more detailed assessment. It seems to be that the RQ value of alachlor is largely due to the very high application rate on small rice fields.

Implications toward the risk of pesticides in Thailand's environment

A central aim of this study was to understand the risks of pesticides in the Thai environment. Thailand is facing a problem of pesticide contamination in the river system from agriculture as a consequence of heavy use of pesticides and inappropriate pesticide application. While data on the occurrence of pesticides in Thailand's surface waters are limited, the available data indicate that concentrations in rivers are much lower than the concentrations examined in this study. Therefore, based on the available data, the risk assessment of mixtures of alachlor with paraquat, are likely to pose a serious threat to organisms in aquatic systems in Thailand. However, the four studied herbicides are sold and used in larger quantities than other herbicides in Thailand (Panuwet et al., 2012b) and the environmental monitoring that has been put in place is limited. It is possible that contaminations to surface water and ground water might be greater in some instances and the risks may be greater in reality.

It is also important to recognize that this thesis focuses only on one species. It is known that different species responds differently to pesticide exposure and that the effects can be influenced by factors such as temperature, rainfall, and agricultural practices (Iwai *et al* 2011b). While *Lemna* occurs in the Thai aquatic environment, it would be valuable to explore other local species to see if their sensitivity to toxicants

differs considerably from that of *Lemna* (Domingues et al., 2007). Differential responses of organisms, representing diverse physiological capabilities and niches in the aquatic system, can help focus field studies where non-target effect due to off-site movement of pesticides are suspected. From our knowledge, there are a few studies that use local organisms such as zooplankton *Moina Micruza* Kurz as test species to evaluate the ecotoxicology of pesticides in Thailand. The results indicated that this species is sensitive to pesticides but it would be helpful to perform the test with other species from a wide range of trophic levels such as plant, plankton, macro-invertebrates or fish. Thus, Thailand needs ecological effect test guidelines with which to derive new data on toxicological responses of organisms to environmental contaminants.

The limitations of this research

1. A data survey was conducted in Thailand during the period of December 2011 to January 2012. The survey asked farmers about the frequency of their pesticide application on rice fields within a 7-day period. The 7-day time frame was set up since the surveyed data would be used in laboratory experiments that were followed by OECD221 (2006) *Lemna* test for 7-day toxicity. Therefore, only the pesticides that were mixed within that time frame could be tested. In reality, however, the farmers apply pesticides in a variety of ways depending on factors such as the label description on the pesticide product, the commercials, the practice of neighboring farms or the type of pests

2. This field study was performed at a small scale and was conducted in December and January, which is outside the rice growing season. Normally, rice is cultivated

during the rainy season from June to August. In the future, if data surveys are conducted during the growing season, the acquired information on pesticide products used by the farmers will likely be more accurate.

3. In terms of the endpoint of the toxicity test, this study used only one endpoint which is the total frond area. Many guidelines and publications suggest using more than one endpoint for duckweed toxicity test such as frond number, chlorophyll, dry-weight, etc. However, as described in the section on species test and test condition in this Chapter, alachlor affected the frond area and not the frond number. It would be valuable for future experiments to perform toxicity test using more than one endpoint.

4. In the mixture experiments, the author used only the 25 and 50% effect level to compare predictions of a mixture interaction model with experimental observations. However, it would be valuable for future research to explore additional effect levels to see whether anatagonism or synergism also occurs as higher effect levels.

5. In terms of the toxicity of sequential pulse exposure, the author has confined discussions only to the herbicides' modes of action and external concentrations without including the internal concentrations of herbicides in duckweed. This is due to time and chemical analysis limitations. This work would have benefited from analysis of residues of the herbicides in the plant tissue. This data might have allowed toxico-kinetic/toxico-dynamic modelling of the pesticide interactions.

6. The analytical method used to determine concentrations of the herbicides in this study may not be the most efficient. Paraquat and alachlor were analysed using the ELISA test kit which is recommended for analysis of low concentrations in ng/ml. Since the ELISA test is very sensitive to changes in concentrations, another test

method should be implemented to confirm the results. However, the method for paraquat is difficult to recheck due to its tendency to stick onto glassware and its requirement of special analytical instrumentation. Therefore, it is recommended for future research to use another method to confirm the results.

7. For the recovery study that aimed to understand the carry-over toxicity from pretreated herbicides, it would be valuable for future research to consider the toxicokinetic and toxicodynamic (TK-TD) models to provide a better understanding of the toxicity of herbicides, sensitivity of organism, organism recovery times and carry-over toxicity.

Conclusion

The effects of herbicide mixtures with different modes of action on the aquatic plant *Lemna minor* are considered in this thesis. It investigates the toxic effects of different combinations of herbicides in different types of mixture, namely, simultaneous mixtures and sequential exposures. In order to predict the effects of binary mixture toxicity, a model based on the Independent Action model (IA) and concentration addition (CA) were used. The results show that the mixture combinations of atrazine with 2,4-D have antagonistic interaction but alachlor with paraquat has synergistic interactions in *L. minor*. For the sequential exposure, the model adopted the OECD221 (2006) Lemna toxicity test from the growth rate model based on the frond area and used the single toxicity data. The results show that the single toxicity data can be used to make predictions in the tests with low concentrations of pre-exposure herbicides. In addition, the study of the potential recovery of *L. minor* from the four

herbicides indicates that there are different types of recovery depending on the type of the herbicide and the time of exposure.

However, the prediction models are not yet perfect since some assumptions were made in the experiment that leads to some limitations. In addition, there are many questions that still need answering as discussed above. Furthermore, it should be possible to further develop the model in more detail with considerations of the mechanisms of the herbicides' interaction with plants and the toxicokinetics and toxicodynamics of plants. (Wang, 1991). A simple risk assessment showed that there is potentially a high risk to aquatic plants from pesticides, particularly alachlor. Therefore more attention should be paid to understanding the occurrence and effects of pesticides in the Thai environment.

Appendix A

Table A: Data survey pesticide used in rice field in small scale Chiang Mai, Thailand from December 2011 to January 2012

Question	Farmer 1	Farmer2	Farmer3	Farmer4	Farmer5
Age	67	71	56	70	46
Gender	Male	Male	Male	Male	Male
District	Mae Rim	Mae Rim	Mae Rim	Mae Rim	Mae Rim
1. Total area of rice field (hectare)	1.28	0.64	5.28	0.48	0.64
2. Do you apply pesticide to your paddy crops	yes	yes	yes	yes	yes
2.1 Chemical I	Gramoxone	Gramoxone	Lannate	Lannate	Lannate
2.1.1 size	5 L	1 L	10 L	2 L	500 cc
2.1.2 Number of product container used each year (bottles)	1	1	1	1	2
2.1.3 Number of occasions when product is applied to a rice field (per crop season)	1	2	1	1	2
2.1.4 When will be used (month)	April	May, July	June	June	August, Januray
2.2 Chemical II	Lannate	-	Furadan	Gramxone	Tamaron
2.2.1 size	1 L	-	5 kgs	1 L	500 cc
2.2.2 Number of product container used each year	1	-	1	1	2
2.2.3 Number of occasions when product is applied to a rice field	1	-	1	1	1
2.2.4 When will be used (month)	June/July	-	August	May	August
3. Do you apply any of the pesticide products together (either in separate application over 1-2 d or as a mixture)?	No	No	No	No	Yes (rate 1:1)
4.Are there use any pesticide products where the timing of application is very close (e.g. both products are applied within 7d)? if yes, please give details (product /timing)	No	No	No	No	No

Table A: (Cont.) Data survey pesticide used in rice field in small scale Chiang Mai, Thailand from December 2011 to January 2012

Question	Farmer 6	Farmer7	Farmer8	Farmer9	Farmer10
General question					
Age	61	63	64	54	56
Gender	Male	Male	Male	Male	Male
District	Mae Rim	Mae Rim	San Pa Thong	San Pa Thong	San Pa Thong
1. Total area of rice field (hectare)	1.92	0.48	5.28	1.6	1.12
2. Do you apply pesticide to your paddy crops	yes	No	yes	yes	yes
2.1 Chemical I	Lannate	-	Glyphosate48	Dimethoate	2,4-D (H-Sonud95)
2.1.1 size	1 L	-	5L	1 L	15 kgs
2.1.2 Number of product container used each year (bottles)	3	-	1	2	1
2.1.3 Number of occasions when product is applied to a rice field (per crop season)	1	-	1	1	1
2.1.4 When will be used (month)	June	-	June	Depend on pest	July
2.2 Chemical II	-	-	-	-	-
2.2.1 size	-	-	-	-	-
2.2.2 Number of product container used each year	-	-	-	-	-
2.2.3 Number of occasions when product is applied to a rice field	-	-	-	-	-
2.2.4 When will be used (month)	-	-	-	-	-
3. Do you apply any of the pesticide products together (either in separate application over 1-2 d or as a mixture)?	No	No	No	No	No
4.Are there use any pesticide products where the timing of application is very close (e.g. both products are applied within 7d)? if yes, please give details (product /timing)	No	No	No	No	No

Table A: (Cont.) Data survey pesticide used in rice field in small scale Chiang Mai, Thailand from December 2011 to January 2012

Question	Farmer 11	Farmer12	Farmer13	Farmer14	Farmer15
General question					
Age	67	60	59	50	54
Gender	Male	Male	Male	Male	Male
District	San Pa Thong	San Pa Thong	San Pa Thong	San Pa Thong	Mae Rim
1. Total area of rice field (hectare)	2.56	2.56	2.88	4.48	8.64
2. Do you apply pesticide to your paddy crops	yes	yes	yes	yes	yes
2.1 Chemical I	2,4-D (H-sonud95)	2,4-D (H-sonud95)	Paraquat	2,4-D (H-sonud95)	Glyphosate48
2.1.1 size	15 kgs	10 kgs	5L	10 kgs	5L
2.1.2 Number of product container used each year (bottles)	2	1	1	1	1
2.1.3 Number of occasions when product is applied to a rice field (per crop season)	1	1	1	1	1
2.1.4 When will be used (month)	June	October	June	August	June
2.2 Chemical II	Paraquat	-	Lannate	Santurn-D	2,4-D 80
2.2.1 size	5L	-	50 g	15 kgs	10 kgs
2.2.2 Number of product container used each year	1	-	1	1	5
2.2.3 Number of occasions when product is applied to a rice field	1	-	1	1	1
2.2.4 When will be used (month)	May	-	May	August	May
3. Do you apply any of the pesticide products together (either in separate application over 1-2 d or as a mixture)?	No	No	No	Yes Hectoana50cc:SanturnD500g:water 15L)	No
4.Are there use any pesticide products where the timing of application is very close (e.g. both products are applied within 7d)? if yes, please give details (product /timing)	No	No	No	No	No

Table A: (Cont.) Data survey pesticide used in rice field in small scale Chiang Mai, Thailand from December 2011 to January 2012

Question	Farmer 16	Farmer17	Farmer18	Farmer19	Farmer20
General question					
Age	63	50	56	57	64
Gender	Male	Male	Male	Male	Male
District	Mae Rim	Mae Rim	San Pa Thong	San Pa Thong	San Pa Thong
1. Total area of rice field (hectare)	3.68	0.64	0.96	0.96	1.12
2. Do you apply pesticide to your paddy crops	yes	yes	yes	yes	yes
2.1 Chemical I	2,4-D 80	Lasso(alachlor48%)	Lasso(alachlor48%)	Glyphosate48	Lannate
2.1.1 size	10 kgs	1 L	1L	1L	50 g
2.1.2 Number of product container used each year (bottles)	4	2	2	1	1
2.1.3 Number of occasions when product is applied to a rice field (per crop season)	1	1	1	1	1
2.1.4 When will be used (month)	May	June	June	June	July
2.2 Chemical II	-	Grammoxone	Grammoxone	Furadan	-
2.2.1 size	-	1 L	1 L	25 kgs	-
2.2.2 Number of product container used each year	-	2	2	1	-
2.2.3 Number of occasions when product is applied to a rice field	-	1	1	1	-
2.2.4 When will be used (month)	-	June	June	October	-
3. Do you apply any of the pesticide products together (either in separate application over 1-2 d or as a mixture)?	No	Lasso 180cc:Grammoxone 100cc:water 20L	Lasso180cc:Grammoxone 100cc:water 20L	No	No
4.Are there use any pesticide products where the timing of application is very close (e.g. both products are applied within 7d)? if yes, please give details (product /timing)	No	No	No	No	No

Table A: (Cont.) Data survey pesticide used in rice field in small scale Chiang Mai, Thailand from December 2011 to January 2012

Question	**Farmer 21**	**Farmer22**	**Farmer23**	**Farmer24**	**Farmer25**
General question					
Age	66	49	54	48	68
Gender	Male	Male	Male	Male	Male
District	Mae Thang	Mae Thang	Mae Thang	Mae Thang	Mae Thang
1. Total area of rice field (hectare)	1.6	0.64	0.8	0.64	0.96
2. Do you apply pesticide to your paddy crops	yes	yes	yes	No	yes
2.1 Chemical I	Glyphosate48	H-sonud95	2,4-D	-	2,4-D
2.1.1 size	1L	15 kgs	10 kgs	-	10 kgs
2.1.2 Number of product container used each year (bottles)	1	1	1	-	1
2.1.3 Number of occasions when product is applied to a rice field (per crop season)	1	1	1	-	1
2.1.4 When will be used (month)	June	July	May	-	May
2.2 Chemical II	-	Grammoxone	-	-	Lannate
2.2.1 size	-	1 L	-	-	50 g
2.2.2 Number of product container used each year	-	1	-	-	1
2.2.3 Number of occasions when product is applied to a rice field	-	1	-	-	1
2.2.4 When will be used (month)	-	June	-	-	July
3. Do you apply any of the pesticide products together (either in separate application over 1-2 d or as a mixture)?	No	No	No	No	No
4.Are there use any pesticide products where the timing of application is very close (e.g. both products are applied within 7d)? if yes, please give details (product /timing)	No	No	No	No	No

Table A: (Cont.) Data survey pesticide used in rice field in small scale Chiang Mai, Thailand from December 2011 to January 2012 (cont.)

Question	**Farmer 26**	**Farmer27**	**Farmer28**	**Farmer29**	**Farmer30**
General question					
Age	32	61	61	47	55
Gender	Female	Female	Female	Female	Female
District	Mae Thang	Mae Thang	Mae Thang	Mae Thang	Mae Thang
1. Total area of rice field (hectare)	1.12	0.64	0.96	1.92	0.64
2. Do you apply pesticide to your paddy crops	yes	No	No	yes	No
2.1 Chemical I	2,4-D	-	-	Round up	-
2.1.1 size	10 kgs	-	-	5 L	-
2.1.2 Number of product container used each year (bottles)	1	-	-	2	-
2.1.3 Number of occasions when product is applied to a rice field (per crop season)	1	-	-	1	-
2.1.4 When will be used (month)	May	-	-	June	-
2.2 Chemical II	Furadan	-	-	-	-
2.2.1 size	25 kgs	-	-	-	-
2.2.2 Number of product container used each year	1	-	-	-	-
2.2.3 Number of occasions when product is applied to a rice field	1	-	-	-	-
2.2.4 When will be used (month)	July	-	-	-	-
3. Do you apply any of the pesticide products together (either in separate application over 1-2 d or as a mixture)?	No	No	No	No	No
4.Are there use any pesticide products where the timing of application is very close (e.g. both products are applied within 7d)? if yes, please give details (product /timing)	No	No	No	No	No

5. Are you aware of any evidence that pesticides are having a negative impact on river systems in your region? (if yes, please give details)

Appendix B

Appendix B1:The results of chemical analysis of binary mixture atrazine and 2,4-D (mean± standard deviation)with three replicates

Mixture	**dilution**	**nominal concentration (mgL-1)**		**initial concentration first day (mgL-1)**		**initial concentration seven-day (mgL-1)**		**% remain**	
		atrazine	***2,4-D***	***atrazine***	***2,4-D***	***atrazine***	***2,4-D***	***atrazine***	***2,4-D***
		std 0.01	std 0.5	0.01±0	0.5±0.01	0.01±0	0.5±0	100	100
		std 0.05	std 5	0.05±0	5.0±0.10	0.05±0	5.1±0.04	100	102
		std 0.1	std 10	0.10±0	10.0±0.22	0.10±0	9.9±0.04	100	99
		std 0.5	std 15	0.50±0	15.0±0.08	0.50±0	15.0±0.09	100	100
		std 1	std 20	1.00±0	19.9±0.06	1.00±0	20.0±0.07	100	101
100/0	1 (x0.25)	0.04		0.04±0	ND	0.04±0	ND	100	ND
	2 (x0.5)	0.09		0.09±0	ND	0.09±0	ND	100	ND
	3 (x0.75)	0.13		0.13±0	ND	0.13±0	ND	100	ND
	4	0.17		0.17±0	ND	0.17±0	ND	100	ND
	5 (x1.25)	0.21		0.21±0	ND	0.22±0	ND	100	ND
	6 (x1.5)	0.25		0.25±0	ND	0.25±0	ND	100	ND
	7 (x2)	0.34		0.34±0	ND	0.35±0	ND	103	ND
83/17	1 (x0.25)	0.04	2	0.04±0	2.0±0.06	0.04±0	2.0±0.03	100	100
	2 (x0.5)	0.07	4	0.07±0	4.1±0.09	0.08±0	4.1±0.03	114	100
	3 (x0.75)	0.11	6	0.11±0	6.2±0.07	0.12±0	6.1±0.03	109	102
	4	0.14	8	0.14±0	8.1±0.01	0.14±0	8.1±0.03	100	100
	5 (x1.25)	0.18	10	0.18±0	10.1±0.04	0.18±0	10.1±0.10	100	100

Appendix B1: (Cont.) The results of chemical analysis of binary mixture atrazine and 2,4-D (mean± standard deviation)with three replicates

Mixture	dilution	nominal concentration (mgL-1)		initial concentration first day (mgL-1)		initial concentration seven-day (mgL-1)		% remain	
		atrazine	2,4-D	atrazine	2,4-D	atrazine	2,4-D	atrazine	2,4-D
	6 (x1.5)	0.21	12	0.21±0	12.3±0.27	0.22±0	12.1±0.05	105	98
	7 (x2)	0.28	16	0.28±0	16.1±0.01	0.29±0	16.0±0.03	104	99
63/37	1 (x0.25)	0.03	4	0.03±0	4.1±0.04	0.03±0	4.0±0.02	100	98
	2 (x0.5)	0.06	9	0.06±0	9.1±0.04	0.06±0	9.0±0.05	100	99
	3 (x0.75)	0.08	13	0.08±0	12.9±0.25	0.08±0	13.1±0.06	100	102
	4	0.11	18	0.11±0	18.0±0.14	0.11±0	18.1±0.01	100	101
	5 (x1.25)	0.14	22	0.14±0	22.2±0.02	0.14±0	22.0±0.03	100	99
	6 (x1.5)	0.17	27	0.17±0	27.2±0.11	0.17±0	27.1±0.02	100	100
	7 (x2)	0.22	36	0.22±0	35.8±0.05	0.22±0	36.1±0.08	100	101
50/50	1 (x0.25)	0.02	6	0.02±0	6.0±0.04	0.02±0	6.0±0.01	100	100
	2 (x0.5)	0.05	12	0.05±0	12.1±0.03	0.05±0	12.0±0.09	100	99
	3 (x0.75)	0.07	18	0.07±0	18.0±0.12	0.07±0	18.0±0.05	100	100
	4	0.09	24	0.09±0	23.7±0.04	0.09±0	24.4±0.01	100	103
	5 (x1.25)	0.11	30	0.11±0	30.1±0.05	0.11±0	30.2±0.09	100	100
	6 (x1.5)	0.14	36	0.14±0	35.7±0.03	0.14±0	36.3±0.04	100	102
	7 (x2)	0.18	48	0.18±0	48.1±0.01	0.18±0	48.2±0.12	100	100
37/63	1 (x0.25)	0.02	8	0.02±0	8.1±0.01	0.02±0	8.0±0.06	100	99
	2 (x0.5)	0.03	15	0.03±0	15.0±0.22	0.03±0	15.0±0.11	100	100
	3 (x0.75)	0.05	23	0.05±0	22.9±0.09	0.05±0	23.0±0.01	100	100
	4	0.06	30	0.06±0	29.7±0.04	0.06±0	29.9±0.1	100	101
	5 (x1.25)	0.08	38	0.08±0	37.8±0.04	0.08±0	38.1±0.07	100	101

Appendix B1: (Cont.) The results of chemical analysis of binary mixture atrazine and 2,4-D(mean± standard deviation)with three replicates

Mixture	dilution	nominal concentration (mgL-1)		initial concentration first day (mgL-1)		initial concentration seven-day (mgL-1)		% remain	
		atrazine	2,4-D	atrazine	2,4-D	atrazine	2,4-D	atrazine	2,4-D
	6 (x1.5)	0.09	45	0.09±0	45.4±0.51	0.09±0	45.0±0.07	100	99
	7 (x2)	0.12	60	0.12±0	60.5±0.1	0.12±0	60.3±0.13	100	100
17/83	1 (x0.25)	0.01	10	0.01±0	10.1±0.02	0.01±0	10.1±0.06	100	100
	2 (x0.5)	0.02	20	0.01±0	20.0±0.02	0.02±0	20.1±0.02	102	101
	3 (x0.75)	0.02	30	0.02±0	30.3±0.27	0.02±0	30.2±0.04	100	100
	4	0.03	40	0.03±0	40.0±0.20	0.03±0	40.3±0	100	101
	5 (x1.25)	0.04	50	0.04±0	50.2±0.19	0.04±0	49.9±0.15	100	99
	6 (x1.5)	0.05	60	0.05±0	60.1±0.22	0.05±0	60.1±0.05	100	100
	7 (x2)	0.06	80	0.06±0	79.9±0.05	0.06±0	79.9±0.03	100	100
0/100	1 (x0.25)		12	ND	12.0±0.12	ND	12.1±0.03	ND	101
	2 (x0.5)		24	ND	24.2±0.13	ND	24.1±0	ND	100
	3 (x0.75)		36	ND	36.3±0.04	ND	36.3±0.04	ND	100
	4		48	ND	47.9±0.20	ND	48.2±0.14	ND	101
	5 (x1.25)		60	ND	60.2±0.24	ND	60.2±0.05	ND	100
	6 (x1.5)		72	ND	71.8±0.03	ND	72.2±0.02	ND	101
	7 (x2)		96	ND	96.1±0.37	ND	96.2±0.47	ND	100

Appendix B2: The results of ELISA test kit analysis of binary mixture alachlor and paraquat

mixture	dilution	nominal concentration (mg/l)		initial concentration				% remain		% mean recovery	
				Alachlor		paraquat		alachlor	paraquat		
		Alachlor	paraquat	day1	day7	day1	day7			alachlor	paraquat
100/0	1(x0.25)	4	0	5.76	13.8	0	0	239	0	244 (9.41)	0
	2(x0.5)	8	0	7.64	NA	0	0	NA	0	95.5 (7.64)	0
	3(0.75)	11	0	15.51	NA	0	0	NA	0	141	0
	4	15	0	13	15.51	0	0	119	0	95	0
	5(x1.25)	19	0	39	NA	0	0	NA	0	205	0
	6(x1.5)	23	0	39	NA	0	0	NA	0	169	0
	7(x2)	30	0	NA	NA	0	0	NA	0	NA	0
83/17	1(x0.25)	3	0.4	8.6	7.63	0.50	0.50	88.72	100	270	125
	2(x0.5)	6	0.9	10	8.55	0.95	0.53	85.5	55	154	82
	3(0.75)	9	1.3	11.73	NA	1.53	0.98	NA	64	130	139
	4	12	1.7	12.31	NA	0.75	1.23	NA	163	102	59
	5(x1.25)	16	2.1	16.27	NA	1.05	2.65	NA	252	102	88
	6(x1.5)	19	2.6	NA	NA	1.45	1.95	NA	134	NA	65
	7(x2)	25	3.4	NA	NA	2.08	2.75	NA	133	NA	71
63/37	1(x0.25)	2	0.9	0.9	2	0.60	0.55	222	92	72.5	64
	2(x0.5)	5	1.9	7	NA	1.55	1.90	NA	123	140	90.8
	3(0.75)	7	2.8	27	10.28	2.23	1.50	38.1	67	266	67
	4	9	3.7	NA	NA	2.60	3.18	NA	122	NA	87
	5(x1.25)	12	4.6	13	NA	2.18	3.03	NA	139	108	57
	6(x1.5)	14	5.6	21	NA	3.90	4.60	NA	118	150	76
	7(x2)	19	7.4	NA	NA	4.03	7.80	NA	194	NA	80

Appendix B2: (cont.) The results of ELISA test kit analysis of binary mixture alachlor and paraquat

mixture	dilution	nominal concentration (mg/l)		initial concentration				% remain		% mean recovery	
		Alachlor	paraquat	Alachlor		paraquat		alachlor	paraquat	alachlor	paraquat
				day1	day7	day1	day7				
50/50	1(x0.25)	2	1.3	0.83	NA	0.98	1.45	NA	149	42	93
	2(x0.5)	4	2.5	9.19	NA	1.43	1.65	NA	116	229	62
	3(0.75)	6	3.8	7.8	10.3	3.28	3.03	132	92	130	83
	4	8	5.0	11.8	15.58	1.80	3.48	132	193	171	53
	5(x1.25)	9	6.3	9.7	NA	4.08	4.00	NA	98	108	64
	6(x1.5)	11	7.5	11.27	NA	3.53	6.53	NA	185	102	67
	7(x2)	15	10.0	NA	15.48	6.75	11.65	NA	173	103	92
63/37	1(x0.25)	1	1.6	NA	NA	1.05	1.80	NA	171	NA	89
	2(x0.5)	3	3.2	18.9	21.57	1.73	3.30	114	191	674	79
	3(0.75)	4	4.7	NA	4.3	3.38	4.63	NA	137	108	85
	4	6	6.3	NA	7.22	4.68	5.98	NA	128	120	85
	5(x1.25)	7	7.9	13.	18.5	5.20	5.98	142	115	225	71
	6(x1.5)	8	9.5	15.4	8.69	8.65	7.00	56.4	81	150	82
	7(x2)	11	12.6	20.6	NA	8.45	10.30	NA	122	187	74
83/17	1(x0.25)	1	2.1	NA	NA	0.73	2.10	NA	290	NA	67
	2(x0.5)	1	4.2	NA	NA	2.20	3.20	NA	145	NA	64
	3(0.75)	2	6.2	2.72	8.14	3.88	4.53	299	117	271	68
	4	3	8.3	NA	NA	5.65	6.93	NA	123	NA	76
	5(x1.25)	3	10.4	5	7.	9.63	7.38	140	77	200	82
	6(x1.5)	4	12.5	10.62	3.91	8.68	14.78	37	170	181	94
	7(x2)	5	16.6	8.56	NA	13.35	10.95	NA	82	171	73

Appendix B2: (cont.) The results of ELISA test kit analysis of binary mixture alachlor and paraquat

mixture	dilution	nominal concentration (mg/l)		initial concentration				% remain		% mean recovery	
		alachlor	paraquat	alachlor		paraquat		alachlor	paraquat	alachlor	paraquat
				day1	day7	day1	day7				
100/0	1(x0.25)	0	2.5	0	0	0.95	3.28	0	345	0	85
	2(x0.5)	0	5.0	0	0	2.60	4.43	0	170	0	70
	3(0.75)	0	7.5	0	0	5.68	6.48	0	114	0	81
	4	0	10.0	0	0	8.03	7.05	0	88	0	75
	5(x1.25)	0	12.5	0	0	9.80	11.53	0	118	0	85
	6(x1.5)	0	15.0	0	0	11.45	8.53	0	74	0	67
	7(x2)	0	20.0	0	0	14.15	24.28	0	172	0	96

Appendix C

Table C1: pH data of sequential exposure I (mean ±standard deviation for three replicates)

Hebicides	concentrations	Day0	Day3.5	Day3.5	Day7
control	-	6.5 (±0)	7.32(±0.03)	6.5 (±0)	7.35(±0.02)
atrazine	0.07	6.5 (±0)	7.21(±0.02)	6.5 (±0)	7.21(±0.02)
	0.11	6.5 (±0)	7.22(±0.03)	6.5 (±0)	7.21(±0.1)
	0.17	6.5 (±0)	7.32(±0.02)	6.5 (±0)	7.21(±0.03)
	0.27	6.5 (±0)	7.24(±0.03)	6.5 (±0)	7.21(±0.02)
	0.42	6.5 (±0)	7.2(±0.1)	6.5 (±0)	7.17(±0.1)
2,4-D	19	6.40 (±0.02)	7.11(±0.02)	6.43(±0.02)	7.11(±0.02)
	22	6.33(±0.01)	7.11(±0.3)	6.34(±0. 2)	7.11(±0.03)
	28	6.32(±0.01)	7.08(±0.1)	6.33(±0.1)	7.08(±0.1)
	32	6.28(±0.01)	7.05(±0.02)	6.26(±0.01)	7.05(±0.02)
	37	6.217(±0.02)	7.00(±0.02)	6.18(±0.01)	7.00(±0)
alachlor	1.9	6.5 (±0)	7.12(±0.01)	6.5 (±0)	7.14 (±0.03)
	5	6.5 (±0)	7.11(±0.03)	6.5 (±0)	7.14 (±0.02)
	16	6.5 (±0)	7.15(±0.03)	6.5 (±0)	7.16 (±0.02)
	46	6.5 (±0)	7.13 (±0.02)	6.5 (±0)	7.17(±0.04)
	100	6.5 (±0)	7.15 (±0.03)	6.5 (±0)	7.15 (±0.04)

Table C1: (cont.) pH data of sequential exposure I (mean ±standard deviation for three replicates)

Hebicides	concentrations	Day0	Day3.5	Day3.5	Day7
Paraquat	1.9	6.5 (±0)	7.15(±0.05)	6.5 (±0)	7.21 (±0.02)
	5	6.5 (±0)	7.16 (±0.02)	6.5 (±0)	7.2 (±0.03)
	13	6.5 (±0)	7.13 (±0.02)	6.5 (±0)	7.16 (±0.03)
	34	6.5 (±0)	7.12(±0)	6.5 (±0)	7.17 (±0.04)
	89	6.5 (±0)	7.17 (±0.01)	6.5 (±0)	7.14 (±0.01)

Table C2: pH data of long sequential exposure (mean ±standard deviation for three replicates)

Herbicide	Concentrations	Day0-3.5	D3.5-7	D7-10.5	D10.5-14
control	-	6.5 (±0)	7.21(0.01)	7.08(±0.05)	7.18(±0.05)
atrazine	0.07	6.5 (±0)	7.28(0.05)	7.10(±0.1)	7.19(±0.04)
	0.11	6.5 (±0)	7.26(0.06)	7.12(±0.1)	7.33(±0.09)
	0.17	6.5 (±0)	7.23(0.11)	7.1(±0.02)	7.32(±0.1)
	0.27	6.5 (±0)	7.26(±0.06)	7.12(±0.01)	7.3(±0.06)
	0.42	6.5 (±0)	7.25(±0.03)	7.29(±0.07)	7.32(±0.04)
2,4-D	19	6.5 (±0)	7.01(±0.02)	7.23(±0.03)	7.23(±0.01)
	22	6.5 (±0)	7.13(±0.01)	7.18(±0.06)	7.26(±0.12)
	28	6.5 (±0)	7.12(±0.12)	7.21(±0.08)	7.13(±0.02)
	32	6.5 (±0)	7.24(±0.12)	7.17(±0.07)	7.17(±0.04)
	37	6.5 (±0)	7.18(±0.06)	7.20(±0.08)	7.13(±0.04)
alachlor	1.9	6.5 (±0)	7.21(±0.05)	7.22(±0.06)	7.17(±0.04)
	5	6.5 (±0)	7.28(±0.06)	7.24(±0.01)	7.28(±0.02)
	16	6.5 (±0)	7.33(±0)	7.26(±0.06)	7.24(±0.03)
	46	6.5 (±0)	7.21(±0.1)	7.25(±0.01)	7.29(±0.13)
	100	6.5 (±0)	7.22(±0.01)	7.22(±0)	7.19(±0.05)
Paraquat	1.9	6.5 (±0)	7.15(±0.01)	7.24(±0.02)	7.25(±0.06)
	5	6.5 (±0)	7.24(±0.02)	7.14(±0.02)	7.21(±0.04)
	13	6.5 (±0)	7.28(±0)	7.15(±0)	7.22(±0.07)
	34	6.5 (±0)	7.25(±0.04)	7.21(±0.05)	7.22(±0.01)
	89	6.5 (±0)	7.18(±0.05)	7.22(±0.06)	7.17(±0.04)

AppendixD

Table D1: pH in short-term recovery test during day0 to day 14 (mean ± standard deviation) with three replicates

Herbicide	**Effective concentration**	**concentration**	**Day0**	**Day3.5**	**Day7**	**Day14**
		Control	6.5 (±0)	7.12 (±0.01)	7.17 (±0.05)	7.21 (±0.05)
Atrazine	EC10	0.07	6.5 (±0)	7.22 (±0)	7.17 (±0.02)	7.26 (±0.4)
	EC20	0.09	6.5 (±0)	7.12 (±0.11)	7.23 (±0.06)	7.22 (±0.07)
	EC25	0.11	6.5 (±0)	7.17 (±0.04)	7.15 (±0.03)	7.21 (±0.04)
	EC30	0.13	6.5 (±0)	7.23 (±0.02)	7.12 (±0.07)	7.25 (±0.14)
	EC50	0.17	6.5 (±0)	7.19 (±0.02)	7.20 (±0.07)	7.17(±0.02)
	EC60	0.20	6.5 (±0)	7.23 (±0.04)	7.22 (±0.06)	7.14(±0.04)

Table D1: (Cont.) pH in short-term recovery test during day0 to day 14 **(mean ± standard deviation) with three replicates**

Herbicide	Effective concentration	concentration	Day0	Day3.5	Day7	Day14
atrazine	EC75	0.27	6.5 (±0)	7.11(±0.05)	7.17 (±0.03)	7.19(±0.03)
	EC80	0.30	6.5 (±0)	7.22 (±0.04)	7.26 (±0.33)	7.23(±0.1)
	EC90	0.42	6.5 (±0)	7.19 (±0.09)	7.26 (±0.03)	7.32(±0.03)
2,4-D	EC10	19	6.5 (±0)	7.27 (±0.06)	7.42 (±0.01)	7.34(±0.04)
	EC20	21	6.5 (±0)	7.25 (±0.02)	7.20 (±0.04)	7.19(±0.02)
	EC25	22	6.5 (±0)	7.22 (±0.04)	7.25 (±0.02)	7.19(±0.11)
	EC30	24	6.5 (±0)	7.22 (±0.06)	7.22 (±0.09)	7.35(±0.03)
	EC50	28	6.5 (±0)	7.22 (±0.06)	7.24 (±0.01)	7.26(±0.1)
	EC60	29	6.5 (±0)	7.25 (±0.03)	7.24 (±0.05)	7.32(±0.09)
	EC75	31	6.5 (±0)	7.20 (±0.09)	7.15 (±0.01)	7.18(±0.04)

Table D1: (Cont.) pH in short-term recovery test during day0 to day 14 **(mean ± standard deviation) with three replicates**

Herbicide	**Effective concentration**	**concentration**	**Day0**	**Day3.5**	**Day7**	**Day14**
2,4-D	EC80	32	6.5 (±0)	7.26 (±0.05)	7.13 (±0.02)	7.24(±0.02)
	EC90	37	6.5 (±0)	7.14 (±0.04)	7.25 (±0.02)	7.20(±0.03)
Alachlor	Control		6.5 (±0)	7.55 (±0.04)	7.27 (±0.01)	7.25(±0.02)
	EC10	1.9	6.5 (±0)	7.29 (±0.04)	7.28 (±0)	7.17(±0.03)
	EC20	4	6.5 (±0)	7.16 (±0.01)	7.22 (±0)	7.22(±0.10)
	EC25	5	6.5 (±0)	7.22 (±0.09)	7.21 (±0.03)	7.17(±0.06)
	EC30	9	6.5 (±0)	7.34 (±0.09)	7.18 (±0.01)	7.40(±0.1)
	EC50	16	6.5 (±0)	7.20 (±0.09)	7.20(±0.06)	7.32(±0.12)
	EC60	23	6.5 (±0)	7.09 (±0.05)	7.21 (±0.08)	7.32(±0.04)
	EC75	46	6.5 (±0)	7.15(±0.06)	7.32(±0.01)	7.19(±0.01)

Table D1: (Cont.) pH in short-term recovery test during day0 to day 14 **(mean ± standard deviation) with three replicates**

Herbicide	**Effective concentration**	**concentration**	**Day0**	**Day3.5**	**Day7**	**Day14**
Alachlor	EC80	62	6.5 (±0)	7.25(±0.04)	7.18(±0.06)	7.26(±0.07)
	EC90	100	6.5 (±0)	7.22(±0.03)	7.18(±0.04)	7.24(±0.06)
Paraquat	EC10	1.9	6.5 (±0)	7.24(±0.05)	7.27(±0.05)	7.16(±0.03)
	EC20	4	6.5 (±0)	7.32(±0.03)	7.13(±0.03)	7.16(±0.03)
	EC25	5	6.5 (±0)	7.16(±0.06)	7.17(±0.02)	7.23(±0.10)
	EC30	8	6.5 (±0)	7.14(±0.06)	7.16(±0.06)	7.12(±0.07)
	EC50	13	6.5 (±0)	7.18(±0.04)	7.17(±0.08)	7.12(±0)
	EC60	19	6.5 (±0)	7.19(±0.03)	7.22(±0.07)	7.17(±0.05)
	EC75	34	6.5 (±0)	7.25(±0.08)	7.12(±0.11)	7.17(±0.07)
	EC80	44	6.5 (±0)	7.20(±0.03)	7.18(±0)	7.13(±0.12)
	EC90	89	6.5 (±0)	7.33(±0.24)	7.21(±0.04)	7.17(±0.11)

Table D2: pH in long-term recovery experiment (mean ± standard deviation) with three replicates

Herbicide	Effective concentration	concentration	Day0	Day3.5	Day7	Day14	Day17.5	Day21	Day24.5	Day28
Atrazine	Control	0	6.5 (±0)	7.22(±0.01)	7.18(±0.09)	7.25(±0.01)	7.31(±0.08)	7.19(±0.03)	7.25(±0.04)	7.23(±0.01)
	EC10	0.07	6.5 (±0)	7.22(±0.01)	7.22(±0)	7.22(±0.05)	7.20(±0.03)	7.13(±0.04)	7.14(±0.03)	7.20(±0.03)
	EC20	0.09	6.5 (±0)	7.23(±0.02)	7.20(±0.04)	7.17(±0.06)	7.22(±0.1)	7.20(±0.04)	7.24(±0.02)	7.12(±0.02)
	EC25	0.11	6.5 (±0)	7.13(±0.02)	7.23(±0.02)	7.26(±0)	7.15(±0.01)	7.25(±0.06)	7.28(±0.06)	7.15(±0.06)
	EC30	0.13	6.5 (±0)	7.22(±0.01)	7.22(±0.01)	7.19(±0.06)	7.27(±0.02)	7.23(±0.01)	7.25(±0.03)	7.23(±0.02)
	EC50	0.17	6.5 (±0)	7.24(±0.01)	7.24(±0.02)	7.22(±0.01)	7.26(±0.14)	7.27(±0.02)	7.22(±0.01)	7.18(±0.02)
	EC60	0.20	6.5 (±0)	7.22(±0.03)	7.17(±0.07)	7.20(±0.06)	7.22(±0.01)	7.24(±0.08)	7.22(±0.05)	7.15(±0.06)
	EC75	0.27	6.5 (±0)	7.21(±0.02)	7.23(±0.01)	7.23(±0.01)	7.25(±0.02)	7.23(±0.03)	7.25(±0.02)	7.14(±0.04)
	EC80	0.30	6.5 (±0)	7.23(±0.01)	7.26(±0.01)	7.16(±0.07)	7.27(±0.09)	7.19(±0.04)	7.21(±0.03)	7.11(±0.01)
	EC90	0.42	6.5 (±0)	7.23(±0.01)	7.22(±0.02)	7.21(±0.01)	7.14(±0.05)	7.24(±0.03)	7.25(±0.01)	7.11(±0.03)

Table D2: (Cont.) pH in long-term recovery experiment (mean ± standard deviation) with three replicates

Herbicide	Effective concentration	concentration	Day0	Day3.5	Day7	Day14	Day17.5	Day21	Day24.5	Day28
2,4-D	EC10	19	6.5 (±0)	7.23(±0.01)	7.16(±0.06)	7.19(±0.06)	7.1(±0.04)	7.27(±0.06)	7.19(±0.04)	7.18(±0.04)
	EC20	21	6.5 (±0)	7.26(±0.06)	7.29(±0.06)	7.18(±0.05)	7.29(±0.27)	7.25(±0.02)	7.21(±0.03)	7.26(±0.07)
	EC25	22	6.5 (±0)	7.19(±0.06)	7.23(±0.01)	7.17(±0.05)	7.17(±0.03)	7.33(±0.01)	7.21(±0.03)	7.21(±0.03)
	EC30	24	6.5 (±0)	7.32(±0.01)	7.22(±0.02)	7.21(±0.06)	7.16(±0.07)	7.24(±0.09)	7.22(±0.03)	7.20(±0.02)
	EC50	28	6.5 (±0)	7.18(±0.05)	7.21(±0.01)	7.26(±0)	7.24(±0.02)	7.26(±0.03)	7.14(±0.02)	7.29(±0.19)
	EC60	29	6.5 (±0)	7.18(±0.04)	7.22(±0.01)	7.21(±0.08)	7.29(±0.06)	7.25(±0.01)	7.20(±0.03)	7.20(±0.05)
	EC75	31	6.5 (±0)	7.18(±0.04)	7.24(±0)	7.22(±0.08)	7.3(±0.12)	7.27(±0.09)	7.36(±0.38)	7.24(±0.01)
	EC80	32	6.5 (±0)	7.21(±0.09)	7.18(±0.02)	7.24(±0.08)	7.31(±0.04)	7.19(±0.08)	7.25(±0.03)	7.20(±0.07)
	EC90	37	6.5 (±0)	7.23(±0.1)	7.20(±0.05)	7.42(±0.08)	7.24(±0.11)	7.29(±0.17)	7.32(±0.12)	7.29(±0.06)
Alachlor	Control	0	6.5 (±0)	7.21(±0.08)	7.21(±0.03)	7.32(±0.15)	7.2(±0.05)	7.11(±0.06)	7.11(±0.09)	7.15(±0.01)

Table D2: (Cont.) pH in long-term recovery experiment (mean ± standard deviation) with three replicates

Herbicide	Effective concentration	concentration	Day0	Day3.5	Day7	Day14	Day17.5	Day21	Day24.5	Day28
Alachlor	EC10	1.9	6.5 (±0)	7.2(±0.08)	7.22(±0.04)	7.28(±0.08)	7.27(±0.08)	7.31(±0.02)	7.13(±0.05)	7.29(±0.17)
	EC20	4	6.5 (±0)	7.18(±0.04)	7.18(±0.04)	7.3(±0.02)	7.25(±0.06)	7.14(±0.02)	7.25(±0.06)	7.25(±0.01)
	EC25	5	6.5 (±0)	7.12(±0.06)	7.23(±0.05)	7.28(±0.12)	7.11(±0.05)	7.16(±0.01)	7.18(±0.03)	7.30(±0.06)
	EC30	9	6.5 (±0)	7.19(±0.04)	7.3(±0.21)	7.19(±0.04)	7.41(±01)	7.34(±0.1)	7.21(±0.04)	7.28(±0.04)
	EC50	16	6.5 (±0)	7.25(±0.08)	7.20(±0.05)	7.19(±0.04)	7.31(±0.09)	7.27(±0.11)	7.22(±0.06)	7.30(±0.1)
	EC60	23	6.5 (±0)	7.26(±0.09)	7.29(±0.05)	7.15(±0.07)	7.23(±0.09)	7.34(±0.07)	7.26(±0.05)	7.24(±0.08)
	EC75	46	6.5 (±0)	7.35(±0.08)	7.32(±0.03)	7.22(±0.05)	7.2(±0.04)	7.22(±0.06)	7.26(±0.02)	7.15(±0.04)
	EC80	62	6.5 (±0)	7.25(±0.02)	7.3(±0.04)	7.24(±0.06)	7.19(±0.1)	7.26(±0.11)	7.23(±0.09)	7.33(±0.11)
	EC90	100	6.5 (±0)	7.21(±0.06)	7.22(±0.05)	7.11(±0.07)	7.28(±0.05)	7.16(±0.02)	7.26(±0.07)	7.29(±0.06)
Paraquat	EC10	1.9	6.5 (±0)	7.20(±0.02)	7.23(±0.05)	7.31(±0.04)	7.20(±0.1)	7.14(±0.06)	7.17(±0.04)	7.16(±0.01)

Table D2: (Cont.) pH in long-term recovery experiment (mean ± standard deviation) with three replicates

Herbicide	Effective concentration	concentration	Day0	Day3.5	Day7	Day14	Day17.5	Day21	Day24.5	Day28
paraquat	EC20	4	6.5 (±0)	7.32(±0.13)	7.22(±0.1)	7.2(±0.07)	7.21(±0.1)	7.25(±0.09)	7.25(±0.09)	7.24(±0.1)
	EC25	5	6.5 (±0)	7.23(±0.05)	7.19(±0.05)	7.24(±0.1)	7.26(±0.08)	7.26(±0.04)	7.23(±0.01)	7.2(±0.02)
	EC30	8	6.5 (±0)	7.23(±0.08)	7.24(±0.02)	7.18(±0.02)	7.14(±0.05)	7.26(±0.1)	7.36(±0.25)	7.28(±0.07)
	EC50	13	6.5 (±0)	7.21(±0.02)	7.29(±0.05)	7.30(±0.03)	7.44(±0.12)	7.26(±0.07)	7.25(±0.1)	7.28(±0.05)
	EC60	19	6.5 (±0)	7.35(±0.07)	7.30(±0.03)	7.22(±0.06)	7.16(±0.04)	7.16(±0.02)	7.23(±0.07)	7.20(±0.04)
	EC75	34	6.5 (±0)	7.29(±0.02)	7.32(±0.06)	7.24(±0.06)	7.27(±0.11)	7.20(±0.07)	7.22(±0.06)	7.25(±0.05)
	EC80	44	6.5 (±0)	7.32(±0.07)	7.28(±0.07)	7.21(±0.06)	7.18(±0.04)	7.21(±0.05)	7.25(±0.01)	7.31(±0.06)
	EC90	89	6.5 (±0)	7.21(±0.05)	7.17(±0.05)	7.31(±0.11)	7.19(±0.05)	7.15(±0.07)	7.17(±0.06)	7.23(±0.01)

AppendixE

Table E1: R^2 and slope of short-term recovery based on ln(area).

Treatment	Short-term	R^2	R^2	slope
Control		Y= 0.255x – 0.7421	0.9481	0.255
Atrazine	EC10	Y= 0.252x - 0.7593	0.951	0.252
	EC20	Y=0.2576x – 0.8273	0.9489	0.257
	EC25	Y= 0.25x – 0.850	0.9572	0.250
	EC35	Y= 0.2712x – 1.0475	0.9672	0.271
	EC50	Y= 0.2815x – 1.1018	0.9764	0.281
	EC60	Y=0.2778x - 1.1248	0.9682	0.277
	EC75	Y=0.2859x – 1.4142	0.9744	0.285
	EC80	Y=0.292x – 1.6357	0.9785	0.292
	EC90	Y=0.2876x-1.7095	0.9669	0.287
Control		Y= 0.255x – 0.7421	0.9481	0.255
2,4-D	EC10	Y=0.211x – 0.8005	0.9583	0.211
	EC20	Y=0.2027x – 0.8134	0.9651	0.203
	EC25	Y=0.1972x-0.7416	0.9423	0.197
	EC35	Y=0.1985x-1.0346	0.9641	0.198
	EC50	Y=0.2069x-0.8643	0.9399	0.206
	EC60	Y=0.2153x-1.1076	0.974	0.215
	EC75	Y=0.2166x-1.2335	0.9597	0.217
	EC80	Y=0.1984x-0.9872	0.965	0.198
	EC90	Y=0.2128x-1.3317	0.979	0.213
	control	Y=0.2577x-1.2491	0.9684	0.257
alachlor	EC10	Y=0.2794x-1.4626	0.978	0.279
	EC20	Y=0.2886x-1.648	0.9769	0.288
	EC25	Y=0.271x-1.4015	0.9728	0.271
	EC35	Y=0.2815x-1.5558	0.9868	0.281
	EC50	Y=0.2848x-1.6455	0.976	0.284
	EC60	Y=0.2912x-1.807	0.9867	0.291

Table E1: R^2 and slope of short-term recovery based on ln(area).

Treatment	Short-term	R^2	R^2	slope
	EC75	Y=0.2363x-1.806	0.9656	0.236
	EC80	Y=0.2589x-1.9507	0.9796	0.258
	EC90	Y=0.2535x-1.9146	0.9829	0.253
	control	Y=0.2577x-1.2491	0.9684	0.257
paraquat	EC10	Y=0.2521x-0.9696	0.9526	0.252
	EC20	Y=0.1794x-1.2448	0.8803	0.179
	EC25	Y=0.1932x-1.2668	0.9385	0.193
	EC35	Y=0.2648x-1.6883	0.9298	0.264
	EC50	Y=0.2996x-1.8206	0.9772	0.299
	EC60	Y=0.26x-1.6069	0.9753	0.260
	EC75	Y=0.2315x-2.0239	0.8645	0.231
	EC80	Y=0.1263x-1.6388	0.9108	0.126
	EC90	Y=0.2284x-1.5829	0.7976	0.228

Table E2: R^2 and slope of short-term recovery based on ln(area)

Treatment	long-term	Period (day)	R^2	R^2	slope
Control	Control1	11-18	Y=0.2382x-0.3665	0.9963	0.255
Atrazine	EC10	11-18	Y=0.2721x-0.5795	0.9956	0.272
	EC20	11-18	Y=0.2736x-0.6271	0.9984	0.274
	EC25	11-18	Y=0.2896x-0.8046	0.9995	0.289
	EC35	11-18	Y=0.2846x-0.8965	0.9964	0.286
	EC50	11-18	Y=0.275x-0.9332	0.9977	0.275
	EC60	11-18	Y=0.2944x-1.2588	0.9977	0.294
	EC75	11-18	Y=0.2931x-1.2995	0.9974	0.293
	EC80	11-18	Y=0.2979x-1.2245	0.9987	0.297
	EC90	11-18	Y=0.2859x-1.5845	0.9903	0.285
	Control2	19-28	Y=0.2058x-0.6088	0.9876	0.205
	EC10	19-28	Y=0.2168x-0.623	0.9846	0.216
	EC20	19-28	Y=0.2045x-0.4408	0.9865	0.204
	EC25	19-28	Y=0.2038x-0.4357	0.9851	0.203
	EC35	19-28	Y=0.2103x-0.3814	0.9776	0.210
	EC50	19-28	Y=0.2182x-0.5594	0.9832	0.218
	EC60	19-28	Y=0.2353x-0.6793	0.9897	0.235
	EC75	19-28	Y=0.2267x-0.5828	0.9872	0.226
	EC80	19-28	Y=0.2128x-0.323	0.9813	0.212
	EC90	19-28	Y=0.1757x-0.3583	0.8888	0.175
	control	19-28	Y=0.2382x-0.3665	0.9963	0.255
2,4-D	EC10	11-18	Y=0.0834x-0.4081	0.9446	0.083
	EC20	11-18	Y=0.0876x-0.4879	0.9341	0.087
	EC25	11-18	Y=0.0884x-0.639	0.9758	0.088
	EC35	11-18	Y=0.0787x-0.8969	0.9633	0.078
	EC50	11-18	Y=0.0749x-0.8719	0.9645	0.074
	EC60	11-18	Y=0.0925x-0.9435	0.9771	0.092
	EC75	11-18	Y=0.0806x-0.8896	0.9465	0.080
	EC80	11-18	Y=0.0934x-1.0169	0.9102	0.093

Treatment	long-term	Period (day)	R^2	R^2	slope
	EC90	11-18	Y=0.0657x-0.6934	0.8902	0.065
2,4-D	Control2	19-28	Y=0.2058x-0.6088	0.9876	0.205
	EC10	19-28	Y=0.1584x-1.3101	0.9747	0.158
	EC20	19-28	Y=0.1682x-1.4559	0.9002	0.168
	EC25	19-28	Y=0.1674x-1.3844	0.9936	0.167
	EC35	19-28	Y=0.0904x-1.2071	0.9657	0.090
	EC50	19-28	Y=0.1262x-1.3203	0.9567	0.126
	EC60	19-28	Y=0.1256x-1.126	0.9695	0.125
	EC75	19-28	Y=0.0808x-1.2616	0.8936	0.080
	EC80	19-28	Y=0.0766x-1.1826	0.9323	0.076
	EC90	19-28	Y=0.1111x-1.1931	0.9454	0.111
alachlor	Control1	11-18	Y=0.1828x-0.3729	0.8963	0.182
	EC10	11-18	Y=0.2278x-0.9993	0.9939	0.227
	EC20	11-18	Y=0.2061x-0.9577	0.9987	0.206
	EC25	11-18	Y=0.2006x-1.1565	0.9907	0.200
	EC35	11-18	Y=0.1946x-1.0452	0.9908	0.194
	EC50	11-18	Y=0.1544x-1.2414	0.9854	0.154
	EC60	11-18	Y=0.1052x-1.3243	0.9641	0.105
	EC75	11-18	Y=0.0665x-1.2694	0.8698	0.066
	EC80	11-18	Y=-0.0032x-1.595	0.0108	-0.003
	EC90	11-18	Y=-0.018x-1.0905	0.2907	-0.018
alachlor	Control2	19-28	Y=0.1904x-0.7115	0.9599	0.190
	EC10	19-28	Y=0.186x-0.6206	0.9756	0.186
	EC20	19-28	Y=0.1994x-1.0254	0.9944	0.199
	EC25	19-28	Y=0.1987x-0.8593	0.9844	0.198
	EC35	19-28	Y=0.2419x-1.5177	0.9947	0.241
	EC50	19-28	Y=0.1958x-1.3949	0.892	0.195
	EC60	19-28	Y=0.1509x-1.2642	0.9729	0.151
	EC75	19-28	Y=0.1016x-1.6818	0.9256	0.101
	EC80	19-28	Y=0.066x-1.5642	0.9109	0.066
	EC90	19-28	Y=0.042x-1.4592	0.2045	0.042

Treatment	long-term	Period (day)	R^2	R^2	slope
Paraquat	Control1	11-18	Y=0.1828x-0.3729	0.8963	0.182
	EC10	11-18	Y=0.2141x-0.6006	0.9958	0.214
	EC20	11-18	Y=0.2294x-0.7151	0.998	0.229
	EC25	11-18	Y=0.2421x-0.8904	0.9976	0.242
	EC35	11-18	Y=0.2063x-0.7557	0.9949	0.206
	EC50	11-18	Y=0.2323x-1.0617	0.9977	0.232
	EC60	11-18	Y=0.2102x-09689	0.9969	0.210
	EC75	11-18	Y=0.0922x-1.0261	0.9769	0.092
	EC80	11-18	Y=-0.00224x-1.09	0.4689	-0.002
	EC90	11-18	Y=-0.027x-1.1605	0.6108	-0.027
Paraquat	Control2	19-28	Y=0.1904x-0.7115	0.9599	0.190
	EC10	19-28	Y=0.2039x-0.9258	0.971	0.204
	EC20	19-28	Y=0.2042x-1.0545	0.9936	0.204
	EC25	19-28	Y=0.1758x-0.8657	0.9867	0.175
	EC35	19-28	Y=0.1912x-1.0626	0.976	0.191
	EC50	19-28	Y=0.2x-0.88	0.9832	0.2
	EC60	19-28	Y=0.1966x-0.9594	0.9914	0.196
	EC75	19-28	Y=0.0793x-0.7891	0.1154	0.079
	EC80	19-28	Y=-0.0247x-1.417	0.1197	-0.024
	EC90	19-28	Y=-0.0355x-1.635	0.4601	-0.035

References

ABD EL-MAGEED, A. E. M. & SHALABY, S. E. M. 2011. Toxicity and Biochemical Impacts of Some New Insecticide Mixtures on Cotton Leafworm Spodoptera littoralis (Boisd.). *Plant Protection Science,* 47**,** 166-175.

ALONSO, A. & CAMARGO, J. A. 2009. Effects of pulse duration and post-exposure period on the nitrite toxicity to a freshwater amphipod. *Ecotoxicology and Environmental Safety,* 72**,** 2005-2008.

ALTENBURGER, R., BOEDEKER, W., FAUST, M. & GRIMME, L. H. 1996. Regulations for combined effects of pollutants: Consequences from risk assessment in aquatic toxicology. *Food and Chemical Toxicology,* 34**,** 1155-1157.

ANGEL, B. M., SIMPSON, S. L. & JOLLEY, D. F. 2010. TOXICITY TO MELITA PLUMULOSA FROM INTERMITTENT AND CONTINUOUS EXPOSURES TO DISSOLVED COPPER. *Environmental Toxicology and Chemistry,* 29**,** 2823-2830.

ARCURY, T. A., GRZYWACZ, J. G., TALTON, J. W., CHEN, H., VALLEJOS, Q. M., GALVAN, L., BARR, D. B. & QUANDT, S. A. 2010. Repeated Pesticide Exposure Among North Carolina Migrant and Seasonal Farmworkers. *American Journal of Industrial Medicine,* 53**,** 802-813.

ARTS, G., DAVIES, J., DOBBS, M., EBKE, P., HANSON, M., HOMMEN, U., KNAUER, K., LOUTSETI, S., MALTBY, L., MOHR, S., POOVEY, A. & POULSEN, V. 2010. AMEG: the new SETAC advisory group on aquatic macrophyte ecotoxicology. *Environmental Science and Pollution Research,* 17**,** 820-823.

ASHAUER, R., AGATZ, A., ALBERT, C., DUCROT, V., GALIC, N., HENDRIKS, J., JAGER, T., KRETSCHMANN, A., O'CONNOR, I., RUBACH, M. N., NYMAN, A. M., SCHMITT, W., STADNICKA, J., VAN DEN BRINK, P. J. & PREUSS, T. G. 2011a. Toxicokinetic-Toxicodynamic Modeling of Quantal and Graded Sublethal Endpoints: A Brief Discussion of Concepts. *Environmental Toxicology and Chemistry,* 30**,** 2519-2524.

ASHAUER, R., BOXALL, A. & BROWN, C. 2006. Predicting effects on aquatic organisms from fluctuating or pulsed exposure to pesticides. *Environmental Toxicology and Chemistry,* 25**,** 1899-1912.

ASHAUER, R., BOXALL, A. B. A. & BROWN, C. D. 2007a. New ecotoxicological model to simulate survival of aquatic invertebrates after exposure to fluctuating and sequential pulses of pesticides. *Environmental Science & Technology,* 41**,** 1480-1486.

ASHAUER, R., BOXALL, A. B. A. & BROWN, C. D. 2007b. Simulating toxicity of carbaryl to Gammarus pulex after sequential pulsed exposure. *Environmental Science & Technology,* 41**,** 5528-5534.

ASHAUER, R. & BROWN, C. D. 2008. Toxicodynamic assumptions in ecotoxicological hazard models. *Environmental Toxicology and Chemistry,* 27**,** 1817-1821.

ASHAUER, R., HINTERMEISTER, A., CARAVATTI, I., KRETSCHMANN, A. & ESCHER, B. I. 2010. Toxicokinetic and Toxicodynamic Modeling Explains

Carry-over Toxicity from Exposure to Diazinon by Slow Organism Recovery. *Environmental Science & Technology,* 44**,** 3963-3971.

ASHAUER, R., THORBEK, P., WARINTON, J. S., WHEELER, J. R. & MAUND, S. 2013. A method to predict and understand fish survival under dynamic chemical stress using standard ecotoxicity data. *Environmental Toxicology and Chemistry,* 32**,** 954-965.

ASHAUER, R., WITTMER, I., STAMM, C. & ESCHER, B. I. 2011b. Environmental Risk Assessment of Fluctuating Diazinon Concentrations in an Urban and Agricultural Catchment Using Toxicokinetic-Toxicodynamic Modeling. *Environmental Science & Technology,* 45**,** 9783-9792.

ASHTON, F. M. & BAYER, D., E 1976. Effects on slute transport and plant constituents. *In:* AUDUS, L. J. (ed.) *Herbicides; Physiology,Biochemistry, Ecology.* London: London, Academic press.

BABEL, M. S., AGARWAL, A., SWAIN, D. K. & HERATH, S. 2011. Evaluation of climate change impacts and adaptation measures for rice cultivation in Northeast Thailand. *Climate Research,* 46**,** 137-146.

BACKHAUS, T. & FAUST, M. 2012. Predictive Environmental Risk Assessment of Chemical Mixtures: A Conceptual Framework. *Environmental Science & Technology,* 46**,** 2564-2573.

BAIRD, C. 1999. *Environmental Chemistry in Society,* London, Taylor&Francis Group.

BEARD, M. J. 2009. *Environmental Chemistry in Society,* London, Taylor&Francis Group.

BEARR, J. S., DIAMOND, J., LATIMER, H. & BOWERSOX, M. 2006. Effects of pulsed copper exposures on early life-stage Pimephales promelas. *Environmental Toxicology and Chemistry,* 25**,** 1376-1382.

BELDEN, J. B., GILLIOM, R. J. & LYDY, M. J. 2007. How well can we predict the toxicity of pesticide mixtures to aquatic life? *Integrated environmental assessment and management,* 3**,** 364-72.

BELDEN, J. B. & LYDY, M. J. 2000. Impact of atrazine on organophosphate insecticide toxicity. *Environmental Toxicology and Chemistry,* 19**,** 2266-2274.

BELGERS, J. D. M., AALDERINK, G. H. & VAN DEN BRINK, P. J. 2009. Effects of four fungicides on nine non-target submersed macrophytes. *Ecotoxicology and Environmental Safety,* 72**,** 579-584.

BISEWSKA, J., SARNOWSKA, E. I. & TUKAJ, Z. H. 2012. Phytotoxicity and antioxidative enzymes of green microalga (Desmodesmus subspicatus) and duckweed (Lemna minor) exposed to herbicides MCPA, chloridazon and their mixtures. *Journal of Environmental Science and Health Part B-Pesticides Food Contaminants and Agricultural Wastes,* 47**,** 814-822.

BLISS, C. I. 1939. The toxicity of poisons applied jointly. *Annals of Applied Biology,* 26**,** 585-615.

BOXALL, A. B. A., FOGG, L. A., ASHAUER, R., BOWLES, T., SINCLAIR, C. J., COLYER, A. & BRAIN, R. A. 2013. Effects of repeated pulsed herbicide exposures on the growth of aquatic macrophytes. *Environmental Toxicology and Chemistry,* 32**,** 193-200.

BRAIN, R. A., ARNIE, J. R., PORCH, J. R. & HOSMER, A. J. 2012a. Recovery of photosynthesis and growth rate in green, blue-green, and diatom algae after exposure to atrazine. *Environmental Toxicology and Chemistry,* 31**,** 2572-2581.

BRAIN, R. A., HOSMER, A. J., DESJARDINS, D., KENDALL, T. Z., KRUEGER, H. O. & WALL, S. B. 2012b. Recovery of duckweed from time-varying exposure to atrazine. *Environmental Toxicology and Chemistry,* 31**,** 1121-1128.

BRIAN, R. 1976. The history and classification of herbicides. *In:* 2ND (ed.) *Herbicides; Physiology, Biochemistry, Ecology.* London: AUDUS.

CALOW, P. 1998. *Handbook of ecotoxicology,* Oxford, Blackwell Science Ltd.

CEDERGREEN, N. 2014. Quantifying Synergy: A Systematic Review of Mixture Toxicity Studies within Environmental Toxicology. *Plos One,* 9.

CEDERGREEN, N., ABBASPOOR, M., SORENSEN, H. & STREIBIG, J. C. 2007a. Is mixture toxicity measured on a biomarker indicative of what happens on a population level? A study with Lemna minor. *Ecotoxicology and Environmental Safety,* 67**,** 323-332.

CEDERGREEN, N., ANDERSEN, L., OLESEN, C. F., SPLIID, H. H. & STREIBIG, J. C. 2005. Does the effect of herbicide pulse exposure on aquatic plants depend on K-ow or mode of action? *Aquatic Toxicology,* 71**,** 261-271.

CEDERGREEN, N., CHRISTENSEN, A. M., KAMPER, A., KUDSK, P., MATHIASSEN, S. K., STREIBIG, J. C. & SORENSEN, H. 2008. A review of independent action compared to concentration addition as reference models for mixtures of compounds with different molecular target sites. *Environmental Toxicology and Chemistry,* 27**,** 1621-1632.

CEDERGREEN, N., KAMPER, A. & STREIBIG, J. C. 2006. Is prochloraz a potent synergist across aquatic species? A study on bacteria, daphnia, algae and higher plants. *Aquatic Toxicology,* 78**,** 243-252.

CEDERGREEN, N., KUDSK, P., MATHIASSEN, S. K., SORENSEN, H. & STREIBIG, J. C. 2007b. Reproducibility of binary-mixture toxicity studies. *Environmental Toxicology and Chemistry,* 26**,** 149-156.

CEDERGREEN, N., KUDSK, P., MATHIASSEN, S. K. & STREIBIG, J. C. 2007c. Combination effects of herbicides on plants and algae: do species and test systems matter? *Pest Management Science,* 63**,** 282-295.

CEDERGREEN, N., SVENDSEN, C. & BACKHAUS, T. 2013. Toxicity prediction of chemical mixtures. *Encyclopedia of Environmental Management.* New York: Taylor&Francis.

CHALERMPHOL, J. & SHIVAKOTI, P. J. 2009. Pesticide use and prevention practices of tangerine growers in Northern Thailand. *Agricultural Education and Extension,* 15**,** 21-38.

CHANDRA, A., RANA, J. & LI, Y. Q. 2001. Separation, identification, quantification, and method validation of anthocyanins in botanical supplement raw materials by HPLC and HPLC-MS. *Journal of Agricultural and Food Chemistry,* 49**,** 3515-3521.

CHEN, D. G. 2009. A quantal statistical isobologram model to identify joint action for chemical mixtures. *Environmetrics,* 20**,** 101-109.

CHESWORTH, J. C., DONKIN, M. E. & BROWN, M. T. 2004. The interactive effects of the antifouling herbicides Irgarol 1051 and Diuron on the seagrass Zostera marina (L.). *Aquatic Toxicology,* 66**,** 293-305.

CHULINTORN, P. 2002. Distribution of pesticides from agriculutural area to the main rivers in Thailand. *Agricultural Toxic Substances Division.* Krabi, Thailand.

CLEUVERS, M. 2003. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. *Toxicology Letters,* 142**,** 185-194.

CONNELL, C., LAM, P., RICHARDSON, B. & WU, R. 1999. *Introduction to ecotoxicology,* Blackwell

CONNICK, J., SIMONEAUX, M. & JACQUELINE, M. 1982. Determination of (2,4-Dichlorophenoxy) acetic acid and 2,6-Dichlorobenonitrile in water by High Performance Liquid Chromatography. *Agricul. Food Chem,* 30**,** 258-260.

DAAM, M. A., RODRIGUES, A. M. F., VAN DEN BRINK, P. J. & NOGUEIRA, A. J. A. 2009. Ecological effects of the herbicide linuron in tropical freshwater microcosms. *Ecotoxicology and Environmental Safety,* 72**,** 410-423.

DAAM, M. A., SANTOS PEREIRA, A. C., SILVA, E., CAETANO, L. & CEREJEIRA, M. J. 2013. Preliminary aquatic risk assessment of imidacloprid after application in an experimental rice plot. *Ecotoxicology and Environmental Safety,* 97**,** 78-85.

DAAM, M. A. & VAN DEN BRINK, P. J. 2010. Implications of differences between temperate and tropical freshwater ecosystems for the ecological risk assessment of pesticides. *Ecotoxicology,* 19**,** 24-37.

DALTON, R. L., NUSSBAUMER, C., PICK, F. R. & BOUTIN, C. 2013. Comparing the sensitivity of geographically distinct Lemna minor populations to atrazine. *Ecotoxicology,* 22**,** 718-730.

DAMALAS, C. A. 2004. Review herbicide tank mixtures: Common interactions. *International Journal of Agriculture&Biology,* 6**,** 208-212.

DAS, A. C., CHAKRAVARTY, A., SUKUL, P. & MUKHERJEE, D. 1995. INSECTICIDES - THEIR EFFECT ON MICROORGANISMS AND PERSISTENCE IN RICE SOIL. *Microbiological Research,* 150**,** 187-194.

DAVIES, J., HONEGGER, J. L., TENCALLA, F. G., MEREGALLI, G., BRAIN, P., NEWMAN, J. R. & PITCHFORD, H. F. 2003. Herbicide risk assessment for non-target aquatic plants: sulfosulfuron - a case study. *Pest Management Science,* 59**,** 231-237.

DECHACHETE, T. & NUTHALL, P. 2002. Organic Farming in Thailand Case Studies on Fruit and Flower Production in Chiangmai, Thailand. *In:* 02/2002 (ed.) *1174-8796.* Farm and horticultural Management. Canterbury, New Zealand: Lincoln University.

DENEER, J. W. 2000. Toxicity of mixtures of pesticides in aquatic systems. *Pest Management Science,* 56**,** 516-520.

DENNIS, N., TIEDE, K. & THOMPSON, H. 2012. Repeated and multiple stress (exposure to pesticides) on aquatic organisms. *In:* 2012:EN-347, S. P. (ed.). York, UK: Food and Environment Research Agency, Sand Hutton, York, UK.

DEWEZ, D., DAUTREMEPUITS, C., JEANDET, P., VERNET, G. & POPOVIC, R. 2003. Effects of methanol on photosynthetic processes and growth of Lemna gibba. *Photochemistry and Photobiology,* 78**,** 420-424.

DIAMOND, J. M., KLAINE, S. J. & BUTCHER, J. B. 2006. Implications of pulsed chemical exposures for aquatic life criteria and wastewater permit limits. *Environmental Science & Technology,* 40**,** 5132-5138.

DROST, W. 2011. *The factor time in assessing ecotoxicity. Studies with herbicides and metals applied singly and in combination to Lemna minor in simple and complex time patterns.* PhD, Bremen.

DROST, W., BACKHAUS, T., VASSILAKAKI, M. & GRIMME, L. H. 2003. Mixture toxicity of s-triazines to Lemna minor under conditions of simultaneous and sequential exposure. *Fresenius Environmental Bulletin,* 12**,** 601-607.

DROST, W., MATZKE, M. & BACKHAUS, T. 2007. Heavy metal toxicity to Lemna minor: studies on the time dependence of growth inhibition and the recovery after exposure. *Chemosphere,* 67**,** 36-43.

ECOBICHON, D. J. 2001. Pesticide use in developing countries. *Toxicology,* 160**,** 27-33.

ESCHER, B. I., ASHAUER, R., DYER, S., HERMENS, J. L. M., LEE, J.-H., LESLIE, H. A., MAYER, P., MEADOR, J. P. & WARNE, M. S. J. 2011. Crucial Role of Mechanisms and Modes of Toxic Action for Understanding Tissue Residue Toxicity and Internal Effect Concentrations of Organic Chemicals. *Integrated Environmental Assessment and Management,* 7**,** 28-49.

EUROPEAN & MEDITERRANEAN PLANT PROTECTION, O. 1997. Guideline for the efficacy evaluation of plant protection products: Phytotoxicity assessment. *Bulletin OEPP,* 27**,** 389-400.

FABRO, L. & VARCA, L. M. 2012. Pesticide usage by farmers in Pagsanjan-Lumban catchment of Laguna de Bay, Philippines. *Agricultural Water Management,* 106**,** 27-34.

FAIRCHILD, J. F., RUESSLER, D. S., HAVERLAND, P. S. & CARLSON, A. R. 1997. Comparative sensitivity of Selenastrum capricornutum and Lemna minor to sixteen herbicides. *Archives of Environmental Contamination and Toxicology,* 32**,** 353-357.

FAUST, M., ALTENBURGER, R., BACKHAUS, T., BLANCK, H., BOEDEKER, W., GRAMATICA, P., HAMER, V., SCHOLZE, M., VIGHI, M. & GRIMME, L. H. 2001. Predicting the joint algal toxicity of multi-component s-triazine mixtures at low-effect concentrations of individual toxicants. *Aquatic Toxicology,* 56**,** 13-32.

FERREIRA, A. L. G., LOUREIRO, S. & SOARES, A. M. V. M. 2008. Toxicity prediction of binary combinations of cadmium, carbendazim and low dissolved oxygen on Daphnia magna. *Aquatic Toxicology,* 89**,** 28-39.

FISHEL, F. 1991. Pesticide and the Environment.

FLORES, F., COLLIER, C. J., MERCURIO, P. & NEGRI, A. P. 2013. Phytotoxicity of Four Photosystem II Herbicides to Tropical Seagrasses. *Plos One,* 8.

FU, R. 2008. Analysis of atrazine in drinking water at the ppb level using new agilent reversed phase LC columns. *Agilent Technologies* [Online], 52.

GEOFFROY, L., FRANKART, C. & EULLAFFROY, P. 2004. Comparison of different physiological parameter responses in Lemna minor and Scenedesmus obliquus exposed to herbicide flumioxazin. *Environ Pollut,* 131**,** 233-41.

GRECO, W. R., BRAVO, G. & PARSONS, J. C. 1995. THE SEARCH FOR SYNERGY - A CRITICAL-REVIEW FROM A RESPONSE-SURFACE PERSPECTIVE. *Pharmacological Reviews,* 47**,** 331-385.

GREEN, J. M. 1989. HERBICIDE ANTAGONISM AT THE WHOLE PLANT-LEVEL. *Weed Technology,* 3**,** 217-226.

GROVERMANN, C., SCHREINEMACHERS, P. & BERGER, T. 2013. Quantifying pesticide overuse from farmer and societal points of view: An application to Thailand. *Crop Protection,* 53**,** 161-168.

GUNSOLUS, L. J.&CURRAN, S. W.2002. *Herbicide mode of action and injury symptoms*[Online]. North Central Regional Extension Publication No. 377: University of Minnesota. [Accessed 2015].

GUO, Y., PUNNASIRI, K. & TONG, S. 2012. Effects of temperature on mortality in Chiang Mai city, Thailand: a time series study. *Environmental Health,* 11.

HADRUP, N., TAXVIG, C., PEDERSEN, M., NELLEMANN, C., HASS, U. & VINGGAARD, A. M. 2013. Concentration Addition, Independent Action and Generalized Concentration Addition Models for Mixture Effect Prediction of Sex Hormone Synthesis In Vitro. *Plos One,* 8.

HE, H., CHEN, G., YU, J., HE, J., HUANG, X., LI, S., GUO, Q., YU, T. & LI, H. 2013. Individual and Joint Toxicity of Three Chloroacetanilide Herbicides to Freshwater Cladoceran Daphnia carinata. *Bulletin of Environmental Contamination and Toxicology,* 90**,** 344-350.

HERTZBERG, R. C. & MACDONELL, M. M. 2002. Synergy and other ineffective mixture risk definitions. *Science of the Total Environment,* 288**,** 31-42.

HOGAN, A., VAN DAM, R., TRENFIELD, M. & HARFORD, A. 2012. Toxicity of single magnesium pluse exposures to tropical freshwater species. Australia: Department of Sustainability, Environment, Water, Population and Communities Supervising Scientist.

HOLZMANN, S., DITTRICH, P. & POCH, C. 1999. Dose response curves of vasorelaxants: computerized calculation of combination effects using the example of nicorandil. *Clin Basic Cardiol,* 2**,** 96.

HOSAMANI, K., U. 2009. *Economic Consequence of Pesticides Use in Paddy Koppal District, Karnataka.* Master of Science, University of Agricultural Sciences.

HOSMER, A. J., WARREN, L. W. & WARD, T. J. 1998. Chronic toxicity of pulse-dosed fenoxycarb to Daphnia magna exposed to environmentally realistic concentrations. *Environmental Toxicology and Chemistry,* 17**,** 1860-1866.

HUDAK, P. F. & THAPINTA, A. 2005. Agricultural pesticides in groundwater of Kanchana Buri, Ratcha Buri, and Suphan Buri provinces, Thailand. *Bulletin of Environmental Contamination and Toxicology,* 74**,** 631-636.

HUGHE, J. S., ALEXANDER, M. M.&BALU, K.1933. An evaluation of appropriate expression of toxicity in aquatic plant bioassay as demostrated by the effects of atrazine on algae and duckweed. *In:*WILLIAM, J. A. (ed.) *Aquatic Toxicology and Hazard Assessment.*ASTM international.

IWAI, C. B., SOMPARN, A.&NOLLER, B.2011a. *Using Zooplankton, Moina Micrura Kurz to Evaluate the Ecotoxicology of Pesticides Used in Paddy Fields of Thailand*.

IWAI, C. B., SOMPARN, A. & NOLLER, B. 2011b. Using zooplankton, *Moina Micrura* Kurz to Evaluate the Ecotoxicology of Pesticides Used in Paddy Fields of Thailand. *In:* STOYTCHEVA, D. M. (ed.) *Pesticides in the Modern World - Risks and Benefits.*

IWAI, C. B., SUJIRA, H., SOMPARN, A., KOMAROVA, T., MUELLER, J. & NOLLER, B. 2007. Monitoring Pesticides in the Paddy Field Ecosystem of North-Eastern Thailand for Environmental and Health Risks. *Rational Environmental Management of Agrochemicals: Risk Assessment, Monitoring, and Remedial Action,* 966**,** 259-273.

JAIPIEAM, S., VISUTHISMAJARN, P., SUTHERAVUT, P., SIRIWONG, W., THOUMSANG, S., BORJAN, M. & ROBSON, M. 2009. Organophosphate

Pesticide Residues in Drinking Water from Artesian Wells and Health Risk Assessment of Agricultural Communities, Thailand. *Hum Ecol Risk Assess,* 15**,** 1304-1316.

JUNGBLUTH, F. 1996. Crop protection policy in Thailand. *In:* JUNGBLUTH, F. (ed.) *Economic and Political Factors Influencing Pesticide Use.* Hannover: The Institute of Horticultural Economics, Herrenhauser Str.2.

JUNGHANS, M., BACKHAUS, T., FAUST, M., SCHOLZE, M. & GRIMME, L. H. 2006. Application and validation of approaches for the predictive hazard assessment of realistic pesticide mixtures. *Aquatic Toxicology,* 76**,** 93-110.

KERLE, E. A., JENKINS, J. J. & VOGUE, P. A. 2007. Understanding pesticide persistence and mobility for groundwater and surface water protection.

KIRBY, M. F. & SHEAHAN, D. A. 1994. Effects of atrazine, isoproturon, and mecoprop on the macrophyte Lemna minor and the alga Scenedesmus subspicatus. *Bull Environ Contam Toxicol,* 53**,** 120-6.

KISS, I., KOVATS, N. & SZALAY, T. 2003. Evaluation of some alternative guidelines for risk assessment of various habitats. *Toxicology Letters,* 140**,** 411-417.

KOVACH, J., PETZOLDT, C., DEGNIL, J. & TETTE, J. 1992. A method to measure the environmental impact of pesticides. *New York's Food and Life Sciences Bulletin.*

KRETSCHMANN, A., ASHAUER, R., HOLLENDER, J. & ESCHER, B. I. 2012. Toxicokinetic and toxicodynamic model for diazinon toxicity-mechanistic explanation of differences in the sensitivity of Daphnia magna and Gammarus pulex. *Environmental Toxicology and Chemistry,* 31**,** 2014-2022.

LAETZ, C. A., BALDWIN, D. H., COLLIER, T. K., HEBERT, V., STARK, J. D. & SCHOLZ, N. L. 2009. The Synergistic Toxicity of Pesticide Mixtures: Implications for Risk Assessment and the Conservation of Endangered Pacific Salmon. *Environmental Health Perspectives,* 117**,** 348-353.

LARRAS, F., LAMBERT, A.-S., PESCE, S., RIMET, F., BOUCHEZ, A. & MONTUELLE, B. 2013. The effect of temperature and a herbicide periphytic algae mixture on freshwater. *Ecotoxicology and Environmental Safety,* 98**,** 162-170.

LARSON, J. S., CAPEL, D. P. & MAJEWSKI, S. M. 1997. *Pesticides in Surface Waters: Distribution, Trends, and Governing Factors (Pesticides in the Hydrologic System),* Ann Arbor Press, Inc. Chelsea, Michigan, U.S., CRC Press.

LENG, R. A. 1999. *Duckweed: A tiny aquatic plant with enormous potential for agriculture and environment,* Rome (Italy), FAO.

LLOYD, C. W., LOWE, S. B. & PEACE, G. W. 1980. The mode of action of 2,4-D in counter acting the elongation of carot cell growth culture. *Cell Scinece,* 45**,** 257-268.

LLOYD, R. 1987. Special tests in aquatic toxicity for chemical mixture: Interactions and modification of response by variation of physicochemical conditions. *Methods for Assessing the Effects of Mixtures of Chemicals***,** 491-507.

LYDEARD, C. & MAYDEN, R. L. 1995. A DIVERSE AND ENDANGERED AQUATIC ECOSYSTEM OF THE SOUTHEAST UNITED-STATES. *Conservation Biology,* 9**,** 800-805.

MACHADO, S. G. & ROBINSON, G. A. 1994. A DIRECT, GENERAL-APPROACH BASED ON ISOBOLOGRAMS FOR ASSESSING THE

JOINT ACTION OF DRUGS IN PRECLINICAL EXPERIMENTS. *Statistics in Medicine,* 13**,** 2289-2309.

MALTBY, L., ARNOLD, D., ARTS, G., DAVIES, J., HEIMBACH, F., PICKL, C.&POULSEN, V.2010. *Aquatic macrophyte risk assessment for pesticides,* SETAC publication, Taylor&Francis Group.

MARVIER, M. 2002. Improving risk assessment for nontarget safety of transgenic crops. *Ecological Applications,* 12**,** 1119-1124.

MATTHEWS, G. A.1979. *Pesticide Application Methods, Third Edition,* Essex, England, Longman Scientific & Technical.

MICHEL, A., JOHNSON, R. D., DUKE, S. O. & SCHEFFLER, B. E. 2004. Dose-response relationships between herbicides with different modes of action and growth of Lemna paucicostata: An improved ecotoxicological method. *Environmental Toxicology and Chemistry,* 23**,** 1074-1079.

MIKKELSEN, L. 2012. Mixture Toxicity Call-for-Action. The Danish Ecological Council: The Danish Ecological Council.

MILNE, I., SEAGER, J., MALLETT, M. & SIMS, I. 2000. Effects of short-term pulsed ammonia exposure on fish. *Environmental Toxicology and Chemistry,* 19**,** 2929-2936.

MINTON, B. W., KURTZ, M. E. & SHAW, D. R. 1989. BARNYARDGRASS (ECHINOCHLOA-CRUS-GALLI) CONTROL WITH GRASS AND BROADLEAF WEED HERBICIDE COMBINATIONS. *Weed Science,* 37**,** 223-227.

MOHAMMAD, M., ITOH, K. & SUYAMA, K. 2008. Comparative effects of different families of herbicides on recovery potentials in Lemna sp. *Journal of Pesticide Science,* 33**,** 171-174.

MOHAMMAD, M., ITOH, K. & SUYAMA, K. 2010. Effects of Herbicides on Lemna gibba and Recovery from Damage After Prolonged Exposure. *Archives of Environmental Contamination and Toxicology,* 58**,** 605-612.

MOHAMMAD, M., ITOH, K. & SUYAMA, K. 2011. Mixture toxicity of herbicides on Lemna gibba and recovery potential after prolonged exposure (PO). *Canadian Technical Report of Fisheries and Aquatic Sciences,* 2949**,** 65-65.

MOHAMMAD, M., ITOH, K., SUYAMA, K. & YAMAMOTO, H. 2006. Recovery of Lemna sp after exposure to sulfonylurea herbicides. *Bulletin of Environmental Contamination and Toxicology,* 76**,** 256-263.

MOHR, S., SCHOTT, J., MALETZKI, D. & HUENKEN, A. 2013. Effects of toxicants with different modes of action on Myriophyllum spicatum in test systems with varying complexity. *Ecotoxicology and Environmental Safety,* 97**,** 32-39.

MULLAR, J. D. 1996. *Pesticide movement in soils-Groundwater protection,* Washington State University.

MULLER, R., BERGHAHN, R. & HILT, S. 2010. Herbicide effects of metazachlor on duckweed (Lemna minor and Spirodela polyrhiza) in test systems with different trophic status and complexity. *J Environ Sci Health B,* 45**,** 95-101.

MUNKEGAARD, M., ABBASPOOR, M. & CEDERGREEN, N. 2008. Organophosphorous insecticides as herbicide synergists on the green algae Pseudokirchneriella subcapitata and the aquatic plant Lemna minor. *Ecotoxicology,* 17**,** 29-35.

NORGAARD, K. B. & CEDERGREEN, N. 2010. Pesticide cocktails can interact synergistically on aquatic crustaceans. *Environmental Science and Pollution Research,* 17**,** 957-967.

NYMAN, A. M., SCHIRMER, K. & ASHAUER, R. 2012. Toxicokinetic-toxicodynamic modelling of survival of Gammarus pulex in multiple pulse exposures to propiconazole: model assumptions, calibration data requirements and predictive power. *Ecotoxicology,* 21**,** 1828-1840.

OFLAZ, H., TURKMEN, A., KOCAMAN, O., ERDOGAN, D., MERIC, M., ONCUL, A., KOYLAN, N., YILMAZ, E., YILMAZ, C., SELCUKBIRICIK, F., KASIKCIOGLU, E. & SEVER, M. S. 2004. Is there a relation between duration of cyclosporine usage and right and left ventricular function in renal transplant patients?: Tissue Doppler echocardiography study. *Transplantation Proceedings,* 36**,** 1380-1384.

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT. 2006. Test No. 221: Lemna sp. Growth Inhabition Test. *OECD Guidelines for the Testing of Chemicals, Section 2: Effects on Biotic Systems,*. Paris: OECD Publishing.

PANUWET, P., PRAPAMONTOL, T., CHANTARA, S., THAVORNYUTHIKARN, P., MONTESANO, M. A., WHITEHEAD, R. D., JR. & BARR, D. B. 2008a. Concentrations of urinary pesticide metabolites in small-scale farmers in Chiang Mai Province, Thailand. *Sci Total Environ,* 407**,** 655-68.

PANUWET, P., PRAPAMONTOL, T., CHANTARA, S., THAVORNYUTHIKARN, P., MONTESANO, M. A., WHITEHEAD, R. D., JR. & BARR, D. B. 2008b. Concentrations of urinary pesticide metabolites in small-scale farmers in Chiang Mai Province, Thailand. *Science of the Total Environment,* 407**,** 655-668.

PANUWET, P., SIRIWONG, W., PRAPAMONTOL, T., RYAN, P. B., FIEDLER, N., ROBSON, M. G. & BARR, D. B. 2012a. Agricultural Pesticide Management in Thailand: Situation and Population Health Risk. *Environ Sci Policy,* 17**,** 72-81.

PANUWET, P., SIRIWONG, W., PRAPAMONTOL, T., RYAN, P. B., FIEDLER, N., ROBSON, M. G. & BARR, D. B. 2012b. Agricultural pesticide management in Thailand: status and population health risk. *Environmental Science & Policy,* 17**,** 72-81.

PAVLAKI, M. D., PEREIRA, R., LOUREIRO, S. & SOARES, A. M. V. M. 2011. Effects of binary mixtures on the life traits of Daphnia magna. *Ecotoxicology and Environmental Safety,* 74**,** 99-110.

PHYU, Y. L., PALMER, C. G., WARNE, M. S. J., HOSE, G. C., CHAPMAN, J. C. & LIM, R. P. 2011. A comparison of mixture toxicity assessment: Examining the chronic toxicity of atrazine, permethrin and chlorothalonil in mixtures to Ceriodaphnia cf. dubia. *Chemosphere,* 85**,** 1568-1573.

PIMENTEL, D., ACQUAY, H., BILTONEN, M., RICE, P., SILVA, M., NELSON, J., LIPNER, V., GIORDANO, S., HOROWITZ, A. & D'AMORE, M. 1992. Environmental and Economic Costs of Pesticide Use. *BioScience,* 42**,** 750-760.

PINTO DE CARVALHO, S. J., NICOLAI, M., FERREIRA, R. R., DE OLIVEIRA FIGUEIRA, A. V. & CHRISTOFFOLETI, P. J. 2009. HERBICIDE SELECTIVITY BY DIFFERENTIAL METABOLISM: CONSIDERATIONS FOR REDUCING CROP DAMAGES. *Scientia Agricola,* 66**,** 136-142.

PLIANBANGCHANG, P., JETIYANON, K. & WITTAYA-AREEKUL, S. 2009. Pesticide use patterns among small-scale farmers: a case study from

Phitsanulok, Thailand. *Southeast Asian J Trop Med Public Health,* 40**,** 401-10.

PRANEETVATAKU, S., SCHREINEMACHERS, P., PANANURAK, P. & TIPRAQSA, P. 2013. Pesticides, external costs and policy options for Thai agriculture. *Environmental Science & Policy,* 27**,** 103-113.

PRANEETVATAKULL, S. & WAIBEL, H. Year. The impact of farmer field school on pesticide use and environment in Thailand. *In:* Annual Meeting of the German Association of Agricultural Economist (Gewisoloa) in Giessen, 4-6 October 2006 2006 Germany.

REESE, D., C, DODSON, W., I, BECKER, L., D & KEMPTER, J., C 1972. Pesticides in the aquatic environment. Environmental Protention Agency.

REUNGLERTPANYAKUL, V. 2001. National Study: Thailand "Exploring the Potential of Organic Agriculutre for Rural Poverty Alleviation in Asia and the Pacific". *In:* REUNGLERTPANYAKUL, V. (ed.) *United Nations Economic and Social Commission for Asia and the Pacific regional Workshop.* Chiang Mai, Thailand, 26-29 Novermber: Organic farming

RICCIARDI, A. & RASMUSSEN, J. B. 1999. Extinction rates of North American freshwater fauna. *Conservation Biology,* 13**,** 1220-1222.

RIDER, C. V. & LEBLANC, G. A. 2005. An integrated addition and interaction model for assessing toxicity of chemical mixtures. *Toxicological Sciences,* 87**,** 520-528.

RODNEY, S. I., TEED, R. S. & MOORE, D. R. J. 2013. Estimating the Toxicity of Pesticide Mixtures to Aquatic Organisms: A Review. *Human and Ecological Risk Assessment,* 19**,** 1557-1575.

ROSENKRANTZ, R. T., BAUN, A. & KUSK, K. O. 2013. Growth inhibition and recovery of Lemna gibba after pulse exposure to sulfonylurea herbicides. *Ecotoxicology and Environmental Safety,* 89**,** 89-94.

SANGCHAN, W., BANNWARTH, M., INGWERSEN, J., HUGENSCHMIDT, C., SCHWADORF, K., THAVORNYUTIKARN, P., PANSOMBAT, K. & STRECK, T. 2014. Monitoring and risk assessment of pesticides in a tropical river of an agricultural watershed in northern Thailand. *Environmental Monitoring and Assessment,* 186**,** 1083-1099.

SANGCHAN, W., HUGENSCHMIDT, C., INGWERSEN, J., SCHWADORF, K., THAVORNYUTIKARN, P., PANSOMBAT, K. & STRECK, T. 2012. Short-term dynamics of pesticide concentrations and loads in a river of an agricultural watershed in the outer tropics. *Agriculture Ecosystems & Environment,* 158**,** 1-14.

SCHREINEMACHERS, P. & TIPRAQSA, P. 2012. Agricultural pesticides and land use intensification in high, middle and low income countries. *Food Policy,* 37**,** 616-626.

SEMATHONG, S., ZAPUANG, K. & KITANA, N. 2008. Pesticide use, farmer knowledge and awareness in Thong Phaphum Region, Kanchanaburi province. *Health Research,* 22**,** 15-20.

SHIMABUKURO, R. A. S., HR. 1969. Atrazine metabolism, selectivity, and Mode of Action. *Symposium.*

SOLOMON, K. R. 1996. Overview of recent developments in ecotoxicological risk assessment. *Risk Analysis,* 16**,** 627-633.

SOLOMON, K. R., GIESY, J. P., LAPOINT, T. W., GIDDINGS, J. M. & RICHARDS, R. P. 2013. Ecological risk assessment of atrazine in North

American surface waters. *Environmental Toxicology and Chemistry,* 32**,** 10-11.

SONG, Y. 2014. Insight into the mode of action of 2,4-dichlorophenoxyacetic acid (2,4-D) as an herbicide. *Journal of integrative plant biology,* 56**,** 106-13.

SORENSEN, H., CEDERGREEN, N., SKOVGAARD, I. M. & STREIBIG, J. C. 2007. An isobole-based statistical model and test for synergism/antagonism in binary mixture toxicity experiments. *Environmental and Ecological Statistics,* 14**,** 383-397.

SORENSEN, H., CEDERGREEN, N. & STREIBIG, J. C. 2010. A Random Effects Model for Binary Mixture Toxicity Experiments. *Journal of Agricultural Biological and Environmental Statistics,* 15**,** 562-577.

SYBERG, K., ELLEBY, A., PEDERSEN, H., CEDERGREEN, N. & FORBES, V. E. 2008. Mixture toxicity of three toxicants with similar and dissimilar modes of action to Daphnia magna. *Ecotoxicology and Environmental Safety,* 69**,** 428-436.

SYBERG, K., JENSEN, T. S., CEDERGREEN, N. & RANK, J. 2009. On the Use of Mixture Toxicity Assessment in REACH and the Water Framework Directive: A Review. *Human and Ecological Risk Assessment,* 15**,** 1257-1272.

TABASHNIK, B. E. 1989. MANAGING RESISTANCE WITH MULTIPLE PESTICIDE TACTICS - THEORY, EVIDENCE, AND RECOMMENDATIONS. *Journal of Economic Entomology,* 82**,** 1263-1269.

TALLARIDA, R. J. 2006. An overview of drug combination analysis with isobolograms. *Journal of Pharmacology and Experimental Therapeutics,* 319**,** 1-7.

TAYLOR, E. J., MAUND, S. J. & PASCOE, D. 1991. TOXICITY OF 4 COMMON POLLUTANTS TO THE FRESH-WATER MACROINVERTEBRATES CHIRONOMUS-RIPARIUS-MEIGEN (INSECTA, DIPTERA) AND GAMMARUS-PULEX (L) (CRUSTACEA, AMPHIPODA). *Archives of Environmental Contamination and Toxicology,* 21**,** 371-376.

TEODOROVIC, I., KNEZEVIC, V., TUNIC, T., CUCAK, M., LECIC, J. N., LEOVAC, A. & TUMBAS, II 2011. Myriophyllum aquaticum vs. Lemna minor: sensitivity and recovery potential after exposure to atrazine. *Environ Toxicol Chem.*

TEODOROVIC, I., KNEZEVIC, V., TUNIC, T., CUCAK, M., LECIC, J. N., LEOVAC, A. & TUMBAS, I. I. 2012. Myriophyllum aquaticum versus Lemna minor: Sensitivity and recovery potential after exposure to atrazine. *Environmental Toxicology and Chemistry,* 31**,** 417-426.

THAPINTA, A. & HUDAK, F. P. 1998. Pesticide use and residual occurence in Thailand. *Environmental Monitoring and Assessment,* 60**,** 103-114.

THAPINTA, A. & HUDAK, P. F. 2003. Use of geographic information systems for assessing groundwater pollution potential by pesticides in Central Thailand. *Environment International,* 29**,** 87-93.

TIRADO, R., ENGLANDE, J. A., PROMAKASIKORN, L. & NOVOTNY, V. 2008. Use of agrochemicals in Thailand and its consequences for the environment. Greenpeace Research Laboratories Technical Note 03/2008.

TIRYAKI, O. & TEMUR, C. 2010. The fate of pesticide in the environment. *environmental Science,* 4**,** 29-38.

TOMLIN, C. 1997. *Pesticide:Handbooks manual,* Fumham, Surrey, British Crop Protect Council.

TREBST, A. 2008. *The Mode of Action of Triazine Herbicides in Plants*.

TU, M., HURD, C. & RANDALL, J. M. 2001. Weed Control Methods Handbook. *The Nature Conservancy* [Online]. [Accessed May 2011].

VALE, J. A. 1998. Toxicokinetic and toxicodynamic aspects of organophosphorus (OP) insecticide poisoning. *Toxicology Letters,* 103**,** 649-652.

VALLOTTON, N., ILDA, R., EGGEN, L., ESCHER, B. I., KRAYENBUHL, J. & CHEVRE, N. 2008a. Effect of pulse herbicidal exposure on Scenedesmus vacuolatus: A comparison of two photosystem II inhibitors. *Environmental Toxicology and Chemistry,* 27**,** 1399-1407.

VALLOTTON, N., MOSER, D., EGGEN, R. I. L., JUNGHANS, M. & CHEVRE, N. 2008b. S-metolachlor pulse exposure on the alga Scenedesmus vacuolatus: Effects during exposure and the subsequent recovery. *Chemosphere,* 73**,** 395-400.

VALLOTTON, P. 2008. Differential aberration correction (DAC) microscopy: a new molecular ruler. *Journal of Microscopy,* 232**,** 235-239.

VAN OORSCHOT, J., LP 1976. Effects in relation to water and carbon dioxide exchange on plants. *In:* AUDUS, L. J. (ed.) *Herbicides; Physiology, Biochemistry, Ecology.* London: London, Academic press.

WANG, W. C. 1990. LITERATURE-REVIEW ON DUCKWEED TOXICITY TESTING. *Environmental Research,* 52**,** 7-22.

WANG, W. C. 1991. LITERATURE-REVIEW ON HIGHER-PLANTS FOR TOXICITY TESTING. *Water Air and Soil Pollution,* 59**,** 381-400.

WANG, W. C. & WILLIAMS, J. M. 1990. THE USE OF PHYTOTOXICITY TESTS (COMMON DUCKWEED, CABBAGE, AND MILLET) FOR DETERMINING EFFLUENT TOXICITY. *Environmental Monitoring and Assessment,* 14**,** 45-58.

WIBOONPONGSE, A. & CHAOVANAPOONPHOL, Y. 2001. Rice marketing system in Thailand. *In:* WIBOONPONGSE, A. (ed.) *Agribusiness Management towards Strengthening Agricultural Development and Trade.* Multiple Cropping Center, Chiang Mai University.

WILSON, P. C. & KOCH, R. 2013. Influence of Exposure Concentration and Duration on Effects and Recovery of Lemna minor Exposed to the Herbicide Norflurazon. *Archives of Environmental Contamination and Toxicology,* 64**,** 228-234.

YEO, R. R. 1967. DISSIPATION OF DIQUAT AND PARAQUAT AND EFFECTS ON AQUATIC WEEDS AND FISH. *Weeds,* 15**,** 42-&.

ZEZULKA, S., KUMMEROVA, M., BABULA, P. & VANOVA, L. 2013. Lemna minor exposed to fluoranthene: Growth, biochemical, physiological and histochemical changes. *Aquatic Toxicology,* 140**,** 37-47.

ZHANG, J. H., HAMILL, A. S. & WEAVER, S. E. 1995. ANTAGONISM AND SYNERGISM BETWEEN HERBICIDES - TRENDS FROM PREVIOUS STUDIES. *Weed Technology,* 9**,** 86-90.

ZHANG, Y.-H., LIU, S.-S., LIU, H.-L. & LIU, Z.-Z. 2010. Evaluation of the combined toxicity of 15 pesticides by uniform design. *Pest Management Science,* 66**,** 879-887.

ZIMDAHL, L. R. 1999. *Fundamentals of Weed Science,* USA, Academic Press.