Mixture Toxicity of Pesticides and Biological Effects in Agricultural Streams

Field and Laboratory Studies

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Cover: A Swedish agricultural stream
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Abstract

The main objective of this thesis was to examine pesticide mixture toxicity and its effects on aquatic biota in agricultural streams. This was accomplished by (1) calculating the ΣTUs for algae and *Daphnia magna* and a pesticide toxicity index (PTI) based on water quality standards (WQS) for long-term monitoring data (2002-2010), (2) by performing algal inhibition tests with *Pseudokirchneriella subcapitata,* using both collected *in situ* water samples and reconstituted water spiked with field-observed pesticide mixtures, and (3) by studying seasonal changes in macroinvertebrate and diatom communities during 2008 in four agricultural streams and determine a set of community-based metrics such as species richness, diversity, ASPT, SPEAR and IPS. Partial Least Squares (PLS) regression was used to analyse community changes.

Long-term data showed that collected stream water samples had peaks of estimated pesticide toxicity, mainly due to herbicides though insecticides and fungicides also contributed. $\sum TU_{algae}$, based on weekly average concentrations, exceeded 0.1 on 28 out of 902 occasions, and 8 of these were higher than 0.2. Only one or a few pesticides contributed to a major part of estimated mixture toxicity in the streams.

The algal growth inhibition studies in spiked water showed negative effects at pesticide concentrations corresponding to those found in stream water samples, indicating that pesticide effects on algae occur under *in situ* conditions. The field study also showed effects of pesticides, suggesting diatom diversity as a sensitive indicator of pesticide effects. Macroinvertebrate community changes were primarily explained by physiochemical conditions, though some non-insect invertebrates decreased (*Asellus aquaticus*) or increased (*Oligochaeta*) with pesticide exposure. Somewhat surprisingly, the $SPEAR_{pesticides}$ index, specifically developed to detect pesticide effects of macro-invertebrate communities in streams, was not related to pesticide toxicity. The SPEAR index may need to be modified to better fit to Swedish conditions.

A combination of chemical and biological monitoring is needed to increase our understanding of the relationship between pesticide stress and the biological diversity of agricultural streams. Specific endpoints and analysis methods are also needed to separate effects of pesticides from effects of other stressors.

Keywords: Pesticides, Macroinvertebrates, Algae, Diatoms, Toxicity tests, Monitoring, Bioassessment, Biodiversity, Mixture effects

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Look deep into nature, and you will understand everything better.

Albert Einstein

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Rydh Stenström, J., Kreuger, J., Goedkoop, W. (2013). Pesticide mixtures in Swedish streams and toxicity to aquatic algae (manuscript).

II Rydh Stenström, J., Gardeström, J., Kreuger, J., Goedkoop, W. (2013). Pesticide mixture toxicity and effects on invertebrate and diatom communities in agricultural streams (manuscript).

The contribution of Jenny Rydh Stenström (JRS) to the papers included in this thesis was as follows:

I JRS planned and designed large parts of the experimental work, was involved in the experimental work and was the lead author for the manuscript.

II JRS planned, designed and performed large parts of the field study, took part in sample processing, and performed statistical analysis. JRS was the lead author for the manuscript.

Abbreviations

ASPT	Average Score Per Taxon
CA	Concentration Addition
CV-ANOVA	Cross-Validation Analysis of Variance
DDT	Dichlorodiphenyltrichloroethane
EC	Effect Concentration
EFSA	European Food Safety Authority
EPT	Ephemeroptera Plecoptera Trichoptera
EQS	Environmental Quality Standards
EtOH	Ethanol
HCH	Hexachlorocyclohexane
IA	Independent Action
IPS	Indice de Polluo-sensibilité Spécifique
KEMI	Kemikalieinspektionen
LC	Lethal Concentration
OECD	Organisation for Economic Co-operation and Development
PLS	Partial Least Squares
PPDB	Pesticide Properties Database
%PT	Frequency of Pollution Tolerant valves
PTI	Pesticide Toxicity Index
SPEAR	Species At Risk
TU	Toxic Unit
VIP	Variable Importance on the Projection
WQS	Water Quality Standards

1 Introduction

The worldwide distribution of toxic substances is of major concern. Emission of anthropogenic pollutants to the environment has increased with progressive industrial development and a multitude of chemicals have been detected in environmental samples during the last decades. Several pollutants have been found at distances far away from the source, which shows a global spread (Unsworth et al. 1999). Chemicals are often developed for human use, with no intentions to make them toxic. For example, flame-retardants are used in flammable materials to limit the development of fire, and plasticizers are used in plastics to make the material softer. Chemicals like these are useful, but may have toxicity as a side effect to non-target organisms (e.g. de Wit 2002). Pesticides, however, have actually been developed with the main purpose to be toxic, e.g. to protect plants from various types of pests. They are used to act toxic on specific target organisms in order to control weeds, fungi or pest organisms. The widespread occurrence of different pest organisms impacting the harvest of agricultural crops makes pesticides in the form of plant protection products important for farmers all over the world. Due to the constantly increasing world population there is also a need for increasing food production (WWF 2012), and the use of pesticides has continuously increased since the 1950s both in number of chemicals and quantities sprayed over the fields (Carvalho 2006).

The pesticides used today have generally a higher selectivity and specificity toward the target-organisms, and are more readily degraded than many of the pesticides used some decades ago, e.g. DDT, dieldrin and HCH (Echobichon

2001). However, due to their inherent toxicity also to certain non-target organisms, the environmental fate of modern pesticides is still of great concern. Pesticides applied in agricultural fields can be transported from the site of application into aquatic ecosystems where they might reach and affect non-target organisms. The transport generally occurs through run-off or subsurface drainage and is induced by rain or irrigation (Liess et al. 1999, Brown and Beinum 2009), but also wind drift or incautious actions during the handling of pesticides are potential sources for pesticides entering stream waters (figure 1). Studies have shown that streams with an agricultural catchment area are susceptible to brief pesticide inputs (Kreuger 1995) and that pesticide concentrations can become considerably higher in streams at runoff events following heavy rains (Liess et al. 1999).

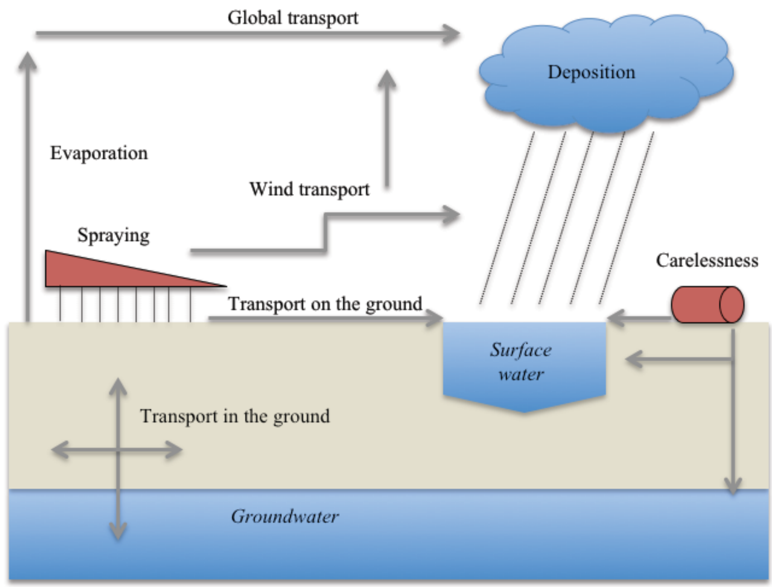

Figure 1. Potential pesticide transport routes to water.

Monitoring results have demonstrated a widespread presence of multiple pesticides in running waters of catchments dominated by agricultural land use (Kreuger 1998, Gilliom 2007, Rasmussen et al. 2011, Ansara-Ross et al. 2012). Biological effects of pesticides have also been reported for different aquatic organism groups, such as macroinvertebrates (Liess and Schulz 1999, Beketov et al. 2008), phytoplankton (Nyström et al. 1999, Debenest et al. 2009) and microbes (DeLorenzo et al. 2001, Widenfalk et al. 2004). In order to protect Europe's surface waters from chemical pollution, the European commission has established a directive on science-based environmental quality standards (EQS) (2008/105/EC), i.e. the highest estimated concentration in surface water at which no adverse ecosystem effects are expected. In addition to these European EQS, including some ten pesticides, the Swedish Chemicals Agency has established national water quality standards (WQS) for an additional hundred pesticides (KEMI 2008).

To coordinate and evaluate effects of chemicals on aquatic organisms internationally, a set of standardised laboratory guideline tests is used, encompassing different organism groups (e.g. OECD 2006). These tests are designed to evaluate the toxicity of single substances and developed to be relatively easy to perform, and thereby increase inter-laboratory comparability. In these tests, most non-treatment factors, e.g. light, temperature and water flow, are controlled in order to establish specific cause-and-effect relationships. A disadvantage of these simplified tests is that results may not reflect the actual toxicity in a complex ecosystem with multiple compounds and all its physical and chemical variations (Baird and Burton 2001, Probst et al. 2005). These standardised tests with single compounds may therefore not reflect field-relevant conditions and could lead to an underestimation of true toxicity, as compounds may interact and have combined effects (Junghans et al. 2003, Lydy et al. 2004). Also, the nutrient amount and dominating vegetation type in an ecosystem could influence the effects of pesticide exposure (Wendt-Rasch et al. 2004). Possibly, standardised tests could lead to overestimations of the toxicity encountered in the field, e.g. due to lower bioavailability of certain pesticides under field conditions than predicted based on laboratory tests (Lundqvist 2011). Prediction of combined pesticide effects is usually based on models of concentration addition (CA, assuming a similar mode of action for the mixture components) or independent action (IA, assuming a dissimilar mode of action). 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. 2004). As concentration addition is the more conservative model, several studies are recommending concentration addition to be used in assessment for both scenarios with similar and dissimilar ways of action in order to achieve a worst case scenario (Belden et al. 2007a, Cedergreen et al. 2008).

Running waters are habitats with environmental characteristics that have given the opportunity for development of a unique flora and fauna. There is a rapid turnover of water and much of the energy is often derived from organic matter produced in terrestrial ecosystems. Streams are in close contact with the surrounding catchment and the soil and geology of the catchment thus have a great impact on the water chemistry (Giller and Malmqvist 1998). Because of the downstream flow of water, most species are associated with the substrate by being attached to it, rooted in it, or living on or in it. Many taxa of freshwater macroinvertebrates are confined to running waters, and Chironomidae (midges), Odonata (dragonflies and damselflies), Ephemoptera (mayflies) and Plecoptera (stoneflies) are believed to have evolved in cool running waters (Giller and Malmqvist 1998). Benthic algae are usually the major primary producers in streams and fill an important ecosystem function as they provide food for higher trophic levels. Diatoms constitute the base of aquatic food webs, supplying consumers with important fatty acids (Brett and Müller-Navarra 1997). Effects on algae may indirectly induce effects on higher trophic levels and important ecosystem processes (Lamberti and Steinman 1997, Fleeger et al. 2003). Also, several studies have shown that long-term and repeated exposure to pesticides can induce tolerance in algae (Molander and Blanck 1992, Berard et al. 2002), which could be considered a long-term effect.

Direct uptake of pesticides from the water or sediment occurs by cells or roots and by gills or integument for aquatic plants and animals, respectively. Also, ingestion of aquatic organisms is a route of exposure (EPA 2011). Many insecticides interfere with membrane transport of different ions, enzyme activities or release of transmitters, affecting the nervous system, while herbicides interfere with mechanisms such as photosynthesis, respiration, growth, cell and nucleus division, or synthesis of proteins, carotenoids or lipids (Echobichon 2001, DeLorenzo et al. 2001). At the individual level, pesticide interference can lead to reduced survival, growth or reproduction of an organism. Reduction in primary productivity or prey availability can secondarily lead to changes along the food chain. At the community level, there could be changes in species number or diversity, reduced number of sensitive species and shifts of dominating species. Also, community changes such as reduced cover can modify the habitat structure (EPA 2011).

Biological macroinvertebrate indices such as Average Score per Taxon (ASPT) or Ephemeroptera-Plecoptera-Trichoptera (EPT) are important tools in surface water quality assessment, providing measures of ecological quality and indicating effects of general organic pollution (EPA 2007). Macroinvertebrates could also provide a bioassessment tool for the quantification of pesticide effects at the community level. E.g. the Species At Risk (SPEAR) concept is a classification system based on ecological traits, where macroinvertebrates are classified according to traits that indicate a high sensitivity to pesticides, life-cycle traits and recovery potential, being at risk or not at risk of negative effects from pesticide exposure (Liess and von der Ohe 2005). Contaminant effects on communities of benthic invertebrates could be more long-lasting and have stronger effects at the ecosystem level than effects on algae, considering the short generation times and subsequent rapid recovery of algal communities in their nutrient-rich habitats (Brain et al. 2012, Debenest et al. 2009).

2 Objectives

The main objective of this thesis was to contribute to the understanding of pesticide mixture toxicity and its effects on aquatic biota in agricultural streams. This was accomplished by the evaluation of long-term pesticide monitoring data and directed studies of the effects on algal growth under laboratory conditions, as well as on benthic macroinvertebrate and diatom communities under field conditions. The major questions addressed were:

- What is the estimated toxicity of pesticide mixtures in Swedish agricultural streams and how does it vary seasonally and among years? (Paper I and II)
- Do the observed pesticide mixtures have negative effects on algal growth? (Paper I)
- Is ecological structure, i.e. community composition, of benthic algae and invertebrates in agricultural streams affected by pesticide exposure? (Paper II)

3 Methods

3.1 Study sites (Paper I and II)

The sites used for the studies in this thesis were four small (8-16 km^2) agricultural streams in southern Sweden (figure 2). The sites, referred to as O18, E21, N34 and M42, are included in the long-term national monitoring programme for pesticides, and have been specifically selected for a high proportion of agricultural land use in their catchment (85-94%). They are all located within the nemoral (M42 and N34) or boreonemoral (O18 and E21) ecoregions, which have similar macroinvertebrate assemblages in Sweden (Sandin and Johnson 2000). Within these areas information is collected annually regarding crops grown, including nutrient and pesticide management practices, on a field level. About 20-60 pesticides have been used in each area per year 2002-2010. Drainage systems have been installed in all four streams, while vegetated buffer strips are absent or only partly present. None of the investigated streams originate from an upstream site without agricultural practices (upstream recovery site).

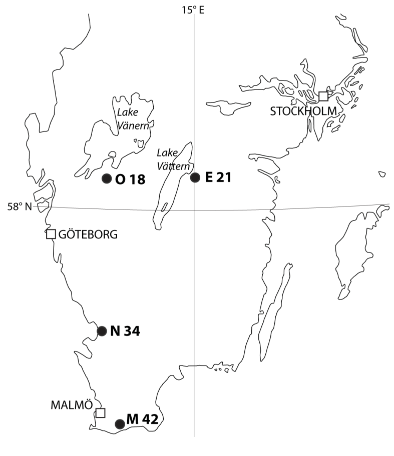

Figure 2. Map of South Sweden showing the location of the sampling sites O18, E21, N34 and M42.

3.2 Water sampling and analysis (Paper I and II)

Water samples were collected as part of our national monitoring program for pesticides from the four study sites. Samples were taken every 80th minute in the streams with an automatic water sampler (ISCO™) and were immediately stored in a built-in refrigerator (4^{o}C). Accumulated water samples were collected for analysis on a weekly basis, thus providing weekly average pesticide concentrations. Samples were analysed for 81-86 pesticides in 2005 and 2006 (paper I) and 82-84 pesticides in 2008 (paper II) at our accredited pesticide laboratory. In 2005-2006, immediately after extraction of the subsample used for pesticide analysis, the remaining stream water samples were frozen in glass beakers and later used in algal inhibition experiments (paper I). Water samples for analysis of inorganic water chemistry variables (paper II) were manually collected as grab samples once per month between April and October 2008. Data on inorganic water chemistry was supplemented with data from monitoring databases (SLU data host 2012). Samples were analysed using standardised methods in accredited laboratories at SLU.

3.3 Organism sampling and processing (Paper II)

Macroinvertebrates were collected from the four sampling sites with a modified Surber sampler with a surface area of 675 cm^2 and a mesh size of 0.5 mm. On each occasion 10 samples were taken over a stream length of approximately 100 m. Macroinvertebrate samples were sieved (0.5 mm) and were preserved in 70% EtOH in the field. Diatom samples were collected in three of the sites (not in N34 due to lack of substrates) by brushing 5 cobbles (area approximately 60 cm^2 per stone mixed in 250 mL tap water) and preserved in 70% EtOH. In the laboratory, invertebrate samples were sorted and identified to the lowest possible taxonomic unit, commonly species level, except for Chironomidae (to family), Oligochaeta (to subclass) and Bivalvia (to class). The diatom valves were boiled in superoxide and embedded in Naphrax®. At least 400 diatom valves per sample were selected for species identification. The procedure followed the EU standard methodology described in SIS 2003 and SIS 2005. Macroinvertebrate endpoints were taxa, family and genera number, number of individuals, Shannon Diversity, Average Score Per Taxon (ASPT), Ephemoptera-Plecoptera-Trichoptera index (EPT) and Species At Risk ($SPEAR_{pesticides}$). Diatom endpoints were species richness, Shannon Diversity, Indice de Polluo-sensibilité Spécifique (IPS) and frequency of pollution tolerant valves (%PT) (for more details on the indices, I refer to paper II).

3.4 Algal growth inhibition tests (Paper I)

We performed a set of alga inhibition tests using both *in situ* stream water samples and spiked reconstituted water. *In situ* water samples were identical to those that were used for pesticide analyses (see above). In tests with *in situ* water samples, growth medium was prepared from stock solutions according to the guideline of freshwater alga growth inhibition test (OECD 2006) and used as control water. Nutrients were added to stream water samples in the same concentrations as in the control water, to exclude effects on algal growth due to nutrient limitation. Alga growth inhibition tests were performed on 61 stream water samples, divided into 15 different test runs. After 0 and 72 h, the number of algae cells was estimated by cell counting using a light microscope or by measuring fluorescence with a fluorometer. Algal growth rates and growth inhibition was calculated according to the guideline (see paper I for more information).

We also performed a set of algal growth inhibition tests using spiked reconstituted water to further quantify pesticide mixture toxicity of the streams under more controlled water chemistry conditions. For these tests, we identified the eight pesticide mixtures with the highest estimated algal toxicity in water samples from the four study sites in 2002-2010. Algal toxicity was calculated as summed toxic units for algae (ΣTU_{algae}) and the eight selected mixtures all had a ΣTU_{algae} higher than 0.2 (see also 3.5). Growth media were spiked with the prepared pesticide mixtures at concentrations of 0 (controls), 0.1, 0.5, 1, 10, 50 and 100 times those found in stream water mixtures. After 0 and 72 h, algae cells were quantified, algal growth rates and inhibition was calculated.

3.5 Pesticide mixture toxicity (Paper I and II)

Stream water samples with estimated high mixture toxicity were identified using weekly average data from long-term pesticide monitoring in the four agricultural stream sites (Graaf et al. 2011). Altogether, we used data for 77-111 pesticides from 902 water samples collected during 2002-2010 (paper I) or 82-84 pesticides from 85 samples collected in 2008 (paper II).

In order to estimate the toxicity of pesticide mixtures, summed toxic units (ΣTUs) were calculated for each water sample. The concept of toxic units (Marking 1985) is based on the concentration of each pesticide in the mixture in relation to an EC_{50}-value for that pesticide, EC_{50} being the concentration when 50% of the organisms exposed were affected according to standardised acute toxicity guideline tests (e.g. OECD 2004, 2006). We calculated ΣTUs based on EC_{50} values for aquatic algae or the water flea *Daphnia magna*:

$$\sum TU = \sum_{i=1}^{n} \frac{C_i}{EC50_i}$$

where ∑TU is the sum of toxic units for the *n* pesticides detected, C_i is the concentration of the pesticide *i* and $EC50_i$ is the EC_{50} for pesticide *i* for the exposed organisms. EC_{50}-values were primarily obtained from the literature used to calculate Swedish Water Quality Standards for pesticides (Andersson and Kreuger 2011, KEMI 2008) and secondarily from established pesticide databases (EU Pesticides database, EFSA or PPDB). Effect concentrations were generally EC_{50}-values from 72-hours or 96-hours green algal growth inhibition tests according to OECD (2006) and from 48-hours Daphnia immobilization tests according to OECD (2004).

Also, a pesticide toxicity index (PTI) was calculated (Belden et al. 2007b, Munn et al. 2006), based on the concentration of each pesticide in the mixture in relation to the Swedish Water Quality Standards (WQS). A WQS is the concentration of a substance for which no effects can be expected on the aquatic environment, determined according to guidelines in the EU Frame Directive for water 2000/60/EEC and internationally accepted methods of EU Technical Guidance Document (KEMI 2004). WQS are based on results from toxicity tests on different aquatic organisms and include a safety factor between 10 and 1000, depending on the number and reliability of the tests (KEMI 2008).

$$\mathrm{PTI} = \sum_{i=1}^{n} \frac{C_i}{WQS_i}$$

where C_i is the concentration of the pesticide *i* and WQS_i is the Swedish Water Quality Standard for the pesticide *i* (Andersson and Kreuger 2011).

In paper I, predictions of effect concentrations for the mixtures by concentration addition (CA) were calculated (see Faust et al. 2001):

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

where ECx_{mix} is the estimated toxic effect of the mixture, p_i is the fraction of component i in the mixture and ECx_i is the individual effect concentrations when applied singly. CA predicts toxicity of mixtures with similar modes of action.

For more specific information on calculations, I refer to the method parts in the papers.

3.6 Statistical analyses (Paper I and II)

Differences in algal growth (paper I) were tested using one-way ANOVA, complemented with Tukey means pairwise comparison test. The relationships between pesticide mixture toxicities were examined with linear regression. When needed, the data was log-transformed for normal distribution. Analyses were performed in JMP10® (SAS Institute Inc.).

When biological responses were tested against pesticide toxicity (paper II), the highest PTI or ΣTU from one month before each organism-sampling occasion, or the highest toxicity value between two sampling occasions, were selected. These values are referred to as PTI*max* and ΣTU*max*. The relative importance of different variables (pesticide toxicity, month and physical and chemical water parameters) for biological endpoints was assessed using partial least squares regression (PLS). This multivariate statistical method is a suitable analytical tool for data sets with fewer observations than variables and a high degree of intercorrelation between the independent variables (ter Braak and Juggins 1993, Eriksson et al. 1995). Extracted components are used to construct a predictive model for the response variable, e.g. diversity, with the relative importance of predictor variables ranked via variable importance on the projection (VIP) values. All significant components were extracted for analysis. Variables with a VIP exceeding 1 are the most important for explaining the variance in community response, though values 0.7-1 can also be considered important (Eriksson et al. 2006). PLS was conducted with SIMCA-P (version 12.0.1; Umetrics AB, Umeå, Sweden).

4 Results and Discussion

4.1 Estimates of pesticide mixture toxicity (Paper I and II)

We analysed data from 902 water samples from four streams that had been analysed for 77-111 pesticides during 2002-2010. In 63% or more of the samples from three of the four sampling sites, more than 10 pesticides were detected, with 90-percentiles ranging 21–29 pesticides. The average number of pesticides in a single sample ranged from 8.8±3.7 in O18 (mean ± SE, used throughout) to 17.8±7.4 in M42, while the maximum was 41 pesticides in a single sample. These results illustrate the complexity of exposure by pesticides in agricultural streams. Our analysis of long-term data on pesticide concentrations showed that $\sum TU_{algae}$, based on weekly average concentrations, exceeded 0.1 on 28 occasions, and 8 of these were higher than 0.2. The number of pesticides in these samples was 21±3 (90-percentile 34) (figure 2). Also, 70% of these occasions with a $\sum TU>0.1$ occurred between May and July. The eight pesticide mixtures with a $\sum TU_{algae}$ >0.2 were selected for laboratory studies with spiked reconstituted water (results in 4.2). A meta-analysis of results from freshwater mesocosm studies suggest that effects of herbicide contamination can occur above a TU of 0.1 for algae (Brock et al. 2000) where TU is based on EC_{50}-values of the most sensitive standard alga, mainly *Pseudokirchneriella subcapitata* or *Scenedesmus subspicatus*. Applied to our study, our analysis shows toxicity on algae in at least 3% of analysed water samples. This is most likely an underestimate of true toxicity as our findings are based on weekly average concentrations that do not capture the peak concentrations that likely occur under shorter intervals (Liess et al. 1999, Xing et al. 2013). Adielsson and Kreuger (2008) showed occasions in M42 with temporary pesticide peaks in concentrations 10 times higher, occasionally even up to 100 times, when momentary samples and runoff-triggered samples were taken at the same time as the regular weekly samples. This was the case e.g. for MCPA, metamitron, quinmerac, metazachlor, isoproturon and glyphosate, all commonly found in our tested stream waters and several also present in the mixtures used in our tests with reconstituted spiked water. In 2002-2010, 396 of the 902 weekly water samples from the streams had a $\sum TU_{algae}$ higher than 0.01, which could according to the results from Adielsson and Kreuger (2008) mean a $\sum TU_{algae}$ of at least 0.1 during a temporary peak these weeks.

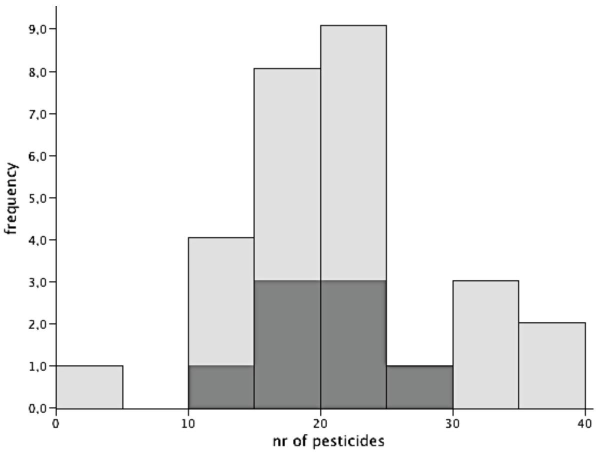

Figure 2. Histogram showing the number of pesticides in the 28 samples with a $\sum TU_{algae}$ >0.1 from four streams in 2002-2010. The darker areas constitute samples with a $\sum TU_{algae}$ >0.2.

Our analysis of long-term monitoring data also shows that the toxicity of pesticide mixtures with a high estimated toxicity frequently is set by one or a few compounds that contribute to more than 90% of mixture toxicity to algae, despite the multitude of pesticides in a single sample (figure 3). Considering our extensive dataset, covering nine years and 77-111 pesticides, the observation that toxicity is set by a few substances could be a general pesticide mixture scenario.

A comparison of $\sum TU_{algae}$ and $\sum TU_{daphnia}$ from the monitoring data of 2008 showed that ΣTU_{algae} was generally higher than $\Sigma TU_{daphnia}$, with means of 0.026±0.007 (highest value 0.58) and 0.0057±0.002 (highest value 0.16), respectively, suggesting that algae were more exposed than invertebrates.

However, the neonicotinoide insecticides imidacloprid and tiacloprid could have occurred at concentrations higher than WQS without showing in the data: The detection limit for imidacloprid (0.2 µg/L) was higher than WQS (0.03 µg/L) until 2008, and tiacloprid was used at site M42 during 2008 but not included in analysis until 2009. Herbicides were most frequently exceeding their WQS, though the largest exceedance of WQS was found for a few insecticides and fungicides (more details in paper II). Linear regression analyses showed that PTI was related to $\sum TU_{algae}$ and $\sum TU_{daphnia}$, (R^2=0.70 and 0.63, respectively, $p<0.0001$). Also, $\sum TU_{algae}$ and $\sum TU_{daphnia}$ were related to each other (R^2=0.51, $p<0.0001$). ∑TUs are most likely a good choice to use in pesticide toxicity investigations of a particular organism group, while PTI, being a more general pesticide toxicity index including a safety factor, can be a useful tool for surface water protection.

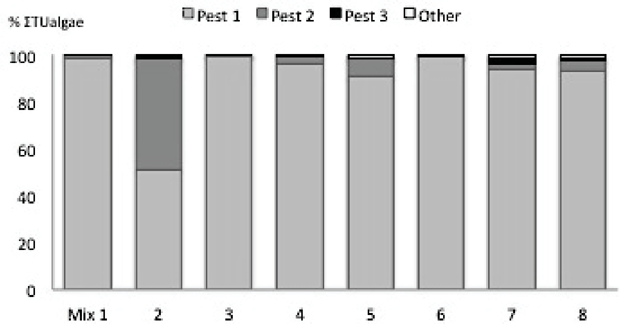

Figure 3. Toxicity distribution for the pesticides in each of the eight mixtures (1-8) with the highest $\sum TU_{algae}$ (>0.2) that were used for algal inhibition tests with reconstituted water. Presented here are the three pesticides that contributed to the most toxicity (Pest 1-3) in each of the mixtures and all other pesticides summed together for each mixture (Other).

4.2 Pesticide growth effects on *Pseudokirchneriella subcapitata* (Paper I)

During 2005–2006 between 6 and 24 pesticides were detected in the 61 time-integrated weekly stream water samples, with an average of 13±2. Laboratory studies on collected stream-water showed that algal growth inhibition occurred in natural pesticide-contaminated water. In 27 of 61 time-integrated samples of stream water we found a 6-66 % significant inhibition of algal growth. PTI for samples that showed inhibition were significantly higher than those lacking inhibition, indicating the reliability of this indicator and suggesting a relation between algal inhibition and pesticide toxicity. Also, results from tests using reconstituted water spiked with eight selected pesticide mixtures showed that inhibition of algal growth occurred at 1–10 times the concentrations found in the stream water. As our findings are based on weekly average concentrations and do not capture the peak concentrations that occur under shorter time intervals (Adielsson and Kreuger 2008), the observed effects from pesticide toxicity is most likely an underestimate of true toxicity in the streams. These findings strongly suggest that toxic effects on algae occur in the field.

The EC_{50}-values for the eight tested pesticide mixtures were 2-17 times higher than estimated EC_{50}-values according to the model of concentration addition (CA), except from two of them, where these were 2-3 times lower. However, mixtures acting strictly similar or dissimilar should be rare in nature. Indeed, our eight mixtures all include pesticides with dissimilar modes of action, and six of them also include similarly acting pesticides. As the CA model predicts higher toxicity than the IA model and as mixture toxicities higher than predicted by CA are rare, CA is suggested to be a better, precautious approach in risk assessment, regardless mechanisms of action of the mixture components (Backhaus and Faust 2012). Our results support this conclusion, since six of the eight most toxic mixtures found in the streams 2002-2010 were less toxic than expected according to CA. However, two mixtures were more toxic than expected, suggesting that the CA model may not always be precautious enough.

4.3 Pesticide effects on diatom and macroinvertebrate community structure (Paper II)

PLS identified PTI*max* and $\Sigma TUmax_{algae}$ as very important factors (VIP>1) for diatom diversity, with negative coefficients showing diversity declines with increasing toxicity (table 1). Additionally, PLS identified several physical and chemical water parameters such as calcium, conductivity and pH, maximum flow and silicon as important for diatom diversity. However, pesticide toxicity was positively related to IPS and negatively related to %PT, i.e. IPS increased and %PT decreased with increasing toxicity. This contradictory result could be related to the fact that the indices are developed to quantify effects of eutrophication and/or organic loading and do not specifically address pesticide effects. Also, the status according to Swedish Water Quality Criteria did not differ much between months; all IPS-values represent good or moderate conditions for all streams and PT% indicate constantly good status in E21, mainly bad status in O18 and bad to moderate status in M42 (EPA 2009). PLS also included other factors, such as calcium, conductivity, aluminium and silicon, as important factors for the responses. These results may partly be due to a few extremely high concentrations of aluminium. Diatom species richness gave a highly non-significant PLS model (p=1.0) and was therefore not included in analyses. A previous study has suggested that the complex biological matrix (biofilm), where benthic diatoms evolve, may protect these algae against pesticide effects (Peres et al. 1996). Our results suggest that diatom diversity is a sensitive indicator of herbicide effects in inland waters. However, due to the short generation time of algae, algal communities may recover rapidly and effects of herbicides may not be very long-lasting.

Most of the variance in macroinvertebrate abundance and community metrics was explained by physical and chemical water parameters such as conductivity, calcium, flow and pH, in PLS analyses (table 1). Also metals were important factors with a VIP>1, showing lower ASPT and SPEAR with higher concentrations of cadmium and copper. Seasonal changes (months) were important factors for several responses, month 9 and 10 (September and October) in particular, and there were also important differences between the sites. PLS did not select pesticide toxicity among the most important variables in explaining macroinvertebrate abundance or metrics variance. The studied streams were highly eutrophicated with generally low macroinvertebrate diversity and index results sometimes below good ecological status according to Swedish Water Quality Criteria (EPA 2007), indicating a fauna affected beyond seasonal pesticide exposure. It is a well-known fact that eutrophication by itself can cause long-term effects on species diversity (e.g. Smith et al. 1999, Hering et al. 2006). Moreover, these streams are small (catchment $\leq$ 16 km^2) and narrow (width below 2m), which contributes to low diversity, due to lower habitat complexity and fewer niches (e.g. Probst et al. 2005). Our results indicate a macroinvertebrate fauna affected by factors other than pesticides, such as stream habitat structure and degradation common in agricultural streams (Rasmussen et al. 2012, von der Ohe and Goedkoop 2013). The occurrence of other contaminants may also affect communities. Our PLS analyses show that several metals were negatively related to both diatom and macroinvertebrate metrics. Also, our analysis of $\sum TU_{daphnia}$ shows only a single value higher than 0.1, suggesting lower toxicity for macroinvertebrates than for algae in the investigated streams.

Table 1. PLS regression results for diatom diversity (Shannon), Indice de Polluo-sensibilité Spécifique (IPS), frequency of pollution tolerant valves (%PT), macroinvertebrate abundance, Average Score Per Taxon (ASPT) and $SPEAR_{pesticides}$, presented with explained variance (R^2), p-values (CV-ANOVA) and number of components. All variables with a VIP (variable importance on the projection) more than 1 are listed in descending order, with positive or negative relations (+/-). Seasonal changes (months) were included as qualitative variables. For more details, I refer to table 5 and 6 in paper II.

	Diatom diversity		**IPS**		**%PT**
	R^2=0.66, p=0.0061		*R^2=0.93, p<0.0001*		*R^2=0.62, p=0.015*
	1 component		*2 components*		*1 component*
-	Ca	+	Ca	-	Ca
-	Conductivity	+	Conductivity	-	PTImax
+	Al	-	Al	-	Conductivity
-	$\Sigma TUmax_{algae}$	+	$\Sigma TUmax_{algae}$	+	Si
-	PTImax	-	Pb	-	$\Sigma TUmax_{algae}$
-	pH	+	PTImax	-	pH
+	Pb	-	Si	+	$Flow_{max}$
+	$Flow_{max}$	-	pH	+	Al
+	Si				
	Macroinvertebrate abundance		**ASPT**		**$SPEAR_{pesticides}$**
	R^2=0.83, p<0.001		*R^2=0.75, p=0.025*		*R^2=0.51, p=0.13*
	2 components		*2 components*		*1 component*
+	Conductivity	+	Month 10	-	TP
+	Ca	-	Si	-	Cu
-	pH	-	Cu	-	Cd
-	Flowmin	-	Month 9	-	Month 9
-	Flowmax	-	Cd	+	Flowmin
-	Al	-	TP	-	Pb
		+	Pb	+	Month 10
		-	Month 7		

The seasonal emergence of aquatic insects confounds the use of macroinvertebrate samples collected during summer since there is a natural decrease of this type of organisms, often at times of elevated pesticide concentrations in streams. One way to avoid this type of bias could be by specifically studying the taxa that are not in the adult emerging stage during pesticide exposure. This is included in $SPEAR_{pesticides}$, where taxa with a flying stage at time of pesticide application is considered not at risk (Liess and von der Ohe 2005). SPEAR index values in our streams were generally low (ASTERICS 2012, Beketov et al. 2009) with seasonal means of 21.1±2.0 in O18, 22.8±3.2 in E21, 25.8±1.9 in N34 and 15.3±3.0 in M42. Interestingly, our PLS results show that pesticide toxicity was not important in explaining the variance of $SPEAR_{pesticides}$. Possibly, the concentrations of insecticides and fungicides in our streams were too low to induce any effect. For example lindane was found at trace levels of 0.001 μg/L in one of our streams, while the highest concentration was 0.5 μg/L in the streams of Liess and von der Ohe (2005). The fact that site was the highest explaining factor shows that there were important differences among the streams, Also, there could be differences in traits such as hatching time between the European streams incorporated in the development of SPEAR and Swedish streams. SPEAR may need some adjustment to be useful to the streams of our climate.

Analysis of common macroinvertebrate taxa that lacked emerging adult at the time of elevated pesticide levels showed that the isopod *Asellus aquaticus* (waterlouse) dropped in abundance on several occasions when PTI or ΣTUs peaked. In N34, the abundance of *Asellus aquaticus* increased from 49±26 ind/m^2 in April to 877±362 ind/m^2 in June, but then showed a sudden population crash in July (p=0.026, t-test), co-occurring with PTI and $\Sigma TU_{daphnia}$ peaks (data not shown). Also in E21, *Asellus aquaticus* abundance decreased by 79% i.e. from 706±279 to 148±65 ind/m^2, between April and July (p=0,046), when PTI and $\Sigma TU_{daphnia}$ values peaked. At the same time, the organism group *Oligochaeta* increased by 808%, i.e. from 332±211 in May to 3013±1161 ind/m^2 in July (p=0.036) (figure 4), maybe due to less habitat or food competition. Asellus is considered sensitive to pesticide peaks, while Oligochaeta is not (Liess and von der Ohe 2005), indicating effects of pesticides for some taxa in the streams. Despite absence of upstream recovery sites, the recovery of *Asellus aquaticus* between July and September may be due to drift from a small bi-flow originating in a forest site in the Eastern part of that catchment area.

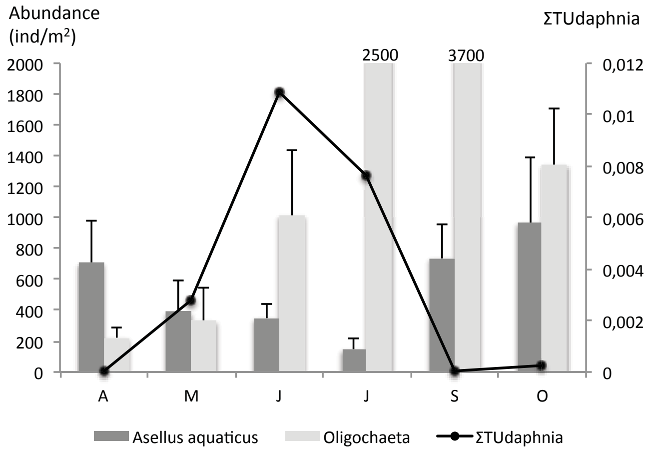

Figure 4. Abundances of *Asellus aquaticus* and *Oligochaeta* with co-occurring highest values of $\Sigma TU_{daphnia}$ at stream E21 in April, May, June, July, September and October 2008. Error bars represent SE.

5 Conclusions

- Temporary peaks of estimated pesticide toxicity occurred in collected stream water samples, mainly between May and July, which is the main season for pesticide application in the fields. Otherwise, most water samples had a low estimated toxicity. The peaks were mainly due to toxicity from herbicides, but a few insecticides and fungicides had the highest toxicity values.
- Our analysis of pesticide monitoring data suggest that only one or a few pesticides contribute to a major part of summed toxicity in streams, despite the large number of pesticides that occur in the samples.
- The laboratory studies showed effects on algal growth at pesticide concentrations found in water samples from the investigated streams, indicating that pesticide effects on algae occur under *in situ* conditions.
- The field study showed effects of pesticide exposure on the diatom communities, indicating that diatom diversity can be a sensitive indicator of pesticide effects. However, diatoms have a short generation time and a high recovery potential, and effects from pesticide pulses may therefore not be long-lasting.
- Effects on the macroinvertebrate communities were primarily explained by physiochemical conditions in the streams, while pesticide toxicity did not contribute. This could be due to a low toxicity for macroinvertebrates or due to a bias caused by other stressors such as eutrophication or low habitat complexity. However, some organisms decreased (*Asellus aquaticus*) or increased (*Oligochaeta*) with pesticide exposure.

- The SPEAR index, specifically developed to detect pesticide effects of macroinvertebrate communities in streams, was not related to pesticide toxicity. $SPEAR_{pesticides}$ specifically addresses pesticide effects. This index may, however, need to be modified to better fit to Swedish conditions.
- The other biological indices (IPS, PT%, ASPT, EPT) were not able to indicate negative changes due to pesticides. Biological indices are important tools in surface water quality assessment, but are probably not specific enough to assess pesticide effects.

6 Future perspectives

A multitude of strategies has been used to assess toxicity of chemical compounds in water, running from standardised toxicity testing, more or less advanced experimental approaches, to complex field studies at the ecosystem scale. In general, the more simple and controlled systems that are used, the clearer results and specific cause-and-effect relationships can be obtained. However, the more complex systems or studies, the more environmentally realistic conditions can be assessed. When possible, a combination should be preferred.

A combination of chemical and biological monitoring is needed to increase our understanding of long-term evolvement of the biological diversity in agricultural streams and possibly to discover changes when new groups of chemicals are introduced on the market. Useful tools, such as specific endpoints (e.g. SPEAR) and analysis methods suitable for data with a high degree of inter-correlation (e.g. PLS) are needed to separate effects of pesticides from effects of other stressors.

Weekly average pesticide concentrations probably do not capture the highest peaks, leading to toxicity underestimations. For a more thorough investigation of peak exposure scenarios, runoff-triggered samples should be collected. Also large-scale pesticide monitoring with the intention to capture the peak exposures that have the largest effects on biota should include runoff-triggered samplers. Pesticide monitoring efforts should focus on hot spots of pesticide effects, i.e. sites with high pesticide use and with high slopes that lack buffer strips, that can be identified using pesticide data (when available) or from modelling approaches.

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