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Mixture and single-compound toxicity using *Daphnia magna*

– Comparisons with estimates of concentration addition and independent action

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Abstract

Aquatic organisms are usually not exposed to single substances but rather to mixtures of toxicants in streams located in agricultural areas. The transport of pesticides used in the agricultural area in Sweden is monitored with continuous environmental supervision every year. During 2002-2008 an average of 10 pesticides were found in each sample and 40% of the samples contained substances with concentrations higher than the Water Quality Standards. Chemical risk management is normally based on single-test evaluations. A problem when assessing mixture toxicities is that the constituents and concentrations of pollutants in the environment vary greatly. Therefore experimental testing of all possible combinations of constituents in a mixture in the environment is not possible. Models such as concentration addition and independent action have been developed that allow an estimation of the toxicity of mixtures on the basis of the toxicity of the single compounds. In most cases, these models give quite accurate estimations of the toxicity of mixtures. This study was aimed at testing three insecticides (pirimicarb, fenitrothion and esfenvalerate) neurotoxic to *Daphnia magna* in order to evaluate if synergistic, antagonistic or strictly additive effects occur when added together in a mixture. The study also aimed at investigating if the mixed exposure toxicity can be predicted with any of the concentration addition and independent action models. The selected insecticides have been used frequently in Swedish agriculture and have been found above the Water Quality Standards in Swedish surface waters for several years during environmental monitoring. The highest concentration found in surface waters for esfenvalerate exceeded the NOEC and therefore negative effects on *Daphnias* can be expected. The highest concentration found in surface waters for all three insecticides were higher than the Water Quality Standards values. Therefore all three insecticides have the potential to be toxic to aquatic life with the concentrations found in agricultural streams during surveys. EC_{50} values obtained from the mixed exposure tests were lower than the EC_{50} values obtained from the single exposure tests for all three insecticides. Esfenvalerate showed the highest increase in toxicity, 80% in EC_{50} value, pirimicarb 50% and fenitrothion 45%. Independent action predicted the toxicity accurately at EC_{50} but the concentration addition model is the preferable model to work with as it generally predicts a higher toxicity than independent action, and therefore gives a "worse case scenario". There is a need for further studies in order to see how these three insecticides interact with each other to see if the combination shows synergism, antagonism or additivity. Studies have found that concentrations that cause biological changes in *Daphnia* and other cladocerans are significantly lower than lethal concentrations. Therefore there is a risk that concentrations found in the environment can lead to changes in the entire ecosystem.

Sammanfattning

Vattenlevande organismer i bäckar i jordbruksområden utsätts oftast inte för en substans utan snarare en kombination av kemikalier. Transporten av pesticider som används inom jordbruket i Sverige övervakas varje år. Under 2002-2008 hittades i genomsnitt 10 pesticider i varje prov och 40% av proverna innehöll kemikalier med koncentrationer högre än sitt riktvärde. Kemiska riskanalyser baseras vanligtvis på utvärderingar från enkeltester. Ett problem vid fastställningen av en blandnings toxicitet är att komponenterna och koncentrationerna av föroreningar i naturen varierar kraftigt. Därför är det praktiskt omöjligt att utföra experimentella tester av alla tänkbara kombinationer av kemikalier i naturen. Modeller som koncentrationsaddition och oberoende effekter har utvecklats för att beräkna toxiciteten av kombinationer av kemikalier, med enskilda ämnens toxicitet som grund. I de flesta fall uppskattar dessa modeller toxiciteten av en blandning på ett tillförlitligt sätt. Syftet med denna studie var att testa tre insekticider (pirimikarb, fenitrotion och esfenvalerat) som är giftiga för *Daphnia magna* för att se om de tillsammans ger synergistiska, antagonistiska eller additiva effekter i en blandning. Syftet var även att undersöka om blandningens toxicitet kan beräknas enligt någon av modellerna koncentrationsaddition och oberoende effekter. De utvalda insekticiderna har varit vanligt förekommande inom svenskt jordbruk och har även påträffats i högre koncentrationer än sina riktvärden under flera år under miljöövervakningen. Den högsta koncentrationen som påträffats av esfenvalerat i ytvatten överskred NOEC och kan därför förväntas ha negativa effekter på *Daphnia*. Den högsta koncentrationen som hittats i ytvatten för alla tre insekticider var högre än riktvärderna. Alltså har alla tre insekticider potential att ha negativ effekt på vattenlevande organimser med de koncentrationer som har uppmätts under miljöövervakningen. EC_{50} värden från kombinationsförsöken var lägre än EC_{50} värden från enkelförsöken för alla tre insekticider. Esfenvalerat ökade mest i toxicitet med 80% i EC_{50} värde, pirimiarb 50% och fenitrotion 45%. Oberoende effekter beräknade toxiciteten bra men föredrar koncentrationsaddition då modellen ger ett “worse case scenario”. Det behövs fler studier för att avgöra hur dessa tre insekticider interagerar med varandra för att avgöra om det sker synergistiska, antagonistiska eller additiva effekter i blandningen. Studier har visat att koncentrationer som orsakar biologiska förändringar hos *Daphnia* och andra cladocerer är signifikant lägre än dödliga koncentrationer. Det finns därför en risk att de koncentrationer som uppmätts i ytvatten kan leda till förändringar i hela ekosystemet.

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1 Introduction

People have been fighting pests by hand and with simple appliances as long as the soil has been used for agriculture. Natural poisons like nicotine were used in the eighteenth century against insect attacks and potatoes were treated with a mixture of copper and sulphur (Perry et al. 1998). In the late nineteenth century the organochlorine DDT was developed. It was widely used during the Second World War, to combat body lice and malaria but was also used by farmers in agriculture (National Pesticide Information Center 2011). DDT was initially considered the perfect insecticide as it seemed to be non-toxic to humans, whilst highly toxic to insects (as cited in Baird & Cann 2005). During the Second World War several varieties of pesticides were developed as a consequence of research on chemical warfare. Organophosphates, chemically related to nerve gases, were amongst the first insecticides developed (U.S. Environmental Protection Agency 2011). A growing global population called for a global increase in the food production and rationalisation within the agricultural sector. Fertilizers and more refined seeds, weeding machines and rotation techniques resulted in enhanced yields (Morell 2001). Additionally, farmers needed fast-acting chemical substances to fight pests. Consequently, the production and use of insecticides increased dramatically after 1945, and with the increased use of chemical pesticides and the new techniques, agricultural yields more than doubled for crops like winter wheat and oat between the 1930s and 1990s (Flygare & Isacson 2001).

Initially, before the negative effects of the pesticides were known, pesticides were applied to fields and forests without proper dosing or protective clothing. But, during the early 1950s, the side-effects of DDT and other organochlorines started to appear such as the pesticides' persistence and bioaccumulation in the environment. In 1962 Rachel Carson released the book ”Silent Spring” in which she was able to show that DDT successively bioaccumulated through the food web, ultimately reaching humans. Carson showed that DDT altered DNA and decreased reproduction capacities through the food web. Many birds of prey were affected, with beak deformation and decreased nesting success due to egg-shell thinning. Soil field monitoring studies revealed that DDT was much more persistent than formerly believed, with half-lives up to 15 years (Mischke et al. 1985). Despite these early signs, DDT was not banned in Sweden until 1970, and many countries followed suit. DDT is still used in some countries in Africa and Asia to control malaria (Swedish EnvironmentalProtection Agency 2011). The use of organophosphates increased as organochlorines were banned and new products entered the market, e.g products like phenoxy acids, carbamates and pyrethroids (Flygare & Isacson 2001). In the 1980s, the actual effects of the pesticides were studied in more detail, and it was found that even these new chemicals were not entirely decomposed in the environment. In fact, they were found in rivers and streams and even in rain- and groundwater (Frank et al. 1982, Clark et al. 1991). The development of chemical pesticides progressed from persistent, fat-soluble substances to more easily degradable water-soluble pesticides (Flygare & Isacson 2001).

Modern pesticides are generally more water-soluble and more easily degradable than former pesticides. Moreover, modern pesticides are also more target-specific and generally have a higher acute toxicity allowing for low-dose applications. A disadvantage of many of these modern pesticides, like the organophosphates and carbamates, is that they bind less easily to soil particles and therefore can be transported relatively quickly through soils to groundwater and surface waters (Schultz et al. 2002). Pesticides that are sprayed over arable land are partly retained and degraded by soil microorganisms, but some are leaked to the surrounding land, transported to the atmosphere and enter groundwaters through volatilization, wind drift, surface runoff and so on (Figure 1) (Torstensson 1987, Kreuger 1998, Liess et al. 1999). The loss of pesticides from arable land can have catastrophic effects on aquatic life, as the pesticides can affect non-target organisms (Hanazato 2001, George et al. 2002, Schulz 2004).

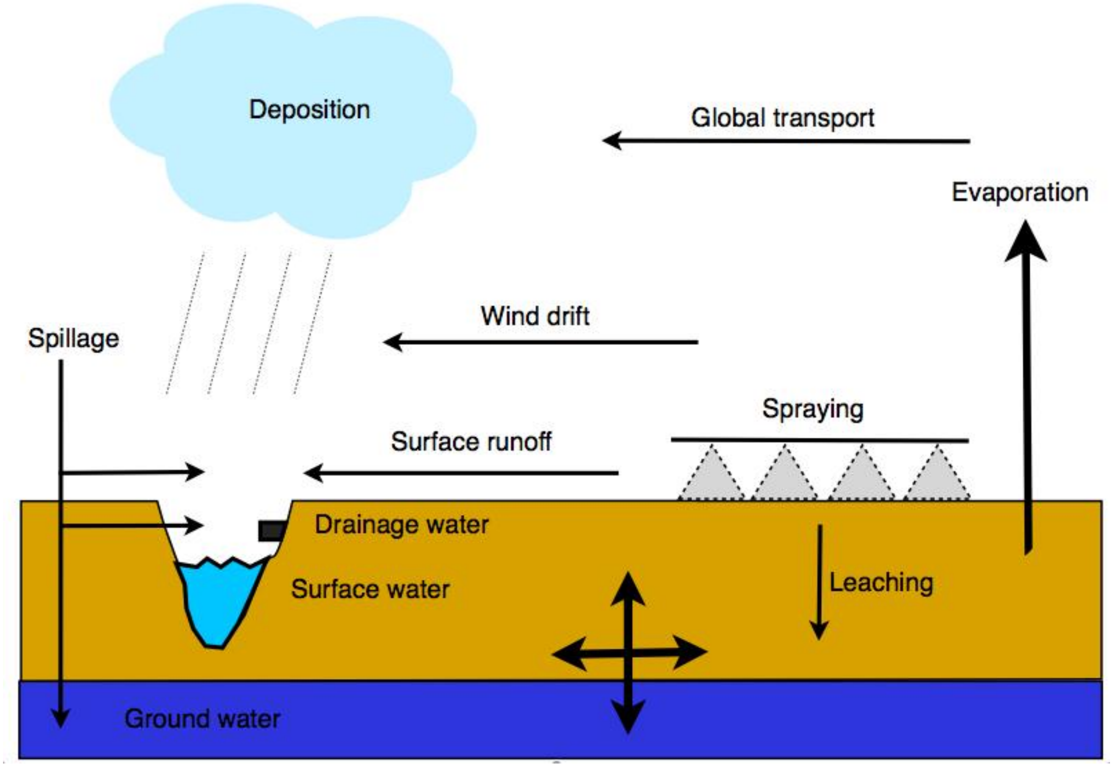


Figure 1: Potential pathways of pesticide transport in landscapes after spraying arable land with pesticides. These pathways include volatilization, surface runoff, deposition, wind drift, drainage water etc.

In 2007, 10 600 tonnes of pesticides were sold in Sweden, of which 1 640 tonnes (or 15%) were used within the agricultural sector (the Swedish Chemical Agency (KemI) 2008). Pesticide use by agriculture has decreased with more than 50% since the beginning of the 1980s. In France, some 65 800 tonnes of pesticides was used in 2007 within the agricultural sector, Germany 40 000, California 78 000, Palestine 79 000, Mexico 64 300 and the Netherlands 11 000 tonnes (Food and Agriculture Organization of the United Nations 2010). In countries and areas with high agricultural load pesticide use is normally high. For example a small country like Palestine uses considerable higher amounts of pesticides than Sweden.

In Sweden, the transport of pesticides used in the agricultural areas has been monitored annually since 2002. Four small catchments, 800-1700 hectare, representing four agricultural regions (>90% agriculture in the catchment), representing different common soil types, have been selected for monitoring. From May through October stream water is sampled weekly, with time-integrated sampling every 80th minute. The contents in one sample therefore represent the weekly average concentration of pesticides. During 2002-2008, detectable concentrations of 93 pesticides were determined. Transport of pesticides to streams is normally around 0.1% of the pesticides used, but could be up to 1% in wet years. However, in dry summers more substances are detected exceeding the Water Quality Standards (WQS). Water Quality Standards state the highest concentration of plant protection products in surface waters not expecting any negative effects on aquatic life (the Swedish Chemical Agency 2010). During 2002-2008, on average 10 pesticides were found in each sample and 40% of the samples contained substances with concentrations exceeding Water Quality Standards. 92% of the transport consisted of herbicides, 1% of insecticides and 7% consisted fungicides (Kreuger et al. 2003-2004, Törnquist et al. 2005, Adielsson et al. 2006-2009).

In order to evaluate the risks and effects of chemicals in Europe, a number of standardized test methods have been developed within the EU (European Chemicals Bureau 2010) and the US (American Society for Testing and Materials 2011). The objective is to identify and assess any adverse effects that chemicals may have and to estimate relationships between exposure and severity of effects (European Chemicals Bureau 2010). To assess the safety of chemical products the Organisation for Economic Co-operation and Development, OECD, has developed a collection of guidelines for the testing of chemicals. Following international standards, these methods are used by governmental agencies, industry and independent laboratories (OECD 2011). For exemple, in aquatic toxicology the *Daphnia magna* acute immobilisation test is commonly used as a measure of chemical toxicity (OECD guidelines for testing of chemicals-*Daphnia* sp. Acute Immobilisation test 202).

Aquatic ecosystems and communities are often exposed to several toxicologically and structurally different pesticides rather than individual substance. (Deneer 2000, Lydy & Austin 2004a, George & Liber 2007, Relyea 2009). This has been shown in surface waters, where mixtures of potentially toxic substances enter the surface waters as a result of human activities (Backhaus et al. 2004a, Verro et al. 2009). Although organisms are rarely exposed to individual chemicals, chemical risk management procedures commonly rely on single-test evaluations (Altenburger et al. 2004, Cedergreen et al. 2008, Syberg et al. 2008) and when determining threshold values, like no observed effect concentrations (NOECs) (Walter et al. 2002). There are therefore concerns regarding the use of knowledge from single substance testing on mixture toxicity evaluations as the mechanisms of action may be poorly understood and the interaction between chemicals hard to determine (Berenbaum 1985). Chemicals can interact with each other during uptake and metabolism or under the influence of a receptor or an organ, to produce an effect greater (synergism) or smaller (antagonism) than expected. An additive effect occurs when the combined effect of chemicals is equal to the sum of the effects of each given chemical alone (Eaton & Klaassen 2001). Mixed exposure tests have demonstrated that exposure to mixtures of pesticides can lead to a toxic effect higher than each pesticide alone, i.e. synergistic effect. Anderson et al. (2002) showed an increase in toxicity when the amphipod *Hyalella azteca* was exposed to three organophosphates (chlorpyrifos, methyl parathion and diazinon) in the presence of atrazine. Laetz et al. (2009) observed addition and synergism, with a greater degree of synergism at higher exposure concentrations of organophosphate and carbamates mixtures, using the salmonid *Oncorhynchus*. Nørgaard & Cedergreen (2009) showed synergism when the water flea *Daphnia magna* was exposed to prochloraz and alpha-cypermethrine. Strictly additive effects were found by Bailey et al. (1997) in experiments where the water flea *Ceriodaphnia dubia* was exposed to the organophosphates diazinon and chlorpyrifos. These studies show that there is a need for further mixture toxicity studies in order to determine the interactions between chemicals in a mixture, instead of relying on the quantification of toxicity from single test evaluations.

Several methods and models for the prediction of combined effects of chemical compounds have been introduced (Berenbaum 1985, Drescher & Boedeker 1995, George et al. 2003). The assessment of the combined pesticide effects is usually based on concentration addition (CA), independent action (IA) and/or interaction. Concentration addition assumes a similar mechanism of action of mixture components were the toxicity is in proportion to the dose of the chemical (Deneer 2000, Junghans et al. 2003a & b, Rider & LeBlanc 2005). On the other hand, when using independent action the components in the mixture are assumed to act on dissimilar systems, i.e. the mixture components have different target sites, and are therefore not affected by the presence of other chemicals within the organism (Backhaus et al. 2003, Lydy et al. 2004b, Cedergreen et al. 2006). The predictive power of concentration addition and independent action with regards to the estimated toxicity in mixtures has been documented in several studies (Faust et al. 2001, Backhaus et al. 2004b, Belden et al. 2007, Cedergreen et al 2008). Independent action has given reasonable predictions for the toxicity of pesticide mixtures consisting of several substances with dissimilar modes of action. Faust et al. (2003) showed that independent action accurately estimated the toxicity of 16 dissimilar acting herbicides and fungicides on the green algae S*cenedesmus vacuolatus*. The same result was achieved by Backhaus et al. (2000a) when exposing the bacteria *Vibrio fischeri* to 14 dissimilar acting substances. In the studies by Faust et al. (2003) and Backhaus et al. (2000a) concentration addition overestimated the toxicity. Concentration addition is commonly used to predict the toxicity of combined effects of similarly acting chemicals. Silva et al. (2002) showed that multi-component mixtures of xenoestrogens on yeast cells could accurately be predicted by the concentration addition model, while predictions made by independent action lead to an underestimation of the mixture effects. Junghans et al. (2003a) exposed *Scenedesmus v.* to eight similar acting herbicides, chloroacetanilides and showed that concentration addition accurately estimated the toxicity of the herbicide mixture. Also in this study independent action underestimated toxicity. Studies have also shown that concentration addition and independent action can equally well predict the toxicity of a mixture. This was shown by Syberg et al. (2008) who tested binary mixtures of similar- and dissimilar-acting chemicals (pirimicarb, dimethoate and linear alkyl benzene sulfonate) on *Daphnia magna.* Several of these studies show that concentration addition almost always predicts a higher toxicity than independent action (Backhaus et al. 2000a, Silva et al. 2002, Faust et al. 2003, Junghans et al. 2003a). As concentration addition is the more conservative model, several studies are recommending concentration addition to be used for both scenarios with similar and dissimilar ways of acting in order to achieve a “worse case scenario” (Belden et al. 2007, Cedergreen et al. 2008).

The aim of this study was to evaluate the mixture toxicity effect of three insecticides, pirimicarb, fenitrothion and esfenvalerate, commonly occurring in Swedish agricultural streams (Adielsson et al. 2009). It was investigated whether the mixture of the three insecticides educed antagonistic, synergic or additive effects. Also the predictability of the mixture effects according to the concepts of concentration addition and independent action was investigated. The study was based on OECD guidelines for testing of chemicals- *Daphnia* sp. Acute Immobilisation Test 202.

2 Material and methods

The toxicity experiments were performed according to the OECD guideline for testing of chemicals- *Daphnia* sp. Acute Immobilisation Test-guideline 202 (OECD 2004). The principle of acute toxicity immobilisation test is to determine the concentration a chemical immobilises 50% of the test organisms after 48h, providing the EC_{50} value. Juvenile *Daphnia*, age <24 hours at the start of the test, are exposed to the test substance at a range of concentrations for a period of 48 hours. Immobilisation is recorded and compared with control values. The results are analysed in order to calculate the EC_{50} at 48 hours.

2.1 Test organism

The laboratory *Daphnia* culture was generously provided by the Institute of Zoology at the University of Cologne. *Daphnia*, commonly called water flea, is a zooplankter that reproduces through parthenogenesis under optimal conditions. It is a suitable test organism because it is easily cultured in the laboratory and has a short generation time (Adema 1978). Other benefits of *Daphnia* are that they have a high sensitivity to toxicants and are genetically constant due to partenogenetic reproduction (ten Berge 1978). It belongs to the order Cladocera and the genus *Daphnia* is distributed worldwide with over 50 species and forms an important link in food chains. As a filter feeder the organisms feeds on algae, bacteria, fungi, protozoa and organic debris. In order to grow *Daphnia* shed by moulting every 2-3 days and can grow up to 5 mm. Reproductive maturity is normally reached 4-5 days after birth and a new clutch of eggs is produced after every moult (Hebert 1978). The life span is dependent on temperature; in 20ºC the daphnids generally lives about 8 weeks (ten Berge 1978). In the laboratory *Daphnia* was cultured in synthetic freshwater, Elendt M4 medium (OECD 2004) in 1-litre glass flasks.

2.2 Test chemicals

Three different insecticides, pirimicarb, fenitrothion and esfenvalerate were selected for this study, all are neurotoxic to *Daphnia*. The insecticides were chosen as they have been frequently used in Swedish agriculture and have been found at concentrations above the Water Quality Standards in Swedish surface waters for several years during environmental monitoring (Adielsson et al. 2009). Pirimicarb belong to the group carbamates which inhibit the enzyme acetylcholinesterase thereby disturbing normal synapses between neural cells. Acetylcholinesterase inhibition leads to a prolonged stimulation of the cholinergic receptors and disruption of the normal transmission of impulses across the synapses (Ecobichon 2001). It is mainly used to combat aphides on crops, fruits, strawberries and vegetables, see Figure 2 for structural formula and Table 1 for physicochemical properties (the Swedish Chemicals Agency 2010). During 2002-2008, pirimicarb dominated the transport of insecticides with 43% (Adielsson et al. 2009).

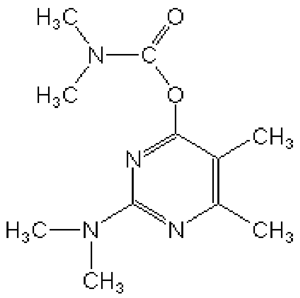


Figure 2. Structural formula of pirimicarb.

Fenitrothion, an organophosphate, also inhibits the enzyme acetylcholinesterase at the synapses. The difference between carbamates and organophosphates is that organophosphates have a phosphorus atom that attacks acetylcholinesterase, while carbamates have a carbon atom, see Figure 3 (Baird & Cann 2005). In Sweden the substance has been used on oil-seed rape to combat the pollen beetle, see Table 1 for physicochemical properties (Ecobichon 2001). In 2007, the Swedish Chemical Agency revoked the endorsement of fenitrothion (KemI 2010). 3% of the total transport from insecticides consisted of fenitrothion during 2002-2008 (Adielsson et al. 2009).

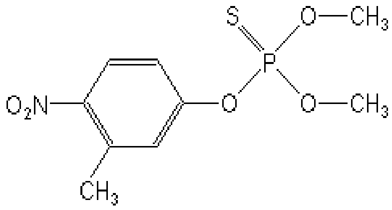


Figure 3. Structural formula of fenitrothion.

Esfenvalerate, a pyrethroid, acts on the nerve impulses by blocking the sodium channels in the central and peripheral nerves. Normally the sodium channels are open for a brief moment, allowing Na^+ ions to flow inward. Pyrethroids delay the closure of the channels, thereby increasing the flow of Na^+ ions. This leads to uncontrolled repetitive and spontaneous discharge along the nerve causing uncoordinated muscular tremors. The low solubility and high log K_{ow} value indicates that the substance is likely to bind to particles in the sediment and has a high potential for bioaccumulation (Table 1). Esfenvalerate is used on several groups of insects like beetles, moths and grasshoppers and on crops like legumes, fruit and cereals (Ecobichon 2001). 6% of the total transport of insecticides consisted of esfenvalerate during 2002-2008 (Adielsson et al. 2009). The structural formula for esfenvalerate is given in Figure 4.

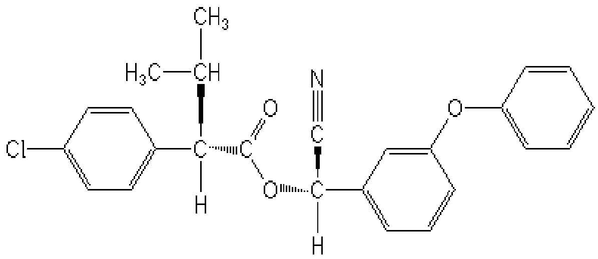


Figure 4. Structural formula of esfenvalerate.

Table 1. Physicochemical properties of pirimicarb, fenitrothion and esfenvalerate from the Swedish Chemicals Agency, [a]Spectrum Laboratories Inc. (2010), [b]Footprint PPDB (2010). [c]the Laboratory for Organic Chemistry at the Swedish University of Agricultural Sciences.

	Pirimicarb	**Fenitrothion**	**Esfenvalerate**
CAS-No.	23103-98-2	122-14-5	66230-04-4
Log K_{ow}	1.7 (20˚C)	3.3 (25˚C)	6.2 (25˚C)
Solubility (mg/l, 20˚C)	3060	19	0.01
Hydrolysis (T½, days)	>23	183[b]	65
Photolysis (T½, days)	3-20	3.5[a]	1.1-2.5
Concentration (µg/ml acetone)	2394[c]	1178[c]	1154[c]
Molecular weight (g/mol)	238	277	419.9

Stock solutions for the three insecticides were prepared by the accredited Laboratory for Organic Chemistry at the Swedish University of Agricultural Sciences. The insecticides were dissolved in acetone (pesticide grade) and stored in the freezer. A Hamilton Microlab® 1000 diluter and aerated Elendt M4 medium were used to obtain the final test concentration. The solutions were prepared shortly before use and frozen shortly after for further analyses. Esfenvalerate solutions were put in a Bransonic® MT5510 ultrasonic bath, 185 W, for five minutes to ensure proper dissolution of the molecules. The purity for all three stock solutions exceeded 98.5%.

2.3 Test procedure

The *Daphnia* culture was kept according to the test requirement with the temperature of 20 ± 2°C, the photoperiod of 16 hours light / 8 hours dark and pH was in the range 6-9. *Daphnia* were fed with a mixture of the green algae's *Scenedesmus* sp. and *Selenastrum* sp. twice a week. The *Scenedesmus* and *Selenastrum* culture was generously provided by the Biological Institute at the University of Oslo. The algal cultures were grown in the medium recommended in the ISO guideline 6341:1996. The algae were kept in a climate chamber with 24 hour light and were inoculated every three to four days.

Three single acute toxicity tests were performed according to the OECD guideline for testing of chemicals- *Daphnia* sp. Acute Immobilisation Test-guideline 202 (OECD 2004) for every insecticide. This was made in order to determine single compound EC_{50} value for pirimicarb, fenitrothion and esfenvalerate. At least five concentrations were used per experiment, with 20 animals per concentration and control. The experiment started by transferring juvenile *Daphnia* (age <24 hours at the start of the test) from the laboratory cultures to a beaker containing Elendt M4 medium (OECD 2004). From this, starting population animals were allocated to 20 ml glass beakers, with Elendt M4 medium, for each concentration and control. For each concentration, the animals were divided into four beakers, five *Daphnia* in each, with 10 ml solution in each beaker. Before the experiments started EC_{50} values were obtained from the Swedish Chemicals Agency in order to set the concentration-range for each pesticide. The concentrations used in the experiments were adjusted, if necessary, after every experiment. The concentrations used for pirimicarb were 12, 14, 15, 16, 18, 20, 21, 22 and 24 µg/l. The concentrations used for fenitrothion were 3, 6, 9, 11, 12, 13, 15, 18 and 21 µg/l. For esfenvalerate the concentration were 0.2, 0.25, 0.5, 0.7, 0.75, 1, 1.2, 1.7, 2, 2.2, 2.7, 3, 4 and 8 µg/l. Controls and solvent controls (only acetone added) were set up as positive controls of *Daphnia* performance in the Elendt M4 medium and to check the animals' sensitivity to acetone, respectively. The solvent control exposed *Daphnia* to the highest concentration of acetone used in the dilution of the three insecticides, with the highest concentration of 17 µl acetone/l Elendt M4 medium. The beakers were covered with Steripropps® to reduce the evaporation of water and avoid entry of dust. The beakers were marked and placed randomly under a light source, and exposed to the same light / dark photoperiod as the stock cultures, i.e. 16 hours light / 8 hours dark. Three tests were made with the reference chemical potassium dichromate, $K_2Cr_2O_7$, one test for every experiment period, to ensure that the test organisms were in a proper condition for the experiments, assuring that the test conditions were reliable (ISO guideline 6341:1996).

After 48h, immobility, abnormalities and changes in the behaviour of *Daphnia* were observed visually and recorded. The animals were considered immobilised when they were unable to swim within 15 seconds after gentle agitation. In accordance with the OECD guideline, movement of the antennas was not scored as swimming activity. *Daphnia* used in the experiments were not fed during the experimental period. Dissolved oxygen and pH were measured in the highest test concentration and in the controls at the start and end of the tests. In order for an experiment to be valid not more than 10% (i.e. 2 of 20 *Daphnia* in a control group) should be immobilised. The EC values and their 95% confidence limits were determined by probit analysis using the EPA probit analysis programme (Version 1.5). From the plotted log concentration-probit curves the EC values were calculated. Corrected response was calculated according to Abbott's formula (Abbott 1925). For fenitrothion, only four concentrations were used in one of the three experiments due to lack of animals.

The mixed exposure tests were performed according to the OECD guideline for testing of chemicals- *Daphnia* sp., Acute Immobilisation Test-guideline 202 (OECD 2004). The mixed exposure concentrations were set up from single toxicity tests results, containing all three insecticides in the mixture. Initially concentrations similar to those in the single compound exposure tests were used but had to be lowered when all animals became immobilised. Therefore compounds were mixed in the ratio of their individual EC-concentrations of 0.1, 0.2, 0.4, 0.7 and 1.0. The concentrations for pirimicarb were 8.5, 9, 9.5, 10 and 10.4 μg/l, for fenitrothion 5.8, 6.1 6.4, 6.6 and 6.8 μg/l and for esfenvalerate 0.11, 0.12, 0.14, 0.16 and 0.17 μg/l. The mixed exposure solutions were put in a Bransonic® MT5510 ultrasonic bath, 185 W, for five minutes to ensure proper dissolution of the molecules.

2.4 Statistics and calculations

Statistical analyses were performed in JMP® 8.0.2 (SAS Institute Inc., 2009). Student's *t*-tests were used to test for differences in EC values between single compound and mixed exposure tests. Predictions of effect concentrations for mixtures by concentration addition were calculated according to Loewe equation (Faust et al. 2003):

$$ECx_{mix} = \sum_{i=1}^{n}\left(\frac{p_i}{ECx_i}\right)^{-1}$$

where ECx_{mix} is the predicted toxic effect of the mixture, p_i is the fraction of component *i* in the mixture. ECx_i is the individual effect concentrations when applied singly. Independent action was calculated according Bliss equation (Berenbaum 1985):

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

$E(c_{\mathrm{mix}})$ is the overall effect, expressed as fractions of a maximum possible effect (scaled from 0-1) of a mixture composed of *i* chemicals, c_i is the concentration of the *i*th compound in the mixture, and $E(c_i)$ describes the effect of chemical *i* if applied singly in a concentration *c* which corresponds to the concentration of that component in the mixture.

3 Results

3.1 Single acute toxicity tests

Concentration-response functions were determined for pirimicarb, fenitrothion and esfenvalerate individually. Figure 5 shows the dose-response curve for the substances. The EC_{50} value for pirimicarb was 80.3 ± 1.0 nmol/l (19.1 ± 0.2 µg/l), the EC_5 was 52.1 ± 4.1 nmol/l (12.4 ± 1.0 µg/l) and the EC_{90} was 112.8 ± 7.5 nmol/l (26.8 ± 1.8 µg/l). For fenitrothion the EC_{50} value was 41.4 ± 3.3 nmol/l (11.5 ± 0.9 µg/l), the EC_5 value was 28.7 ± 4.5 nmol/l (7.9 ± 1.2 µg/l) and the EC_{90} was 55.3 ± 3.2 nmol/l (15.3 ± 0.9 µg/l). The EC_{50} value for esfenvalerate was 1.9 ± 0.5 nmol/l (0.8 ± 0.2 µg/l), EC_5 0.6 ± 0.1 nmol/l (0.3 ± 0.1 µg/l) and EC_{90} 4.6 ± 1.6 nmol/l (1.9 ± 0.7 µg/l). The results show that pirimicarb had the lowest toxic effect on *Daphnia*, whilst esfenvalerate had the highest effect. According to current regulatory rules, all three pesticides tested are classified as highly toxic to *Daphnia*.

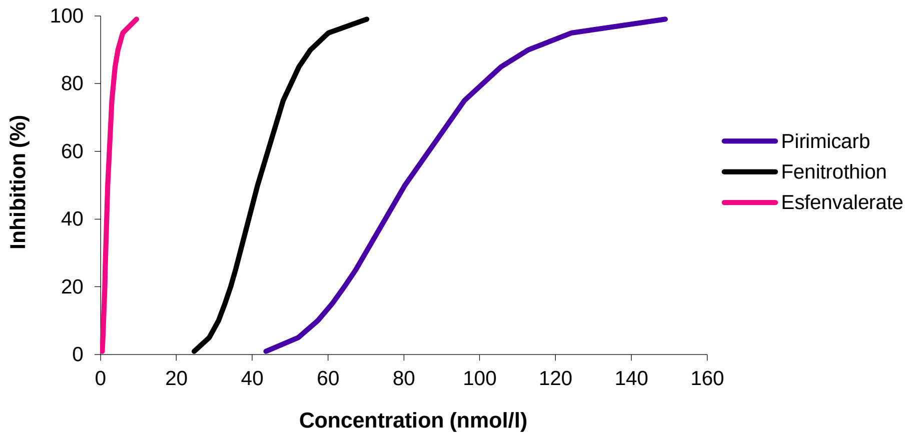


Figure 5. Concentration-response relationship curve for the *Daphnia* toxicity from single compound toxicity tests of esfenvalerate, fenitrothion and pirimicarb respectively. The response is the reduction of mobility, i.e. inhibition (%).

The EC_{50} values obtained for pirimicarb and esfenvalerate corresponded well with the values compiled by the Swedish Chemicals Agency (Table 2). By contrast, the EC_{50} value for fenitrothion was 30% higher. However, EC_{50} values for all three pesticides were much higher than the Water Quality Standards (WQS) and also higher than NOEC values and the highest concentration found in surface waters in the environment. For pirimicarb the NOEC value exceeded both the WQS value and the highest concentration found in surface waters. The highest concentration of pirimicarb found in surface water was 40 times higher than the value for WQS. For fenitrothion the NOEC value was higher than the highest concentration found in surface waters and the WQS value. The highest concentration of fenitrothion found in surface water was 30 times higher than the WQS. Concentrations of esfenvalerate found in surface waters exceeded the WQS 2000 times and the NOEC value by 80%.

Table 2. Comparison between EC_{50} values from the experiments (observed), EC_{50} values complied by the Swedish Chemicals Agency (KemI), Water Quality Standards, NOECs and the highest peak concentration measured in surface water. Values for EC_{50} are given as mean ± standard deviation. All values are in µg/l. [a]The Swedish Chemicals Agency; pirimicarb found 1985-1988, esfenvalerate 1994. [b]The Swedish environmental supervision of pesticides 2007 (Adielsson et al. 2008).

Substance	**EC_{50} observed**	**EC_{50} (KemI)**	**WQS**	**NOEC**	**Peak environmental concentration**
Pirimicarb	19.1 ± 0.24	19.0	0.09	6.2	3.7[a]
Fenitrothion	11.5 ± 0.91	8.6	0.009	2.0	0.3[b]
Esfenvalerate	0.8 ± 0.19	0.9	0.0001	0.11	0.2[a]

3.2 Mixed exposure tests

Concentration-response curves from the single exposure tests of pirimicarb, fenitrothion and esfenvalerate showed a significant ($p<0.05$) decrease in EC values in mixed exposure tests compared to the single exposure tests (Figure 6). The largest decrease in EC_{50} values between single and mixed exposures were found for esfenvalerate, where the EC_{50} value changed from 1.9 ± 0.5 nmol/l to 0.3 ± 0.01 nmol/l. This implies a >80% increase in toxicity for esfenvalerate in the mixed expose tests. The EC_5 for esfenvalerate changed from 0.6 ± 0.1 nmol/l to 0.2 ± 0.01 nmol/l (65% decrease) and the EC_{90} from 4.6 ± 1.6 nmol/l to 0.5 ± 0.02 nmol/l (90% decrease), respectively, between single and mixed exposures. The decrease in EC_{50} value for pirimicarb was 50%, where the EC_{50} value changed from 80.3 ± 1.0 nmol/l to 38.7 ± 0.2 nmol/l. The EC_5 for pirimicarb changed from 52.1 ± 4.1 nmol/l to 30.0 ± 0.3 nmol/l (42% decrease) and EC_{90} from 112.8 ± 7.5 nmol/l to 47.3 ± 0.6 nmol/l (58% decrease). Fenitrothion showed the lowest decrease in EC_{50} values between single and mixed exposure tests; a decrease of 45%, from 41.4 ± 3.3 nmol/l to 22.3 ± 0.1 nmol/l. The EC_5 value changed from 28.7 ± 4.5 nmol/l to 18.0 ± 0.2 nmol/l (37%). The EC_{90} value changed from 55.3 ± 3.2 nmol/l to 26.4 ± 0.3 nmol/l (52%). These results show that there were large differences in toxicity between exposures with single compounds and in mixed exposures and that the differences increased with higher concentrations, suggesting that the three insecticides become more toxic when added together in a mixture.

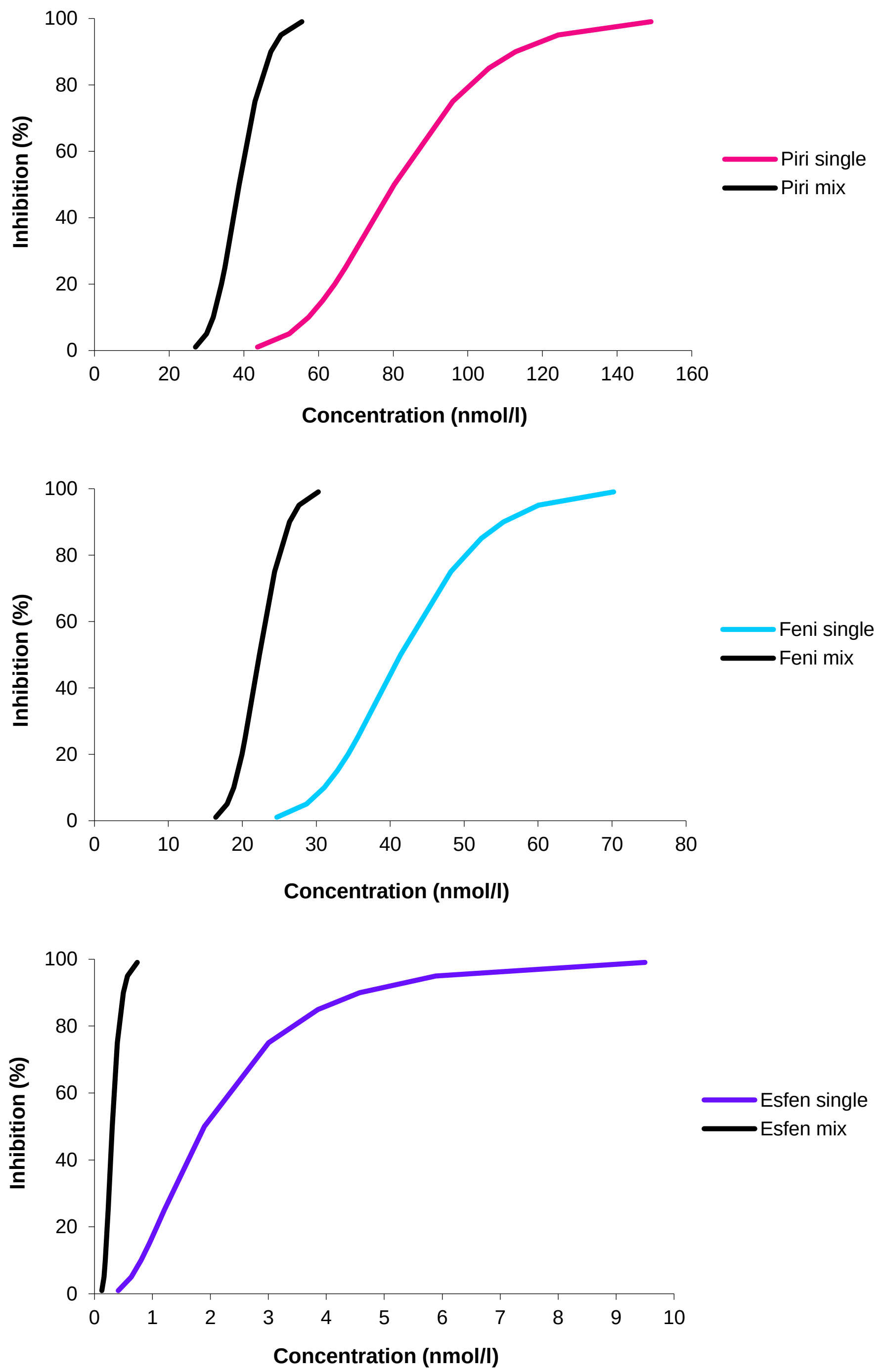
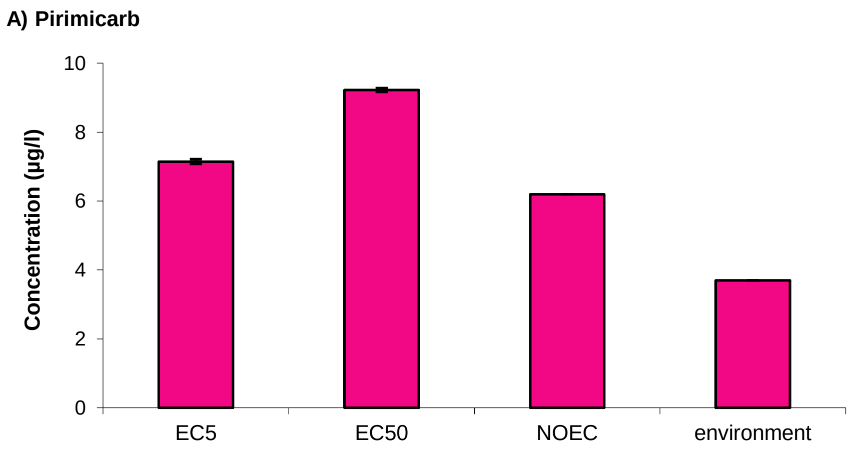
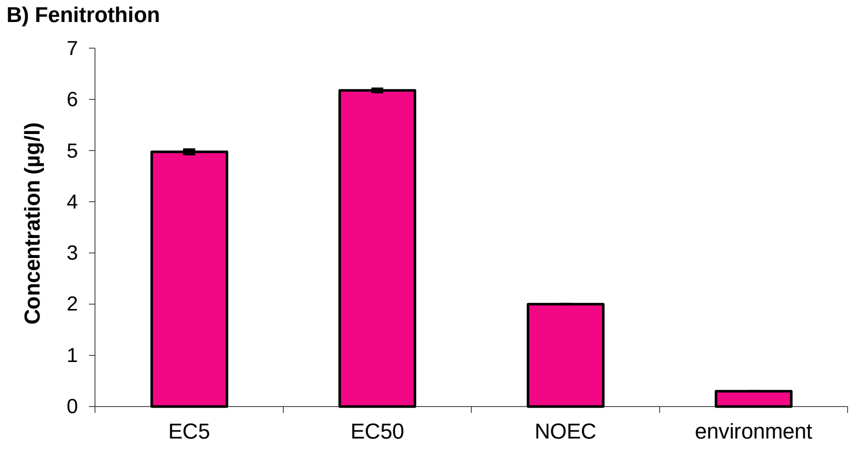


Figure 6. Concentration-response curves from the single exposure tests of pirimicarb, fenitrothion and esfenvalerate are compared with the concentration-response curve obtained from the mixed expose tests. The coloured lines indicate the concentration-response curves for the single expose tests whereas the black lines indicate concentration-response curve for the mixed expose tests.

3.3 Concentration addition and independent action

The observed EC_5 and EC_{50} values for pirimicarb and fenitrothion were higher than the NOEC values, and the highest concentration found in surface waters (Figure 7A and B). The NOEC values for pirimicarb and fenitrothion were also higher than the highest concentration found in surface waters. For esfenvalerate the concentration found in the environment was three times higher than the EC_5 value ($p<0.05$) (Figure 7C). The concentration found in the environment was also higher than the EC_{50} value (50% higher) ($p<0.05$). The EC_5 value was 40% lower than the NOEC value ($p<0.05$) whereas the EC_{50} was slightly higher (15%).





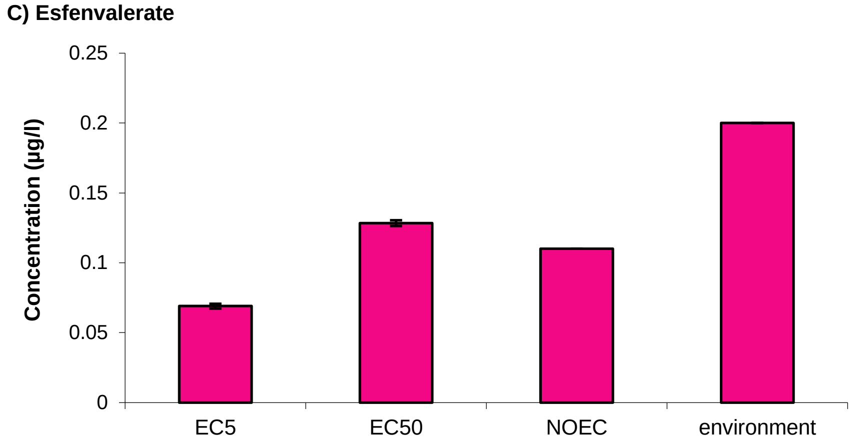


Figure 7. EC_5 and EC_{50} values from mixed expose tests for pirimicarb (A), fenitrothion (B) and esfenvalerate (C) compared to the highest concentration found in surface waters in the environment and NOEC values.

The effects of the three pesticides when applied as single compounds were equal to their individual $EC_{0.26}$ value in order to receive the observed EC_{50} effect in the mixed exposure test (Figure 8). The observed EC_{50} value was 61.3 ± 0.4 nmol/l for the mixed expose test. The predicted joint effects calculated according to independent action predicted an EC_{50} value of 63.0 nmol/l and therefore estimated the toxicity accurately. The EC_{50} value according to the concentration addition model was calculated as 41.2 nmol/l. Hence, the concentration addition model overestimated toxicity by 33% and showed a significant difference ($p<0.05$) from the observed EC_{50} value. Concentration addition and independent action predictions were compared with the observed mixed exposure inhibition of 50% of the *Daphnia* population. Concentration addition predicted an inhibition by 66% at the same concentration that caused the observed 50% inhibition. Independent action predicted an inhibition by 48.7% at the observed 50% inhibition concentration.

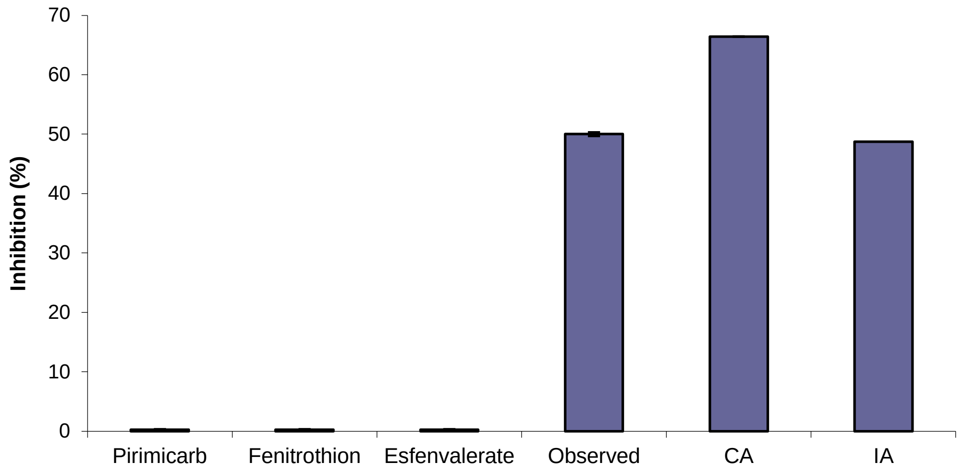


Figure 8. Comparison of the total effects of pirimicarb, fenitrothion and esfenvalerate with the single concentrations equal individual $EC_{0.26}$ values that gave the mixed exposure toxicity inhibiting 50% of the *Daphnia* population, i.e. the observed effect. The predicted joint effects of concentration addition (CA) and independent action (IA) were compared at the same concentration that caused the observed 50% inhibition.

The observed mixed exposure concentration-response curve differed ($p<0.05$) compared to the curve for predicted joint effects according to concentration addition (Figure 9). Concentration addition on avage overestimated toxicity by 32.2 ± 11.7%. Independent action, on the other hand, provided accurate estimates of toxicity, with inhibition around 50%. At a lower range of inhibition (<25%) independent action overestimated toxicity by on average 11.0 ± 6.2%. At higher range of inhibition (>75%) the predictions of independent action underestimated toxicity by on average 20.5 ± 8.9%. These results show that concentration addition predicts a higher mixture toxicity than the independent action model, and that the independent action model is good at predicting the observed toxicity, especially at EC_{50}.

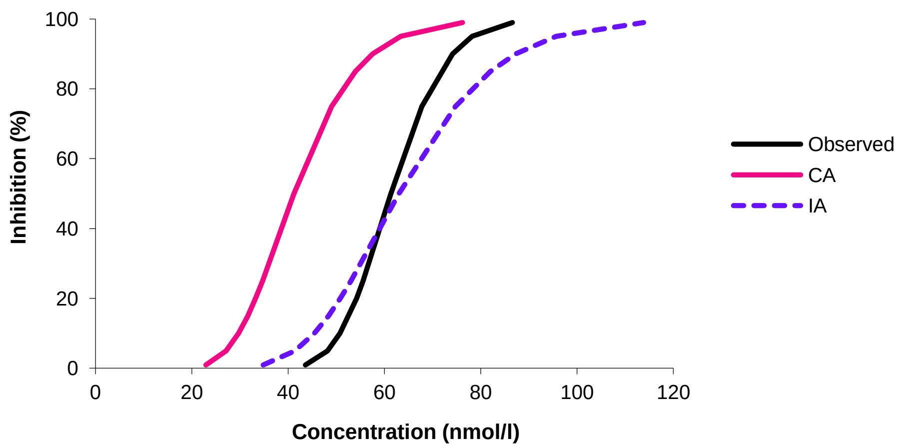


Figure 9. Concentration response curves of the observed inhibition (%) and the calculated concentration addition and independent action.

Water quality variables measured during acute toxicity tests were satifactory according to the OECD test requirements, except for temperature in one single exposure toxicity test of fenitrothion (temperature ranged from 21.4-22.6ºC). The pH during the experiments ranged from 7.57 to 8.93, the oxygen concentrations were 6.6-8.5 mg/l and the temperature varied between 20.5 and 22.6ºC. Over 90% of the control animals survived in each experiment. Several tests with the reference chemical potassium dichromate during the experiment periods showed that the *Daphnia* culture was in good condition.

4 Discussion

4.1 Single acute toxicity tests

The estimated EC_{50} values for pirimicarb and esfenvalerate in this study corresponded well with the values given by the Swedish Chemicals Agency. The reasons why the EC_{50} value for fenitrothion from this study deviated from the stated value from the Swedish Chemicals Agency are unclear as the experiments were performed under the same conditions as for pirimicarb and esfenvalerate. In one of our experiments the temperature exceeded the test requirement temperature, but the concentration-response curve was similar to the other two tests performed on fenitrothion. The *Daphnia* culture was in good condition, which was apparent after several tests with the reference chemical potassium dichromate made during the experiment periods. All three insecticides have the potential to be toxic to *in situ* aquatic life as the highest concentration found in the environment was higher than the Water Quality Standard values. As the highest concentration found in the environment for esfenvalerate exceeded the NOEC, negative effects on *Daphnia* can be expected. This shows that each of the three insecticides can have negative effects on aquatic life with the concentrations found in agricultural streams during pesticide surveys.

4.2 Mixed exposure tests

As expected from other studies, the EC values obtained from the mixed exposure tests were lower than the EC values obtained from the single exposure tests for all three insecticides. The highest increase in toxicity was found for esfenvalerate, with an increase by 80% in EC_{50} value. As both EC_5 and EC_{50} for esfenvalerate are lower than the NOEC value, it can be expected that esfenvalerate potentially has negative effects on *Daphnia* in surface waters when applied together with pirimicarb and fenitrothion in concentrations similar to those used in this study, or together with other substances from the organophosphate- and carbamates groups. The low solubility and high log K_{ow} value of esfenvalerate indicates the compound's tendencies to dissipate from the water-phase and instead be absorbed into organic matter and sediment. During

2002-2008, esfenvalerate was found in 40% of the samples taken from sediments in agricultural streams (Adielsson et al. 2009). Esfenvalerate can therefore be a potential threat to organisms living and feeding in the sediments.

4.3 Concentration addition and independent action

In this study, independent action accurately estimated the EC_{50} mixed exposure concentration, whereas concentration addition overestimated toxicity by 30%. The toxicity of this mixed exposure study is therefore best predicted by independent action. In previous studies it has been argued that concentration addition is the best model to work with as it is the more conservative of the two models and therefore gives a worse case scenario (Altenburger et al. 1996, Junghans et al. 2003b, Belden et al. 2007). Concentration addition can also be used when calculating risks below individual NOEC values as opposed to independent action where no combined effects are expected to occur at these concentrations (Altenburger et al. 1996, Backhaus et al. 2000b). An implication of the use of concentration addition and independent action models is that few pesticide combinations have exactly the same mode of toxic action, or act strictly independent (Berenbaum 1985, Junghans et al. 2006, Syberg et al. 2008). For example organophosphates and carbamates share the same receptor site at the synapses but can have different affinities for the receptor (Lydy et al. 2004b). Moreover, the models are not considering uptake kinetics, transportation, metabolism and excretion of the chemicals that can have potentially large effects on the mixture toxicity (Deneer et al. 1988, Altenburger et al. 2003, Junghans et al. 2003a). In many cases information is often lacking on the modes of action of the chemicals entering the watercourse in order to divide the chemicals into groups of similar- and dissimilar action (Drescher & Boedeker 1995, Faust et al. 2001, Walter et al. 2002). Warne & Hawker (1995) suggested that non-additive interactions are only appearing in chemical mixtures with few components. As the number of components in a mixture increases, the range of deviation from toxic additivity decreases. This is called the Funnel Hypothesis. Therefore, deviations from concentration addition are more common with few components in the chemical mixture. It has also been suggested that concentration addition predicts the toxicity more accurately when dose-response slopes are steep, which is likely for most aquatic pesticides (Drescher & Boedeker 1995, Lydy et al. 2004b, Syberg et al. 2008). As contaminants of surface waters normally consists of both similar- and dissimilar-acting toxicants concentration addition seems to be the preferred model for calculating mixed exposure toxicities in risk assessment.

4.4 Synergism, antagonism, additivity

When a model accurately predicts the toxicity of a mixture, the question remains if the combination shows zero interaction, synergy or antagonism (Berenbaum 1985). Even if the independent action model accurately predicted toxicity of the three insecticides used in this study, there are questions as to whether the pesticides have different target sites (Backhaus et al. 2003, Lydy et al. 2004b, Cedergreen et al. 2006). The mechanism of action is similar for pirimicarb and fenitrothion, as they both inhibit acetylcholine esterase, but uses different atoms to attack the acetylcholine esterase. Esfenvalerate has a different mode of action as it disturbs nerve impulses by blocking sodium channels. Denton et al. (2003) showed that a combination of esfenvalerate and the organophosphate diazinon induced greater than additive toxicity on fathead minnow larvae (*Pimephales promelas*). The carboxylesterase activities were examined as a possible explanation for the greater than additive effects, as carboxylesterases are inhibited by organophosphates and carbamates. Carboxylesterases are therefore inhibited from detoxifying pyrethroids. It can therefore be assumed that diazinon inhibits carboxylesterases, which leads to the prevention of hydrolysing esfenvalerate. From this experiment it would be expected that esfenvalerate would have a greater than additive effects on *Daphnia* in the presence of organophosphates and/or carbamates. To be able to assess any interaction further studies have to be made on these three insecticides, preferably with binary mixtures in order to see how each insecticide interact with others.

4.5 Swedish pesticide monitoring

The transport of pesticides used in the agricultural area in Sweden is monitored with continuous environmental supervision every year as mentioned earlier. The contamination of pesticides to surface waters may range from a few minutes to several hours or days. A rapid decrease of pesticide concentrations in the water course follows as the water is renewed in streams and adsorption and degradation of pesticides occurs (Liess et al. 1999). The concentrations of pesticides, especially in smaller streams, can therefore vary significantly from day to day. Consequently, the time of sampling can be crucial for the results. After heavy rain during the spraying season there is a risk of high transportation of pesticides. As the pesticides in one sample are the weekly average concentration there is a chance of pesticide concentration dilution. Weekly sampling can therefore give an erroneous depiction of the true concentrations of pesticides reaching the stream. Pesticide monitoring should therefore have shorter intervals between samples, especially during periods of spraying and heavy rainfall. 24 hour or 48 hour sampling should give a more accurate representation of transport. Also the risk assessment of pesticides must be based on more realistic exposure regimes, e.g. episodic, to reflect the pesticides transport to surface water. The duration and breakdown rates must also be considered when trying to predict the impacts of pesticides exposure. If additive and synergistic effects occur, even pesticide concentrations that are present at concentrations well below toxic levels can lead to major effects on aquatic biota.

4.6 Biological effects

When *Daphnia* and other cladocerans are exposed to pesticides several biological disturbances can appear. Studies have found that the concentrations that cause biological changes are significantly lower than lethal concentrations (Relyea & Hoverman 2006). Pesticides can have effects on the clutch size and also produce smaller juveniles. Barry et al. (1995) exposed *Daphnia* to sublethal concentrations of the organochlorine endosulfan and reported a reduction in clutch sizes. Hanazato & Dobson (1995) noted a reduction in juvenile growth rate when *Daphnia* were exposed to high concentrations of the carbamate carbaryl. The reduction in growth rate resulted in smaller body size when reaching maturation, which in turn led to smaller clutches and the production of smaller juveniles. Pesticides can also reduce the filtration activity. When *Daphnia galeata, Ceriodaphnia lacustris* and the copepod *Diaptomus organensis* were exposed to sublethal concentrations of the pyrethroid fenvalerate feeding rates were reduced by half (Day & Kaushik 1987). This can lead to reduced growth rate and reduced reproduction (Hanazato 2001). The swimming behaviour can also be affected by pesticides. Dodson et al. (1995) recorded behavioural changes, spinning and irritation, when *Daphnia* was exposed to carbaryl. Spinning behaviour was caused by acute toxic concentrations, whereas irritation from sublethal concentrations. The spinning behaviour made the *Daphnia* more vulnerable as they were easier to spot by predators. Reduced swimming ability was seen when *Daphnia* were exposed to sublethal concentrations of the organochlorine lindane and in turn made them easy prey for *Hydra oligactis* (Taylor et al. 1995). When affected animals are preyed on to larger extent it may lead to biomagnification in the food web (Hanazato 2001). There are also reports on increases in phytoplankton abundance when zooplankton populations are reduced (Rand et al. 2001), which indirectly can lead to phytoplankton bloom (Relyea & Hoverman 2006). Other studies have shown that pesticides can strongly affect other species. Gray tree frogs and leopard frogs were exposed to a mixture of 5 herbicides and 5 insecticides as tadpoles. 99% of the leopard frogs died, whereas all of the gray tree frogs survived. Furthermore, the gray tree frog grew twice as large without the competition from the leopard frog (Relyea 2009). From these studies it is evident that species have different sensitivities to pesticides and can lead to competitive release and changes in the structure of the ecosystem.

The highest concentration of pesticides from one weekly sample during pesticide monitoring in Sweden was 31 µg/l (Adielsson et al. 2009). The impact of this concentration on the aquatic life is obviously dependent on the toxicity of the pesticides in the sample. For example, the same total concentration of pirimicarb, fenitrothion and esfenvalerate gives a 50% inhibition on

Daphnia in this study. Moreover, 40% of the samples from the pesticide survey contained pesticides with concentrations higher than the Water Quality Standard concentration. Pesticide concentrations are expected to be higher when sampling is made more often to avoid dilution and degradation of pesticides. Many countries that have a higher agricultural load compared to Sweden use considerable higher amounts of pesticides on a smaller land area. These countries would be expected to have higher transport of pesticides as well. The effects on aquatic biota in these areas will therefore be greater than in Sweden. As studies have shown that low concentrations of pesticides can cause biological changes in organisms, there is a chance that concentrations found in the environment can lead to changes in the entire ecosystem.

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