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Testing biological pesticide indices for Danish streams

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List of abbreviations and central terms used in the report

Ecological Quality Ratio (EQR) = Ecological Quality Ratio is a measure of the relative deviation of an ecological community from the reference scenario. Values range from 0 to 1 with higher values being indicative of closer resemblance to the reference scenario.

%SPEAR = % SPEcies At Risk of being affected by periodic pesticide. SPEAR is based on macroinvertebrate data from streams.

Toxic Units (TU) = Toxic Units is a measure for pesticide toxicity towards selected standard test species which can be used to predict the total toxicity of a complex pesticide mixture. The standard test species is used as surrogate for selected groups of organisms occurring in the field (i.e. *D. magna* is used as benchmark for macroinvertebrates, and *P. subcapitata* is used for all photosynthesising organisms). This approach is used as pesticide concentrations is no useful predictor for ecological effects, as the effect basically depends on the potency of active ingredient. This approach additionally allows the inclusion of all pesticide groups (herbicides, fungicides, insecticides) in the analysis of results as all pesticides are toxic to all organisms when concentrations are sufficiently high.

SumTU = The sum of Toxic Units in a water or sediment sample. The Toxic Unit is calculated for each pesticide component in the sample. The SumTU represents the sum of all individual TU values in the sample. The SumTU concept relies on the assumption of toxic additivity (no toxic interactions among active ingredients).

MaxTU = The maximum Toxic Unit of a single pesticide in a sample containing multiple pesticides. I.e. the pesticide in the sample containing the highest single Toxic Unit.

EC50 = The concentration of a chemical causing effect in 50% of the tested organisms.

LC50 = The concentration of a chemical causing mortality in 50% of the tested organisms.

Content

1. Introduction

Pesticides frequently occur in Danish streams (Bøgestrand 2007; Rasmussen *et al.* 2011b; Petersen *et al.* 2012), and the highest concentrations are proposed to originate from surface runoff and tile drain flow facilitated by heavy precipitation events (Kronvang *et al.* 2003a; Petersen *et al.* 2012). However, chronic occurrence of especially herbicides and fungicides in low concentrations (in the ng L^{-1} scale) is a basic element of stream base flow conditions in agricultural catchments (Nanos, Boye & Kreuger 2012). Furthermore, a significant proportion of pesticides with low water solubility, such as many insecticides, are retained as sorption complexes in the bed sediment (Kuivila *et al.* 2012; Nanos, Boye & Kreuger 2012). Consequently, stream biota in especially agricultural streams is continuously exposed to pesticides via water and sediments punctuated by shorter peak concentrations in stream water (Nanos, Boye & Kreuger 2012).

Multiple controlled experiments have documented effects of environmentally realistic concentrations of pesticides on stream biota with special emphasis on insecticide effects on stream macroinvertebrates (e.g. Liess & Schulz 1996; Beketov & Liess 2008b; Nørum *et al.* 2010; Rasmussen *et al.* 2013b; Agatz, Ashauer & Brown 2014). However, recent studies show that fungicides affect freshwater fungi communities (Feckler, Kahlert & Bundschuh 2015; Fernandez *et al.* 2015; Zubrod *et al.* 2015), and these effects may propagate through the food chain (Bundschuh *et al.* 2011; Rasmussen *et al.* 2012a). Moreover, herbicides as well as fungicides at environmentally realistic concentrations may affect biofilm communities (Artigas *et al.* 2014; Andrus *et al.* 2015; Feckler, Kahlert & Bundschuh 2015; Lorente *et al.* 2015). Importantly, pesticide effects may propagate beyond the directly impacted organisms and influence other organisms through changed community interaction (Schäfer, Van den Brink & Liess 2011).

In spite of the multiple controlled experiments showing pesticide effects on various groups of stream organisms within the range of environmentally realistic concentrations, extrapolation of these results to the field level is strongly challenged by multiple factors of uncertainty. Firstly, there is an alarming scarcity of ecotoxicological field studies (Beketov & Liess 2012) which confounds the quantification of pesticide-response mechanisms at the level of communities and ecosystems due to insufficient amounts of data. Moreover important, agricultural streams are influenced by multiple concomitant stressors including channelization, dredging, weed cutting, removal of riparian vegetation, and eutrophication. Many of these stressors are likely to impose effects similar to those of pesticides on especially stream macroinvertebrates (Friberg 2014). In recognition of the significant effects of pesticides on stream biota, stream managers are in need of tools which can reliably assess these effects. Pesticide effect assessments are necessary to provide valuable feedback to the current pesticide regulation (retrospective risk assessment) and additionally in context of the Water Framework Directive where causes for significant deviations from the good ecological status in surface water bodies need diagnosis to optimize targeted mitigation efforts. Therefore, the development of pesticide specific indicators should thoroughly address i) a thorough characterization of pesticide exposure for water and sediment and some estimation of concentration ranges between stream flow regimes, and ii) the influence of confounding stressors should be minimized in order to promote the pesticide response signal.

Benthic stream macroinvertebrates have traditionally been used as indicators for various anthropogenic stressors since their sensitivity to these is high and their life span sufficiently long to integrate effects (Rosenberg & Resh 1993). In European countries, the ecological quality assessment is typically based on indices/metrics reflecting the effect of oxygen depletion due to

the degradation of non-toxic organic substances. One example is the ASPT (Average Score Per Taxon) developed in the U.K. (Armitage *et al.* 1983) and modified for use in several other European countries. The Danish Stream Fauna Index (DSFI) (Skriver, Friberg & Kirkegaard 2000) also belongs in this category. Unfortunately, indices of this type generally have a low capability to capture the effects of toxicants (Beketov & Liess 2008a; McKnight *et al.* 2012). One plausible explanation for this is that they are based on species indicative of high to low oxygen concentrations, which is not necessarily linked to similar sensitivity for toxicants, although this remains to be clearly verified in scientific literature.

One decade ago the macroinvertebrate based SPEAR index (SPEcies At Risk) was introduced by Liess & von der Ohe (2005) aiming to pinpoint pesticide effects in macroinvertebrate stream communities. SPEAR was developed in small streams and macroinvertebrate samples were specifically sampled on hard substrate types (riffle sequences). The index considers approximated pesticide sensitivity (values are extrapolated from the few taxa for which ecotoxicity data exists), generation time, and migration ability. The selected traits are used to combine the sensitivity of a species and the recovery potential of the population after significant pesticide pollution events. The index has been validated in Central and West European, and Australian streams showing clear and significant correlations to measured pesticide toxicity (quantified as Toxic Units) in storm flow water samples (Liess & von der Ohe 2005; Schäfer *et al.* 2007; von der Ohe *et al.* 2007; Schletterer *et al.* 2010; Schäfer *et al.* 2011).

Although the SPEAR index has been proposed to be stressor specific and appears to capture community changes occurring as a function of pesticide exposure, we have identified several drawbacks and elements that need further validation before this indicator can be proved to act with high specificity to pesticide exposure.

1. Traits have been identified and selected based on a priori expert judgement (Liess & von der Ohe 2005), and a separate analysis of which traits respond to pesticide concentration gradients have not been performed. In other words, there is a need to validate the optimal set of traits to maximize the predictive power of the pesticide indicator.
2. Rasmussen et al. (2012b) showed that stream sites with degraded physical properties consistently scored lower SPEAR values compared to less degraded sites and that this difference was independent of pesticide exposure. This indicates that the life cycle and migration based traits exert strong influence on the SPEAR values and, more importantly, that SPEAR additionally responds to overall disturbance regimes. The pesticide sensitivity trait could therefore be the single trait framing the ability of SPEAR to differentiate between pesticides and other types of stress.
3. The pesticide sensitivity values in SPEAR are obtained using ecotoxicity data for a few hundred species to extrapolate values to all other remaining species. One important remaining question is which level of uncertainty such extrapolation introduces. Ecotoxicity data will never exist for all macroinvertebrate species, but realizing the potential levels of uncertainty affiliated with an indicator is prerequisite for correct usage and interpretation of results.
4. The SPEAR index is built on the same mathematical principles as the Central European Saprobic index (used as indicator for organic pollution). This mathematical approach has been criticized for the low ability to obtain scores at the lowest and highest end of the numerical gradient for index scores (Metcalfe-Smith 1996). In order to optimize the functionality of an ecological indicator, the full gradient of responses must be included to maximize the correlation strength to environmental variables.

In this project we aim to test the applicability of pesticide indicators for Danish streams. The project contains an extensive amount of data analyses, and laboratory and field studies. In order to optimize clarity, we subdivided the report into four overall chapters each addressing a well identified part of the project. Each chapter consists of a short introduction with specific

study aims followed by methods, results and discussion. In chapter 2 we re-analyse existing data from field and laboratory studies. In chapter 3 we present a laboratory study addressing the specific separation of pesticide effects and habitat degradation. In chapter 4 we present a comprehensive field study addressing ecosystem responses to various measures (including SPEAR) of pesticide exposure. In chapter 5 we synthesize the obtained knowledge and discuss pros and cons with currently existing indices compared to suggested modifications of these. Moreover we thoroughly discuss which level of information regarding pesticide pollution is necessary in order to generate a useful benchmark for a pesticide indicator.

2. Identifying traits and uncertainty for a pesticide indicator

2.1 Introduction

This chapter is based in part on previous field work (Rasmussen et al. 2011b; Rasmussen et al. 2012b) and ecotoxicity tests (Wiberg-Larsen et al. 2013). We use the field data to address the selection of traits for a pesticide indicator and to check alternative calculation procedures for SPEAR. The study conducted by Wiberg-Larsen et al. (2013) (RAINTOP) provides a large set of ecotoxicity data for non-standard macroinvertebrate test species exposed to a pyrethroid insecticide. This data will be used to evaluate i) how measured pesticide sensitivity correlates to extrapolated sensitivity from the taxonomically closest relative (as done in SPEAR), and ii) analyse which morphological traits are strongest indicators for the observed species specific sensitivities. In congruent order, our aims are to:

1. Evaluate alternative calculation procedures for SPEAR to optimize its performance
2. Analyse pesticide specific traits responses to pesticide pollution for macroinvertebrates sampled at streams sites with degraded and less degraded physical conditions
3. Evaluate the level of uncertainty introduced when extrapolating ecotoxicity values among taxonomic identities
4. Analyse which physiological macroinvertebrate traits that respond most strongly to insecticide exposure

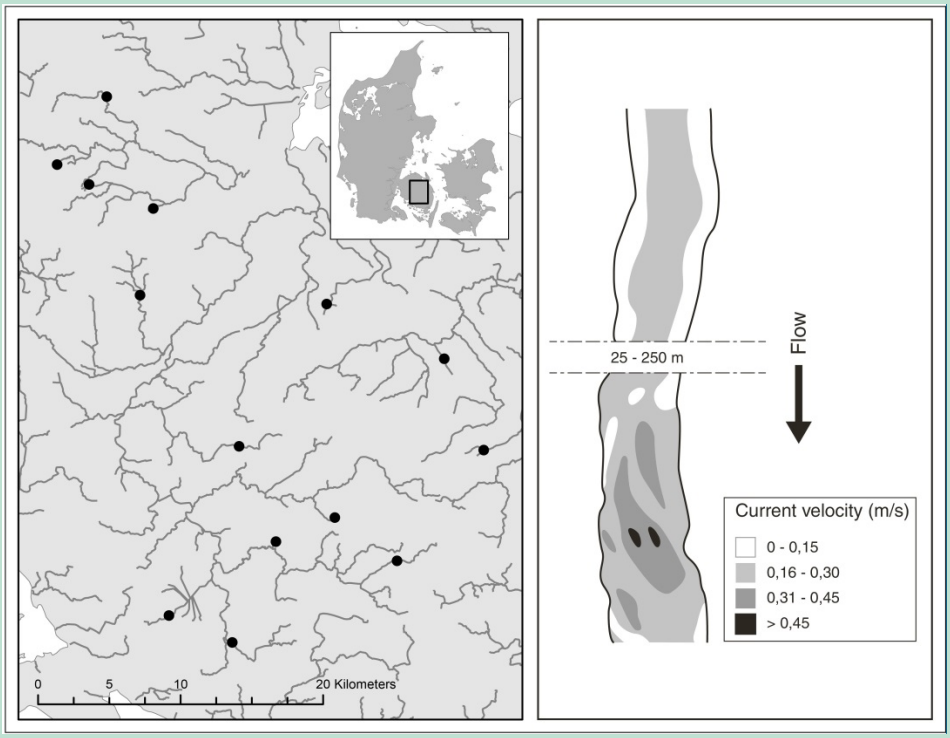
Aims 3 and 4 are additionally published in the form of an article published in Environmental Science and Technology (Wiberg-Larsen *et al.* 2016), and this report provides a summary of the contents of this article. Therefore, we refer Wiberg-Larsen *et al.* (2016) for detailed information in terms of methods, results and discussion of these results.

2.2 Methods

2.2.1 Alternative calculation procedures for SPEAR

We used data provided in Rasmussen *et al.* (2012b) as basis for evaluating different calculation procedures to optimize the performance of SPEAR. The study by Rasmussen *et al.* (2012b) was conducted in 14 1st and 2nd order streams located on Funen. Two neighbouring sampling sites were sampled in each stream, and the two sampling sites represented contrasting physical conditions with the upstream site having homogenous substrate composition dominated by sand and mud whereas the downstream sampling site had more heterogeneous substrate composition with higher proportions of hard substrate types (Fig. 2.1).

FIGURE 2.1. Overview of the location of the 14 study streams (left) and the location and physical complexity (represented by current velocity profiles). The figure is additionally published in (Rasmussen *et al.* 2012b).



Pesticide pollution (herbicides, fungicides and insecticides included) was measured in May and June 2009 using event triggered sampling representing storm flow episodes, and these results are reported in Rasmussen et al. (2011b). Moreover, macroinvertebrate communities were collected at all sampling sites using surber sampling in June 2009. The full details of the study streams, sampling techniques, and taxa lists are provided in Rasmussen *et al.* (2012b).

We used the taxa lists from Rasmussen *et al.* (2012b) to evaluate if the reported correlation between measured pesticide pollution (converted to $TU_{(D.magna)}$) and %SPEAR could be further improved through changing the calculation procedure in the SPEAR metric. We evaluated multiple alternative SPEAR calculations such as i) calculating the ecological quality ratio (EQR) of the SPEAR values (each SPEAR value was translated into a fraction of the highest SPEAR value of all sites), ii) ranking the present SPEAR taxa in each sample according to their abundance (predefined abundance categories (≤ 10 individuals = 1 point, 10 < individuals ≤ 100 = 2 points, 100 < individuals ≤ 1000 = 3 points, and 1000 < individuals ≤ 10000 = 4 points) and summing all points from each sample, and iii) calculating the EQR of the abundance based ranking procedure. Multiple additional versions were produced, but we chose to only show the alternative SPEAR versions that provided the best fits. The explanatory power of the alternative calculation procedures were compared to the original SPEAR version by comparing Pearson correlation coefficients.

2.2.2 Macroinvertebrate traits responses to pesticides and physical habitat degradation

We extracted data for ecological and physiological traits from the traits data base available in Tachet et al. (2002) (referred to as the Tachet database). We selected traits that directly (toxicity) or indirectly (population recovery) could be important for pesticide effects. In more detail, we extracted data for maximum potential size (important for the relative surface area available for chemical uptake), respiration type (important for the relative proportion of highly permeable respiratory surfaces), substrate preferendum, current preferendum and temperature preferendum (all related to oxygen requirements which could be correlated to metabolic activity and the

fraction of respiratory surfaces), generation time (important for population recovery), dispersal technique (important for population recovery). Similar to SPEAR, we supplied these traits with species specific measures for pesticide sensitivity by extrapolating sensitivity values from the taxonomically closest relative (von der Ohe & Liess 2004). The physiological and ecological traits available in the Tachet database are stored using a fuzzy coding approach. The fuzzy coding was transformed into fractions according to the method described in Chevenet et al. (1994). Hereby, the sum of all fractions within one trait category for a species equals 1. Subsequently, we combined this traits matrix with the species abundances in the macroinvertebrate samples collected at each of the paired reaches in the 14 study streams on Funen (Rasmussen *et al.* 2012b) generating relative traits abundances normalized according to the actual abundance of each species. This new abundance weighted traits matrix was then used to analyze specific traits responses to the measured gradient in pesticide pollution (maximum $TU_{(D.magna)}$) for the sampling sites with heterogeneous and homogenous physical properties, respectively.

In order to reveal the biological and physiological traits providing highest explanatory power for the measured maximum $TU_{(D.magna)}$, we used the software EUREQA to develop linear and non-linear models based on traits as dependent parameters and the maximum $TU_{(D.magna)}$ as independent parameter. With EUREQA, the space of mathematical expressions is automatically searched while minimizing the mean squared error of the found models (Eureqa, Nutonian, USA (Schmidt & Lipson 2009)). The result of EUREQA is a predictive ability (squared error) – parsimony (model complexity) Pareto front. Such a Pareto front tends to have a cliff, where the predictive ability significantly increases with a minimum increase in model complexity, and where further increasing model complexity generates only insignificant improvements of predictive abilities of the models (Schmidt & Lipson 2009). We used the cliff criterion to select a model. Subsequently, we validated the selected model within R (R version 3.1.3 (R *et al.* 2015)). To do that, we correlated the modelled maximum $TU_{(D.magna)}$ with the measured maximum $TU_{(D.magna)}$ (lm function, stats package). We checked for significance of the correlation and explained variance (summary.lm function, stats package). Moreover, we checked for homogeneous variances (plot function, base package) and normal distribution (Shapiro-Wilk test, shapiro.test function, stats package) of the model residuals. Finally, we tested, if all samples had similar leverage on the model (Cook's distance, plot.lm function, stats package). The maximum $TU_{(D.magna)}$ resembled a log normal distribution and hence were log transformed before model definition and validation. Finally, we checked for intercorrelation among trait variables to improve interpretative abilities of the model output. Specific traits that were only present in < 5 samples were removed from the analysis to reveal the signals of the overall dominating trends.

2.2.3 Re-analysis of RAINTOP data

Wiberg-Larsen *et al.* (2013) investigated the sensitivity of 34 species of macroinvertebrates to a 90 min pulse of the pyrethroid insecticide; lambda-cyhalothrin, and mortality was registered 7 days post-exposure. For details regarding test-species, sampling of test individuals, and overall experimental details, please consult Wiberg-Larsen *et al.* (2013) and Wiberg-Larsen *et al.* (2016).

In brief, dry weights and body sizes (length, width and height) of all test organisms were measured, and the surface area of each test species was estimated from the measured body dimensions using three-dimensional mathematical models representing the shape of each test species (e.g. ellipsoid or cylindrical). Details regarding model choice and potential correction factors representing large extremities (e.g. gills, antennae, and legs) can be found in Wiberg-Larsen *et al.* (2016). Furthermore, we extracted data for available physical and ecological traits from the Tachet database (Tachet *et al.* 2002). All extracted and calculated traits values are freely available via the online supplementary material to Wiberg-Larsen *et al.* (2016).

In addition, potential indicator values in the macroinvertebrate-based ecological index currently used in Denmark (Danish Stream Fauna Index (DSFI)), as well as indicator values in the dominant indices used in EU: Biological Monitoring Working Party Index (BMWP), and the Saprobic Index (SI), were extracted from the freely available and online database (www.freshwaterecology.info).

We re-analysed the mortality based data for all test species using two-parameter concentration-response models (mortality as a function of lambda-cyhalothrin concentration) in R according to Ritz and Streibig (2005):

$$\%\text{mortality} = \frac{100}{(1+(\frac{c}{\text{EC50}})^b}$$

where *c* is the nominal lambda-cyhalothrin concentration, EC50 is the concentration reducing survival by 50%, and *b* is proportional to the slope around the EC50. Upper and lower limits were fixed at 100 and 0, respectively. Based on these concentration-response models, we derived the lambda-cyhalothrin concentration causing 50% mortality (LC50) for each species.

Introduced uncertainty when extrapolating ecotoxicity values among taxonomic identities
We extracted relative species sensitivity scores (for organic toxicants, e.g. insecticides) from von der Ohe and Liess (2004). The work by von der Ohe and Liess (2004) forms the basis of the SPEAR index, where all species are provided with a value representing sensitivity insecticide exposure. If no test results are available for a given species, it is assigned the value of its closest relative. In this dataset, the species specific LC50 value is rank-ordered according to the 48h LC50 for *Daphnia magna*. Hence, in order to compare actual measured LC50 values for our test species with the relative species sensitivity scores provided in von der Ohe and Liess (2004), we rank-ordered the measured species-specific LC50 values relative to the 48h LC50 for *Daphnia magna*. Importantly, actual measured LC50 values only existed for a few of our test-species in the SPEAR database. Hence, the species sensitivity scores which could be extracted from the SPEAR database for our test species were mainly extrapolated values. We compared the two species sensitivity scores (measured vs extrapolated) using a Spearman Rank correlation in Sigma Plot 11.0 for Windows. Please consult Wiberg-Larsen *et al.* (2016) for details.

2.2.3.1 Physiological macroinvertebrate traits responses to pyrethroid exposure

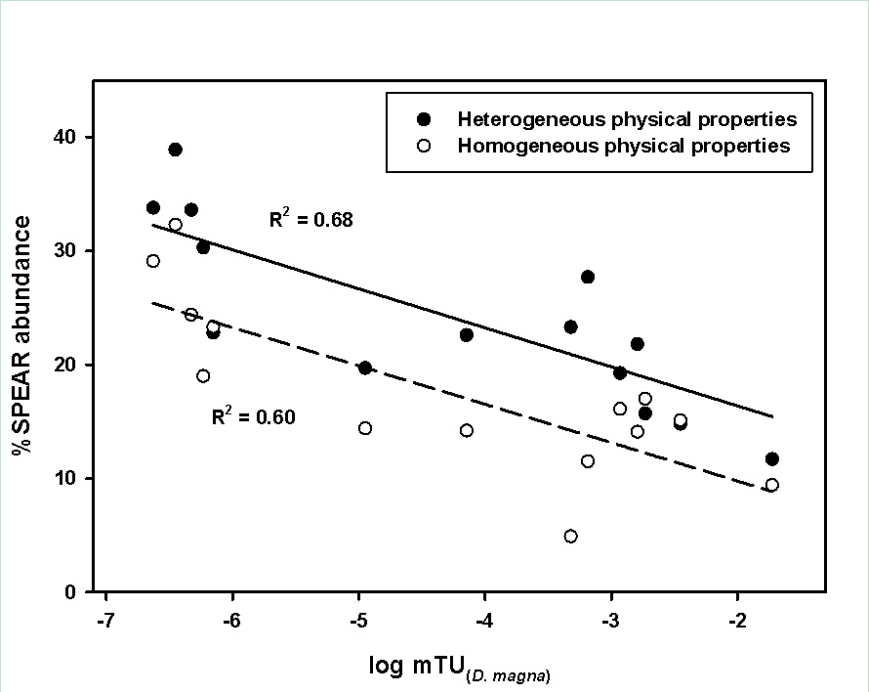
In order to reveal the biological and physiological traits, mentioned above, providing the highest explanatory power for the observed species-specific LC50 values, we used the software EUREQA to produce linear models containing trait values as independent parameters and LC50 concentrations as dependent parameters. Similar to section 2.2.2, we used the cliff-criterion to select the simplest model containing highest explanatory power. The LC50 concentrations resembled a log-normal distribution; hence we used log-transformed LC50 concentrations in the linear model. Finally, we tested the selected in R by correlating measured against modelled LC50 values (lm function, stats package). Please consult Wiberg-Larsen *et al.* (2016) for details.

2.3 Results

2.3.1 Alternative calculation procedures for SPEAR

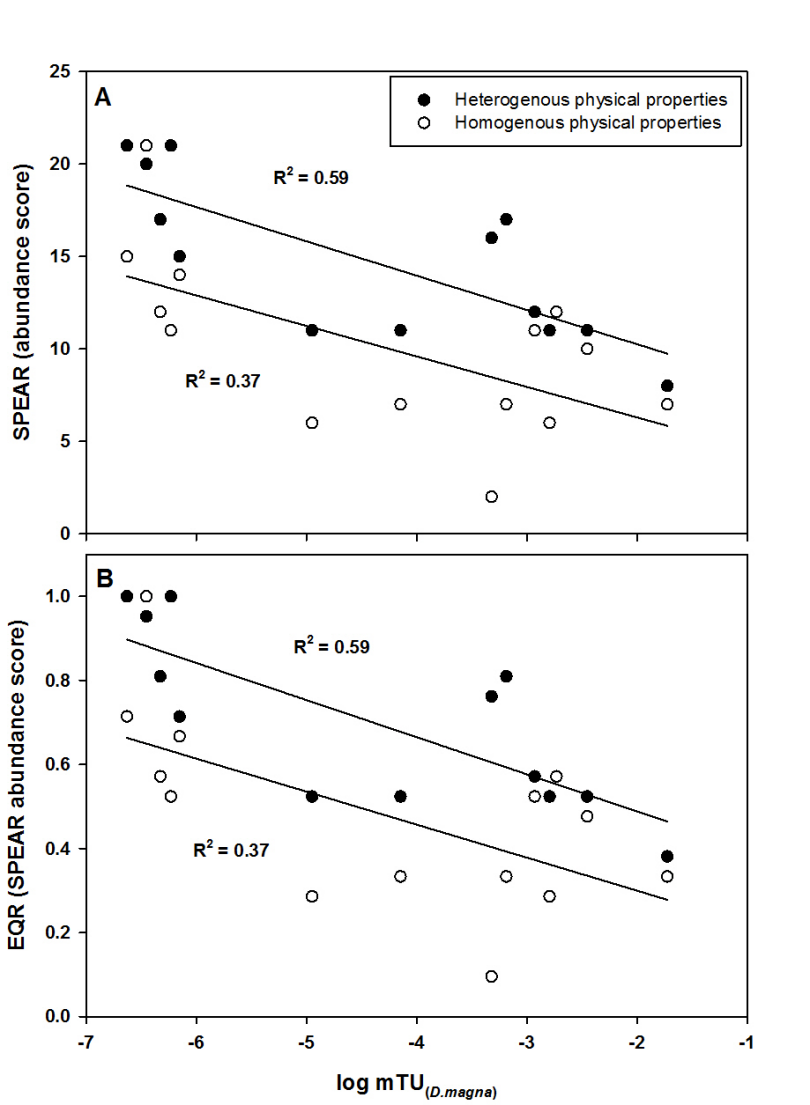
For clarity, we re-present the original results from Rasmussen et al. (2012b) showing a significant correlation between the maximum $TU_{(D.magna)}$ obtained from the pesticide concentrations in event triggered water samples and SPEAR values (Fig. 2.2). SPEAR values were significantly lower for sites with homogenous compared to heterogeneous physical properties, and the correlation between the maximum $TU_{(D.magna)}$ and SPEAR values was significant (Pearson product moment, $P < 0.05$) for each of the two site categories.

FIGURE 2.2. %SPEAR abundance as a function of log maximum TU (log $mTU_{(D.\ magna)}$). The figure is re-presented from Rasmussen et al. (2012b).



None of the alternative versions of SPEAR increased the explanatory power of SPEAR values (Fig. 2.3). Especially the correlation coefficients for alternative SPEAR versions as a function of maximum $TU_{(D.magna)}$ for the sites with homogenous physical properties was lower compared to the original SPEAR version (Fig. 2.3). We additionally note that, similar to the original SPEAR, the mathematical functions of the regression lines for the SPEAR alternatives suggest that sites with highest maximum $TU_{(D.magna)}$ obtained SPEAR values 40-50% below those for the lowest maximum $TU_{(D.magna)}$.

FIGURE 2.3. Alternative SPEAR values (abundance scores (A) and EQR based on abundance scores (B)) as a function of log maximum TU (log mTU(D. magna)) for the paired reaches in the 14 streams used in the work of Rasmussen et al. (2012b). Regression coefficients are indicated.



2.3.2 Macroinvertebrate traits responses to pesticides and physical habitat degradation

The model selection was based on a clear cliff in the Pareto front for the homogenous sites whereas three distinct Pareto fronts were identified for the heterogeneous sites (Fig. 2.4). The occurrence frequencies of traits variables for the homogenous and heterogeneous sites are presented in Fig. 2.5. Number of life cycles per year < 1 was the only trait variable that was used in predictive models for both the homogenous and heterogeneous sites, and the abundance of this trait was consistently decreasing with increasing maximum $TU_{(D.magna)}$ (Table 2.1). Number of life cycles per year < 1 was clearly the most frequently used trait variable for the heterogeneous sites and among the most frequently used trait variables for homogenous sites. The predicted pesticide sensitivity (sensu von der Ohe & Liess 2004) was never used in predictive models for homogenous or heterogeneous sites. Food source, feeding preference and flow

preference were frequently used in models for homogenous sites, whereas spiracle respiration and preference for litter as substrate were frequently used in predictive models for heterogeneous sites.

FIGURE 2.5. Number of models each trait variable occurs in for the homogenous and heterogeneous sites, respectively.

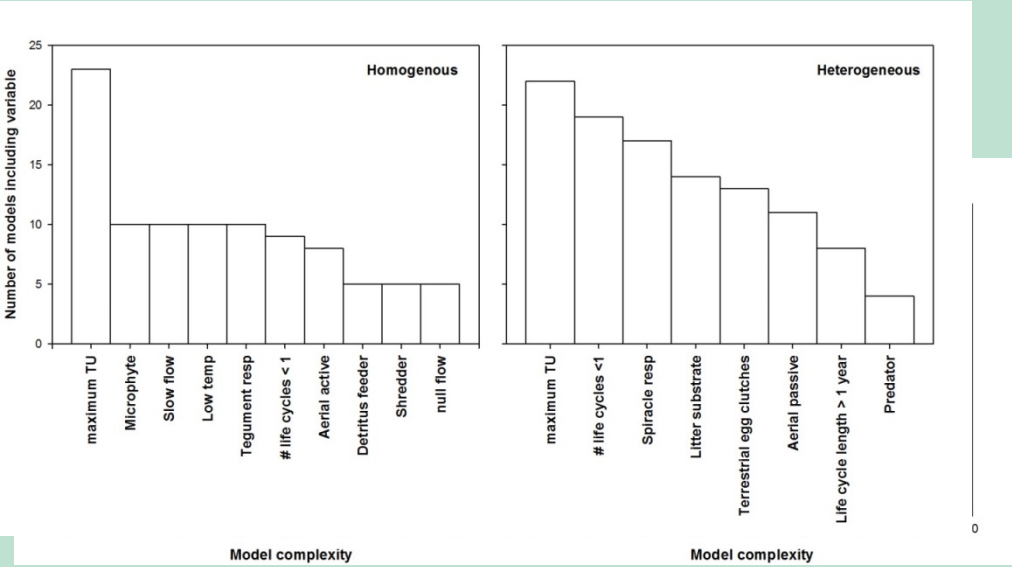
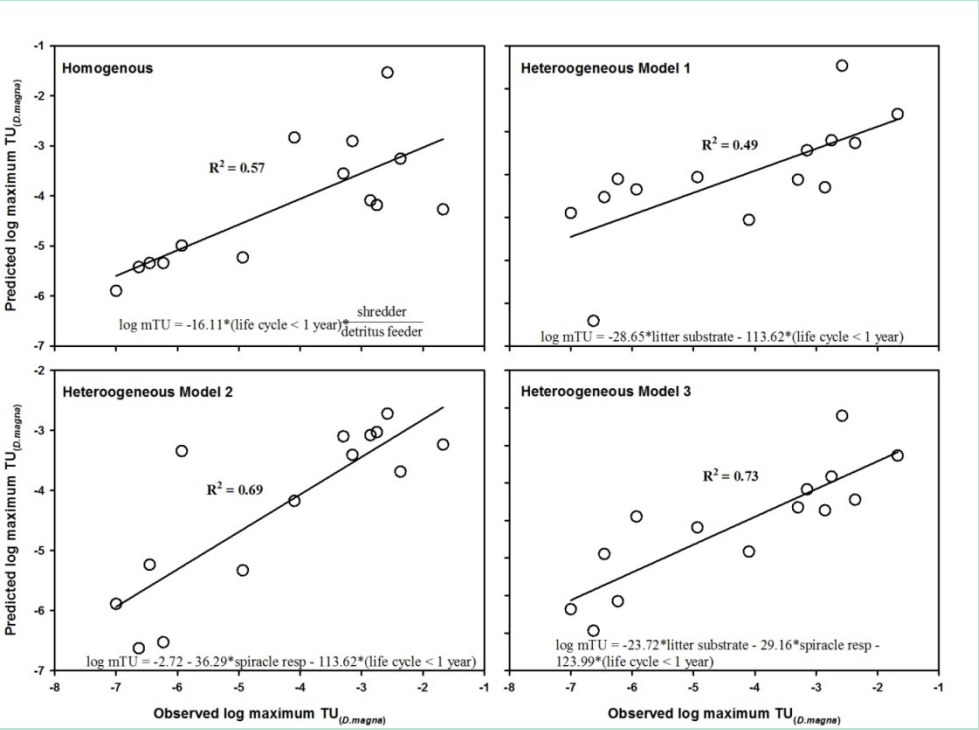


TABLE 2.1. Overview of the operational signs of the correlations between each of the dominating trait variables (Fig. 2.5) in the predictive models. Positive and negative correlations indicate increasing and decreasing, respectively, abundance with increasing log maximum $TU_{(D.magna)}$.

Trait	Homogenous	Heterogeneous
# Life cycles < 1 per year	Negative	Negative
Microphytes (food)	Positive	
Shredder	Negative	
Detritus	Positive	
Predator		Negative
Litter (habitat)		Negative
Null flow preference	Positive	
Slow flow preference	Negative	
Aerial passive dispersal		Positive
Aerial active dispersal	Negative	
Preference for low temperatures	Positive	
Tegument respiration	Positive	
Spiracle respiration		Negative
Ovoviparity	Negative	
Terrestrial egg clutches		Positive

The predictive models were all highly significant (P < 0.001, linear model). The selected model for homogenous sites included three trait variables: (i) number of life cycles < 1 per year, (ii) shredder, and iii) detritus feeder. The abundance of individuals having < 1 life cycles per year and shredder were negatively correlated to maximum $TU_{(D.magna)}$ whereas detritus feeder was positively correlated to maximum $TU_{(D.magna)}$ (Fig. 2.6, Table 2.1). The abundance of individuals having < 1 life cycles per year was used in all selected models for heterogeneous sites whereas spiracle respiration and preference for litter as substrate were both used twice. All trait variables were negatively correlated with maximum $TU_{(D.magna)}$ (Fig. 2.6, Table 2.1).

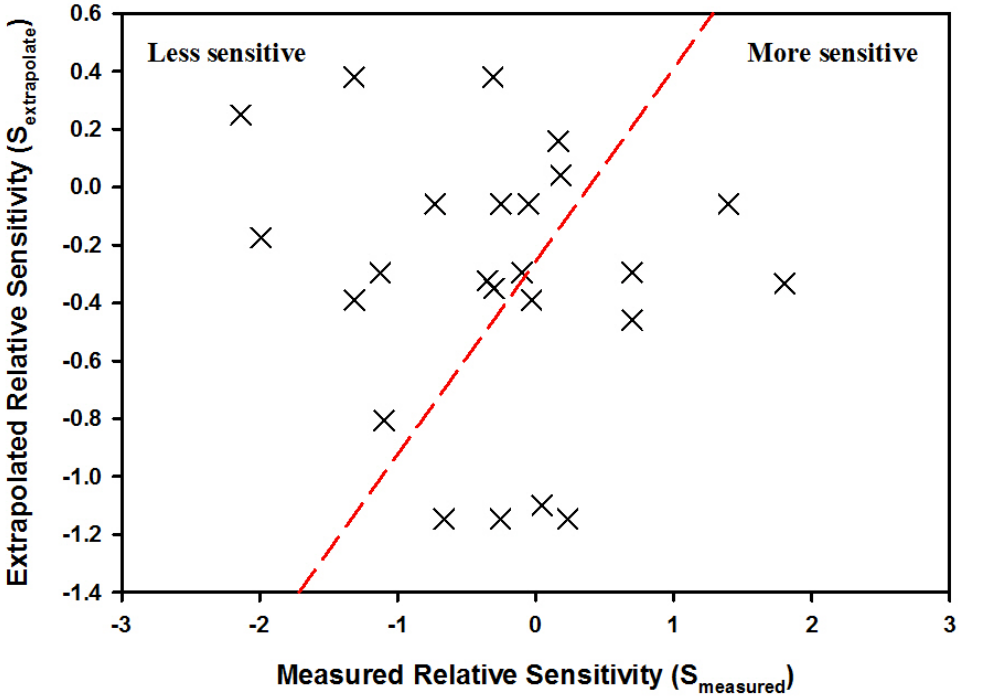
FIGURE 2.6. Predicted log maximum $TU_{(D.magna)}$ as a function of the observed log maximum $TU_{(D.magna)}$. The parameterized models are given along with the correlation coefficients for the linear regressions (linear models, P < 0.001). The model numbers refer to Fig. 2.4.



2.3.3 Introduced uncertainty when extrapolating ecotoxicity values among taxonomic identities

We found no significant correlation between the measured species specific sensitivities to lambda-cyhalothrin exposure and the sensitivities for the same species towards organic toxicants derived by extrapolating sensitivity values from the closest taxonomic relative (n = 24, Spearmans ρ=0.004, P=0.98) (Fig. 2.7). The values which were extrapolated from the closest taxonomic relative deviated by up to a factor > 100 (note that the axes in Fig. 2.7 are logarithmic).

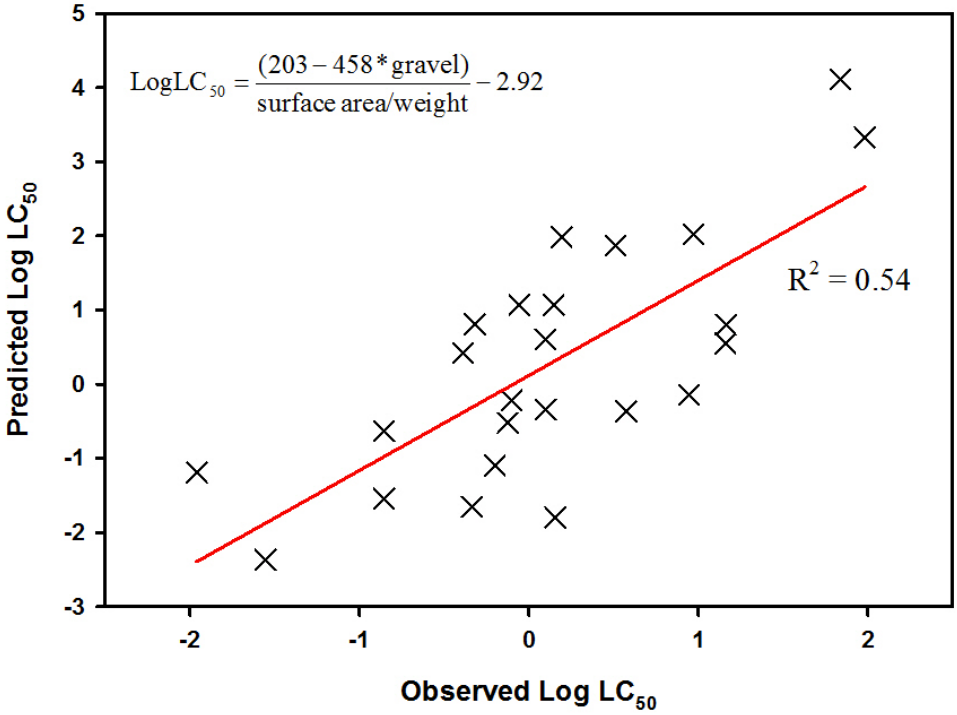
FIGURE 2.7. Extrapolated sensitive score as a function of measured sensitivity score. The dashed line indicates the 1:1 relationship. Sensitivity scores represent extrapolated or measured LC50 concentrations rank-ordered according to 48h LC50 for D. magna. The figure is modified from Wiberg-Larsen et al. (2016).



2.3.4 Physiological macroinvertebrate traits responses to pyrethroid exposure

The model selection was based on a clear cliff in the Pareto Front (see Supplementary Material in Wiberg-Larsen *et al.* 2016), and the validated model was highly significant (Linear regression, $R^2 = 0.54$, $P < 0.001$) (Fig. 2.8). Two traits variables were included in the final model; i) surface area:weight ratio and ii) preference for gravel substrates (Fig. 2.8). Both traits variables were negatively correlated with the LC50. Moreover important, the same traits occurred most frequently in the overall compilation of models (data not shown).

FIGURE 2.8. Predicted Log LC50 concentrations as a function of observed Log LC50. The equation for the selected model is presented in the graph area, and the fitted regression line is indicated with red font. The figure is modified from Wiberg-Larsen et al. (2016).



2.4 Discussion

2.4.1 Alternative calculation procedures for SPEAR

Log-transformation centralizes data and reduces the data range (gradient). Therefore, log-transforming ecological response variables in environmental gradient analyses could confound the results by underestimating the responses at the gradient extremes. The SPEAR indicator values (macroinvertebrate community responses) are log-transformed, but we could not improve the correlation to measured pesticide toxicity in the 14 streams on Funen through alternative and non-log-transformed calculation methods. Our findings indicate that the variation in the non-log transformed SPEAR abundance is substantial and that centralization of this data is necessary in order to produce significant correlations to measured gradients in pesticide pollution. However, our most qualified alternative (EQR based on SPEAR abundance categories) produced correlations to measured gradients in pesticide pollution with similar high correlation coefficients. Consequently, an index scoring system, which to a larger extent uses the full gradient of SPEAR abundances, does likely not provide a substantially improved alternative to the existing SPEAR approach.

2.4.2 Macroinvertebrate traits responses to pesticides and physical habitat degradation

Our results clearly suggest that the abundance of macroinvertebrates with long life cycles (> 1 year) was the strongest predictor for macroinvertebrate community responses to measured pesticide pollution for the streams on Funen, and that this trait was rather independent of the physical habitat quality of the streams. Congruently, the abundance of macroinvertebrates with multiple life cycles per year increased with increasing pesticide pollution in the homogenous and heterogeneous physical habitat types. Short life cycle is an essential trait that partly defines

r-strategists (disturbance tolerant) in general community ecology (Grime 1979). A short life cycle is a prerequisite for fast population recovery subsequent to significant disturbance events. As such, this trait cannot be viewed as pesticide-specific since multiple stressors occur with increasing frequency and magnitude in streams increasingly embedded in agricultural landscapes (Friberg 2014). However, in combination with more pesticide-specific traits (i.e. pesticide sensitivity), the number of life cycles per year could provide important information on the pesticide specific population recovery dynamics.

None of the predictive models used the predicted pesticide sensitivity based on extrapolated values from the few taxa for which ecotoxicity data exists. Since laboratory and mesocosm studies clearly reveal macroinvertebrate population effects within the range of environmentally realistic pesticide concentrations (Rasmussen *et al.* 2013b; Feckler, Kahlert & Bundschuh 2015; Stehle & Schulz 2015), and since the measured pesticide toxicities in the study streams were within the range of those generating environmental effects, we suggest that the absence of predicted macroinvertebrate pesticide sensitivity in the predictive models is likely due to the uncertainty introduced when extrapolating sensitivity values from few to multiple taxa. This receives further support from our work with the RAINTOP data (Appendix Manuscript 1). Our finding strongly suggests that the extrapolation of pesticide sensitivity should not be performed based on taxonomic entities and that this data should not be used as continuous variables in environmental gradient analyses. However, aggregating data, similar to the SPEAR index, provides one opportunity to overcome such variation in data (Beketov, Kattwinkel & Liess 2013). As such, the SPEAR approach is based on the aggregation of macroinvertebrate taxa into two sensitivity categories; insensitive and sensitive using one cut-off value. Since we could not build models with such criteria, we could not reject or confirm that data aggregation provides a more robust measure for the overall abundance of sensitive taxa in a community.

The abundance of shredders (homogenous) and macroinvertebrates preferring litter as substrate (heterogeneous) were frequently incorporated in the parameterized models and both decreased with increasing pesticide pollution. However, the study streams not only represented a gradient in pesticide pollution, but the pesticide pollution was additionally positively correlated to the proportion of conventional agriculture in the stream catchments and negatively correlated to the width of unmanaged buffer zones (containing trees and bushes) (Rasmussen *et al.* 2011b). Therefore, the abundance of twigs, branches and leaves likely decreased with increasing pesticide pollution which provides a logical explanation for the observed pattern in the abundances of these traits as the availability of food and habitat types are paramount filters for macroinvertebrate community structure (i.e. facultative shredders are absent when leaf material is missing due to forest removal in the riparian zones (Hladyz *et al.* 2010)).

In addition, the predictive models frequently incorporated flow preferences and feeding preferences for microphytes and detritus (< 1mm) for the homogenous sites, and our results suggest that the abundance of macroinvertebrates preferring for zero flow, microphytes and detritus increased with increasing pesticide pollution. However, we suggest that the increasing abundance of these traits with increasing pesticide pollution is not causative. More likely these results suggest that flow variation increased with increasing land use (due to drainage of agricultural fields) leading to more frequent zero flow episodes in the agricultural streams. Consequently, epiphytes and fine detritus likely increasingly dominated the food selection in the agricultural streams causing increasing abundance of macroinvertebrates preferring these types of food (Hladyz *et al.* 2010).

2.4.3 Introduced uncertainty when extrapolating ecotoxicity values among taxonomic identities

The observed discrepancy between measured sensitivities towards lambda-cyhalothrin and the sensitivity values extrapolated from the closest taxonomic relative clearly shows that extrapolation of ecotoxicity data from few to multiple species introduces comprehensive uncertainty in

risk and effect assessments when such assessments are performed at the species level. However, in spite of the substantial variation, a comparable amount of species were associated with under- and overestimated values, respectively (i.e. sensitivity overestimated by a factor > 10 in 7 species, and sensitivity underestimated by a factor > 10 in another 7 species) (Fig. 2.7). The underestimations and overestimations appeared to counterbalance each other (average extrapolated LC50 = 1.5 µg L^{-1}, and average measured LC50 = 4.2 µg L^{-1}). This finding suggests that data aggregation, similar to the SPEAR index (Liess & von der Ohe 2005), provides stronger statistical power in terms of interpreting risk and effect in multispecies ecological communities. Nevertheless, the substantial discrepancy between measured and extrapolated sensitivity values should not be disregarded, and we emphasise the further need to explore cause-effect relationships in macroinvertebrate species exposed to pesticides in order to improve the mechanistic understanding of community effects.

2.4.4 Physiological macroinvertebrate traits responses to pyrethroid exposure

Our results suggest that organismal size is tightly coupled to the sensitivity of the organism towards pyrethroid exposure with smaller organisms being more sensitive than larger ones. This finding probably reflects that smaller organisms are subjected to higher relative uptake of the insecticide due to higher surface area relative to body volume leading to lower LC50 concentrations. Similar findings have been reported for heavy metals (Buchwalter *et al.* 2008) and other groups of insecticides (e.g. carbamates (Rubach, Baird & Van den Brink 2010) and organophosphates (Rubach *et al.* 2012; Rico & Van Den Brink 2015)). Rico and Van Den Brink (2015) and Rubach, Baird and Van den Brink (2010) additionally searched for relationships between body size and macroinvertebrate sensitivity towards pyrethroid insecticides but found no significant correlations. The work of these authors was based on literature values for species-specific organismal sizes extracted from the Tachet database (Tachet *et al.* 2002). By contrast, Rubach *et al.* (2012) measured the organismal sizes in the 17 macroinvertebrate species exposed to an organophosphate insecticide and found strong and highly significant correlations to uptake and bioaccumulation in the organisms. These findings in combination with our results emphasise the need to measure sizes of test organisms instead of using literature values in the search of cause-effect relationships. This further suggests that there may be a dependency of life stage on insecticide sensitivity with earlier and smaller life stages being more sensitive towards insecticide exposure than later and larger life stages. This notion receives support from a laboratory study on the freshwater shrimp *Gammarus pulex* showing that early life stages are more sensitive towards pyrethroid exposure than late life stages (Cold & Forbes 2004). In consequence, retrieving detailed information concerning the occurrence of early life stages in late spring and summer, coinciding with the main temporal window for agricultural insecticide application, could benefit the assessments of risk and effects of agricultural insecticides in streams.

2.4.5 Synthesis and applications for testing a Danish pesticide indicator

Our work with the RAINTOP data clearly showed that the surface area / volume ratio was the most important parameter driving mortality among the studied macroinvertebrate species. Similar results have been reported for other toxicants such as organophosphate insecticides (Rubach *et al.* 2010) and Cadmium (Buchwalter *et al.* 2008). In addition, we showed that – given the currently low abundance of actual pesticide sensitivity data – pesticide sensitivity data should not be extrapolated among taxonomic entities to create gradual data. Hence, in the absence of extensive data on pesticide sensitivities, measures for organismal sizes could provide a useful proxy for this. However, the size-related macroinvertebrate information available in published traits databases (Tachet *et al.* 2002) conveys only maximum potential sizes for each species.

Our work with macroinvertebrate traits for the 14 streams on Funen revealed that the maximum potential size was never used in predictive models and hence did not provide useful information

for the community based trait profiles. Not surprisingly, this suggests that the actual organismal size during pesticide exposure – and not the maximum potential size at the end of the life cycle – is determining chemical uptake. Using this information for field investigations is, however, not straight forward since measured sizes of all organisms are not available. One option to circumvent this obstacle is to focus on stream insects with terrestrial adult stages and use information on emergence timing to estimate life stages. Assuming constant growth rates for all focal organisms, the combination of emergence timing and measures for potential maximum size could provide a useful estimate of actual sizes (converted to surface area / volume ratio) during the primary insecticide spraying season in Denmark (May – July). Moreover, using size measures as proxy for pesticide sensitivity could provide a feasible option to use this data as gradual data rather than categorical or transformed.

Our work with the 14 streams on Funen pinpointed multiple life cycles per year as a strong response parameter to the measured gradient in pesticide pollution. The SPEAR index additionally uses the trait “life cycle duration” to characterize species sensitive to pesticide pollution incidents and uses the same cut-off value as identified in our predictive models (number of life cycles per year < 1). Hence, we conclude that this trait and this cut-off value provide useful information on the ability of populations to recover from pesticide pollution events and other types of disturbance.

Our work on RAINTOP and the 14 streams on Funen moreover suggest that sampling efforts should focus on streams sites with higher fractions of hard substrate types (gravel, pebble etc.). Wiberg-Larsen *et al.* (2016) showed that riffle associated macroinvertebrate communities tend to be more sensitive to pesticide pollution than macroinvertebrates more strongly affiliated with vegetation and soft substrate types. Since pesticide molecules follow the main water flow paths (in the main stream channel), riffle based macroinvertebrate communities are additionally more likely to be exposed to higher pesticide concentrations (Beketov & Liess 2008c). In conclusion, riffle communities could exert larger – although not necessarily temporally persistent – responses to pesticide pollution events. Our work with the 14 streams on Funen showed that the selected models based on heterogeneous sites were characterized by higher predictive power compared to the models based on the homogenous sites. This could suggest that macroinvertebrate community responses in the heterogeneous sites were more dramatic which further suggests that macroinvertebrate samples collected at riffle-like habitats should be prioritized when possible in order to optimize the diagnostic abilities regarding pesticide effects.

3. Experimental separation of the effects of pesticides and habitat degradation

3.1 Introduction

Streams are subjected to a wide range of anthropogenic stressors which confounds disentangling the separate effects of a single stressor (Friberg *et al.* 2011; Friberg 2014). The studies of single stressors are not only confounded by the presence of multiple co-occurring stressors, but additionally by the potential interactions among the stressors. In other words, the potential interaction of different anthropogenic stressors may prompt altered effects (Matthaei, Piggott & Townsend 2010; Wagenhoff *et al.* 2011; Rasmussen *et al.* 2012b; Wagenhoff, Townsend & Matthaei 2012). As the effect of one stressor may reduce the fitness of an organism, the effect of another stressor may increase. Conversely, the effect of one stressor may result in competitive release for one organism (e.g. elimination of predators) increasing its fitness and potentially reducing its sensitivity to other stressors (Pedersen & Friberg 2009). One such potential stressor that often interacts with the presence of pesticides in agricultural areas is the intensive disturbance of the natural hydrology and morphology of streams by channelization and management-dredging ensuring optimal flow conveyance from field to stream. One side-effect of these activities is a severe degradation of the diversity and quality of physical habitats in the streams. The physical structure of heavily modified streams is often characterised by higher proportions of soft substrates, larger transport of fine particulate sediment, and low variability in depth and width of the streams (Pedersen 2009). In addition, the degradation of physical habitats results in more laminar and in general slower flow which reduces the reaeration capacity and periodically the total oxygen content in the stream water (Friberg, Sandin & Pedersen 2009; Pedersen 2009).

Significant variation in the macroinvertebrate community structure has been ascribed to substrate characteristics and habitat heterogeneity (Brunke, Hoffmann & Pusch 2001; Pedersen & Friberg 2009). Many lotic Ephemeroptera, Plecoptera and Trichoptera (EPT) require high current velocities, well oxygenated water and coarse substrate (Giller & Malmqvist 1998). Moreover, several EPT taxa have narrower niche requirements than taxa related to physically uniform streams with soft sediments (Dunbar *et al.* 2010). Therefore, high habitat heterogeneity is essential if the site is to support a larger proportion of the generally pollution sensitive EPT taxa.

Rasmussen et al. (2012b) showed that the streams sites with relatively poor and homogenous physical habitat quality consistently obtained lower SPEAR values compared to sites with more heterogeneous substrate composition along a gradient in pesticide pollution. Such effects may originate from interactions between stressors (physical habitat quality and pesticide pollution) or from a preliminary filtration/exclusion of some macroinvertebrate taxa (especially EPT) due to inappropriate habitat conditions.

3.2 Aims

In this study we aim to elucidate the potential interaction between physical habitat deterioration and pesticide stress on four macroinvertebrate species. These represent organisms that are i) tolerant to fine sediment and tolerant to pesticide stress, ii) tolerant to fine sediment and sensitive to pesticide stress, and iii) sensitive to fine sediment and sensitive to pesticide stress. We used the pyrethroid lambda-cyhalothrin as model pesticide in sublethal concentrations. Further,

we used mortality rates, storage lipids (fitness) and food consumption (algae biomass accrual and leaf litter decomposition) as endpoints.

We hypothesized that i) the addition of fine sediment will decrease macroinvertebrate fitness and potentially increase mortality for the species that are strongly affiliated with coarse substrate types, ii) lambda-cyhalothrin exposure will increase mortality and decrease fitness for the species with expected high sensitivity to pesticide pollution, iii) food consumption may increase as a function of fine sediment addition, since fine sediment addition will eliminate interstitial spaces between coarse substrate types and hence increase interspecies and intraspecies competition, and iv) fine sediment addition will increase the effects of lambda-cyhalothrin for the species sensitive to both fine sediment addition and pesticide stress.

3.3 Methods

3.3.1 Experimental set-up

We applied a full factorial design including two stressors: fine sediment addition (particle diameter 0.1-0.5 mm) and lambda-cyhalothrin exposure (75 ng L^{-1}). Hence, the following treatments were used: control, fine sediment, lambda-cyhalothrin, and a treatment receiving both fine sediment and lambda-cyhalothrin (Pest x Sed). The experiment was conducted in October and November 2014 and lasted 29 days. The specific concentration of lambda-cyhalothrin was selected to negatively influence the most sensitive of the test species but not cause acute mortality.

The experiment was conducted in 48 plastic aquaria (n = 12 per treatment) at 15 ºC and using a 14:10 h light:darkness cycle (Fig. 3.1). Each aquarium contained 5 L of artificial freshwater (AFW) (Table 3.1). Moreover, gravel (diameter 1 – 5 cm) was deployed as substrate in each aquarium with a substrate depth of 3 cm. The substrate was collected in Voel Bæk in order to secure the presence of natural biofilm on the substrate. The substrate was rinsed prior to deployment to exclude attached macroinvertebrates. The AFW was continuously aerated using aquaria pumps (Elite Maxima Air pump 113.6 L).

FIGURE 3.1. Photo of experimental set-up.

TABLE 3.1. Ionic composition of the artificial freshwater used in the experiments.

Salt	Concentration (mg L^{-1})	Salt	Concentration (mg L^{-1})	Salt
$CaCl_2 \cdot 2H_2O$	294	$CaCl_2 \cdot 2H_2O$	294	$CaCl_2 \cdot 2H_2O$
$MgSO_4 \cdot 7H_2O$	123.25	$MgSO_4 \cdot 7H_2O$	123.25	$MgSO_4 \cdot 7H_2O$
$NaHCO_3$	64.75	$NaHCO_3$	64.75	$NaHCO_3$
KCl	5.75	KCl	5.75	KCl

3.3.2 Lambda-cyhalothrin exposure and sediment

Prior to the experimental start, we added 250 mL of fine sediment to 24 of the aquaria ensuring that all interstitial spaces were fully covered.

A dilution series of lambda-cyhalothrin (Pestanal, 98% purity) was produced in 96% Ethanol. The macroinvertebrates for each aquarium were distributed into 500 mL glass beakers containing 475 mL AFW. Five mL of the lambda-cyhalothrin stock solution was pipetted into 24 glass beakers using a glass syringe. The remaining 24 glass beakers received an equivalent amount of 96% Ethanol. The exposure duration was 90 min. After ended exposure we rinsed the macroinvertebrates in clean AFW and introduced them to the respective aquaria.

3.3.3 Validation of nominal lambda-cyhalothrin exposure concentrations

We collected 40 mL of exposure medium from each of exposure beakers and pooled these samples into two 1 L samples, one representing lambda-cyhalothrin exposure and one representing the untreated controls. The exposure medium was collected at exposure start as well as after 90 min.

The water samples were shipped to the University of Koblenz-Landau for analysis. Initially, the samples were up-concentrated on SPE columns (Chromabond C18, 500 mg). Subsequently, the SPE columns were flushed with 6 mL MeOH), and 20 µL of the sample was injected into a LC-MS for quantification of lambda-cyhalothrin. The LC system consisted of a combipal autosampler (CTC Analytics, Zwingen, Switzerland) connected to a U-HPLC-system consisting of an Acela pump and a Hypersil Gold C18 column (40x2.1 mm, particle size 1.9 µm) (both Thermo Fisher Scientific, Dreieich, Germany). The MS system was a benchtop orbitrap system (Exactive, Thermo Fisher Scientific, Dreieich, Germany) (Scan range: 100-2000 m/z). Lambda-cyhalothrin was separated using chromatography (eluent A: milliQ water, eluent B: methanol (both purchased from Sigma Aldrich, Seelze, Germany, puriss p.a. grade). The following composition of eluents was used: 0 to 2 min (5% eluent B), 2 to 7 min (100% eluent A), 7 to 8.5 min (5% eluent B). Quantification of lambda-cyhalothrin concentrations was performed using the accurate mass (m/z: 467.1339) and external calibration between 0.5 and 40 µg L^{-1}. The limits for quantification and detection were 20 and 10 ng L^{-1}, respectively.

3.3.4 Macroinvertebrates

We used four species of macroinvertebrates in our experiment: *Gammarus pulex* (shredder), *Agapetus ochripes* (grazer), *Leuctra nigra* (shredder) and *Radix balthica* (grazer) (Table 2.2). All species were sampled in the field one week before experimental start, and the collected macroinvertebrates were stored in large plastic aquaria containing AFW at 15 ºC and using a 14:10 h light:darkness cycle.

On experimental days 8 and 16 we removed three replicate aquaria from each treatment to monitor the abundance of living macroinvertebrates. On experimental day 29 we recorded the abundance of living macroinvertebrates in the remaining 6 replicate aquaria per treatment.

3.3.5 Leaf packs

We collected alder leaves (*Alnus glutinosa*) from trees just before abscission. The leaves were dried to constant mass at 60 ºC. The leaves were enclosed in coarse meshed leaf bags (mesh size 1 cm) allowing shredding macroinvertebrates to enter. A total leaf mass of 0.74 g DW were allocated to each leaf bag, and petioles were removed from all leaves. Prior to deployment in the aquaria, the leaf bags were submerged in clean AFW for 48h to leach potentially toxic compounds such as phenols. One leaf bag was submerged into each aquarium at experimental days 1, 8 and 16. Additional leaf bags were produced to provide measures for starting weight after the leaching process.

On experimental days 8, 16 and 29 the leaf bags were retrieved and attached macroinvertebrates re-introduced in the respective aquarium. The leaf material was stored at -18 ºC until DW measurements. It was then dried at 60 ºC to constant mass and the DW was measured with 0.001 g accuracy. For each batch of leaves the leaf mass loss was calculated as the difference between initial DW (after leaching) and final DW.

3.3.6 Chlorophyll a

We used chlorophyll a as proxy for epiphytic biomass on the gravel substrate. Prior to experimental start we measured the chlorophyll a content on 10 pieces of gravel to provide a measure for initial chlorophyll a concentration.

On experimental days 8, 16 and 29 we sampled three pieces of gravel from each aquarium for chlorophyll a quantification. The gravel was fully submerged in 96% ethanol and stored in darkness at room temperature (20 ºC) for 20h to extract the pigment. Subsequently, we transferred 10 mL of the ethanol to centrifuge tubes and centrifuged for 10 min (2000 rpm). Subsequent to centrifugation we measured the absorbance in the centrifuge tubes in a spectrophotometer (UV-Visible Recording Spectrophotometer UV-160, Shimadzu) at wavelengths 665 and 750 nm.

The chlorophyll a concentrations were adjusted for available surface areas on the gravel and calculated as:

$$\text{Chlorophyll a } (\text{mg m}^{-2}) = \left(\frac{\text{Abs(665-750)*Evol}}{0.0834*\text{A}}\right) * \text{F} * 10 \quad (1)$$

where Abs(665-750) is the light absorption at wavelengths 665 and 750 nm, Evol is the extraction volume (mL), 0.0834 is the specific absorption coefficient for chlorophyll a, and A is the surface area of the gravel. The surface area of the gravel was calculated as:

$$\text{Surface area } (\text{cm}^{-2}) = \frac{1}{2} * \pi * \frac{(\text{L}*\text{B})+(\text{L}*\text{H})+(\text{B}*\text{H})}{3} \quad (2)$$

where L is the length, B is the width and H is the height of the gravel. We multiplied the surface area by 0.5 assuming that photosynthetically active biofilm was restricted to the upper parts of the gravel.

3.3.7 Macroinvertebrate fitness

We estimated macroinvertebrate fitness using glycogen (*G. pulex*) and fatty acid methyl esters (*G. pulex*, *A. ochripes*, and *L. nigra*) (Koop *et al.* 2011). We only measured storage lipids and glycogen in macroinvertebrates collected at the end of the experiment (experimental day 29).

Glycogen content was determined in 48 individuals of *G. pulex* (n = 12 per treatment). Individuals intended for glycogen quantification were shock frozen in liquid nitrogen and freeze dried. The DW of the animals was measured with 0.0001 g accuracy. The individuals were then stored at -80 ºC until analysis. Each individual was dissolved in 1 mL 1M NaOH using a 5 mm stainless steel ball in a Tissue Lyser II, QIAGEN, at 20 l/s for 30 seconds. The dilution was heated at 80 ºC for 3h, and 200 µL of the sample was transferred to an Eppendorf tube along with 2 mL acetate buffer and 2 mg amyloglycosidase. The samples were then cooled in a water bath at 25 ºC for 2h. During these 2h, the samples were shaken every 20 min.

Glycogen quantification was performed on spectrophotometer (UV-Visible Recording Spectrophotometer UV-1650pc, Shimadzu) at 25 ºC. The absorbance of the sample was measured at 334 nm. Initially two reagents were mixed in a 4:1 ratio (Glucouse Gluc-DH FS Kit). We transferred 1 mL of the 4:1 dilution to a cuvette and acclimatized the dilution to 25 ºC. After adding 200 µL of the sample, the absorbance was measured every 30 seconds for 20 minutes to quantify the enzymatic decomposition of glycogen to smaller glucose molecules. The actual glycogen concentration could be calculated as:

$$\text{Glycogen concentration } (\text{mg mL}^{-1}) = \text{a} * \text{Abs334+b} \quad (3)$$

where Abs334 is the absorbance at 334 nm, a is the slope of the standard curve, and b is the intersection of the standard curve with the y-axis.

Contents of fatty acid methyl esters were determined for 36 individuals of *G. pulex* and 12 pooled samples of *L. nigra* (n = 9 and n = 3, respectively). Lipid analyses were not performed on *A. ochripes* due to low recovery or on *R. balthica* since these macroinvertebrates were not expected to be sensitive to sediment or lambda-cyhalothrin treatments in the applied range of stressor intesitiy. Pooling samples were necessary for *L. nigra* to meet minimum requirements for sample mass.

Collected macroinvertebrates were shock frozen in liquid nitrogen and freeze dried immediately after sampling. The DW of freeze dried animals was measured with 0.0001 g accuracy and stored at -80 ºC until analysis.

Each sample was added 0.5 mL PO^{4-} buffer and shaken with a 5 mm stainless steel ball in a Tissue Lyser II, QUIAGEN at 20 l/s for 30 seconds. Subsequently, an additional amount of 1 mL PO^{4-} buffer was added. Each sample was then transferred to a glass tube using a glass pipette. We added 1.5 mL chloroform to the sample, and the glass tube was shaken for 1 minute. After shaking the sample was stored at 20 ºC for 3h. An additional 1.5 mL PO^{4-} buffer was added, and the sample mixture was stored at 20 ºC for 12 h.

The samples were centrifuged for 10 min at 3500 rpm, and the sample supernatant was removed using a syringe connected to a vacuum pump. The remaining sample was transferred to a new glass tube, and the chloroform in the sample was evaporated under nitrogen gas flow. The sample was re-diluted with 3x300 µL chloroform and added to a 100 mg silica column pretreated with chloroform under vacuum. The sample passing through the column was accumulated in a new glass tube. Subsequently, 6 mL of acetone was added to the column, and this part of the sample collected in a separate glass tube. Hereby, the original sample was split into one sample eluted with chloroform and one sample eluted with acetone. The two samples were evaporated under nitrogen gas flow.

We added 0.5 mL methanol, 0.5 mL toluene, and 1 mL 0.2M KOH in ethanol to the evaporated samples. The samples were shaken for 1 min and transferred to a water bath at 37 ºC for 15 min. Then we added 2 mL heptane, 300 µL 1M acetic acid, and 2 mL Elga water and the samples were shaken for 1 min. Subsequently, the samples were centrifuged for 5 min at 1500 rpm. The supernatant was transferred to new glass tubes using glass syringes and evaporated under nitrogen gas flow.

The fatty acid methyl esters were re-dissolved using 3x500 µL heptane and transferred to brown glass bottles. The lipid quantification was performed using GC-MS (GCMS-QP2010 with GC-MS solution 2.70 software). One µL of each sample was injected at 220 ºC. The column used in the GC-MS was based on silica (Omegawax 320, diameter 0.32 mm and length 30cm). The samples passed the silica column at 24.9 kPa with a flow rate of 22.7 mL min^{-1}, column flow of 1.79 mL min^{-1}, and a split ratio of 10.0. Starting temperature for the column was 50 ºC and heating progressed with 20 ºC min^{-1} until the target temperature of 240 ºC. The target temperature was maintained for 6.5 minutes.

3.3.8 Data treatment

Bartletts test was used to check for homogenous variances in macroinvertebrate abundances, leaf decomposition rates, algae biomass accrual, and fitness parameters. The Shapiro-Wilk test was used to check for normality.

Differences in macroinvertebrate abundance between the temporal replications (after 8, 16 and 29 experimental days) were analyzed using One-way ANOVA ($\alpha = 0.05$). Data for *L. nigra* was fourth-root transformed to obtain homogeneity of variance. Moreover, we compared the species specific abundances among treatments within each temporal sampling point (after 8, 16 and 29 experimental days) using Two-way ANOVA ($\alpha = 0.10$). In the cases of significant ANOVAs, we used the Tukey test to test the difference between all pairwise comparisons ($\alpha = 0.05$ for temporal replications and $\alpha = 0.10$ for treatment effects).

Due to low recovery of *L. nigra* we only normalized the leaf weight loss according to the abundance of *G. pulex*. We checked for temporal differences in leaf mass loss using the Wilcoxon Rank test ($\alpha = 0.05$), since non-transformed or transformed data could not meet the assumptions of homogeneity of variance. We used two-way ANOVA to test for treatment effects on leaf mass loss for experimental days 8, 16, and 29 ($\alpha = 0.05$). In the cases of significant Wilcoxon Rank test and ANOVA, we used the Tukey test to perform all pairwise comparisons ($\alpha = 0.05$).

We used one-way ANOVA to test for differences in area-specific chlorophyll a concentrations between temporal replicates within the same treatment (α = 0.05). Moreover, we used two-way ANOVA to test for treatment effects on area-specific chlorophyll a concentrations for experimental days 8, 16, and 29 (α = 0.05). Area specific chlorophyll a concentrations for experimental day 16 and 29 were square root transformed and fourth root transformed, respectively. We used the Tukey test to perform all pairwise comparisons (α = 0.05).

We tested treatment effects on glycogen concentrations (normalized according to the untreated controls) and lipid concentrations (relative to the total body weight) using two-way ANOVA (α = 0.05). Lipid data was log-transformed in order to obtain homogeneity of variance. We compared total relative amount of lipids as well as the dominant fatty acid methyl ester, palmitic acid (Koop *et al.* 2011). The Tukey test was used to perform all pairwise comparisons in the cases of significant ANOVAs. Due to insufficient replication, the fitness parameters could not be statistically analyzed for *A. ochripes*.

3.4 Results

3.4.1 Pesticide concentrations

The nominal lambda-cyhalothrin concentrations were validated to 77.4 ng L^{-1} and 71.2 ng L^{-1} at exposure start and after 90 min, respectively. No lambda-cyhalothrin was detected in the untreated controls.

3.4.2 Macroinvertebrate abundances

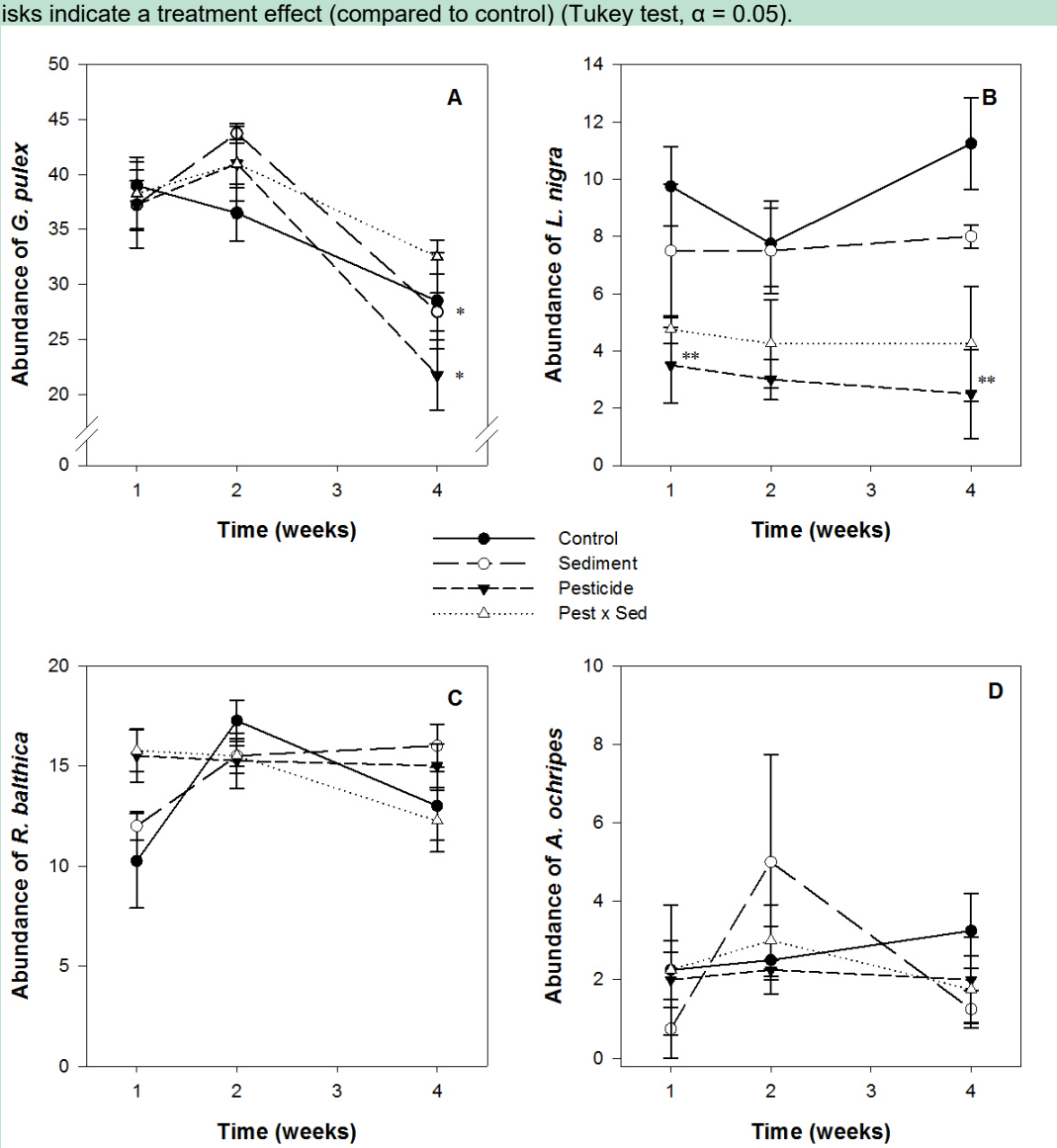
In general, the abundance of *G. pulex* decreased as a function of time, although the decrease was only significant for sediment treatment (one-way ANOVA, P = 0.002) and for the lambda-cyhalothrin treatment (one-way ANOVA, P = 0.089) (Fig. 3.2A). We found no effect of treatments on the abundance of *G. pulex* within each sampling time (two-way ANOVA, P > 0.05).

There was no significant effect of time on the abundance of *L. nigra* (one-way ANOVA, P > 0.05) (Fig. 3.2B). However, we found a significant effect of treatment for experimental days 8, 16, and 29 (two-way ANOVA, P = 0.012, P = 0.012, and P = 0.010, respectively). The effect of lambda-cyhalothrin was significant (P < 0.10), but there was no significant effect of sediment and no significant interaction between sediment and lambda-cyhalothrin (P > 0.10). The abundance of *L. nigra* was significantly lower in the lambda-cyhalothrin treatments compared to controls for experimental days 8 and 29 (Tukey test, P < 0.10) (Fig. 3.2B).

The abundance of *R. balthica* was not significantly different among temporal subsamples within each treatment (one-way ANOVA, P > 0.05) although a considerable variation was evident with highest abundances for experimental day 16 (Fig. 3.2C). The abundance of *R. balthica* was significantly different among treatments for experimental day 8 (two-way ANOVA, P = 0.059), but not for experimental days 16 and 29 (two-way ANOVA, P > 0.10). For experimental day 8, there was a significant effect of lambda-cyhalothrin (P < 0.10), while the effect of sediment and interaction between sediment and lambda-cyhalothrin were not significant (P > 0.10) (Fig. 3.2C). The pairwise comparisons for experimental day 8 showed that the abundance of *R. balthica* was significantly higher in the sedxpest treatment compared to the untreated control (P < 0.10).

The abundance of *A. ochripes* was not significantly different among temporal samples (one-way ANOVA, P > 0.05). Moreover, the abundance of *A. ochripes* was not significantly different among treatments for experimental days 8, 16, and 29 (two-way ANOVA, P > 0.10 for all) (Fig. 3.2D).

FIGURE 3.2. Mean abundance of the four macroinvertebrate species at experimental days 8, 16, and 29 (Weeks 1, 2, and 4). Error bars indicate SE. Single asterisks represent significant differences between temporal subsamples within the same treatment (one-way ANOVA, α = 0.05), and two asterisks indicate a treatment effect (compared to control) (Tukey test, α = 0.05).

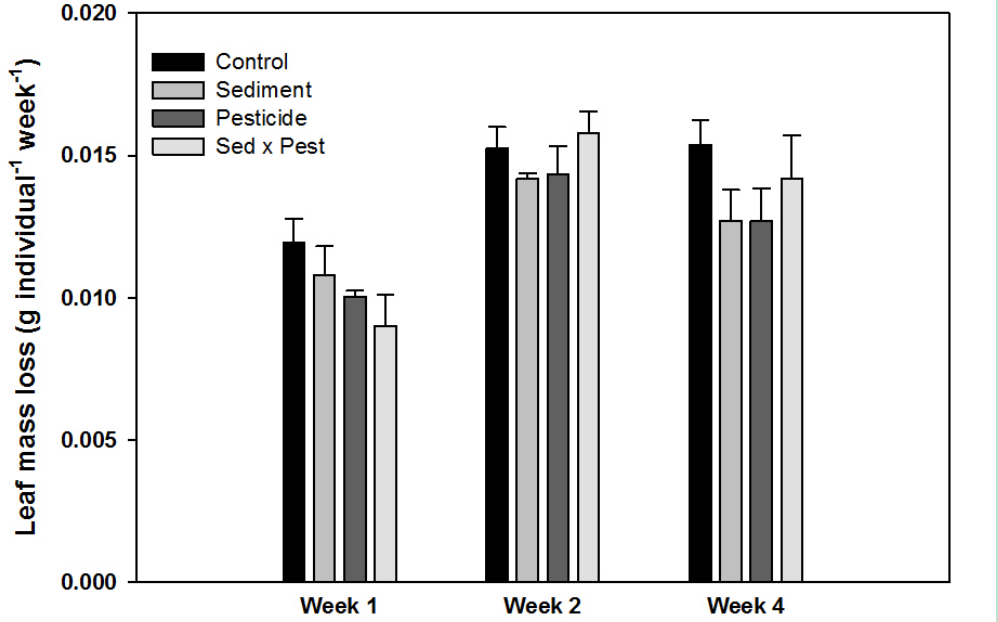


3.4.3 Leaf decomposition

The leaf mass loss (g individual^{-1} experimental week^{-1}) within treatments was significantly different among the sampling dates for the untreated controls, lambda-cyhalothrin, and SedxPest treatments (one-way ANOVA, $P < 0.05$) whereas the temporal variance among sampling dates was not significant for the sediment treatment (one-way ANOVA, $P > 0.05$) (Fig. 2.3). In general, the leaf mass loss was lowest during the first experimental week and increased to a stable level maintained during the remaining weeks. This increase was significant for the controls, lambda-cyhalothrin, and SedxPest treatments (Tukey test, $P < 0.05$) (Fig. 3.3).

The difference in leaf mass loss among treatments was not significant (two-way ANOVA, $P > 0.05$) for all experimental weeks (Fig. 3.3).

FIGURE 3.3. Treatment specific average leaf mass loss per G. pulex during the 29 days experiment. Leaf material was sampled (n = 4) on experimental days 8, 16, and 29 corresponding to experimental weeks 1, 2, and 4, respectively. Error bars indicate SE.

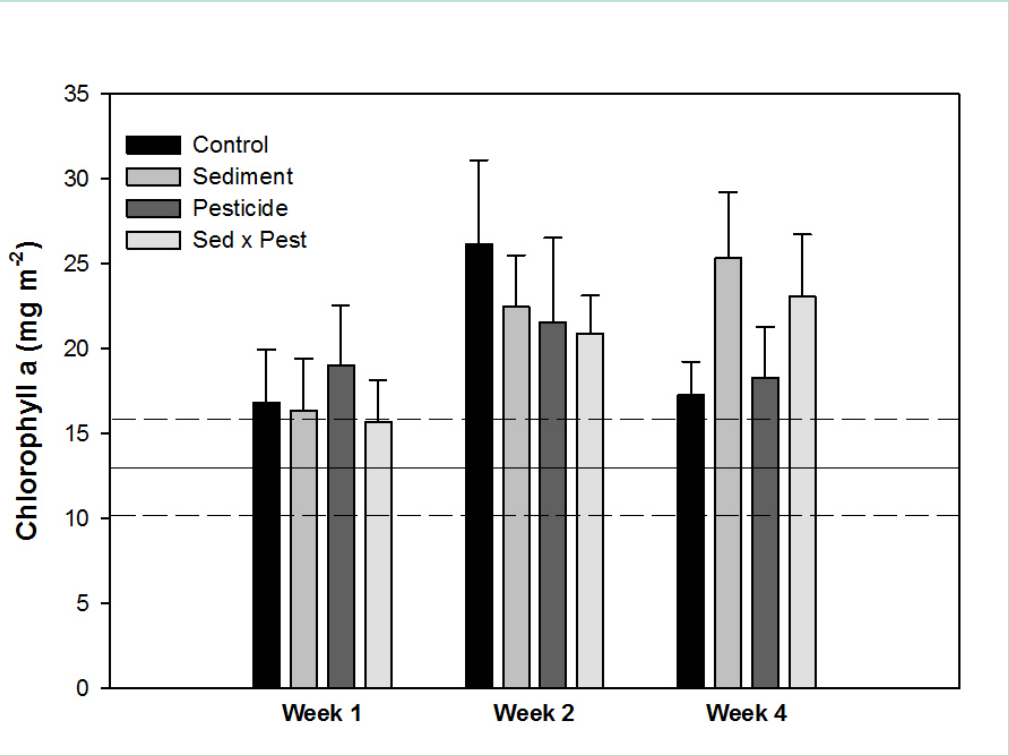


3.4.4 Algae biomass accrual

The chlorophyll a concentration was not significantly different among sampling dates within treatments (one-way ANOVA, P > 0.05) (Fig. 3.4). In general, the chlorophyll a concentrations were associated with large within group variation.

Chlorophyll-a concentrations were significantly different among treatments for experimental week 4 (two-way ANOVA, P = 0.042) with a significant effect of sediment (P = 0.012) while there was no significant effect of lambda-cyhalothrin or a significant interaction link (P > 0.05) (Fig. 3.4). The chlorophyll-a concentrations in the sediment and SedxPest treatments were higher, although not significantly, compared to the untreated controls (Tukey test, P = 0.060 and 0.071, respectively) and the lambda-cyhalothrin treatment (Tukey test, P = 0.079 and 0.092, respectively) (Fig. 3.4).

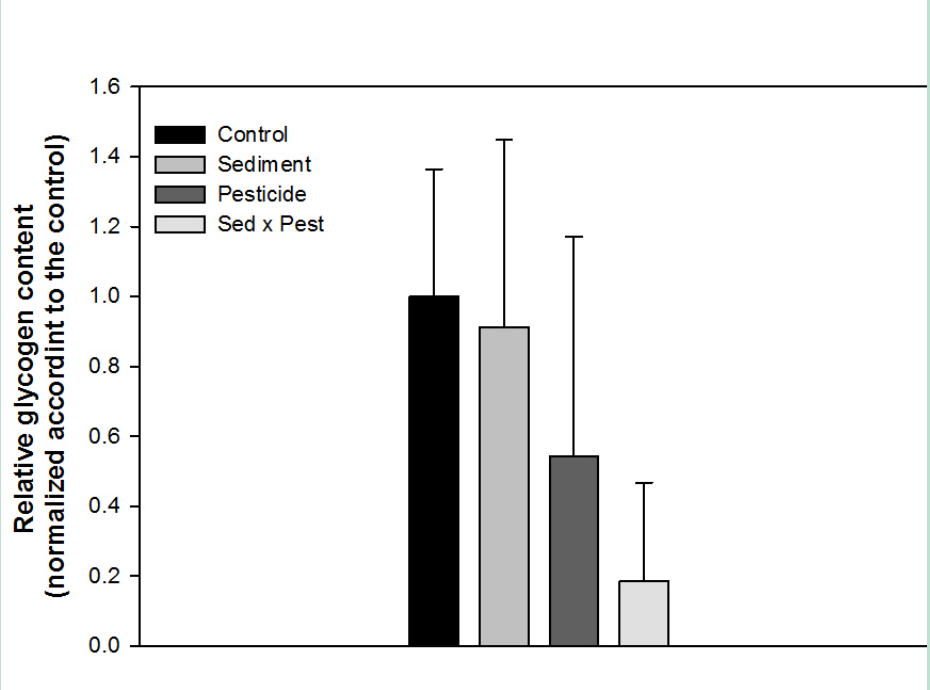
FIGURE 3.4. Treatment specific average chlorophyll a concentration on gravel sampled on experimental days 8, 16, and 29 corresponding to week 1, 2, and 4, respectively. Three gravel was sampled from each treatment replicate (n = 4). Error bars indicate SE. The solid and dashed lines indicate average starting concentration of chlorophyll a and SE, respectively.



3.4.5 Macroinvertebrate fitness

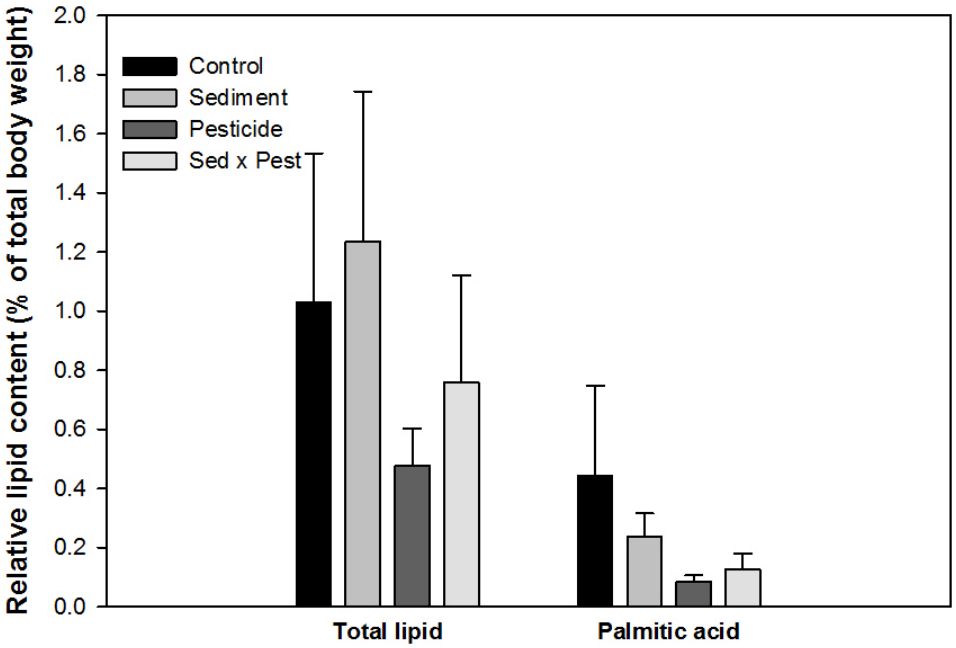
The relative glycogen content in *G. pulex* was highest in the untreated controls and lowest in the Sed x Pest treatment, but we found no statistically significant effect of treatments (two-way ANOVA, P = 0.51) (Fig. 3.5).

FIGURE 3.5. Average relative glycogen content normalized according to the untreated controls in *G. pulex* (n = 12). Animals were collected at the end of the experiment (day 29). Error bars indicate SE.



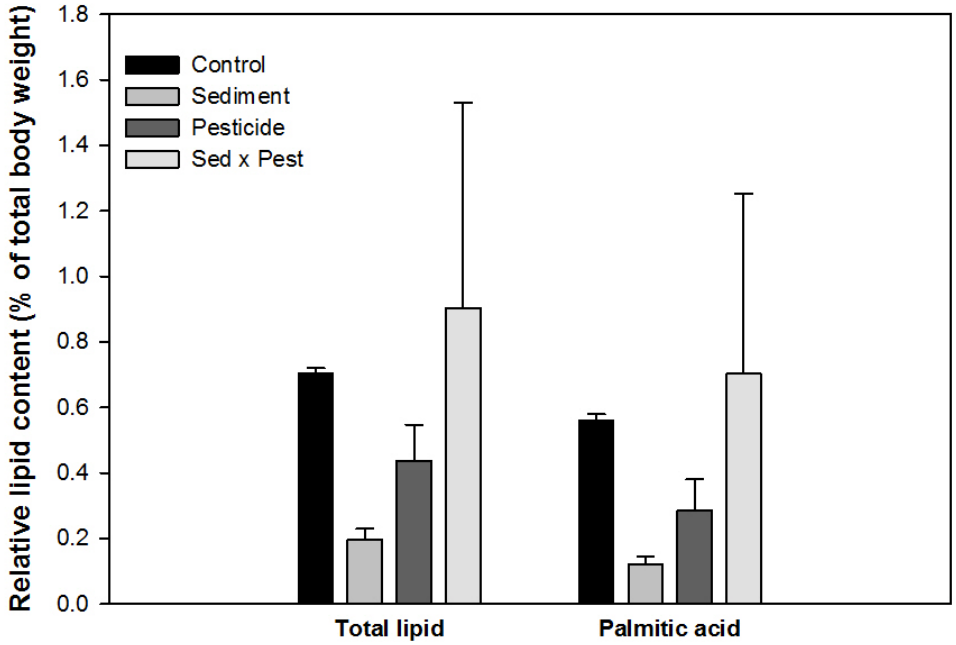
The relative content of palmitic acid and total lipids in *G. pulex* was not significantly different among treatments (two-way ANOVA, P = 0.92 and P = 0.47, respectively) (Fig. 3.6). Although not significant, the relative content of palmitic acid and total lipids was lower in the lambda-cyhalothrin and Sed x Pest treatments compared to the control and sediment treatments (Fig. 3.6).

FIGURE 3.6. Average relative lipid content in *G. pulex* (n = 9). Animals were collected at the end of the experiment (day 29). Error bars indicate SE.



The relative content of palmitic acid and total lipids in *L. nigra* was not significantly different among treatments (two-way ANOVA, P = 0.055 and P = 0.12, respectively) (Fig. 3.7). Although not significant the highest lipid content was found in *L. nigra* from the SedxPest treatment.

FIGURE 3.7. Average relative lipid content in *L. nigra* (n = 3). Animals were collected at the end of the experiment (day 29). Error bars indicate SE.



3.5 Discussion

3.5.1 Macroinvertebrate abundance

We found no overall effect of treatments on the abundance of *G. pulex*, although the abundance of *G. pulex* significantly decreased as a function of time in the sediment and lambda-cyhalothrin treatments. The applied nominal exposure concentration of lambda-cyhalothrin (75 ng L^{-1}) is below expected mortality effects of pyrethroids (Beketov & Liess 2008b) but within the range of expected behavioral effects (Nørum *et al.* 2010; Nørum, Frederiksen & Bjerregaard 2011; Rasmussen *et al.* 2013a). Moreover, *G. pulex* is characterized by high plasticity in habitat preferences and is often one of the dominant macroinvertebrate species in Danish agriculturally impacted streams (Rasmussen *et al.* 2012c). Consequently, the mobility of some *G. pulex* could be reduced in the lambda-cyhalothrin treatments which could prompt increased cannibalistic activity exerted by the less affected individuals (MacNeil, Dick & Elwood 1997). However, this explanation is contradicted by the lacking effects in the SedxPest treatment. The decreasing abundance of *G. pulex* through time in the sediment treatment is unlikely to be due to a direct negative effect of the added sediment but could be caused by increased encounters among *G. pulex* (due to less available refuges) leading to increasing cannibalism. However, this explanation is additionally contradicted by the lacking effect in the SedxPest treatment. In consequence, we fall short of logic explanations for the lacking significant reduction in the abundance of *G. pulex* in the SedxPest treatment.

The abundance of *L. nigra* was significantly reduced in treatments containing lambda-cyhalothrin compared to the control and sediment treatments. This effect was likely due in part to increased predation of *L. nigra* by rather unaffected individuals of *G. pulex* (Rasmussen *et al.* 2013a) and in part due to lambda-cyhalothrin mediated mortality. Although we documented that the acute LC_{50} for *L. nigra* exposed to lambda-cyhalothrin for 90 min was 1.43 µg L^{-1} (Appendix paper 1), this result was obtained using larger individuals collected just before the adult stage. In the current experiment we used individuals collected in October. These were approximately 50% smaller (body mass DW) compared to the individuals used in the Appendix paper 1 which

could severely reduce the acute LC_{50}. Hence, we cannot preclude that the exposure of *L. nigra* to 75 ng L^{-1} lambda-cyhalothrin for 90 min caused mortality as well as reduced mobility.

We found no significant effect of sediment addition or interaction between stressors clearly confirming that *L. nigra* was unaffected by the physical habitat alterations imposed by fine sediment addition. This is surprising considering the species preference for silty environments. Therefore, *L. nigra* may have potential as indicator organism for pyrethroid pollution. The pragmatic value of this is however constrained by the clear preference of forested tiny headwater streams (close to the spring) for this species. In previous work, we showed that the close relative and more widespread, *Leuctra fusca*, was significantly less abundant in physically homogenous sites dominated by sand and mud compared to heterogeneous sites dominated by gravel in Danish streams covering a gradient in pesticide pollution (Rasmussen *et al.* 2012b). Hence, using the *Leuctra* genus as general indicator for pesticide pollution is likely not feasible.

The abundance of *R. balthica* was significantly higher in treatments containing lambda-cyhalothrin in experimental week 1, but this significant difference disappeared in experimental weeks 2 and 4. Moreover, the abundance of *R. balthica* significantly increased as a function of time in treatments containing fine sediment. The concentrations of lambda-cyhalothrin used in this experiment was at least three orders of magnitude below those causing behavioral effects (Appendix paper 1), and we therefore suggest that the observed temporary significant effect of lambda-cyhalothrin on *R. balthica* abundance was coincidental. The observed increasing abundance with time in the sediment treatments was most likely due to a slowly establishing biofilm on the vertical sides of the aquaria attracting increasingly more (and therefore more detectable) individuals. This was probably particularly evident in the sediment treatments where the substrate surfaces containing biofilm were reduced.

We found no significant of treatment or time on the abundance of *A. ochripes* which was most likely due to the low retrieval success of this species. The low recovery was mainly due to the small size of the animals and their cases consisting of coarse sand grains providing strong camouflage for all substrate types used in the experiment. In Appendix Paper 1 we showed that the closely related *Agapetus fuscipes* was the most sensitive test species to lambda-cyhalothrin exposure with acute LC_{50} of 28 ng L^{-1}. We can therefore not conclude anything concerning the sensitivity of *A. ochripes* to lambda-cyhalothrin.

3.5.2 Leaf decomposition

The leaf mass loss adjusted per individual of *G. pulex* increased during the experiment, but we found no significant effect of treatments. The abundance of *G. pulex* was not statistically different among treatments and hence only marginally influenced the individual based leaf mass loss. The increasing leaf mass loss as a function of time could relate to increased levels of stress imposed on the macroinvertebrates due to handling and changing environments leading to i) suppressed normal feeding behavior or/and ii) increased metabolic requirements facilitating increased feeding behavior. Our findings contrast previous studies showing that a short pulse of pyrethroid insecticides of concentrations down to 10 ng L^{-1} significantly reduce shredding activity in *Gammarus* sp., *L. nigra* and several species of caddisflies, and that this reduction persists at least one to two weeks (Lauridsen, Kronvang & Friberg 2006; Rasmussen, Friberg & Larsen 2008; Rasmussen *et al.* 2012a; Bundschuh *et al.* 2013). We speculate that the abundance of especially *G. pulex* may have been too high leading to food limitation in the control and sediment treatments.

3.5.3 Algae biomass accrual

Algae biomass accrual was significantly higher at the end of the experiment in the treatments containing fine inorganic sediment, whereas the algae biomass was maintained at a stable level in the control and lambda-cyhalothrin treatments. This may be due to the grazing activity of *R. balthica*, since this species appeared to prefer feeding on the established biofilm on the vertical

sides of the aquaria in the sediment treatments probably due to the addition of fine sediment limiting algae biomass. This change in substrate preference by *R. balthica* could also have reduced the grazing pressure on coarse substrate enabling increased algae biomass accrual. The grazing activity of *A. ochripes* was expected to exert lower influence on the algae biomass accrual in part caused by lower grazing pressure and in part caused by generally low survivability of this species. Our findings contrast the results of Rasmussen et al. (2008) who showed that algae biomass accrual significantly increased during a 10 day study period in stream channels containing *Heptagenia sulphurea* and *Ancylus fluviatilis* previously exposed to 100 ng L^{-1} for 90 min. One plausible explanation for this incongruence in results could be that the dominant grazer in terms of grazing efficiency and area specific abundance was *R. balthica* which is highly tolerant to pyrethroids, whereas in the study of Rasmussen et al. (2008), the dominant grazer was *H. sulphurea* showing high sensitivity to pyrethroids.

3.5.4 Macroinvertebrate fitness

We found no significant treatment effect on glycogen content in *G. pulex*, or on lipid contents in *G. pulex* or *L. nigra*. However, the glycogen content in *G. pulex* was lowest in the SedxPest treatment and highest in the control treatment indicating that the metabolic requirement was higher for *G. pulex* in the SedxPest treatment. Importantly, glycogen likely provides the most important energy storage depot for crustaceans (Koop *et al.* 2008). Since the majority of pesticide concentrations occurring in the field are below those causing acute mortality on stream macroinvertebrates (Bundschuh, Goedkoop & Kreuger 2014; Moschet *et al.* 2014; Rasmussen *et al.* 2015), and since the measured pesticide concentrations tend to strongly correlate to changes in macroinvertebrate community structure, it is broadly believed that sublethal endpoints, such as molecular biomarkers, could provide essential information on the mechanisms behind the observed macroinvertebrate community changes (Koop *et al.* 2011). However, in order to optimize comparability in such biomarkers for fitness which rapidly change in response to various environmental parameters and life cycle stages (Rozsypal *et al.* 2014), it is likely necessary to streamline ecotoxicological studies more towards comparable sizes and life stages under more controlled conditions.

3.5.5 Synthesis and applications for the development of a Danish pesticide indicator

Although highly likely that certain macroinvertebrate species respond more strongly to physical habitat deterioration than environmentally realistic pesticide concentrations and *vice versa* (Rasmussen et al. 2012b), this experiment provided no clear insight into the underlying mechanisms behind these differences, nor provided unambiguous results aiding to reveal potential interactions between these two important stressors (fine sediment deposition and pesticide pollution). One way to improve our understanding of the importance of each of these stressors on macroinvertebrate communities would be to use available global data containing measures for both habitat characteristics. Such data have been produced by specific groups of researchers (e.g. in this specific research project) but are also available at larger scales although confounded by lower quality in quantified chemical concentrations (Malaj *et al.* 2014; Stehle & Schulz 2015). This data invites to analyze specific species-response relationships to the stressors in combination or alone.

4. Macroinvertebrate community responses and functional characteristics of Danish streams covering a gradient in pesticide pollution

4.1 Introduction

In chapter 2 we showed that extrapolating pesticide sensitivity from few to many macroinvertebrate species (gradual data) is affiliated with significant uncertainty but that specific measures for surface area / volume ratios for macroinvertebrates provided a significant proximate of their measured pesticide sensitivity. However, the SPEAR index does not use the estimated sensitivity data as gradual data. Instead, species are subdivided into two groups using the median sensitivity of all tested species as cut off value to distinguish between sensitive and non-sensitive species. This method is proposed to significantly increase the statistical strength of the correlation between measured pesticide pollution in the field and macroinvertebrate community responses (Beketov, Kattwinkel & Liess 2013). In addition, we showed in chapter 2 that extrapolating pesticide sensitivity from few to many species provided an equal amount of over- and underestimations of actual sensitivities which could suggest that the SPEAR approach is not as biased as indicated by analyzing predicted and measured sensitivities as gradual data. In chapter 2 we additionally showed that taxa specific generation time was a central parameter for predicting community responses to measured gradients in pesticide pollution in the field. This finding lends support to the SPEAR approach although a measure for average generation time in macroinvertebrate communities is probably not responding specifically to pesticide pollution but rather is an indicator of general disturbance tolerance (opportunist vs competitive species).

The engine room of SPEAR consists of three elements: predicted pesticide sensitivity, generation time and migration potential. Migration potential is, similar to generation time, a general indicator for the disturbance tolerance of a species with stronger migration potential rendering a species more likely to recolonize habitats where populations have been reduced or eradicated due to disturbance, such as pesticide pollution. Hence, the single parameter that is proposed to prompt the specificity towards pesticide pollution is the predicted taxa specific pesticide sensitivity. A thorough investigation of the data basis behind SPEAR reveals that migration potential is only characterized as strong for the freshwater shrimp *Gammarus pulex*. Hence, in practice only predicted pesticide sensitivity and generation time actively discriminate the SPEAR grouping of macroinvertebrate taxa. These two parameters are, as described above, the very same parameters identified in chapter 2 as detrimental for characterizing pesticide specific macroinvertebrate community responses.

In addition, Wiberg-Larsen *et al.* (2016) showed that the rank ordering of sensitive macroinvertebrate species (towards organic pollution) in DSFI was not correlated to the measured pyrethroid sensitivity of the same species. However, this prediction that DSFI does not respond to a gradient in pesticide pollution needs field-based validation. In order to validate this prediction, it is imperative that a field study contains a careful selection of sites where pesticide pollution is

the strongest acting environmental stressor and that this gradient is not confounded by co-occurring stressors known to influence DSFI scores (especially organic pollution and physical habitat deterioration) (Wiberg-Larsen *et al.* 2010).

A previous study revealed that the SPEAR index, similar to DSFI, additionally responded to strong physical habitat deterioration (Rasmussen *et al.* 2012b). Moreover, DSFI and SPEAR were strongly intercorrelated in a broad set of Danish streams covering a wide set of environmental stressors (Rasmussen *et al.* 2011a). Therefore, SPEAR additionally needs testing in a Danish field set-up where the pesticide gradient is the strongest acting environmental stressor and needs here to be compared to DSFI in order to pinpoint if SPEAR is superior to DSFI in quantifying macroinvertebrate community responses to pesticide pollution. Equally important, pesticide pollution needs to be thoroughly quantified in order to understand the level of detail necessary for generating sufficiently strong correlations between pesticide pollution and macroinvertebrate indicator responses. These are crucial first steps that need to come before opening and modifying the SPEAR engine.

An additional aspect that receives strongly increasing scientific and political interest is the response of central ecosystem functions, some providing profound services for humans, to anthropogenic stress – and whether these responses in ecosystem function can be predicted from biodiversity or ecological indicators. Land use is an important factor governing stream ecosystem functions such as decomposition of organic matter (Lecerf & Chauvet 2008; Piscart *et al.* 2009; Hladyz *et al.* 2011) and metabolism (Bernot *et al.* 2010). Many factors related to land use influence ecosystem functions either positively or negatively depending on stressor type and intensity, but functional redundancy may mask some or all such influences (Schäfer, Van den Brink & Liess 2011; Woodward *et al.* 2012). Although ecosystem functions have been highlighted as essential focal points for stream ecosystem conservation (MEA 2005), and although studies on ecosystem functions have increased exponentially during the past decade (Mulder *et al.* 2015), very few studies have focused on pesticide effects on ecosystem functions at the ecosystem scale (but see Rasmussen *et al.* 2012c; Schäfer *et al.* 2012a; Fernandez *et al.* 2015; Voss, Fernandez & Schäfer 2015). In fact, no studies have addressed potential effects of pesticides on ecosystem metabolism in streams.

In this part of the project we conducted a comprehensive field campaign in 19 Danish streams aiming to address the knowledge gaps identified above. We collected data allowing a detailed quantification of pesticide contamination in these streams, and the streams were carefully selected to minimize the influence of co-occurring stressors. The streams were thoroughly described in terms of their general physical and chemical composition and temporal dynamics in order to document the specific influence of pesticides on stream biota. We collected samples allowing a highly detailed quantification of macroinvertebrate community composition and recolonization ranging from before to several months subsequent to the main insecticide application season, and we measured whole-stream metabolism to investigate the potential link between structural ecological indicators and stream ecosystem functions potentially influenced by the gradient in pesticide pollution. We use the collected data to examine the ability of currently existing macroinvertebrate indicators (SPEAR and DSFI) to quantify ecological effects of pesticide pollution and inform the progression and improvement of pesticide-specific macroinvertebrate indicators.

4.2 Methods

4.2.1 Site selection and characteristics

We selected 19 stream sites covering a gradient in agricultural intensity in the stream catchments. All streams were selected according to the following selection criteria: 1) streams sizes restricted to 1st or 2nd order (Strahler), 2) riparian zones of the sampling sites must be characterized by minimal trees and shrubs ensuring comparable input of allochtonous material and irradi-

ation, and 3) instream physical conditions must be comparable, hence strongly incised, channelized, and mud-dominated stream sections were deselected. Moreover, we selected 8 streams in Jutland, 6 on Funen and 5 on Zealand to represent the major Danish biogeographical areas. An overview map of the stream sites is presented in Fig. 4.1, and the overall catchment characteristics in Table 4.1. The sampling sites represented stream sections of 100 m and was selected to be representative of the overall hydromorphological conditions of the stream.

FIGURE 3.1. Schematic overview of the Danish study sites. Subdivisions into agricultural and reference sites indicate that proportions of agriculture in a two-sided 100 m buffer zone extending 2,000m upstream of the sampling site are > 60% and <50%, respectively. The figure is an excerpt of the Supplementary material in Rasmussen et al. (2015).

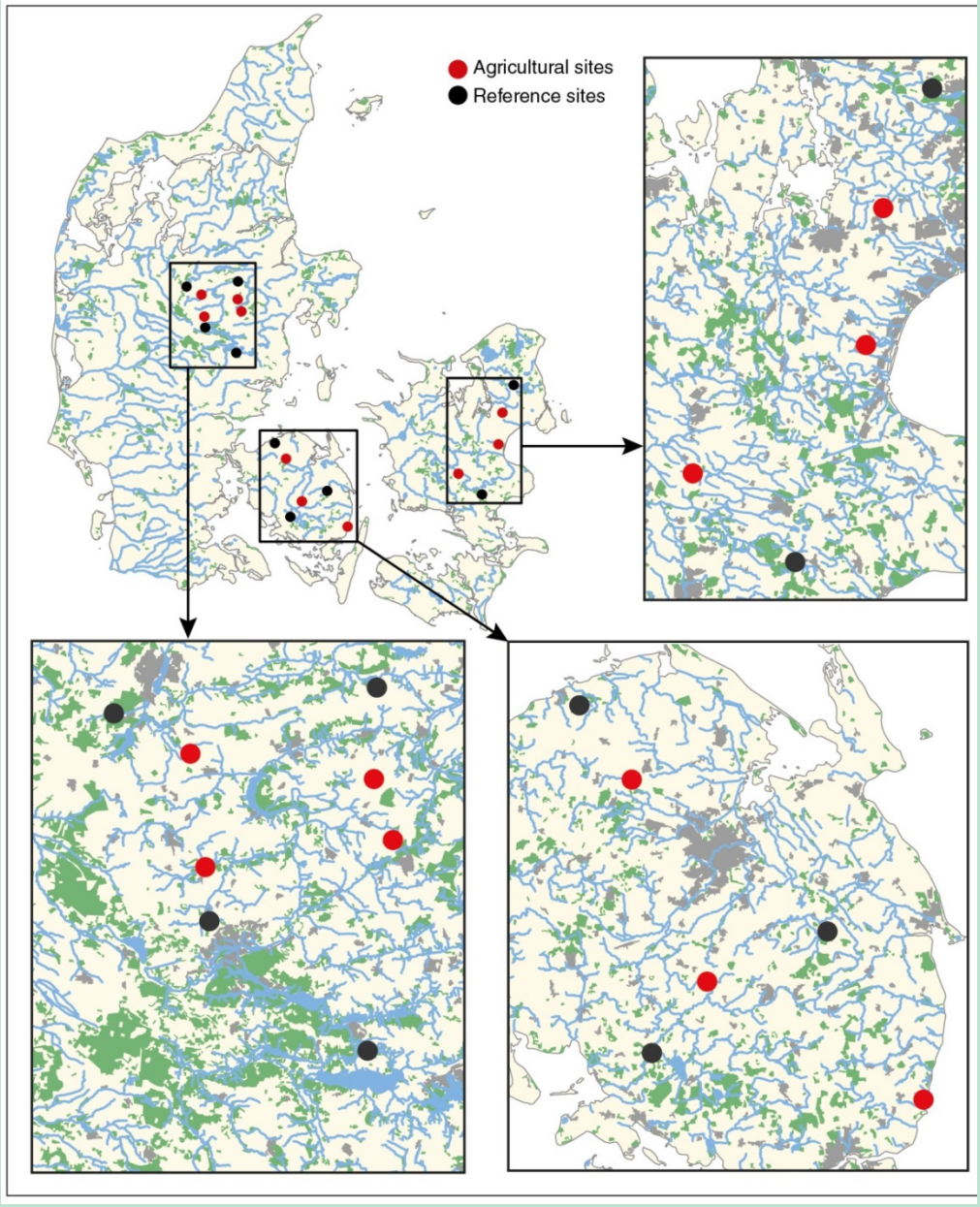


TABLE 4.1. Summary table of relevant stream and catchment properties for the study streams. The stream ID is composed of a letter (C = Control streams, A = Agricultural streams), followed by the stream number. The buffer zone represents a double sided 100m buffer extending from the sampling site and 2,000 m upstream. The table is an excerpt of the Supplementary material in Appendix paper I (Rasmussen *et al.* 2015). Baseflow values represent spot-measurements in August after minimum 7 days without rain.

Parameter/stream code	C1	C2	C3	C4	C5	C6	C7	C8	C9	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
Catchment (km^2)	8.40	10.3	3.52	2.48	4.61	4.26	1.85	0.94	10.2	3.09	0.96	25.6	8.62	4.30	19.4	14.7	18.1	23.2	10.7
% agriculture in catchment	48.4	37.3	80.7	55.8	46.0	54.9	34.5	75.5	84.1	90.9	74.3	89.4	85.7	90.6	82.8	86.6	80.3	59.4	85.4
% agriculture in buffer zone	12.5	38.8	34.7	16.4	38.5	47.1	5.8	16.1	49.0	93.2	60.0	68.9	68.9	83.7	78.2	90.5	85.1	65.0	67.9
Baseflow ($L\ s^{-1}$)	44	31	13	9	13	46	112	37	61	7	16	37	8	7	41	17	95	37	29
Yearly mean discharge ($L\ s^{-1}$)	>44	93	28	13	16	>46	>112	>37	101	22	>16	182	91	33	156	89	222	126	106

4.2.2 Physical site characteristics

The physical site characteristics (substrate composition, other physical structures such as aquatic vegetation and woody debris, width, depth and stream flow parameters) were recorded in April/May and in September/October for each sampling site. We measured width of ten stream transects positioned with 10 m intervals along the 100 m stream section. Each transect was subdivided into four subsections corresponding to 1-25%, 26-50%, 51-75%, and 76-100% of the total stream width. Depth and current velocity (at 0.6 x stream depth) were recorded at the center point of each transect subsection. Moreover, we registered substrate composition (boulder, pebble, gravel, sand, and mud) and coverage of other biological/physical structures (aquatic vegetation and coarse organic debris, such as leaf material and wood fragments) within each rectangular area formed between transects and transect subsections (length of each area = 10 m and width corresponding to 25% of the total stream width). The aquatic plants were additionally identified to lowest taxonomic level (species or genus).

We additionally quantified the width of unmanaged buffer zones (average and minimum), formation of secondary stream profile due to previous channelization activity, and the areal coverage of woody vegetation in the riparian zone (two sided 10 m buffer zone along the 100 m stream section).

4.2.3 General water chemistry

Water samples (2 x 1L) were collected at the downstream end of each stream section in April, June, August and November. The samples were consistently collected in well-mixed zones of the stream. All water samples were immediately transported to the laboratories of Aarhus University, Department of Bioscience in Silkeborg for analysis of biological oxygen demand (BOD_5), nitrate-N, ammonium-N, total-N, orthophosphate-P, and total-P. The following parameters were analysed according to European standards: BOD_5 (DS/EN1899 1999), ortho-phosphate (DS/EN 1189 1997), and ammonia-N (DS 11732 2005). Nitrate-N was analysed using Lachat-methods (Lachat Instruments, USA, Quickchem no. 10-107-06-33-A (Salycate method)). Concentrations of total N and P (unfiltered samples) were measured applying the Kjeldahl-N method (Kjeldahl 1883) and Danish standard (DS-291), respectively.

4.2.4 Pesticide samples and analysis

Samples for quantification of pesticide pollution were collected using four different methods in the period from May to August 2013. In all streams we collected base flow water samples in August, event-triggered storm flow water samples in May, June and July, sediment samples in August, and time-integrated suspended sediment samples representing the period from May to August. The details on sampling methods and pesticide quantification in the respective sample types are described in Appendix paper I (Rasmussen *et al.* 2015).

4.2.5 Macroinvertebrate communities

Information on macroinvertebrate community composition was collected in April/May, June, July, September, and November representing the ecological scenarios before, during and after the primary insecticide application season (May to July). In April/May, July, September, and November, the samples were collected using a modified version of the semi-quantitative NOVANA kick-sampling procedure. The modification was implemented to separate substrate specific sub-samples, whereas all sub-samples are pooled in the NOVANA kick-sampling procedure. We identified the three dominating substrate types and collected four sub-samples on each substrate type. The sub-samples collected on the same substrate types were pooled. In June, the macroinvertebrates were collected using randomized surber sampling (n = 5) in order to quantify species specific abundances.

Macroinvertebrates belonging to Ephemeroptera, Plecoptera, Trichoptera, Coleoptera, Hirudinea, Tricladia, Amphipoda, and Isopoda were identified to species level, Diptera were identified to genus level except Chironmidae (identified to subfamily) and Simuliidae (identified to family). Oligochaeta and Arachnida were identified to the level of order.

4.2.6 Macroinvertebrate recolonization

Macroinvertebrate drift samples were collected in all streams before (April/May) and after (September) the primary insecticide application period. Drift sampling was conducted only during base-flow conditions to avoid overestimating the recolonization potential of the streams. One drift net was deployed at the most upstream transect of the stream section in the main stream channel for three hours starting from the time of sunset. Discharge was quantified for this transect at the beginning and end of drift sampling based on four depth and current velocity measurements (0.6 x depth) representing four transect subsections; 1-25%, 26-50%, 51-75%, and 76-100% of the stream width, respectively. Discharge for each transect was then calculated as the sum of width x depth x current velocity for the four transect subsections. Moreover, the current velocity was measured at time 0 and 3h after deploying the driftnet at the opening of the driftnet (0.5 x height of the driftnet) at three points corresponding to 25%, 50%, and 75% of the width of the driftnet. Using these data, the proportion of stream discharge entering the driftnet can be calculated, and total drift activity can be calculated for the entire transect. The discharge proportion entering the driftnet during the three hours of sampling was assumed to equal the mean of the start and end measurements.

4.2.7 Stream metabolism

Daily gross primary production (GPP), community respiration (CR_{24}), and net daily metabolism (NDM) were measured using the diurnal upstream-downstream dissolved oxygen (DO) change technique (Bott 2007). Oxyguard (model 840) probes and loggers were used, and data (DO concentration, oxygen saturation and temperature) was logged at one minute intervals. The probes were deployed in each stream for minimum 48h at the up- and downstream ends of each stream section and fixated in well-mixed areas of the stream.

The gas exchange rate between stream water and the atmosphere (reaeration) is a necessary parameter for proper estimation of stream community metabolism. Reaeration was estimated using the surface renewal model (SRM) (Bott 2007):

$$f_{(20^{\circ}C)} = 50.8 \times V^{0.67} \times H^{-0.85}$$

where $f_{(20^{\circ}C)}$ is the gas exchange rate (cm h^{-1}), V is the current velocity (cm s^{-1}), and H is the mean water depth measured as weighted mean for minimum ten cross sectional transects (cm). This performance of the SRM model is highest for stream with current velocities exceeding 3 cm s^{-1} and mean water depth exceeding 12 cm (Bott 2007).

Travel time (T_t) for a parcel of water was estimated by adding NaCl to the stream water at the upstream transect. A conductivity meter was deployed at the downstream transect, and stream water conductivity was logged at 15 seconds intervals until the conductivity level stabilized at the initial background level. Stream water conductivity was plotted as a function of time, and the time to 50% of the added NaCl having passed the downstream transect (T_t) was calculated integrating the area under the curve.

Rates of net DO change (net metabolism) were determined as the difference in DO concentration between the deployed upstream and downstream probes corrected for reaeration. Community respiration (CR_{24}) was calculated as the sum of respiration from 24:00 to 03:00 and extrapolated to the full 24h of the day. GPP was determined as the sum of the difference between respiration during daytime and measured net metabolism. Consult Bott (2007) for further details on methodology and calculations.

4.2.8 Quantifying pesticide toxicity to stream biota

Pesticide concentrations in all sample types were converted to measures of potential toxicity to freshwater biota using the Toxic Units approach (TU) using daphnia, algae and fish as benchmark organisms for water samples and *Chironomus riparius* as benchmark organism for sediment and suspended sediment samples. See Appendix paper I (Rasmussen *et al.* 2015) for details on the calculations and ecotoxicological assumptions using this approach.

4.2.9 Statistical methods for environmental variables

We grouped the sites according to the agricultural proportion in a two sided 100 m buffer zone extending 2,000 m upstream of the stream sections (A: agricultural proportion > 60%, C: agricultural proportion < 50%) (Appendix paper I, Rasmussen *et al.* 2015). The use of such a two sided 100 m buffer zone extending 2,000 m upstream is based on previous work suggesting that the near-stream area is a stronger predictor for pesticide concentrations in stream water compared to larger buffer zones and total catchment characteristics (Schriever, von der Ohe & Liess 2007; Rasmussen *et al.* 2011b). The following statistical analyses were based on this grouping or raw gradients in environmental data.

Potential effects of stream group and time (spring vs autumn) on physical stream properties and general water chemistry variables were analyzed using Two-Way ANOVA in JMP 12.1 for Windows. Data normality and homogeneity of variance were tested using Shapiro-Wilk test and Bartlett's test, respectively. In the cases of significant ANOVA ($P<0.05$), the Student's t-test was used to pairwise compare all groups. Moreover, we correlated all physical site parameters and general water chemistry variables to stream catchment size and proportions of agriculture in the catchment and in the two sided 100 m buffer zone extending 2,000 m upstream of the stream sections using Spearman Rank in JMP 12.1 for Windows.
We used Spearman rank test to quantify correlations between proportions of agriculture in stream catchments and in the two sided 100 m buffer zone extending 2,000 m upstream of the stream sections and measures for pesticide pollution (number of pesticides, pesticide concentrations and TU). Moreover, these measures for pesticide pollution were compared between stream groups, and pesticide compounds of concern were identified (Appendix paper I, Rasmussen *et al.* 2015).

4.2.10 Statistical methods for macroinvertebrate community responses

We calculated SPEAR values for pooled macroinvertebrate samples (n = 3 for kick samples and n = 5 for surber samples) using the online and freely available SPEAR calculator (http://www.systemecology.eu/spear/spear-calculator/). Moreover, we calculated DSFI values for the same samples. The SPEAR and DSFI values were correlated to log $sumTU_{D.magna}$ for storm flow samples and log $sumTU_{C.riaparius}$ for sediment samples using Pearson product moment in JMP 12.1 for Windows. The correlations were performed for samples collected in May, June, July, and September, respectively. Linear regressions were fitted to the correlations between log $sumTU_{D.magna}$ for storm flow samples and SPEAR values for samples collected in May, June, July, and September, respectively. We used Analysis of Covariance (ANCOVA) to test for significant differences ($P < 0.05$) in slopes and intercepts between these regressions to identify the temporal persistence of the SPEAR performance. The ANCOVA test was performed in JMP 12.1 for Windows. Two sampling sites, A1 and C5 were removed from this analysis due to extremely low values in SPEAR and DSFI in May samples indicating major disturbance events prior to our sampling. Hence, absolute values and relative changes to these values should be omitted.

We grouped the study streams according to the number of storm flow episodes with $sumTU_{D.magna} > 0.001$ and compared the temporal development of average SPEAR and DSFI values between groups using repeated measures ANOVA in JMP 12.1 for Windows. Data normality and homogeneity of variance was tested using were tested using Shapiro-Wilk test and

Bartlett's test, respectively. In the case of significant difference in average SPEAR values between groups, we conducted a One-Way ANOVA to compare average SPEAR values between groups for samples collected in May, June, July, and September, respectively. The Tukey test was used to perform all pairwise comparisons in the cases of significant ANOVA ($P < 0.05$). Two sampling sites, A1 and C5 were removed from this analysis due to extremely low values in SPEAR and DSFI in May samples indicating major disturbance events prior to our sampling. Hence, absolute values and relative changes to these values should be omitted. Sites A1 and C5 were excluded from this analysis due to reasons described above.

We calculated the relative change in SPEAR values from May to June, July, and September, respectively. The average relative change in SPEAR values was compared between stream groups (described above) using One-Way ANOVA in JMP 12.1 for Windows. Data normality and homogeneity of variance were tested as described above. The Tukey test was used to perform all pairwise comparisons in the cases of significant ANOVA ($P < 0.05$). Sites A1 and C5 were excluded from this analysis due to reasons described above.
Macroinvertebrate recolonization potential for the study streams was quantified using drift samples and further categorized as i) abundance of SPEAR taxa, ii) abundance of Ephemeroptera, Plecoptera, and Trichoptera (EPT), and iii) total macroinvertebrate abundance. These abundances were calculated for drift samples collected in spring and autumn and as the mean of spring and autumn samples. The drift abundances were scaled to the transect level to optimize comparability among streams. Subsequently, the abundance of SPEAR, EPT, and total macroinvertebrate abundance in drift samples were correlated to the agricultural proportion in catchments and two-sided 100 m buffer zones extending 2,000 m upstream of the sampling site. Moreover, these drift parameters were correlated with SPEAR index scores for May, June, July, and September, respectively to quantify the influence of recovery potential on SPEAR index scores. All correlations were performed using the Pearson product moment test (linear correlations) and Spearman rank test (non-linear correlations) in JMP 12.1 for Windows.

4.2.11 Statistical methods for functional ecosystem responses

We analysed the correlation between ecosystem metabolism parameters (ER_{24}, GPP, and NPP) using Pearson product moment in JMP 12.1 for Windows. All metabolism parameters were correlated with stream width, depth, macrophyte coverage, proportional coverage of coarse organic debris, proportional coverage of mud, agricultural intensity in stream catchments and in buffer zones, and concentrations of ammonia-N, nitrate-N, total N, phosphate-P, and total P. Moreover, metabolism parameters were correlated with pesticide concentrations in stormflow water samples and sediment samples, $sumTU_{P.subcapitata}$ and $sumTU_{D.magna}$ in storm flow water samples, and $sumTU_{C.riaparius}$ in sediment samples. These environmental parameters were selected as relevant drivers for ecosystem metabolism rates both in terms of autotrophic and heterotrophic processes. In addition, we correlated GPP with SPEAR index values representing macroinvertebrate samples collected in June concomitantly with metabolism measurements. We used Pearson product moment to test for significance in JMP 12.1 for Windows.

4.3 Results

4.3.1 Physical stream characteristics and general water chemistry

We found a significant effect of stream group on stream width, current velocity, and proportional coverage of gravel (ANOVA P = 0.024, P = 0.018, and P = 0.027, respectively) with agricultural sites characterized by significantly lower values for all these parameters (Student's t-test P = 0.012, P = 0.009, and P = 0.027, respectively) compared to control sites. Moreover, we found a significant overall effect of time on current velocity (ANOVA P = 0.038) with mean current velocities being significantly higher in spring compared to autumn (Student's t-test P = 0.038). No other physical parameters were significantly influenced by site type or time, and we found no significant interaction between site type and time for any physical parameter (ANOVA P > 0.05) (see Table 4.2 for site specific values). Additionally, stream width, current velocity and proportional coverage of gravel significantly decreased with increasing proportions of agriculture in the two sided 100 m buffer zone (Pearson correlation, P = 0.016, P = 0.012, and P = 0.039, respectively), whereas correlations to proportional agriculture in the catchments were all insignificant (Pearson correlation, P > 0.05).

TABLE 4.2. Overview table of the physical stream characteristics. The stream ID is composed of a letter (C = Control streams, A = Agricultural streams), followed by the stream number. The stream categorisation is based on proportion of agriculture in a two sided 100 m buffer zone extending 2,000 m upstream. See Appendix paper II (Rasmussen *et al.* 2015) for details.

Parameter/stream code	C1	C2	C3	C4	C5	C6	C7	C8	C9	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
April																			
Width (cm)	166	254	164	154	182	168	261	118	171	78	176	217	152	130	201	151	187	157	111
Current velocity (m s^{-1})	0.21	0.14	0.09	0.07	0.08	0.39	0.62	0.43	0.34	0.16	0.14	0.09	0.08	0.06	0.20	0.15	0.25	0.25	0.19
Depth (cm)	14	13	11	5	13	10	14	8	10	8	8	14	9	9	16	9	25	14	18
% Boulder	7	33	4	3	4	6	5	1	3	28	0	15	5	14	13	4	8	18	3
% Pebble	30	14	15	15	12	36	22	49	20	16	27	10	57	28	24	10	22	21	2
% Gravel	12	5	7	22	9	31	10	15	16	5	9	6	9	5	6	17	7	13	9
% Sand	31	25	29	52	15	22	43	34	57	14	48	24	19	47	56	63	59	40	79
% Mud	20	23	45	9	60	6	21	2	4	37	16	46	10	6	1	6	5	8	7
% Debris	16	23	4	10	24	0	1	2	0	22	3	16	0	10	3	1	1	0	1
% Macrophyte coverage	36	0	10	12	7	0	6	1	0	1	41	19	2	0	7	19	7	10	2
September																			
Width (cm)	148	233	147	157	169	161	269	119	198	78	182	129	130	134	157	140	195	134	107
Current velocity (m s^{-1})	0.21	0.07	0.09	0.18	0.04	0.13	0.18	0.32	0.31	0.09	0.09	0.00	0.07	0.09	0.14	0.09	0.14	0.11	0.12
Depth (cm)	11	9	8	5	14	8	12	9	10	6	8	7	8	7	7	7	24	13	11
% Boulder	3	19	5	1	0	6	5	2	15	11	5	0	4	15	7	4	12	23	7
% Pebble	25	19	4	15	12	30	13	24	18	10	15	3	47	15	15	12	10	6	8
% Gravel	13	7	10	21	7	25	15	23	9	3	17	4	11	16	15	17	8	7	5
% Sand	33	27	49	54	8	28	29	45	50	2	39	12	26	36	55	49	57	38	62
% Mud	27	28	33	9	73	12	38	7	8	75	24	82	12	18	8	18	14	27	19
% Debris	7	29	10	5	5	8	6	6	3	8	11	6	4	6	5	6	9	0	3
% Macrophyte coverage	36	0	17	23	32	3	42	0	0	62	56	14	7	0	2	20	9	11	4

We found no significant effects of stream group, time, or interaction between stream group and time on any of the general water chemistry variables (ANOVA $P > 0.05$). However, nitrate-N and total N concentrations significantly increased with increasing proportions of agriculture in the catchment (Pearson correlation, $P = 0.0008$ and $P = 0.0005$, respectively) and in the two sided 100 m buffer zone extending 2,000 m upstream of the stream sections (Pearson correlation, $P = 0.0003$ and $P = 0.0002$, respectively) (see Table 4.3 for site specific values).

TABLE 4.3. Overview table of general water chemistry. The stream ID is composed of a letter (C = Control streams, A = Agricultural streams), followed by the stream number. The stream categorisation is based on proportion of agriculture in a two sided 100 m buffer zone extending 2,000 m upstream. See Appendix paper II (Rasmussen *et al.* 2015) for details.

Parameter/stream code	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**	**A9**	**A10**
April																			
Suspended matter (mg L^{-1})	2.7	3.1	2.4	6.9	6.1	4.0	3.4	14.7	7.7	12.7	40.4	1.1	3.1	1.9	8.0	2.7	5.3	5.7	8.6
Ammonia-N (mg L^{-1})	0.029	0.016	0.020	0.025	0.424	0.015	0.057	0.010	0.011	0.074	0.004	0.007	0.021	0.025	0.155	0.023	0.020	0.014	0.192
Nitrate-N (mg L^{-1})	0.13	0.87	3.33	2.17	2.94	2.30	0.52	1.44	6.36	8.30	4.63	2.35	1.92	4.00	2.25	7.48	1.41	0.33	1.13
Total N (mg L^{-1})	0.64	1.07	3.11	2.12	3.62	2.20	0.62	1.41	5.94	8.07	4.69	2.23	1.90	3.98	2.28	7.46	1.43	0.76	1.41
Orthophosphate-P (mg L^{-1})	0.025	0.007	0.010	0.024	0.051	0.010	0.013	0.023	0.027	0.074	0.005	0.001	0.007	0.026	0.020	0.010	0.007	0.013	0.012
Total P (mg L^{-1})	0.10	0.07	0.05	0.09	0.23	0.05	0.04	0.40	0.07	0.19	0.13	0.06	0.04	0.09	0.11	0.03	0.05	0.07	0.10
BOD_5 (mg L^{-1})	1.44	0.79	0.46	1.22	3.30	0.99	0.89	0.26	0.69	1.11	1.03	1.27	1.96	0.90	1.58	0.67	1.23	2.61	1.16
June																			
Suspended matter (mg L^{-1})	9.8	6.2	2.9	22.8	5.6	9.3	3.1	6.6	11.9	9.6	27.2	3.6	7.3	8.6	15.0	9.0	3.0	14.0	5.9
Ammonia-N (mg L^{-1})	0.017	0.068	0.027	0.063	0.530	0.011	0.026	0.008	0.027	0.070	0.024	0.045	0.048	0.105	0.062	0.042	0.031	0.042	0.055
Nitrate-N (mg L^{-1})	0.29	0.99	3.47	1.43	0.75	2.67	0.54	1.19	6.70	0.02	6.08	1.05	1.30	3.89	1.88	8.32	1.46	0.45	0.80
Total N (mg L^{-1})	0.57	1.10	3.36	1.67	2.57	2.59	0.62	1.30	6.55	9.81	5.58	1.20	1.44	3.93	1.80	8.60	1.57	0.71	1.02
Orthophosphate-P (mg L^{-1})	0.035	0.073	0.015	0.007	0.390	0.036	0.010	0.023	0.048	0.238	0.020	0.074	0.025	0.072	0.044	0.012	0.012	0.048	0.021
Total P (mg L^{-1})	0.09	0.13	0.09	0.22	0.57	0.10	0.08	0.14	0.13	0.30	0.16	0.10	0.12	0.19	0.17	0.10	0.09	0.18	0.12
BOD_5 (mg L^{-1})	1.17	0.77	0.69	6.94	3.59	0.61	0.84	0.36	1.18	1.61	0.85	0.74	0.83	1.47	1.70	6.35	1.17	1.07	0.90
August																			
Suspended matter (mg L^{-1})	6.6	2.9	1.5	4.2	14.6	10.8	4.2	6.2	14.7	104.0	22.3	8.4	6.0	7.3	10.3	4.7	5.5	8.7	5.7
Ammonium-N (mg L^{-1})	0.023	0.013	0.025	0.068	0.500	0.010	0.026	0.009	0.012	0.005	0.017	0.139	0.020	0.033	0.063	0.052	0.024	0.022	0.036
Nitrate-N (mg L^{-1})	0.37	0.36	2.54	1.47	3.29	2.38	0.50	1.18	6.07	6.80	5.08	0.15	0.56	3.12	0.81	6.88	1.26	1.14	0.63
Total N (mg L^{-1})	0.91	0.85	2.85	1.65	4.77	2.55	0.55	1.23	6.43	7.63	5.69	0.95	0.85	3.42	1.02	7.35	1.59	1.53	0.96
Orthophosphate-P (mg L^{-1})	0.018	0.042	0.020	0.056	0.181	0.042	0.019	0.024	0.044	0.215	0.021	0.055	0.018	0.083	0.050	0.013	0.013	0.047	0.020
Total P (mg L^{-1})	0.05	0.09	0.04	0.11	0.35	0.07	0.04	0.12	0.12	0.74	0.11	0.11	0.07	0.15	0.16	0.05	0.05	0.12	0.09
BOD_5 (mg L^{-1})	0.67	0.65	0.27	1.24	2.88	0.41	0.73	0.61	0.71	1.18	0.68	1.44	0.72	0.52	0.94	0.50	0.52	0.96	1.02
Parameter/stream code	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**	**A9**	**A10**
April																			
Suspended matter (mg L^{-1})	2.7	3.1	2.4	6.9	6.1	4.0	3.4	14.7	7.7	12.7	40.4	1.1	3.1	1.9	8.0	2.7	5.3	5.7	8.6

Ammonia-N (mg L^{-1})	0.029	0.016	0.020	0.025	0.424	0.015	0.057	0.010	0.011	0.074	0.004	0.007	0.021	0.025	0.155	0.023	0.020	0.014	0.192
Nitrate-N (mg L^{-1})	0.13	0.87	3.33	2.17	2.94	2.30	0.52	1.44	6.36	8.30	4.63	2.35	1.92	4.00	2.25	7.48	1.41	0.33	1.13
Total N (mg L^{-1})	0.64	1.07	3.11	2.12	3.62	2.20	0.62	1.41	5.94	8.07	4.69	2.23	1.90	3.98	2.28	7.46	1.43	0.76	1.41
Orthophosphate-P (mg L^{-1})	0.025	0.007	0.010	0.024	0.051	0.010	0.013	0.023	0.027	0.074	0.005	0.001	0.007	0.026	0.020	0.010	0.007	0.013	0.012
Total P (mg L^{-1})	0.10	0.07	0.05	0.09	0.23	0.05	0.04	0.40	0.07	0.19	0.13	0.06	0.04	0.09	0.11	0.03	0.05	0.07	0.10
BOD_5 (mg L^{-1})	1.44	0.79	0.46	1.22	3.30	0.99	0.89	0.26	0.69	1.11	1.03	1.27	1.96	0.90	1.58	0.67	1.23	2.61	1.16
June																			
Suspended matter (mg L^{-1})	9.8	6.2	2.9	22.8	5.6	9.3	3.1	6.6	11.9	9.6	27.2	3.6	7.3	8.6	15.0	9.0	3.0	14.0	5.9
Ammonia-N (mg L^{-1})	0.017	0.068	0.027	0.063	0.530	0.011	0.026	0.008	0.027	0.070	0.024	0.045	0.048	0.105	0.062	0.042	0.031	0.042	0.055
Nitrate-N (mg L^{-1})	0.29	0.99	3.47	1.43	0.75	2.67	0.54	1.19	6.70	0.02	6.08	1.05	1.30	3.89	1.88	8.32	1.46	0.45	0.80
Total N (mg L^{-1})	0.57	1.10	3.36	1.67	2.57	2.59	0.62	1.30	6.55	9.81	5.58	1.20	1.44	3.93	1.80	8.60	1.57	0.71	1.02
Orthophosphate-P (mg L^{-1})	0.035	0.073	0.015	0.007	0.390	0.036	0.010	0.023	0.048	0.238	0.020	0.074	0.025	0.072	0.044	0.012	0.012	0.048	0.021
Total P (mg L^{-1})	0.09	0.13	0.09	0.22	0.57	0.10	0.08	0.14	0.13	0.30	0.16	0.10	0.12	0.19	0.17	0.10	0.09	0.18	0.12
BOD_5 (mg L^{-1})	1.17	0.77	0.69	6.94	3.59	0.61	0.84	0.36	1.18	1.61	0.85	0.74	0.83	1.47	1.70	6.35	1.17	1.07	0.90
August																			
Suspended matter (mg L^{-1})	6.6	2.9	1.5	4.2	14.6	10.8	4.2	6.2	14.7	104.0	22.3	8.4	6.0	7.3	10.3	4.7	5.5	8.7	5.7
Ammonium-N (mg L^{-1})	0.023	0.013	0.025	0.068	0.500	0.010	0.026	0.009	0.012	0.005	0.017	0.139	0.020	0.033	0.063	0.052	0.024	0.022	0.036
Nitrate-N (mg L^{-1})	0.37	0.36	2.54	1.47	3.29	2.38	0.50	1.18	6.07	6.80	5.08	0.15	0.56	3.12	0.81	6.88	1.26	1.14	0.63
Total N (mg L^{-1})	0.91	0.85	2.85	1.65	4.77	2.55	0.55	1.23	6.43	7.63	5.69	0.95	0.85	3.42	1.02	7.35	1.59	1.53	0.96
Orthophosphate-P (mg L^{-1})	0.018	0.042	0.020	0.056	0.181	0.042	0.019	0.024	0.044	0.215	0.021	0.055	0.018	0.083	0.050	0.013	0.013	0.047	0.020
Total P (mg L^{-1})	0.05	0.09	0.04	0.11	0.35	0.07	0.04	0.12	0.12	0.74	0.11	0.11	0.07	0.15	0.16	0.05	0.05	0.12	0.09
BOD_5 (mg L^{-1})	0.67	0.65	0.27	1.24	2.88	0.41	0.73	0.61	0.71	1.18	0.68	1.44	0.72	0.52	0.94	0.50	0.52	0.96	1.02

4.3.2 Pesticide pollution

The raw data from the pesticide sampling campaign is presented in Appendix 2, Tables 1-4. Pesticide toxicity (TU) to aquatic biota are reported in detail in Appendix paper I (Rasmussen *et al.* 2015) and summarized in Table 4.4.

We found a significant positive relationship among pesticide concentrations in all combinations of sample types ($P < 0.05$) (Appendix paper I, Rasmussen *et al.* 2015). Thus, streams with high peak concentrations during storm flow episodes were additionally characterized by high pesticide concentrations in base flow water samples and in suspended and bed sediment samples (Appendix paper I, Rasmussen *et al.* 2015).

The proportion of agriculture in the two sided 100 m buffer zone extending 2,000 m upstream of the stream sections was generally a strong and often significant predictor for the amount of pesticides, pesticide concentrations and especially sumTU in all sample types (Table 4.5, Fig. 4.2), whereas the proportion of agriculture in the stream catchment was only a significant predictor for the amount of pesticides and pesticide concentrations in water samples collected during storm flow (Table 4.5).

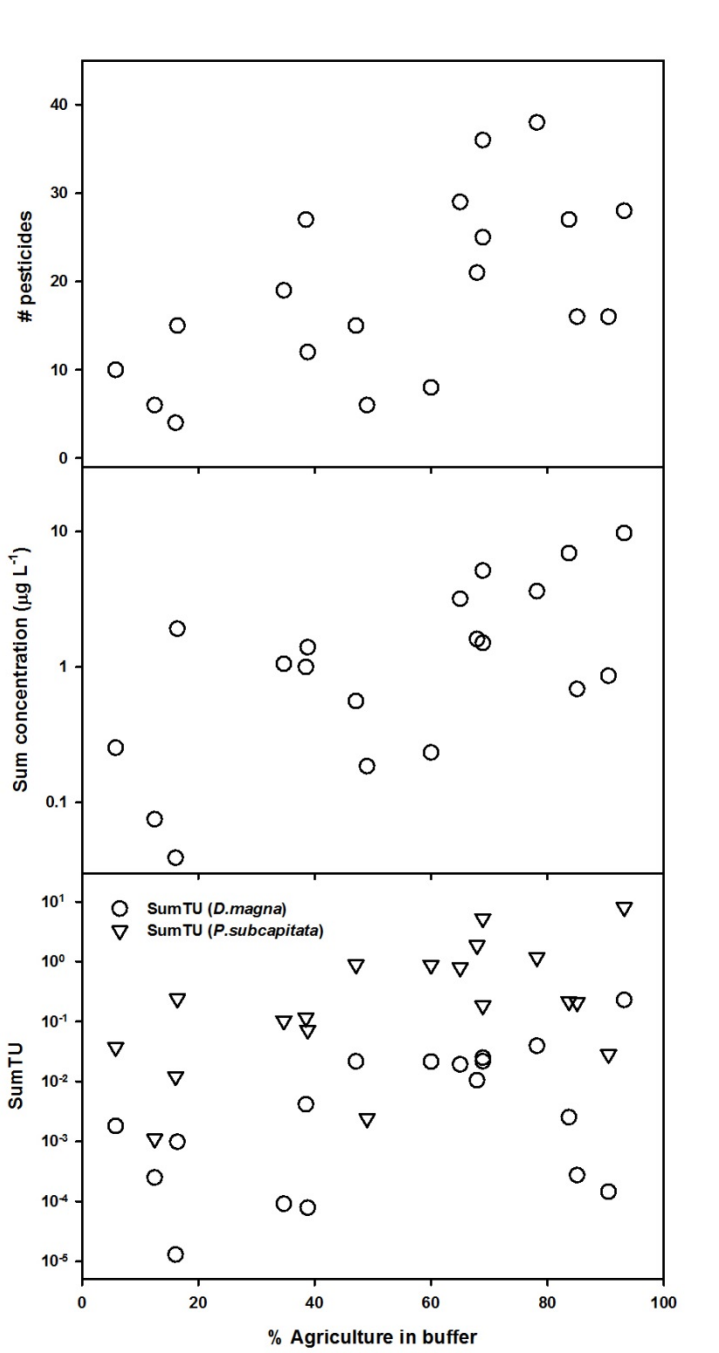
TABLE 4.4. Summary table of pesticide concentrations and predicted toxicity to freshwater biota in the four different sample types. The stream ID is composed of a letter (C = Control streams, A = Agricultural streams), followed by the stream number. The stream categorisation is based on proportion of agriculture in a two sided 100 m buffer zone extending 2,000 m upstream. See Appendix paper II (Rasmussen *et al.* 2015) for details.

Parameter/stream code	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**	**A9**	**A10**
April																			
Suspended matter (mg L^{-1})	2.7	3.1	2.4	6.9	6.1	4.0	3.4	14.7	7.7	12.7	40.4	1.1	3.1	1.9	8.0	2.7	5.3	5.7	8.6
Ammonia-N (mg L^{-1})	0.029	0.016	0.020	0.025	0.424	0.015	0.057	0.010	0.011	0.074	0.004	0.007	0.021	0.025	0.155	0.023	0.020	0.014	0.192
Nitrate-N (mg L^{-1})	0.13	0.87	3.33	2.17	2.94	2.30	0.52	1.44	6.36	8.30	4.63	2.35	1.92	4.00	2.25	7.48	1.41	0.33	1.13
Total N (mg L^{-1})	0.64	1.07	3.11	2.12	3.62	2.20	0.62	1.41	5.94	8.07	4.69	2.23	1.90	3.98	2.28	7.46	1.43	0.76	1.41
Orthophosphate-P (mg L^{-1})	0.025	0.007	0.010	0.024	0.051	0.010	0.013	0.023	0.027	0.074	0.005	0.001	0.007	0.026	0.020	0.010	0.007	0.013	0.012
Total P (mg L^{-1})	0.10	0.07	0.05	0.09	0.23	0.05	0.04	0.40	0.07	0.19	0.13	0.06	0.04	0.09	0.11	0.03	0.05	0.07	0.10
BOD_5 (mg L^{-1})	1.44	0.79	0.46	1.22	3.30	0.99	0.89	0.26	0.69	1.11	1.03	1.27	1.96	0.90	1.58	0.67	1.23	2.61	1.16
June																			
Suspended matter (mg L^{-1})	9.8	6.2	2.9	22.8	5.6	9.3	3.1	6.6	11.9	9.6	27.2	3.6	7.3	8.6	15.0	9.0	3.0	14.0	5.9
Ammonia-N (mg L^{-1})	0.017	0.068	0.027	0.063	0.530	0.011	0.026	0.008	0.027	0.070	0.024	0.045	0.048	0.105	0.062	0.042	0.031	0.042	0.055
Nitrate-N (mg L^{-1})	0.29	0.99	3.47	1.43	0.75	2.67	0.54	1.19	6.70	0.02	6.08	1.05	1.30	3.89	1.88	8.32	1.46	0.45	0.80
Total N (mg L^{-1})	0.57	1.10	3.36	1.67	2.57	2.59	0.62	1.30	6.55	9.81	5.58	1.20	1.44	3.93	1.80	8.60	1.57	0.71	1.02
Orthophosphate-P (mg L^{-1})	0.035	0.073	0.015	0.007	0.390	0.036	0.010	0.023	0.048	0.238	0.020	0.074	0.025	0.072	0.044	0.012	0.012	0.048	0.021

Total P (mg $^{-1}$)	0.09	0.13	0.09	0.22	0.57	0.10	0.08	0.14	0.13	0.30	0.16	0.10	0.12	0.19	0.17	0.10	0.09	0.18	0.12
BOD_5 (mg L^{-1})	1.17	0.77	0.69	6.94	3.59	0.61	0.84	0.36	1.18	1.61	0.85	0.74	0.83	1.47	1.70	6.35	1.17	1.07	0.90
August																			
Suspended matter (mg L^{-1})	6.6	2.9	1.5	4.2	14.6	10.8	4.2	6.2	14.7	104.0	22.3	8.4	6.0	7.3	10.3	4.7	5.5	8.7	5.7
Ammonium-N (mg L^{-1})	0.023	0.013	0.025	0.068	0.500	0.010	0.026	0.009	0.012	0.005	0.017	0.139	0.020	0.033	0.063	0.052	0.024	0.022	0.036
Nitrate-N (mg L^{-1})	0.37	0.36	2.54	1.47	3.29	2.38	0.50	1.18	6.07	6.80	5.08	0.15	0.56	3.12	0.81	6.88	1.26	1.14	0.63
Total N (mg L^{-1})	0.91	0.85	2.85	1.65	4.77	2.55	0.55	1.23	6.43	7.63	5.69	0.95	0.85	3.42	1.02	7.35	1.59	1.53	0.96
Or-thophosphate-P (mg L^{-1})	0.018	0.042	0.020	0.056	0.181	0.042	0.019	0.024	0.044	0.215	0.021	0.055	0.018	0.083	0.050	0.013	0.013	0.047	0.020
Total P (mg L^{-1})	0.05	0.09	0.04	0.11	0.35	0.07	0.04	0.12	0.12	0.74	0.11	0.11	0.07	0.15	0.16	0.05	0.05	0.12	0.09
BOD_5 (mg L^{-1})	0.67	0.65	0.27	1.24	2.88	0.41	0.73	0.61	0.71	1.18	0.68	1.44	0.72	0.52	0.94	0.50	0.52	0.96	1.02

*Represents the stormflow episode with highest value

FIGURE 4.2. Number of pesticides, sum concentration, and SumTU as functions of the proportion of agriculture in a two-sided 100m buffer extending 2,000 m upstream of the stream sections. Data represent storm flow water samples (highest value selected in the cases of multiple storm flow water samples, see Table 3.4).



The legislative safety thresholds for daphnia (1/100 of the 48h LC_{50}), fish (1/100 of the 48h LC_{50}) and algae (1/10 of the 48h EC_{50}) were not exceeded in base flow water samples for any stream, whereas during storm flow events these safety thresholds were exceeded in 8 (1 control + 7 agricultural streams), 7 (1 control + 6 agricultural streams), and 13 (4 control + 9 agricultural streams) streams for daphnia, fish, and algae, respectively (Table 4.5). Although the legis-

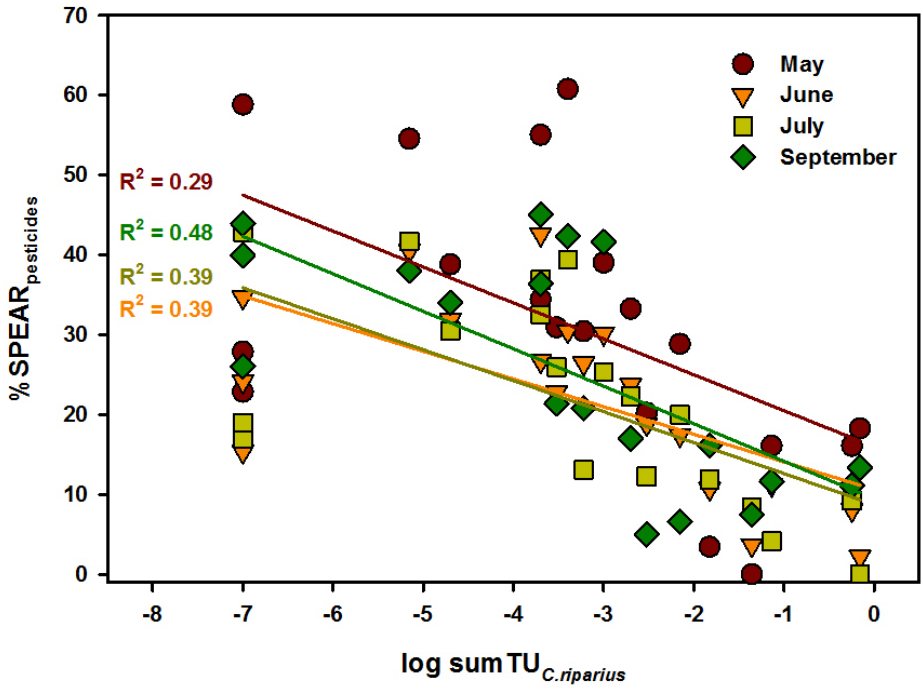
lative threshold for algae was not exceeded in base flow water samples, the values were generally close to the threshold especially in agricultural streams (sumTU values a factor of 2-10 below the threshold) (Table 4.5).

TABLE 4.5. Spearman's ρ values for the correlations between agricultural proportions in stream catchments and in two-sided 100m buffer zones extending 2,000m upstream of the stream section, and selected measures for pesticide pollution subdivided according to sample type. Significance levels are given for each correlation, and significant correlations are highlighted in bold.

4.3.3 Macroinvertebrate community responses

The $SPEAR_{pesticides}$ index values were significantly correlated to log $sumTU_{D.magna}$ during storm flow events for macroinvertebrate samples collected in May, June, July, and September (Pearson correlation: r = -0.635 and P = 0.004, r = -0.749 and P < 0.001, r = -0.660 and P = 0.002, and r = -0.713 and P < 0.001, respectively) (Fig. 4.3). Further, the $SPEAR_{pesticides}$ index values were significantly correlated to log $sumTU_{C.riparius}$ (sediment samples) for macroinvertebrate samples collected in May, June, July, and September (Pearson correlation: r = -0.541 and P = 0.017, r = -0.622 and P = 0.005, r = -0.627 and P = 0.004, and r = -0.690 and P = 0.001, respectively) (Fig. 4.3). Slopes and intercepts of the regression lines fitted to $SPEAR_{pesticides}$ as a function of log $sumTU_{D.magna}$ for macroinvertebrate samples collected in May, June, July, and September were not significantly different (P > 0.05) (Fig. 4.3).

FIGURE 4.3. %SPEARpesticides as function of log sumTUC.riparius based on bed sediment s
Linear regressions are fitted to the data based on macroinvertebrate samples collected in May,
July, and September, respectively. Regression coefficients are indicated and represent the regr
lines of similar color.

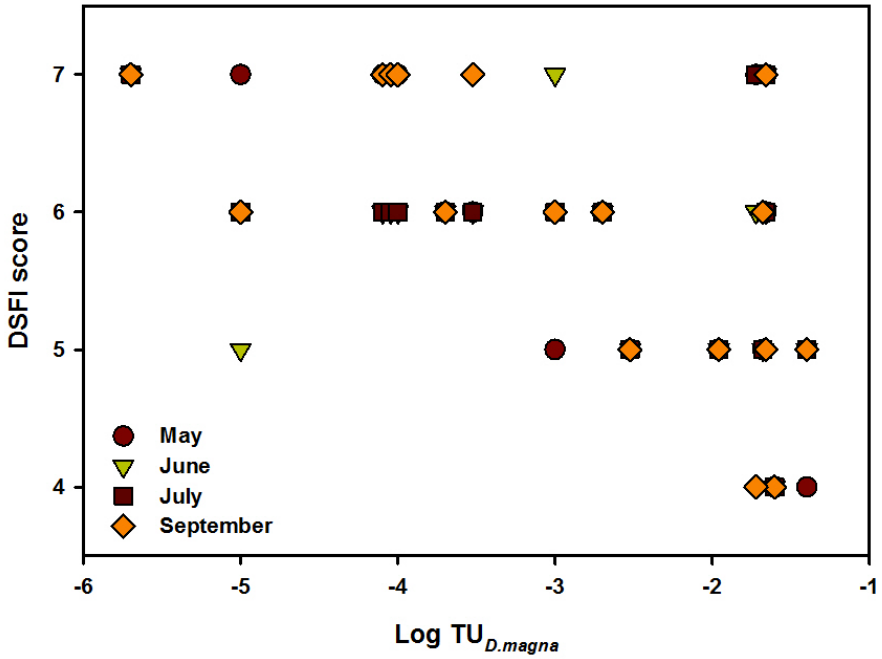


Slopes of the regression lines fitted to $SPEAR_{pesticides}$ as a function of log $sumTU_{C.riparius}$ for macroinvertebrate samples collected in May, June, July, and September were not significantly different (P > 0.05) (Fig. 4.3), whereas the intercept of the regression line fitted to May data was significantly higher compared to regression lines fitted to June, July, and September data (P < 0.05) (Fig. 4.3).

DSFI scores ranged between 4 and 7 for all streams and sampling dates, although DSFI scores of 4 only occurred in two streams (A3 and A9). DSFI scores for the remaining streams therefore ranged between 5 and 7 classified as minimum good ecological status according to the WFD. In spite of the generally high DSFI scores, the correlation between DSFI and log $sumTU_{D.magna}$ (storm flow) was significant for samples collected in May and September (Pearson correlation: r = -0.606 and P = 0.010, and r = -0.658 and P = 0.004, respectively) (Fig. 4.4). Similarly, the correlation between DSFI and log $sumTU_{C.riparius}$ was significant for

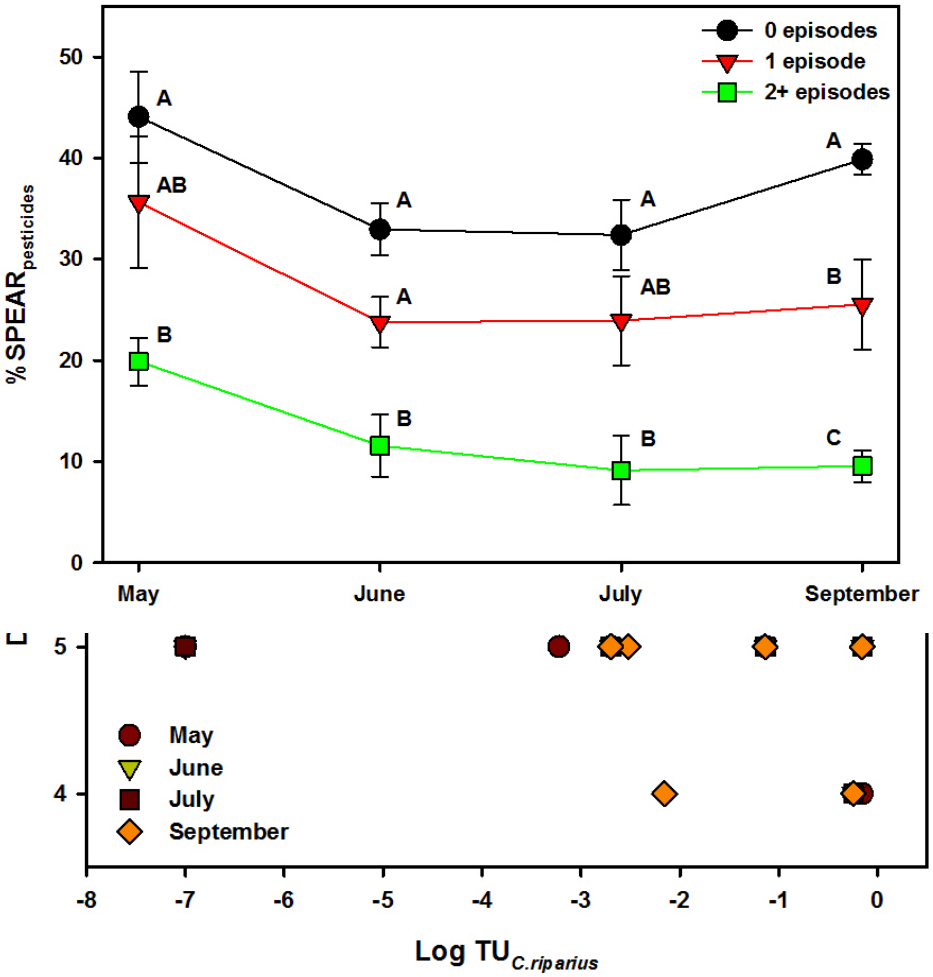
samples collected in May and September (Pearson correlation: r = -0.563 and P = 0.018, and r = -0.655 and P = 0.004, respectively) (Fig. 4.5). We found no significant correlation between DSFI and log sumTU$_{D.magna}$ or log sumTU$_{C.riparius}$ for samples collected in June and July (Pearson correlation: r < 0.300 and P > 0.05 for all) (Fig. 4.4 and Fig. 4.5).

FIGURE 4.4. DSFI scores as function of log sumTU$_{D.magna}$ based on storm flow water samples. In the cases of multiple storm flow water samples in a stream, the highest log sumTU$_{D.magna}$ value was selected. The data represents macroinvertebrate samples collected in May, June, July, and September, respectively.



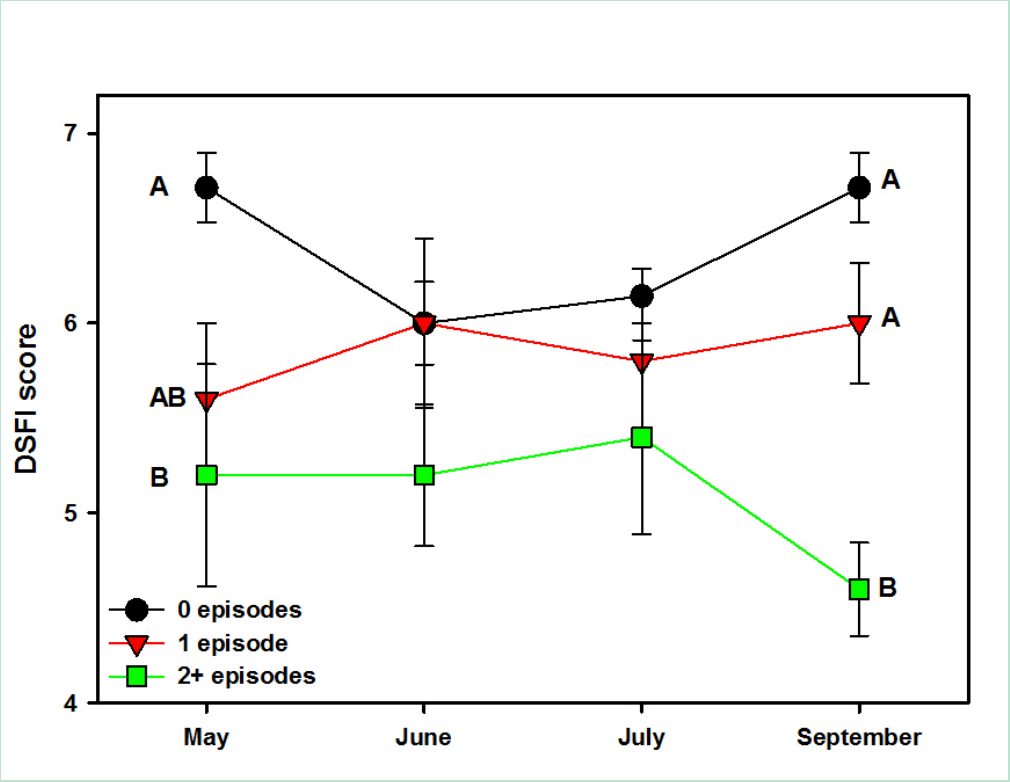
The RM ANOVA revealed a significant effect of invertebrate sampling time (P < 0.001) and pesticide exposure grouping (P < 0.001) on the average SPEARpesticides index values where streams were subdivided into groups reflecting the frequency of storm flow episodes with sumTUD.magna > 0.001, but the interaction link between time and pesticide grouping was not significant (P = 0.783) (Fig. 4.6). For all sampling dates the average SPEARpesticides index values were significantly reduced in streams receiving two or more storm flow episodes with sumTUD.magna > 0.001 compared to streams receiving no such storm flow episodes (ANOVA P < 0.05, Tukey test: P = 0.007, P < 0.001, P = 0.002, and P < 0.001 for May, June, July, and September, respectively) (Fig. 4.6). Moreover, in September, the average SPEAR pesticides values for streams receiving one storm flow episode with sumTUD.magna > 0.001 was significantly higher compared to streams receiving two or more storm flow episodes with sumTUD.magna > 0.001 (Tukey test: P = 0.003), but significantly lower compared to streams receiving no storm flow episodes with sumTUD.magna > 0.001(Tukey test: P = 0.004) (Fig. 4.6).

FIGURE 4.6. Average %SPEARpesticides for stream groups experiencing sumTUD.magna > 0.001 in zero, one, or ≥2 storm flow episodes. SPEAR values are based on macroinvertebrate samples collected in May, June, July, and September, respectively. Average SPEAR values not connected with identical letters within each sampling time are significantly different.



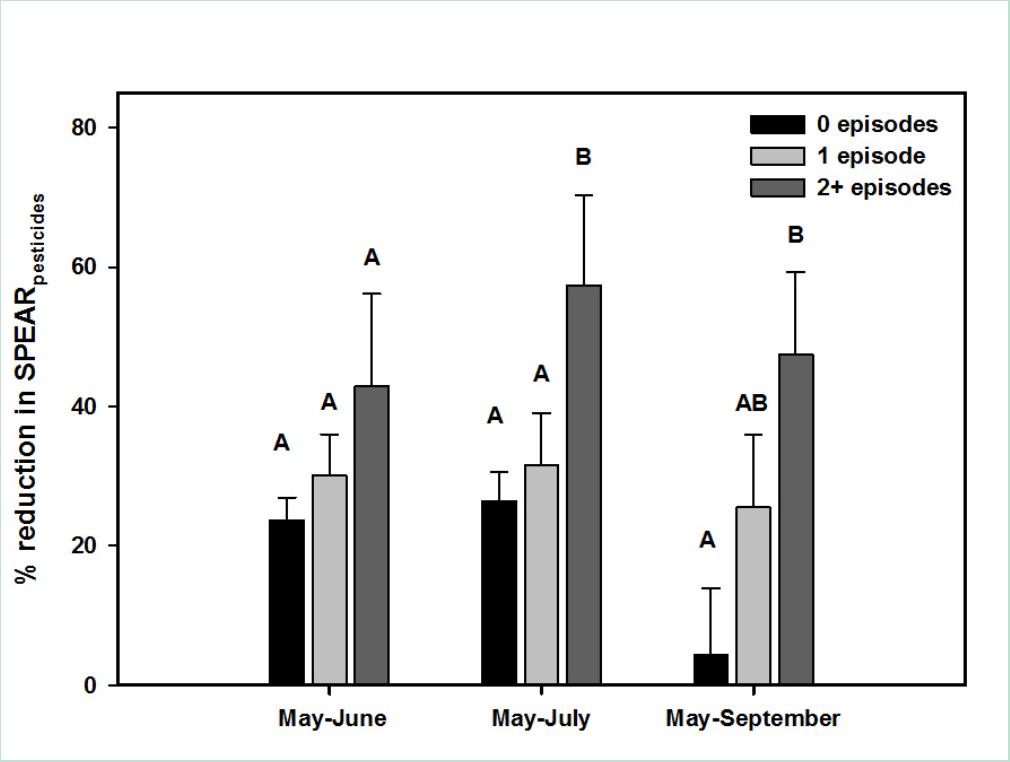
We found a significant effect of macroinvertebrate sampling time ($P < 0.001$) and pesticide exposure grouping ($P < 0.001$) on the average DSFI scores where streams were subdivided into groups reflecting the frequency of storm flow episodes with $sumTU_{D.magna} > 0.001$. We found no significant interaction link between time and pesticide grouping ($P = 0.523$) (Fig. 4.7). In May and September the average DSFI score was significantly reduced in streams receiving two or more storm flow episodes with $sumTU_{D.magna} > 0.001$ compared to streams receiving no such storm flow episodes (ANOVA $P < 0.05$, Tukey test: $P = 0.032$ and $P < 0.001$, respectively) (Fig. 4.7). Moreover, in September, the average DSFI score for streams receiving one storm flow episode with $sumTU_{D.magna} > 0.001$ was significantly higher compared to streams receiving two or more such storm flow episodes (Tukey test: $P = 0.005$) (Fig. 4.7).

FIGURE 3.8. Average DSFI scores for stream groups experiencing sumTU$_{D.magna}$ > 0.001 in zero, one, or ≥2 storm flow episodes. DSFI scores are based on macroinvertebrate samples collected in May, June, July, and September, respectively. Average DSFI scores not connected with identical letters within each sampling time are significantly different. Letters are only indicated in the cases of significant differences (one-way ANOVA, P < 0.05) between stream groups for the same sampling date.



Relative reductions in SPEAR$_{pesticides}$ scores (benchmarked against May samples) were significantly higher in streams receiving two or more storm flow episodes with sumTU$_{D.magna}$ > 0.001 compared to streams receiving no storm flow episodes with sumTU$_{D.magna}$ > 0.001 in July and September (ANOVA P < 0.05, Tukey test: P = 0.040 and P = 0.027), whereas the increase was not significant in June (ANOVA P = 0.233) (Fig. 4.8).

FIGURE 4.8. Average relative reduction in SPEARpesticides using May samples as benchmark. Streams are subdivided according to frequency of storm flow events where sumTU$_{D.magna}$ > 0.001. Average relative reduction in SPEARpesticides was compared between stream groups within each sampling time. Values not connected with identical letters within each sampling time are significantly different.



The drift density of SPEAR taxa, EPT taxa and total amount of macroinvertebrates varied up to several orders of magnitude among streams and sampling times (Table 4.6). The total drift density of all macroinvertebrates and the drift densities of SPEAR and EPT taxa were not significantly correlated to agricultural proportions in stream catchments or in the two-sided 100m buffer zone extending 2,000 m upstream of the sampling site (Pearson correlation: $P > 0.05$, data not shown). SPEAR index values for samples collected in May, June, July, and September were significantly negatively correlated with the mean total abundance of drifting macroinvertebrates (Spearman correlation: $\rho = -0.754$ and $P < 0.001$, $\rho = -0.711$ and $P < 0.001$, $\rho = -0.796$ and $P < 0.001$, and $\rho = -0.744$ and $P < 0.001$, respectively) (see Fig. 4.9 for June correlation). However, SPEAR index values were not significantly correlated with drift densities of SPEAR and EPT taxa (Spearman correlation: $P > 0.05$, data not shown).

FIGURE 4.9. %SPEARpesticides as function of the average total drift density in the study streams. A 2nd order exponential decay model is fitted to the data (R^2 = 0.53).

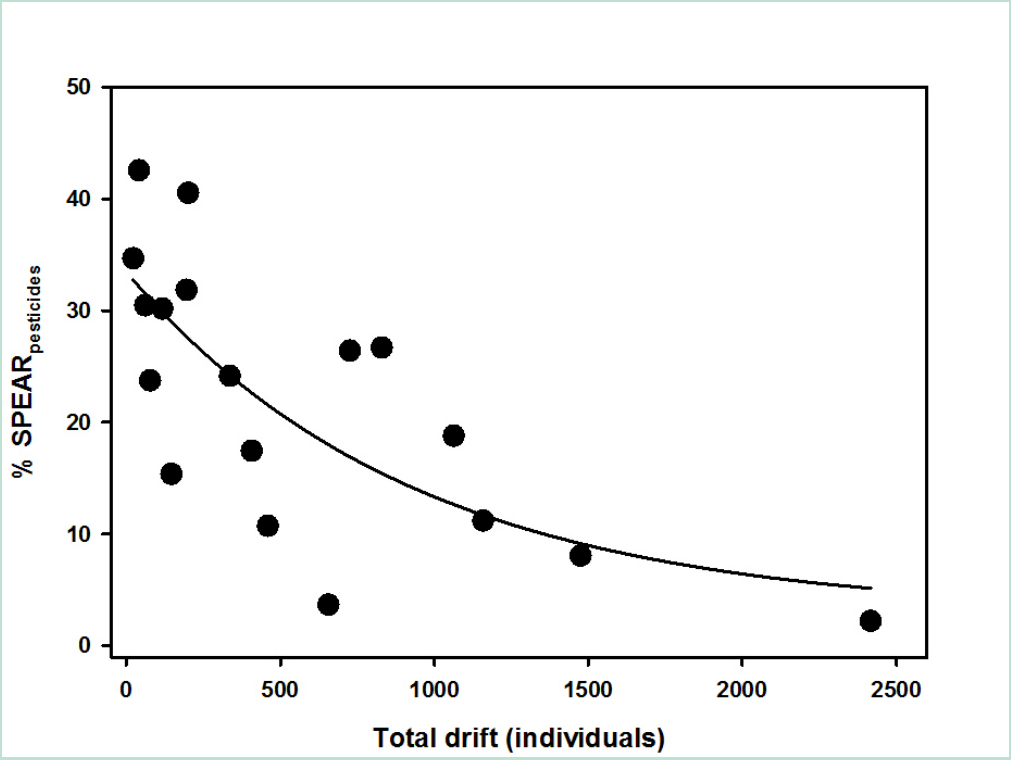


FIGURE 4.10. Panel figure depicting ER_{24}, GPP, and NPP as functions of selected environmental variables. Linear regressions and correlation coefficients are given. Ecosystem metabolism was measured in all study streams in June-July using the upstream-downstream diurnal oxygen change technique.

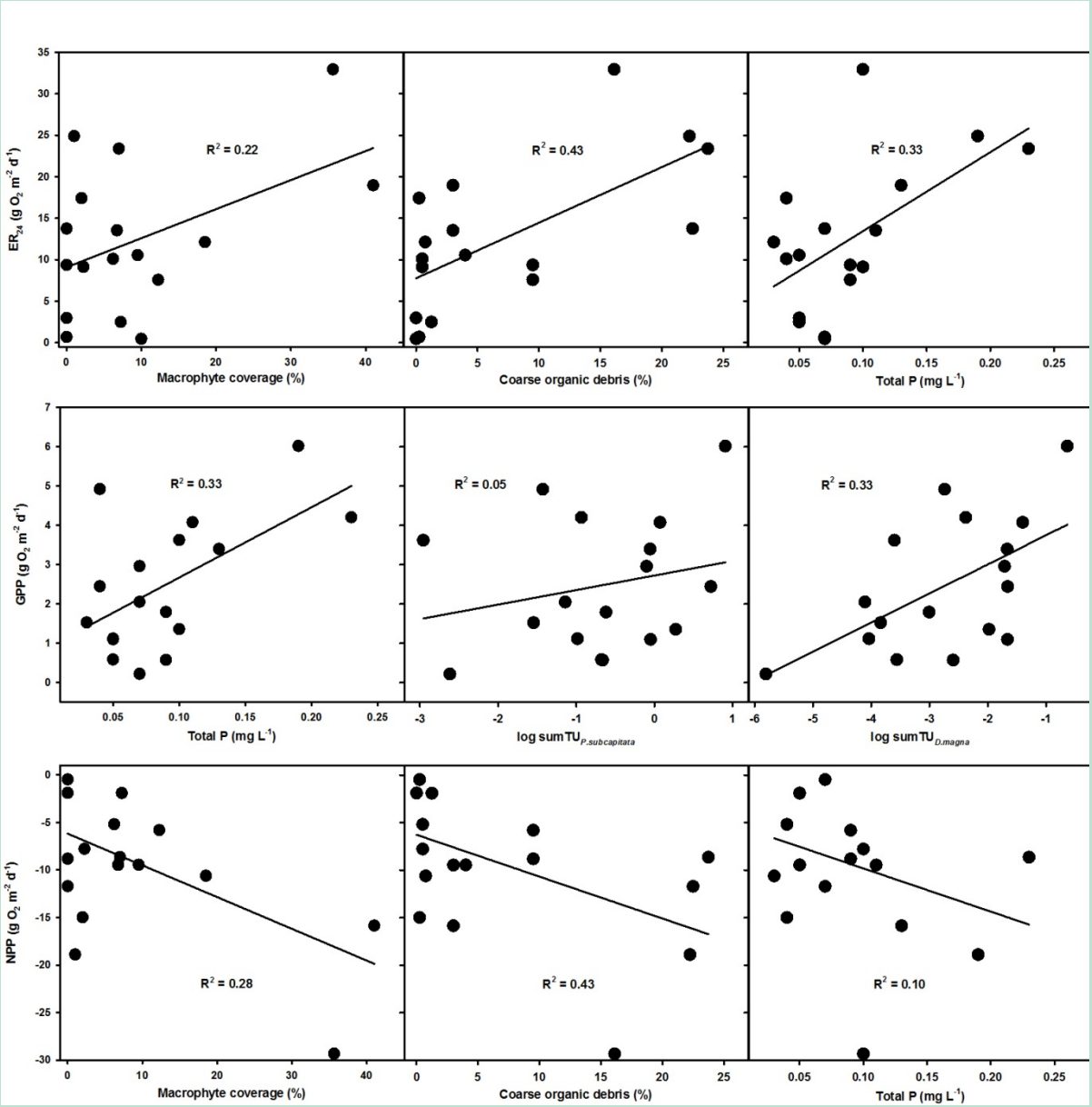


TABLE 4.6. Drift densities in spring, autumn and the overall mean density in the study streams. Samples were consistently collected during sunset. Drift densities were upscaled from drift samples to the cross sectional area of transects using ratios between stream water flow through drift nets and transects in order to optimize comparability of recolonization potential of the respective streams. Drift densities of macroinvertebrates are grouped into i) individuals characterized as SPEAR, ii) number of individuals belonging to EPT, and iii) the total sum of individuals in drift.

Parameter/stream code	C1	C2	C3	C4	C5	C6	C7	C8	C9	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
Spring																			
SPEAR taxa	79	0	0	9	47	3	100	8	3	2	0	325	39	6	6	0	6	0	3
EPT taxa	57	0	0	3	33	3	100	8	3	7	0	63	13	6	6	0	6	0	3
Total individuals	243	73	138	266	493	42	12,533	13	17	278	62	1,475	261	128	118	27	50	180	91
Autumn																			
SPEAR taxa	13	39	58	30	28	20	3	7	8	65	0	0	14	0	18	10	37	9	9
EPT taxa	13	78	40	33	31	20	6	7	23	65	28	0	25	0	61	10	42	97	9
Total individuals	148	600	1,521	1,185	819	80	326	32	385	641	231	0	1,866	2,189	4,716	55	184	634	63
Mean																			
SPEAR taxa	46	20	29	20	37	12	51	8	6	34	0	163	26	3	12	5	21	4	6
EPT taxa	35	39	20	18	32	12	53	8	13	36	14	31	19	3	34	5	24	49	6
Total individuals	196	336	829	725	656	61	6,430	22	201	460	146	738	1,063	1,159	2,417	41	117	407	77

4.3.4 Functional responses - ecosystem metabolism

All study streams, except A9, were heterotrophic characterized by negative values for NPP (Table 4.7), and NPP spanned approximately two orders of magnitude. ER_{24} significantly increased with increasing proportions of macrophyte coverage, coarse organic debris, proportional coverage of mud, and total P concentration (Pearson correlation, r = 0.47 and P = 0.048, r = 0.66 and P = 0.003, r = 0.54 and P = 0.021, and r = 0.57 and P = 0.013, respectively) (Fig. 4.10, top panel). GPP significantly increased with increasing concentration of phosphate-P, total P and increasing $sumTU_{D.magna}$ (Pearson correlation, r = 0.54 and P = 0.020, r = 0.57 and P = 0.013, and r = 0.57 and P = 0.012, respectively) (Fig. 4.10, central panel). NPP significantly decreased with increasing macrophyte coverage and proportional coverage of coarse organic debris (Pearson correlation, r = -0.53 and P = 0.024, and r = -0.50 and P = 0.031, respectively) (Fig. 4.10, lower panel). Stream width, depth, agricultural intensity in catchments and buffer zones, ammonia-N concentration, nitrate-N concentration, total N concentration, $sumTU_{P.subcapitata}$, and $sumTU_{C.riparius}$ were not significantly correlated with ER_{24}, GPP or NPP (Pearson correlation: P > 0.05, data not shown). We found a significant negative correlation between GPP and SPEAR index values representing macroinvertebrate samples collected in June (Pearson correlation, r = -0.579, P = 0.015) (Fig. 4.11).

FIGURE 4.11. GPP as a function SPEARpesticides index values. Ecosystem metabolism was measured in June, and the SPEAR data represents macroinvertebrate samples additionally collected in June. A linear regression is fitted to the data, and the correlation coefficient is indicated.

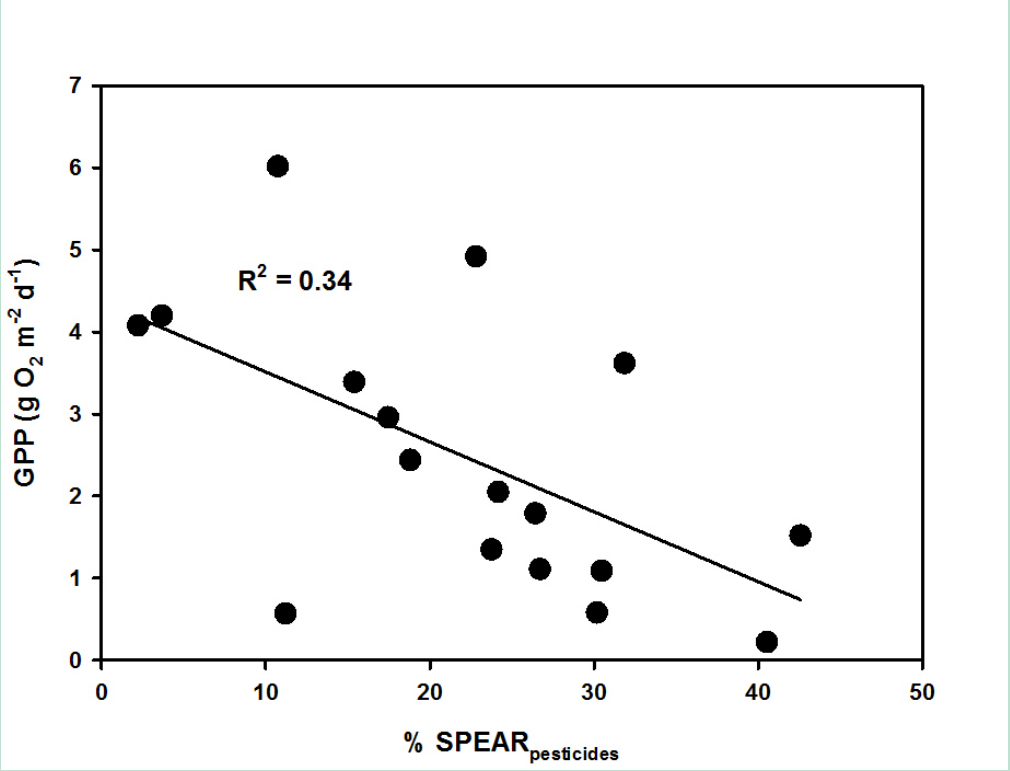


TABLE 4.7. Average ecosystem respiration (ER_{24}), gross primary production (GPP), and net primary production (NPP) for the 19 study streams. Metabolism was measured in June-July for 1-4 consecutive days using the upstream-downstream diurnal oxygen change technique. NA indicates that the specific measurements were defective and hence not available.

Stream code	n	ER_{24} (g O_2 m^{-2} d^{-1})	GPP (g O_2 m^{-2} d^{-1})	NPP (g O_2 m^{-2} d^{-1})
C1	1	32.94	3.62	-29.32
C2	2	13.75	2.05	-11.70
C3	3	10.56	1.11	-9.45
C4	1	7.59	1.79	-5.80
C5	2	23.38	4.20	-8.63
C6	1	2.98	1.09	-1.89
C7	3	10.10	4.92	-5.18
C8	1	NA	NA	NA
C9	1	0.69	0.22	-0.470
A1	3	24.89	6.02	-18.88
A2	4	18.97	3.39	-15.85
A3	1	NA	NA	NA
A4	1	17.42	2.44	-14.98
A5	3	9.38	0.57	-8.81
A6	3	13.54	4.08	-9.46
A7	1	12.14	1.52	-10.61
A8	2	2.49	0.58	-1.91
A9	1	0.47	2.96	2.48
A10	1	9.13	1.35	-7.78

4.4 Discussion

4.4.1 Physical stream properties and general water chemistry

The study streams were selected following a detailed set of criteria regarding especially physical site properties intended to maximize the relative importance of pesticide pollution gradients compared to other co-existing and potentially confounding environmental gradients. In general, the physical site characteristics were not significantly influenced by the gradient in agricultural intensity in stream catchments or in 100m buffer zones. Although significantly different, the proportional coverage of gravel was, according to our expert judgement, comparable between the agricultural and control sites (mean values of 9% and 14% in agricultural and control streams, respectively). Importantly, hard substrate types, including boulder, pebbles and gravel, were dominating in all study streams (mean values of 41% and 44% in agricultural and control streams, respectively). Therefore, we suggest that the observed significant influence of agricultural intensity in stream catchments on width and current velocities is an artefact of the stream selection procedure and not due to an overarching cause-effect relationship.

The influence of the agricultural gradient generated a highly significant concentration gradient in nitrate-N and total-N in the study streams (nitrate-N typically constituting > 90% of total-N), but the observed concentrations in nitrate-N and total-N is not expected to prompt significant negative direct effects on stream macroinvertebrates (Friberg *et al.* 2010). Concentrations of total-P and BOD_5 were not significantly correlated to agricultural intensity. In one stream, BOD_5, exceeded critical levels (> 3 mg L^{-1}) for some species of macroinvertebrates (Friberg *et al.* 2010), probably reflecting an influence of untreated wastewater from scattered dwellings or small sewage effluents.

4.4.2 Pesticide pollution

Summed pesticide concentrations, number of detected pesticides and sumTU for stormwater samples, suspended particle samples and bed sediment samples generally exceeded previous findings for Danish streams up to several orders of magnitude (Kronvang *et al.* 2003b; Rasmussen *et al.* 2011b; Boutrup *et al.* 2015), although the most frequently and commonly used pesticide, glyphosate, was not included as analyst (Danish EPA 2014). In spite of a still increasing amount of pesticide active ingredients traded in Denmark (Danish EPA 2014), we propose that our findings should not be interpreted as reflecting this increase. Our results most likely reflect several improved initiatives regarding sampling methodology and chemical analyses improving quantification of the level of pesticide contamination in Danish streams. Firstly, event triggered water sampling is a simple and cost-effective method providing insight into worst-case scenarios, since the dominant routes for pesticide transport in agricultural dominated catchments, tile drain flow and surface runoff, are mainly triggered by heavy rain episodes (Liess *et al.* 1999; Bundschuh, Goedkoop & Kreuger 2014). Since maximum concentration is a stronger determinant for ecological effects compared to exposure duration (Schulz & Liess 2000; Bundschuh *et al.* 2013), this method likely provides more robust and meaningful estimates of pesticide contamination causing ecological effects compared to time-integrated sampling and random grab sampling. Secondly, increasingly comprehensive analysis programs (number of analysts) proportionally increase sum pesticide concentrations, sumTU, and amounts of pesticide active ingredients in field samples (Moschet *et al.* 2014). All samples in our study were analyzed in accordance with the comprehensive Swedish pesticide monitoring program for streams (Nanos, Boye & Kreuger 2012) which likely contributed to the quantified increase in pesticide contamination in Danish streams. Thirdly, levels for detection and quantification at the laboratory of Swedish University of Agricultural Sciences, Dept. of Aquatic Sciences and Assessment were up to 100 times lower compared to previous studies (Kronvang *et al.* 2003b; Rasmussen *et al.* 2011b) and monitoring results (Boutrup *et al.* 2015) which increased the number of detected pesticide active ingredients.

The measured levels of pesticide contamination in relation to existing political guidelines are discussed in detail in Appendix paper I (Rasmussen *et al.* 2015), including the identification of pesticide active ingredients of particular concern. However, we emphasise that this sampling campaign does not provide the data necessary to pinpoint specific sources for the observed pesticide pollution; neither does it provide data to exclude specific sources. Overall, this project generated one of the most comprehensive data sets regarding pesticide toxicity and occurrence in streams at the global level, and our findings portrait streams covering a complete gradient in pesticide pollution with the majority of agricultural impacted streams experiencing both background and peak exposures well within the range of expected ecological impact (Schäfer *et al.* 2012b; Malaj *et al.* 2014; Orlinskiy *et al.* 2015; Stehle & Schulz 2015).

4.4.3 Macroinvertebrate community responses – the SPEAR index

$SPEAR_{pesticides}$ significantly decreased with increasing $sumTU_{D.magna}$ and $sumTU_{C.riparius}$ before (May), during (June) and after (July and September) the primary insecticide application season indicating that pesticide pollution overall reduced the fraction of macroinvertebrate taxa characterized as sensitive to pesticide exposure. Although $SPEAR_{pesticides}$ values were generally higher in May compared to June, July and September, the consistent significant correlation through time indicates that disturbance history, such as previous pesticide pollution incidents, exerted strong influence on present macroinvertebrate community structure. The strong intercorrelation between TU values representing different pesticide sample types, especially between storm flow water samples and sediment samples (Appendix paper I, Rasmussen *et al.* 2015), and the significant contribution of legacy pesticides to predicted sediment toxicity, suggest that the streams currently exposed to highest pesticide concentrations additionally have the strongest history of previous pesticide pollution pressures. This clearly emphasizes that ecological effects of current pesticide exposure cannot be disentangled from the history of pesticide pollution. The majority of water and sediment samples collected during the course of this field study are not expected to generate acute lethality in most macroinvertebrate taxa, and we therefore propose that pesticide pollution is more likely to slowly divert macroinvertebrate communities from their potential reference state through multiple and recurring exposures to sublethal concentrations.

Importantly, the measured pesticide pollution gradient in our study streams is comparable to previous studies in German, French, Swedish and Australian streams (Liess & von der Ohe 2005; Schäfer *et al.* 2007; Schäfer *et al.* 2011; Bundschuh, Goedkoop & Kreuger 2014), and the macroinvertebrate community response measured as $SPEAR_{pesticides}$ are similarly comparable to these studies. Hence, obtained $SPEAR_{pesticides}$ index values range from levels corresponding to European reference streams (von der Ohe *et al.* 2007) in the least contaminated study streams to a complete absence of SPEAR taxa in the most contaminated study streams corresponding to the most contaminated European streams (Liess & von der Ohe 2005; Schäfer *et al.* 2007). One important conclusion is therefore that the studied Danish streams represent a gradient in pesticide exposure and macroinvertebrate community responses that is fully comparable to other European countries with less strict rules for agricultural pesticide use such as Germany and France.

We subdivided the study streams into three groups according to the frequency of measured significant pesticide exposure events ($sumTU_{D.magna} > 0.001$). Although this value of $sumTU_{D.magna}$ is a factor of 10 below the regulatory accepted concentration (RAC), previous studies have pinpointed this level of $sumTU_{D.magna}$ as threshold causing significant reductions in $SPEAR_{pesticides}$ index values in the field (Liess & von der Ohe 2005; Schäfer *et al.* 2012b; Orlinskiy *et al.* 2015). Our data supports the contention that pesticide effects occur below the RAC, and we show that average $SPEAR_{pesticides}$ index values were significantly lower in streams receiving two or more storm flow episodes exceeding a $sumTU_{D.magna}$ of 0.001. In addition, we have showed that average $SPEAR_{pesticides}$ index values were generally lower in streams receiving just one of such storm flow episodes, although only significant in September, compared to streams where the $sumTU_{D.magna}$ never exceeded this critical level during storm flow episodes. These findings suggest that the frequency of significant pesticide exposures is an important factor influencing the magnitude of macroinvertebrate community response to pesticide exposure. Our findings receive support from mesocosm studies on neonicotinoid and pyrethroid insecticides showing that multiple pesticide exposures exacerbate effects on macroinvertebrate communities compared to single exposures (Bottger *et al.* 2013; Wiberg-Larsen, Nørum & Friberg 2013). We therefore emphasize that a comprehensive characterization of pesticide exposures is crucial to reveal the full potential of pesticide effects in the field.

$SPEAR_{pesticides}$ index values were reduced by approximately 50% from May to June in streams receiving two or more storm flow episodes exceeding a $sumTU_{D.magna}$ of 0.001, and this reduction persisted at least until September. In comparison, $SPEAR_{pesticides}$ index values were only reduced by 20-30% from May to June in streams receiving one or no storm flow episodes exceeding a $sumTU_{D.magna}$ of 0.001, and this reduction persisted at least until September in streams receiving one storm flow episode exceeding this threshold whereas $SPEAR_{pesticides}$ index values approximated full recovery in September. This indicates that long-term sublethal effect mechanisms, as suggested above, is likely the primary factor driving pesticide induced macroinvertebrate community changes. Although stream group specific average $SPEAR_{pesticides}$ index values (discussed above) were not significantly more reduced from May to June in streams with higher frequency of significant pesticide exposure episodes, our results suggest that potential effects of pesticides may be revealed when benchmarking $SPEAR_{pesticides}$ index values against the index values obtained before the primary insecticide spraying season. Therefore, our findings suggest that a revelation of ecological effects of current pesticide exposure not only requires detailed characteristics of recurring pesticide pollution events but additionally a thorough understanding of macroinvertebrate community structure prior to the pesticide application season.

One factor that potentially influences the temporal development of $SPEAR_{pesticides}$ index values is the sequential transport of new macroinvertebrates via drift from upstream and potentially less exposed upstream sections of the streams. However, the drift density of SPEAR taxa was not correlated to agricultural intensity in the stream catchments or in the buffer zones suggesting that the recolonization potential via downstream drift was comparable among streams. Assuming that the drift sampling efforts sufficiently represented the actual flux of new macroinvertebrates to the studied stream sections we suggest that recolonization via drift may be of minor importance to the ecological recovery potential of the streams. Macroinvertebrate drift distances are typically restricted to few hundred meters (Brittain & Eikeland 1988), and since stream sections located few hundred meters upstream of the sampling sites likely have strongly

comparable environmental conditions, including pesticide pollution characteristics (McKnight *et al.* 2012), we find it plausible that the importance of macroinvertebrate drift as source of recolonization is of marginal importance. An important alternative explanation to the observed $SPEAR_{pesticides}$ recovery of study streams receiving no significant pesticide exposure, especially the decline in June and subsequent recovery, is emergence before the episodes and egg laying and immediate juvenile development among several EPT taxa. Further, it should not be ignored that alongside streams and not at least over-land recolonization between streams may be important for some taxa, although such aerial dispersal may be dependent on especially favourable weather condition.

The total density of macroinvertebrates in drift samples increased with decreasing $SPEAR_{pesticides}$ index values, and the macroinvertebrate taxa actively drifting were dominated by the freshwater shrimp, *Gammarus pulex* (L.) and Chironomidae. In other words, the closer the macroinverebrate community composition was to its reference state the lower flux of *G. pulex* and Chironomidae via drift. This likely reflects that *G. pulex* and Chironomidae often increasingly dominate macroinvertebrate communities in Danish streams with increasing agricultural pressure as long as BOD_5 concentrations remain reasonably low (≤ 2 mg L^{-1}) (Rasmussen *et al.* 2012b), and further that both taxa are species characterized as not being at risk according to SPEAR.

4.4.4 Macroinvertebrate community responses – DSFI vs SPEAR

DSFI scores remained high among the study streams and sampling months with only few cases of DSFI scores below 5. Importantly, the DSFI index value 5 characterises the threshold for "good ecological status". This strongly implies that the DSFI index is rather insensitive to pesticide pollution as additionally indicated by Wiberg-Larsen *et al.* (2016). Our study provided several examples of macroinvertebrate samples obtaining very low $SPEAR_{pesticides}$ index values (< 10) while meeting the obligatory requirements of "good ecological status" according to DSFI. Logically, this is somewhat controversial with respect to the management and environmental protection of Danish stream systems, since the macroinvertebrate fauna can remain obviously impaired according to measured pesticide pollution and the $SPEAR_{pesticides}$ index while still obtaining sufficient ecological status according to the only macroinvertebrate index used in Denmark as part of the implementation of the Water Framework Directive. However, in extreme cases of pesticide pollution, all EPT taxa and all amphipods may disappear (Thompson *et al.* 2015) which will obviously trigger a stronger response in the DSFI index than observed in this study. Such extreme events of pesticide pollution are well documented for streams in Funen two decades ago (Wiberg-Larsen *et al.* 1997), but may occasionally still occur.

The DSFI index was significantly correlated to log $sumTU_{D.magna}$ (during storm flow events) outside the main insecticide application season (May and September), and average DSFI score in September was significantly lower in streams receiving two or more storm flow episodes exceeding a $sumTU_{D.magna}$ of 0.001 compared with streams receiving only one or no such episodes, indicating that DSFI after all has a potential to reflect pesticide impact. Importantly however, the strongest correlation between $SPEAR_{pesticides}$ and log $sumTU_{D.magna}$ was obtained using June samples additionally coinciding with highest pesticide pollution. This indicates that the $SPEAR_{pesticides}$ index responded as could be expected to the observed pesticide pollution whereas this was not the case for DSFI.

However, using larger stream data sets covering a broader and more complex combination of environmental stressors reveals that DSFI and $SPEAR_{pesticides}$ is strongly intercorrelated when the pesticide gradient is not the dominant environmental gradient (Rasmussen *et al.* 2011a). In fact, SPEAR appears to respond as strongly as DSFI to e.g. gradients in physical stream impairment when the pesticide gradient is less strong or at least insufficiently quantified (Rasmussen *et al.* 2011a). In the case of concurrent and strong gradients in pesticide pollution and physical habitat deterioration, the $SPEAR_{pesticides}$ index responded to both stressors, whereas DSFI responded only to physical habitat quality (Rasmussen *et al.* 2012b). This clearly indicates that DSFI can only be used as disturbance indicator in streams mainly impacted by physical habitat deterioration or organic pollution, but also that the $SPEAR_{pesticides.}$index should not be used as specific indicator for pesticide pollution in streams with heavy physical habitat deterioration.

4.4.5 Functional responses – ecosystem metabolism

All but one study stream was heterotrophic (ER > GPP), and ecosystem respiration was significantly and positively correlated with proportional coverage of macrophytes, coarse organic debris and mud. This suggests that both allochthonous and autochthonous material strongly governed rates of ecosystem respiration. Existing literature confirms that open-land streams are predominantly heterotrophic, even when stream beds are covered by dense macrophyte stands (Edwards & Owens 1962; Alnoee *et al.* 2015).

Gross primary production significantly increased with increasing concentrations of phosphate-P and total P indicating that phosphorous rather than nitrogen was the limiting nutrient element for phototrophs and heterotrophic microorganisms in the study streams. Stream macrophyte traits generally respond stronger to gradients in phosphate-P concentrations compared to ammonia-N and nitrate-N concentrations in Danish streams (Baattrup-Pedersen *et al.* 2016) confirming that phosphorous rather than nitrogen influence the structure and function of phototrophs in Danish streams. However, our study streams were characterized by low water depth, large fractions of coarse substrate types, and most of macrophyte coverage of the majority of the streams was low (< 25%) suggesting that benthic algae were the main phototrophic components governing nutrient uptake and community metabolism. Numerous Danish open headwater streams draining agricultural catchments are subjected to eutrophication and support high biomasses of benthic epiphytic microalgae (Sand-Jensen, Moller & Olesen 1988), and the microalgae in such streams may be highly important regulators for ecosystem-based processes such as metabolism and nutrient spiraling (Sand-Jensen, Moller & Olesen 1988; Levi *et al.* 2015).

Gross primary production was not significantly influenced by the predicted toxicity to algae of detected pesticides in storm flow water samples indicating that potential direct effects of pesticides on autotrophic productivity was minimal or reduced by functional redundancy. Herbicide mixtures can produce significant effects on the structure and productivity of lotic biofilm communities within the range of environmental realistic concentrations (Paule *et al.* 2013; Bayona *et al.* 2014; Feckler, Kahlert & Bundschuh 2015), but these effects may be clouded or even reversed when nutrient concentrations are sufficiently elevated (Andrus *et al.* 2015). We therefore suggest that the stimulating effect of phosphate-P likely overruled the potential negative effect of herbicides on primary productivity in the study streams.

Gross primary production was significantly increased with increasing predicted toxicity to macroinvertebrates of detected pesticides in storm flow water samples which could be due to an indirect negative effect of especially insecticides (mainly driving $sumTU_{D.magna}$) (Appendix paper I, Rasmussen *et al.* 2015) on macroinvertebrate grazers. Supporting this notion is our additional finding that gross primary production significantly increased with decreasing $SPEAR_{pesticides}$ index values which could suggest that especially abundances of insect grazers could be reduced in streams with highest $sumTU_{D.magna}$. Pesticide mediated reductions of abundances of macroinvertebrate grazers could increase the biomass of benthic microalgae due to released grazing pressure as observed in previous mesocosm studies (Rasmussen, Friberg & Larsen 2008; Pristed, Bundschuh & Rasmussen 2016). Moreover, streams subjected to accidental spillage of potent insecticides such as organophosphates and pyrethroids typically change in trophic structure towards increasing thickness and cell size of benthic microalgae (Thompson *et al.* 2015) and increasing coverage of thread-forming green algae (Fyn County 2001) likely due to reduced abundance of insect and crustacean macroinvertebrate taxa suggesting that reduced grazing pressure is the primary cause of the observed substantial changes in phototrophic stream communities. Similar findings have been reported for another functional attribute of streams, leaf decomposition, where reduced decomposition rates were attributed to insecticide-mediated reductions in macroinvertebrate shredder abundance (Schäfer *et al.* 2007). Importantly, these findings indicate that structural and functional ecosystem properties can be strongly interlinked especially when the stressor acts with high specificity. Moreover, our findings emphasize the importance of understanding indirect effect mechanisms in field-based ecotoxicological research.

5. Considerations regarding operationalization of a pesticide indicator

Ideally, the risk assessment of pesticides in surface waters contains a prospective as well as a retrospective part (Brock 2013). The retrospective risk assessment is particularly relevant since the prospective risk assessment using exposure models is affiliated with substantial uncertainties (Knäbel *et al.* 2012; Knäbel *et al.* 2014) leading to significant underestimation of pesticide exposure especially during storm flow episodes in streams. Consequently, it is imperative that actual exposure scenarios are described allowing a proper retrospective risk assessment. The proper characterization of pesticide exposure is additionally an imperative prerequisite for proper interpretation of pesticide induced ecological effects. Therefore, the development and modification of pesticide indicators cannot be decoupled from the characterization of pesticide exposure scenarios. In consequence, we scrutinize in this chapter both elements and construction of a pesticide indicator as well as the necessary requirements for the characterization of pesticide exposure allowing a sufficient correlation between pesticide exposure and ecological effects.

5.1 Quantification of pesticide pollution as benchmark for pesticide indicators

Several studies have pinpointed that maximum exposure concentrations and to a lesser extent temporal exposure duration drive the magnitude of ecological effects (Schulz & Liess 2000; Bundschuh *et al.* 2013). Moreover, the scientific development of the SPEAR indicator clearly reveals that correlations between the SPEAR index and quantified pesticide toxicity to macroinvertebrates ($TU_{D.magna}$) increases when measures for pesticide toxicity specifically represents storm flow peak concentrations rather than base flow water samples (Liess & von der Ohe 2005; Schäfer *et al.* 2007; Rasmussen *et al.* 2012b). In other words, any pesticide indicator unequivocally depends on a proper and sufficiently detailed quantification of pesticide pollution. This means that, especially for macroinvertebrates characterized by a relatively long generation time (compared to microorganisms) and low dispersal ability (compared to fish), the short but significant pesticide (in particular insecticide) pulses need to be properly quantified in order to provide a pesticide benchmark of sufficient quality to shed light on cause-effect mechanisms and to optimize the link between pesticide pollution and a pesticide indicator for stream macroinvertebrate communities. We draw attention to the fact that especially insecticides, but additionally fungicides and in few cases herbicides were significant contributors to the summed toxicity ($SumTU_{D.magna}$) of water samples to macroinvertebrates in our field campaign. This reveal a need to include all pesticide groups for a proper interpretation of the pesticide mediated toxic pressure in streams.

In this project we showed that pesticide concentrations and predicted toxicity to aquatic biota was significantly intercorrelated among sample types (base flow water, storm flow water, suspended particles, and bed sediment), but correlations to the SPEAR index were strongest for storm flow water samples, suspended particle samples and bed sediment samples. The strong correlations between SPEAR and predicted pesticide toxicity in suspended particle and bed sediment samples are likely due in part to especially bed sediments acting as a sink for pesticides with low water solubility (Hladik & Kuivila 2012; Kuivila *et al.* 2012) and in part that pesticide sorption often significantly increases pesticide half-lives (Xu *et al.* 2008; Li *et al.* 2013) meaning that sediments may provide a historic fingerprint of previous pesticide pollution. Hence, pesticide concentrations in sediments should not be interpreted as unambiguous measures for actual toxicity. Rather, sediment pesticide concentrations provide a complex picture of historic and current pesticide pollution of which some pesticides may still exert significant toxic pressure to macroinvertebrates (Domagalski *et al.* 2010; Ding *et al.* 2011; Ensminger *et al.* 2011; Weston *et al.* 2013).

Our field study revealed a substantial variation in pesticide concentrations and predicted toxicities to aquatic biota between consecutive storm flow episodes within the same stream. Moreover, we showed that the frequency of significant pesticide exposures strongly influenced SPEAR index values of the streams. Not only do our findings highlight the magnitude of environmental variability regarding pesticide fate and occurrence, but more importantly our findings strongly suggest that quantification of one storm flow episode is insufficient for characterization and understanding of ecological effects. Thorough characterization of pesticide pollution during storm flow episodes is likely the best available option for benchmarking current toxic pressure originating from conventional agriculture. Such a characterization should include analysis of a series of pesticide active ingredients pinpointed as significant causes of predicted ecotoxicity from pre-screening studies. For pyrethroids, the group of insecticides applied in largest quantities in Denmark (Danish EPA 2014), it is paramount that the level of quantification is sufficiently low in the analytical laboratory as the concentration range found in our field study remained within the low ng L^{-1} scale. Importantly, such concentrations were predicted to cause substantial toxicity in the water samples (Appendix paper I, Rasmussen *et al.* 2015), and these are additionally within the range of observed ecological effects in controlled laboratory and mesocosm studies (Rasmussen *et al.* 2013b; Wiberg-Larsen *et al.* 2016).

We propose that pesticide monitoring should consist of a combination of storm flow water samples, sediment samples which could be supplemented with base flow samples depending on the purpose of the screening. To optimize relevance for pesticide exposure characterization for macroinvertebrate community responses, storm flow water samples and sediment samples should be prioritized. These samples should be screened for a comprehensive list of all currently used and selected legacy pesticides with sufficiently low limits for detection and quantification. All storm flow episodes occurring from May to July should be included – potentially even longer depending on the agricultural pesticide application pattern. In urban streams and streams subjected to effluents from ornamental greenhouses, the temporal window of interest is probably much extended.

Due to the currently applied national pesticide monitoring strategy under the NOVANA program, where one sediment sample collected in each of five streams per year and analysed for three insecticides and 12 random grab samples of water collected in each of five streams and analysed for 24 herbicides, the available pesticide occurrence data in NOVANA cannot be used to quantify pesticide exposure with sufficient level of detail and hence not be used as benchmark for a macroinvertebrate based pesticide indicator. In consequence, a thorough revision of this monitoring strategy needs to be performed in order to collect useful data for calibration of pesticide indicators and upscale the quantification of the overall magnitude of pesticide pollution in different types of Danish streams.

Importantly, this proposed sampling for pesticides is necessarily applicable to other environmental contaminants such as metals, PAH's and endocrine disruptors.

5.2 Scrutinizing pros and cons with the SPEAR index

Based on a broad set of Danish headwater streams carefully selected to minimize the influence of stressors other than pesticide pollution, the SPEAR index performed strongly. The correlation between SPEAR and predicted pesticide toxicity to macroinvertebrates was highly significant from May to September, although lowest index values were obtained during the primary insecticide application season. Obtained SPEAR values were comparable to those found in other unpolluted European streams (von der Ohe *et al.* 2009) and the study streams with highest predicted toxicity were characterized by very low SPEAR values ranging down to the minimum SPEAR value (zero) which is additionally comparable to other equally polluted streams in Germany, France and Sweden (Liess & von der Ohe 2005; Schäfer *et al.* 2007; von der Ohe & Goedkoop 2013). The SPEAR index revealed that reductions in the relative abundance of species characterized as sensitive to pesticide pollution was strongest in streams with highest and most frequently recurring pesticide pollution incidents. Moreover, SPEAR provided insight into the temporal dynamics of macroinvertebrate communities following significant pesticide pollution incidents suggesting that observed reductions in SPEAR persisted longer in the most contaminated streams. In the context of our field study,

the SPEAR index provided highly convincing results suggesting that SPEAR could be considered for implementation in its current form, although it is probably an essential prerequisite for correct data interpretation that SPEAR values are obtained both in spring and summer and that the change in SPEAR value from spring to summer is used to supplement the mechanistic understanding of potentially low SPEAR values in summer.

The remaining question regarding SPEAR is to which extent the index can perform diagnostic evaluations of ecological stream status, i.e. which information is provided by SPEAR in the absence of pesticide pollution data. This question is particularly relevant in the presence of stronger gradients of co-occurring stressors concomitant with pesticide pollution. One previous study showed that surber samples collected on mud-dominated microhabitats consistently scored very low SPEAR values, approximating maximum pesticide effect (Schletterer *et al.* 2010). Moreover, Rasmussen et al. (2012b) showed that uncontaminated stream sections dominated by soft substrate types (sand and mud) provoked strong negative response in the SPEAR metric. This clearly indicates that SPEAR is not strictly stressor specific and responds to other gradients of disturbance.

As previously discussed, the engine room of SPEAR consists of three traits: (i) predicted species sensitivity to pesticides and two general traits characteristics representing overall disturbance tolerance, (ii) generation time and (iii) migration ability. Streams are per definition highly dynamic and unstable systems, but this is particularly the case in highly anthropogenic utilized catchments such those dominated by agricultural and urban areas. Upscaling to the national level, agricultural streams are subjected to multiple co-occurring stressors. Besides pesticide pollution, stream biota experience stronger daily amplitudes in oxygen and temperature (due to increased solar radiation and primary production), increasing variability and magnitude in hydrological response curves (due to drainage systems), physical habitat deterioration with increasing fractions of stream substrates comprised by soft types such as sand and mud, recurring and intense weed cutting etc. (to promote drainage and transport of water through streams). All such factors should in theory favour taxa with strong dispersal abilities and short generation times (r-strategists). Hence, it is less surprising that SPEAR may act, in addition to pesticide stress, as a general indicator of disturbance. The other trait, migration ability, seems however to be of relatively minor importance, as it actually only reflects two “groups”: *Gammarus pulex* (being assessed as being highly mobile) and all other taxa (having poor dispersal abilities).

The remaining element in the engine room of SPEAR which is proposed to be pesticide specific is the predicted taxonomic pesticide sensitivity. We discussed this thoroughly in Chapter 2 and in Wiberg-Larsen *et al.* (2016) showing that the pesticide sensitivity of macroinvertebrate species with no existing ecotoxicity data could not be predicted safely by obtaining sensitivity values from the taxonomically closest relative for which such a sensitivity value existed. However, our statistical analysis was based on gradual data, and SPEAR uses instead nominal values for sensitivity where a cut-off value is used to subdivide macroinvertebrate taxa into two groups: sensitive and insensitive (Liess & von der Ohe 2005). In Appendix paper I we additionally showed that, although the correlation between measured and extrapolated sensitivity was highly insignificant, the overall underestimation of pesticide sensitivity equaled the overall overestimation when extrapolating pesticide sensitivities between taxa. This could suggest that overall characterizations of community sensitivity using field samples that contain a large number of different taxa is less biased than suggested by our work.

5.3 Potential modifications of SPEAR

In Chapter 2 we used existing field data to analyse consistent macroinvertebrate traits responses to pesticide pollution and aimed to filter out traits that responded exclusively to pesticide stress and not to physical habitat deterioration. Our analysis pinpointed that taxa with short generation time (< 1 year) increased in abundance with increasing pesticide pollution and that this pattern was consistent in physically complex as well as homogeneous and mud-dominated stream sections. Although this indicates an independence of this trait to the physical habitat conditions, our expert judgement definitely does not support this contention. Agricultural stream sections dominated by fine sediment may surely be more prone to physical dis-

turbance compared to more complex physical habitats containing larger fractions of hard and stable substrate types. Importantly, this trait already is integrated in SPEAR using the same cut-off value (< 1 year) to separate pesticide sensitive from insensitive species. Also it must be mentioned that SPEAR samples are recommended to be taken primarily on hard substrates (Liess & von der Ohe 2005).

In Chapter 2 and in Wiberg-Larsen *et al.* (2016) we identified the ratio between total surface area and body volume as a highly significant proxy for measured pesticide sensitivity. Consequently, this measure could be used as improved proxy for the pesticide sensitivity of taxa with no ecotoxicity data. Although highly challenging, such a proxy could be established using maximum potential size (e.g. available in the Tachet database) in combination with emergence timing (needs expert judgement). As stream insect larvae/nymphs with terrestrial adult stages obtain their maximum potential size just prior to the adult stage, the actual size (surface area to volume ratio) of the macroinvertebrate larvae/nymphs occurring during the main insecticide application season (May-June) may be estimated using information on lifecycles, growth pattern, and main flight period. For species with a fully aquatic life cycle, a mean or median size during main insecticide application season may be used. However, this estimation if size “at spraying” is not straight forward. Thus, the method may introduce uncertainty as actual size and even maximum potential size are influenced by several abiotic and biotic factors with temperature, food availability and disturbance intensity considered among the more important ones thereby introducing some uncertainty in the application of this trait. However, the within taxa plasticity in the surface area to volume ratio is likely significantly lower that the among taxa plasticity. Therefore, it remains to be tested whether this proxy for taxa specific pesticide sensitivity can be used as diagnostic tool. Secondly, the surface area / volume ratio is not stressor specific, as r-strategists generally are small in size (i.e. large surface area / volume ratio). If pesticide pollution at the field site is highly significant, our results suggest that larger animals (lower surface / volume ratio) should be favoured in the stream environment. Conversely, if the stream site is highly disturbed by multiple stressors, the advantage of being small (higher surface / volume ratio) and fast reproducing (strong recolonization potential) may overrule the disadvantage of being small in the context of pesticide exposure. Hence, although we pinpointed relevant morphological mechanisms controlling the pesticide sensitivity of macroinvertebrates, this mechanism may be promoted or converted depending on the field scenario. Consequently, using the surface area / volume ratio as single proxy for pesticide sensitivity may not be as promising as indicated by our analyses.

5.4 Perspectives

Based on these considerations we suggest two non-mutually exclusive approaches forward in the development of methodologies aiming at detecting pesticide stress in Danish streams. Firstly we suggest applying SPEAR as an effect-measure for pesticide exposure in streams which are not strongly dominated by fine sediment since this index adequately quantifies effects of pesticide pollution in such systems. Secondly we suggest analysing further the strength of using a combination of relevant traits including the surface area to volume ratio, and the r-strategy that were both promising, together with some additional traits e.g. to diagnose pesticide stress. This will require i) that a database is established holding taxa specific information on these traits and ii) that they are adequately described and characterized for macroinvertebrate communities in minimally disturbed sites thereby making a benchmarking of the traits possible and with the additional possibility of disentangling co-occurring stress.

Furthermore, the overall results of our project are a summed product of comprehensive research efforts, and very few similarly comprehensive studies have been conducted to date (but see Liess & von der Ohe 2005; Schäfer *et al.* 2007; Schäfer *et al.* 2011; Rasmussen *et al.* 2012b). However, since SPEAR is not strictly stressor specific, detailed data on toxicant concentrations is prerequisite for correct interpretation of such results. Due to generally inappropriately collected pesticide samples (in the majority of EU member states, water samples are collected according employee working schedules and not specifically aiming at worst-case scenarios), researchers and managers generally fall short in the attempts to upscale pesticide effects in streams to national or regional level (but see Malaj *et al.* 2014). Hence, there is an urgent need to modify and improve our current monitoring strategy to allow researchers and stream managers access to sufficient data to reliably predict the magnitude of pesticide effects at larger spatial scales. With our current understanding of the strengths and weaknesses of the current SPEAR index it is not meaningful to screen NOVANA stream stations using SPEAR to evaluate current pesticide effects, as especially physical habitat quality would likely confound the results.

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Appendix 1. Rasmussen et al. 2015

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The legacy of pesticide pollution: An overlooked factor in current risk assessments of freshwater systems

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Streams

ABSTRACT

We revealed a history of legacy pesticides in water and sediment samples from 19 small streams across an agricultural landscape. Dominant legacy compounds included organochlorine pesticides, such as DDT and lindane, the organophosphate chlorpyrifos and triazine herbicides such as terbutylazine and simazine which have long been banned in the EU. The highest concentrations of legacy pesticides were found in streams draining catchments with a large proportion of arable farmland suggesting that they originated from past agricultural applications. The sum of toxic units ($SumTU_{D.magna}$) based on storm water samples from agriculturally impacted streams was significantly higher when legacy pesticides were included compared to when they were omitted. Legacy pesticides did not significantly change the predicted toxicity of water samples to algae or fish. However, pesticide concentrations in bed sediment and suspended sediment samples exceeded safety thresholds in 50% of the samples and the average contribution of legacy pesticides to the $SumTU_{C.riparius}$ was >90%. Our results suggest that legacy pesticides can be highly significant contributors to the current toxic exposure of stream biota, especially macroinvertebrate communities, and that those communities were primarily exposed to legacy pesticides via the sediment. Additionally, our results suggest that neglecting legacy pesticides in the risk assessment of pesticides in streams may severely underestimate the risk of ecological effects.

1. Introduction

Publication frequency of articles characterising the contamination dynamics of freshwater systems in space and time has increased over the past decade in recognition of the need to increase realism of current exposure and risk assessments to support an informed management of these systems. Pesticides in particular have received increasing attention given their suggested important role in the global loss of freshwater biodiversity and ecosystem functioning (Beketov et al., 2013; Malaj et al., 2014; Rasmussen et al., 2012; Schäfer et al., 2012). In this article, we subdivide pesticides into those still registered for agricultural use in the European Union and in Denmark (referred to as contemporary pesticides) and those that have been discontinued or banned for usage in conventional agriculture (referred to as legacy pesticides).

Pesticides applied to agricultural fields may reach surface water through a series of different pathways with surface runoff and tile drains being widely accepted as the most important routes for contemporary pesticides (Schulz, 2004). These transport routes are primarily initiated during heavy precipitation events and lead to transient peak concentrations often exceeding current ecological quality criteria (Bundschuh et al., 2014; Liess and von der Ohe, 2005; Schulz, 2004). In contrast, legacy pesticides may enter surface water continuously via groundwater inflow (Barth et al., 2007; Gilliom, 2007; McKnight et al., 2015), atmospheric deposition

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(Konstantinou et al., 2006; Weber et al., 2010) or through continuous leaching from agricultural soils and landfills (Aliyeva et al., 2013). Consequently, legacy pesticides may generate a relatively constant exposure regime in surface waters. The yearly flux of legacy pesticides to freshwater ecosystems may comprise up to several percent of the historical yearly applied amounts in a catchment (Barth et al., 2007). Importantly, pesticides and their residues may persist and even accumulate in sediments of freshwater ecosystems (Dai et al., 2014; Kuivila et al., 2012; Nowell et al., 2013).

Factors controlling the fate of a pesticide in agricultural landscapes include a variety of chemical and environmental properties of the pesticide (e.g. degradation rate, adsorption to organic carbon and water solubility), climatic factors (e.g. temperature and precipitation), soil characteristics, topography and agricultural practices (Leonard, 1990; Wauchope, 1978). More than 20,000 pesticide products have entered the market since registration became legislatively required in 1947, and it is therefore not surprising that the combined effect of multiple factors influencing the environmental transport and fate of each pesticide generates highly complex exposure profiles of pesticide mixtures in time and space (Konstantinou et al., 2006; Wauchope, 1978). However, pesticides that are currently applied in the highest quantities are also those that occur most often in surface waters with the more water soluble and persistent compounds reaching the highest concentrations (Bundschuh et al., 2014; Kreuger and Tornqvist, 1998; Li et al., 2013; Moschet et al., 2014). Therefore, current pesticide usage is often used to guide the prioritisation of active ingredients included in monitoring programmes and research activities. Moschet et al. (2014) showed that a stringent focus on EU priority pollutants or a subset of the active ingredients applied in the highest quantities on the national level may seriously underestimate predicted toxic pressures in streams. Whereas Moschet et al. (2014) aimed to document that an extensive pesticide screening (249 active ingredients) translates into significantly higher predicted mixture toxicities compared to screenings restricted to fewer pesticides ($\leq$36), the authors did not distinguish between the toxic contribution of contemporary and legacy pesticides. Based on water samples mainly analysed for herbicides and four sediment samples mainly analysed for insecticides, McKnight et al. (2015) suggested that legacy pesticides could still be prominent players driving observed impairments of freshwater invertebrates, and the authors urged for more extensive studies that allow for quantifying the predicted toxicological potency of legacy pesticides in comparison to current use pesticides. To our knowledge, such an extensive study of the potential toxicity of legacy pesticides to aquatic biota relative to that of contemporary pesticides is still lacking despite a substantial body of literature addressing the occurrence, concentrations and predicted toxicities of selected legacy pesticides (Aliyeva et al., 2013; Gilliom, 2007; McKnight et al., 2015; Weber et al., 2010). The novelty element is therefore to quantify the possible toxicity of legacy pesticides as an integral part of current risk assessment. Such an integration has a number of potentially vital implications for the usability of risk assessment, including that i) contemporary regulatory actions are only targeting substances that are still in use; ii) it gives an increased explanatory power in river quality assessment by quantifying the impact of current unknowns, which will additionally reduce the potential underestimation of the role of pesticides as stressors in stream ecosystems, which is currently most likely the case (Beketov et al., 2013; Malaj et al., 2014), and iii) it provides highly needed insight into pesticide exposure profiles in time and space that may be used as improved benchmarks for the interpretations of ecological response parameters.

This article aims to compare the toxicity of legacy pesticides and their metabolites to those of contemporary pesticides in 19 Danish 1st and 2nd order streams situated in agricultural landscape covering a range of agricultural intensity, local climate and soil types. Water samples were collected during base flow and peak flow for pesticide analyses, and bulk sediment and suspended sediment were sampled to optimize detections of pesticides with low water solubility. In more detail, our objectives were to: i) characterize the occurrence of legacy pesticides in Danish headwater streams, ii) estimate the predicted toxicity of legacy pesticides and their residues using the Toxic Units (TU) approach, iii) evaluate the relative contribution of legacy pesticides and their residues to the summed TU of contemporary pesticides, and iv) evaluate which legacy pesticides are of highest ecotoxicological concern.

2. Methods

2.1. Study streams

Nineteen Danish 1st and 2nd order streams (Fig. S1) were sampled for pesticide occurrences. Nine streams with <50% agricultural land-use in a two-sided buffer extending 2000 m upstream of the sampling site were selected in addition to 10 streams with expected high impact of pesticides (conventional agriculture >60% in the two-sided 100 m buffer). Furthermore, all study sites complied with the following selection criteria: i) forest should occupy <50% of a two-sided 50 m buffer extending from the study site and 2000 m upstream, ii) proportional coverage of silt and mud in stream substrates (indicative of drainage ditches) should be <50%, and iii) no influence from waste water treatment plants, but scattered settlements may influence the chemical water quality. Detailed information on the study streams and catchments is provided in Table S1). In this article, we refer to the nine streams with expected low agricultural impact as controls and the ten streams with expected high agricultural impact as agricultural streams. All catchments are characterised by loam or sandy loam, low elevation and precipitation ranges from ca. 800–850 mm year^{-1} for central Jutland and on Funen and 700–750 mm year^{-1} on Zealand.

Base flow discharge was calculated as the product of the mean stream width, mean depth and mean water velocity, based on measurements at ten transects along a 100 m stream reach extending upstream from the sampling point (depth and velocity measured at 0, 25, 50 and 75% of the width of each transect). Moreover, yearly mean discharge was estimated as the product of yearly mean discharge coefficients (L s^{-1} km^{-2}), calculated for national hydrological monitoring stream sites geographically/geologically selected as representative for the study streams, and catchment area for the study streams (km^2). In a few cases national monitoring sites could not be regarded as truly representative, and yearly mean discharge was designated as > base flow (Table S1). The proportion of conventional agriculture was quantified for the catchments of each study stream and for a two-sided 100 m buffer extending 2000 m upstream of the sampling site were quantified in ArcGis 10.1 for windows.

2.2. Pesticide sampling

Sampling was conducted during May–August in 2012 coinciding with the main pesticide application season in this part of Europe. Dissolved phase pesticides were sampled with: i) manual grab samples in August during low flow conditions to optimize detections of pesticides originating from groundwater inflow (one sample per stream) and ii) event-triggered water samplers

designed to capture water during storm flow (Liess et al., 1999). Manual collection of water samples during low-flow conditions were consistently preceded by one week without precipitation. Event-triggered water samplers were checked every week during May, June and July and collected if full, resulting in 64 storm flow water samples. The event-triggered water samplers strategically collect water representing a temporal point measurement during the first hours of a heavy rain incident (Liess et al., 1999).

Sediment associated pesticides were sampled with two different methods. Bed sediment was collected (top 1 cm) in depositional areas using Kajak corers (8 cm in diameter). Each bed sediment sample was comprised of 20–30 subsamples to obtain samples representative for the stream reach. Bed sediment was collected in all streams in mid-August reflecting newly deposited material during the summer period. Suspended sediment was additionally collected since the mobile sediment fraction may provide a stronger estimate for worst case scenarios (Liess et al., 1996). The Suspended Particle Samplers (SPS) used in this study are described in detail elsewhere (Laubel et al., 2001).

2.3. Chemical analyses

Water samples were screened for 70 pesticides and metabolites comprising 42 contemporary pesticides, 26 legacy pesticides and 2 metabolites (Table S2). The 68 active ingredients included 35 herbicides, 16 fungicides and 17 insecticides. Bed sediment and suspended sediment samples were screened for 38 pesticides and residues comprising 16 contemporary pesticides, 18 legacy pesticides and 4 metabolites (Table S3). The 34 active ingredients included in the screening included 12 herbicides, 5 fungicides and 17 insecticides.

Analysis of water samples for the non-polar compounds was done by liquid/liquid extraction followed by gas chromatography mass spectrometry (GC–MS). For the polar and semi-polar compounds online solid-phase extraction followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed as described by Jansson and Kreuger (2010).

Wet sediment sample (20 g) was mixed with a drying agent (10 g). A sub-sample of the mixture (9 g, corresponding to 6 g sediment) was placed in pre-cleaned (400 °C) glass fibre cartridges and extracted together with the internal standards ethion and terbuthylazin-D5 by a Soxtec Avanti 2050 Auto System using dichloromethane and acetone (1:1). The extract was evaporated and diluted in cyclohexane and dichloromethane (1:1) before purification by Gel Permeation Chromatography (GPC), followed by evaporation and dilution in cyclohexane and acetone (9:1). The volume was adjusted to 1 ml. The extract was injected on two separate GC–MS systems, one in negative chemical ionization (NCI) mode (Agilent Technologies GC 7890, MS 5975C) and one in electron impact (EI) mode (Agilent Technologies GC 6890, MS 5973), quantifying against an external standard calibration. In order to enhance the sensitivity of the DDTs, a part of the initial extract was purified with sulphuric acid and with the internal standards added once again before injection. The standards used were obtained from Dr Ehrenstorfer GmbH. Dry-weight measurements of sediment were performed in a dry oven (105 °C) during ca. 16 h, with analytical results presented as μg per kg of dry weight.

Values between the limit of detection (LOD) and the limit of quantification (LOQ) were given as trace concentrations. At this level, the uncertainty of the concentration might be higher than stipulated (i.e. above 30%), but the identity of the compound has been confirmed and was therefore considered appropriate to be included in the subsequent data analysis.

2.4. Data analysis

All pesticide properties including effect concentrations (Tables S1 and S2) were acquired from the Pesticide Properties Database (http://sitem.herts.ac.uk/aeru/ppdb/en/ accessed 18.08.2014) and from the US EPA Ecotox Database (http://cfpub.epa.gov/ecotox/ accessed on 25.08.2014). In the cases where more than one effect concentration was available for a pesticide, the lowest value was selected. Legal status of the pesticides in Denmark and the EU was acquired from the Danish Pesticide Database (http://middeldatabasen.dk/Middelvalg.asp accessed on 04.09.2014) and the EU Pesticides Database (http://ec.europa.eu/sanco_pesticides/public/?event=homepage accessed on 04.09.2014), respectively (Tables S1 and S2).

For all water samples and sediment samples with pesticide detections above the LOD, the sum of toxic units (SumTU) was calculated to standardise exposure concentrations according to a benchmark organism. For water samples we used 96 h growth inhibition tests on the green algae *Pseudokirchneriella subcapitata* to benchmark sample toxicity to primary producers. In the cases where no data existed, we used data for *Scenedesmus subspicatus* as an alternative. Acute 48 h mortality tests on *Daphnia magna* were used to benchmark the toxicity to invertebrates and 96 h mortality tests on *Oncorhynchus mykiss* were used to benchmark sample toxicity to fish. *Lepomis macrochirus* was used as an alternative species in the few cases where no data was available for *O. mykiss*.

The sum of toxic units (SumTU) is calculated as:

$$\text{Sum TU} = \sum_{i=1}^{n} \frac{C_i}{EC50_i} \quad (1)$$

where C_i is the concentration of pesticide *i* in the sample, and $EC50_i$ is the concentration of chemical *i* causing a 50% effect to the benchmark organisms.

Bed sediment and suspended sediment pesticide concentrations were converted to TU using 96 h acute mortality tests for the sediment dwelling non-biting midge *Chironomus riparius* supplemented with 28 d chronic exposure tests on emergence success for *C. riparius* in the cases where no 96 h acute mortality test data existed. Often, only one of the tests was available for a pesticide, but in the few cases where data for both acute and chronic tests existed, we selected the lowest effect concentration. Effect concentrations in the *C. riparius* tests were based on measured pore water concentrations. In the cases where no sediment test data existed for a pesticide, we used the 48 h LC50 for *D. magna* as surrogate measure for sediment toxicity. Plotting the *C. riparius* toxicity data as a function of 48 h LC50 for *D. magna* for the pesticide compounds having both sets of toxicity data revealed that the deviation from the 1:1 line rarely exceeded one order of magnitude (Fig. S2).

Measured sediment-associated pesticide concentrations were converted to pore-water concentrations according to the equilibrium-partitioning approach to comply with the sediment benchmark toxicity tests that are based on dissolved phase pesticides in pore water. Moreover, pore water concentrations are superior predictors of sediment toxicity to invertebrates compared to pesticides adsorbed to sediment particles (Xu et al., 2007).

Pore water concentrations from bed sediment and suspended sediment were calculated according to Ditoro et al. (1991) as:

$$C_{PW} = \frac{C_S}{K_d} \quad (2)$$

where K_d is the partitioning coefficient, C_S is the sediment concentration and C_{PW} the pore water concentration of the pesticide.

K_d was calculated as:

$$K_d = K_{OC} \times f_{OC} \quad (3)$$

where K_{OC} is the dimensionless organic carbon–water partitioning coefficient for the pesticide and f_{OC} is the fraction of total organic carbon measured in the sediment sample. Kronvang et al. (2003) found the fraction of total organic carbon in bed sediments from 27 Danish agricultural streams to range from 5.5 to 16.1% with an average of 8.5%. Hence, the f_{OC} was set to 0.085 in our study. The K_{OC} was calculated as:

$$\log K_{OC} = a \times \log K_{OW} + b \quad (4)$$

where K_{OW} is the octanol–water partitioning coefficient. The constants a and b were set to 0.72 and 0.49, respectively, according to Schwarzenbach and Westall (1981).

We tested correlations between pesticide concentrations (ppm) among sample types (n = 19) using Spearman-Rank analysis. Stream specific (arithmetic) mean concentrations of storm flow samples were used. The number of storm flow samples ranged between two and five among streams (Table S4). Moreover, we tested correlations between SumTU of legacy pesticides and sum TU of contemporary pesticides within base flow, storm flow and sediment samples. For water samples, the correlations were based on data for all benchmark organisms. All data used in the Pearson correlation analyses were log-transformed to obtain normal distribution. The Spearman Rank correlation analyses were conducted in JMP 11.1.1 for Windows.

We tested if the addition of legacy compounds significantly increased the SumTU of water and sediment samples in control and agricultural streams, respectively, by comparing the SumTU of contemporary pesticides to the SumTU of all pesticides using Mann–Whitney tests in JMP 11.1.1 for Windows.

3. Results and discussion

3.1. Pesticide occurrence and toxicity patterns

We found a significant positive relationship among pesticide concentrations in all combinations of sample types ($P < 0.05$) (Table 1, Fig. S3). The strongest correlations were obtained between suspended sediment and bed sediment samples, between storm flow water and suspended sediment and between storm flow water and bed sediment (Table 1). Thus, streams with high pesticide concentrations in especially storm flow samples also had a high probability of having high pesticide concentrations in sediments and to a lesser extent during base flow. Importantly, SumTU based on contemporary pesticides was additionally a strong indicator for SumTU based on legacy pesticides in base flow samples (daphnia: $r = 0.724$, $P < 0.001$; fish: $r = 0.578$, $P = 0.009$; algae: $r = 0.460$, $P = 0.046$), storm flow samples (daphnia: $r = 0.603$, $P < 0.001$; fish: 0.468, $P < 0.001$; algae: $r = 0.359$, $P = 0.009$), suspended sediment samples (chironomids: $r = 0.563$, $P = 0.012$) and sediment samples (chironomids: $r = 0.696$, $P < 0.001$) (Fig. 1). This indicates that streams which are currently the most impacted by contemporary pesticide pollution, have probably also been so in the past. This is perhaps not surprising as areas with productive conventional agriculture rarely are converted into non-farming activities (Harding et al., 1998).

Table 1
Results from the Spearman Rank analyses comparing the summed pesticide concentrations (ppm) between all sample types. The correlation coefficients (r, first line) and significance levels (P, second line) are given.

	Base-flow water	Storm flow water	Suspended sediment	Bed sediment
Base-flow water		0.658 0.002	0.523 0.026	0.694 <0.001
Storm flow water			0.794 <0.001	0.782 <0.001
Suspended sediment				0.984 <0.001
Bed sediment				

3.2. Quantification of pesticide toxicity

In 11 (≈17%), 12 (≈18%) and 35 (≈55%) of the storm water samples, pesticide concentrations exceeded safety thresholds for daphnia (1/100 48 h LC50), fish (1/100 96 h LC50) and algae (1/10 96 h EC50), respectively (Panel, 2013) (Fig. 2, Table 2). Concentrations of legacy pesticides alone exceeded the safety thresholds for daphnia and fish in six and three of the storm flow water samples, respectively, while none of the samples contained legacy pesticide concentrations exceeding the safety threshold for algae. Note however, that the average SumTU for daphnia, fish and algae in agricultural streams all exceeded the respective safety thresholds (Table 2). Importantly, and confirming the early findings of McKnight et al. (2015), the addition of $SumTU_{D.magna}$ based on legacy pesticides to the $SumTU_{D.magna}$ based on contemporary pesticides significantly increased the $SumTU_{D.magna}$ in storm water samples from agricultural streams (Fig. 2B, $P = 0.039$). None of the base-flow water samples exceeded existing guideline values for invertebrates, fish or algae (Fig. 2A, Table 2).

Sediment and suspended sediment samples contained pesticide concentrations exceeding safety thresholds in 10 of 20 samples from agricultural streams. In seven of these samples, legacy pesticide concentrations alone exceeded the safety threshold, and the addition of $SumTU_{C.riparius}$ for legacy pesticides to the $SumTU_{C.riparius}$ for contemporary pesticides significantly ($\alpha = 0.1$) increased the $SumTU_{C.riparius}$ in suspended sediments (Fig. 3, $P = 0.038$) as well as in bed sediments (Fig. 3, $P = 0.064$). In fact, the average contribution of legacy pesticides to $SumTU_{C.riparius}$ for bed sediments and suspended sediments was >90%, and the average $SumTU_{C.riparius} > 0.1$ (Table 2).

Our results suggest that legacy pesticides can be highly significant contributors to the contemporary toxic exposure of stream biota, especially macroinvertebrate communities, and that those communities were primarily exposed to legacy pesticides via the sediment. However, Liess and von der Ohe (2005) and Schäfer et al. (2012) showed that stream dwelling macroinvertebrate communities were significantly different in streams containing peak flow concentrations of pesticides at 1/1000 48 h $LC50_{D.magna}$, and this threshold was exceeded in approximately 50% of the storm water samples in our study (30% for legacy pesticides alone) (data not shown). This clearly suggests that the exposure of stream biota to dissolved phase legacy pesticides as well as legacy pesticides adsorbed to sediment particles are likely both important stressors in these streams. Integrating past land use should therefore improve the prediction of pesticide impacts on macroinvertebrate communities compared to the stringent focus on current use chemicals in the water and sediment phases (Harding et al., 1998). Highly important is the fact that our results, supported by the findings of McKnight et al. (2015), strongly suggest that disregarding legacy pesticides, in particular those adsorbed to sediment particles, in ecotoxicological field studies and pesticide monitoring programs probably leads to significant underestimations of total risk and significant underestimations of the relative importance of pesticides compared to other important anthropogenic stressors (Harding et al., 1998; Matson et al., 1997). However, we recognize that the bioavailability of the highly

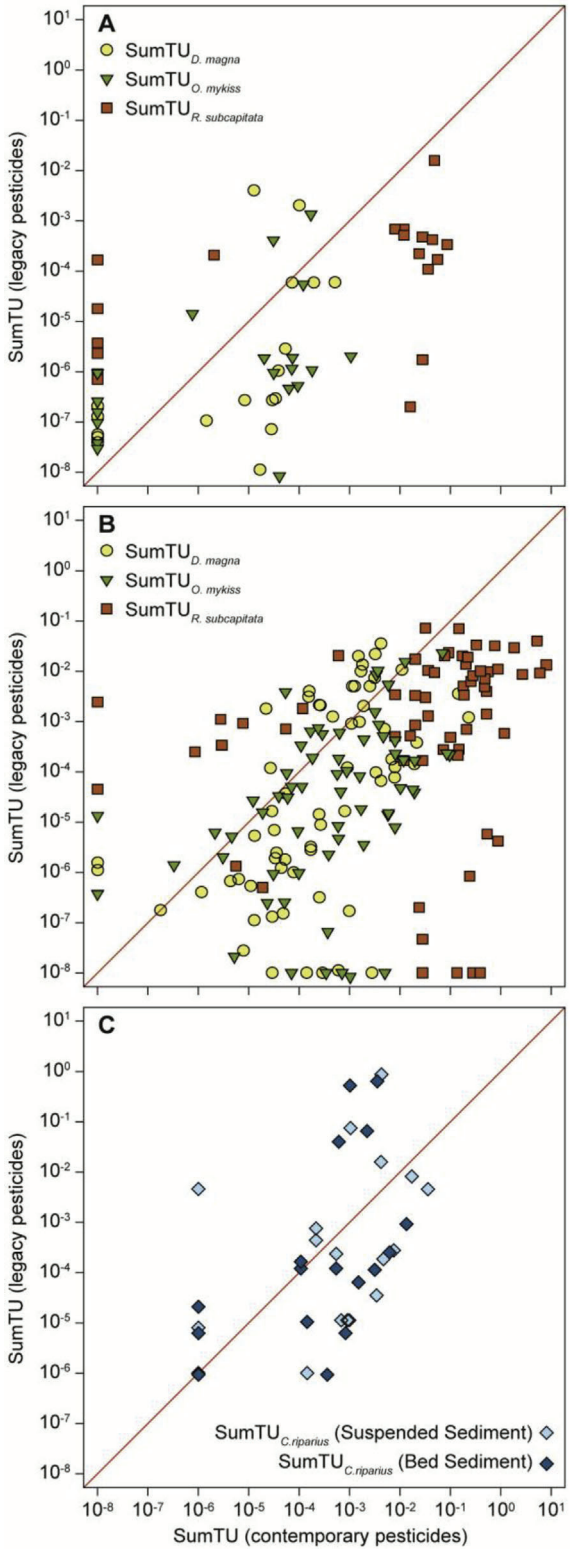

Fig. 1. SumTU for legacy pesticides as a function of the SumTU for contemporary pesticides for base flow water samples (A), storm flow water samples (B) and sediment samples (C). Sediment was sampled with two methods representing the bed sediment and suspended sediment. The diagonal lines indicate 1:1 relationships. For all water samples, the SumTU was calculated for algae (*R. subcapitata*), fish (*O. mykiss*) and invertebrates (*D. magna*), whereas SumTU calculations for sediment samples were based on *C. riparius*.

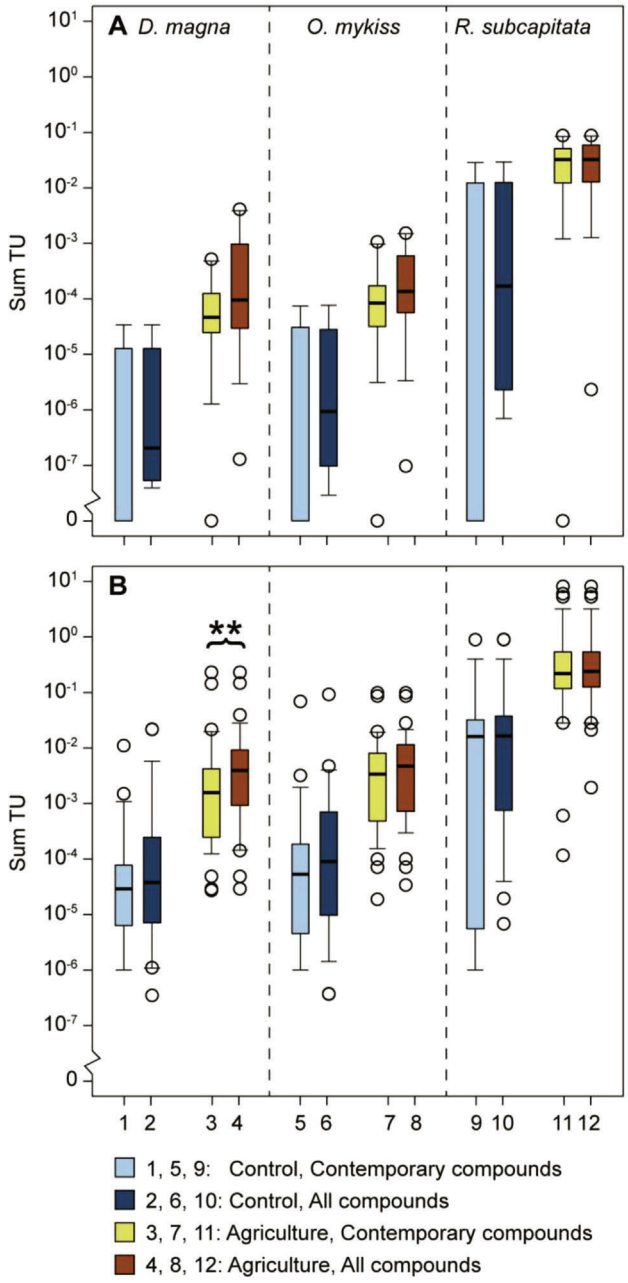

Fig. 2. Average SumTU for base-flow water samples (A) and storm flow water samples (B). SumTU is grouped according to stream category (control, n = 9; agricultural, n = 10) and according to benchmark organisms (*D. magna*, *O. mykiss* and *R. subcapitata*). Asterisks indicate significant differences in the pairwise tests at the 5% level (**). The boxplots display the median (bold line), first and third quartiles (upper and lower end of box) and the 1.5-fold interquartile range (error bars). Outliers are indicated with open circles.

lipophilic pesticides adsorbed to particles may decrease with increasing age of the pesticide-particle complex (Xu et al., 2008). Hence the predicted SumTU for sediment-dwelling organisms may be overestimated when large proportions of the pesticide-particle complexes have been long-established.

Predicting the toxicity of pesticide mixtures based on the assumption of toxic additivity (Concentration Addition, CA), as done in the present study, may be problematic when the pesticides in the sample have dissimilar Modes Of Action (MOA) (Belden et al., 2007; Cedergreen et al., 2013). However, CA appears to be a slightly

Table 2
Overview of central parameters for the pesticides monitored during base-flow and storm flow as well as in bed sediments (BS) and suspended sediments (SS). Parameter values are given ± SE for control streams (n = 9) and agricultural streams (n = 10).

Parameter	Control streams	Agricultural streams
Base-flow water samples		
Average# compounds (all)	3.1 ± 0.9	8.8 ± 1.6
Average# compounds (legacy)	2.1 ± 0.4	4.1 ± 0.9
Average sum conc. (μg L^{-1}) (all)	0.033 ± 0.014	0.192 ± 0.099
Average sum conc. (μg L^{-1}) (legacy)	0.003 ± 0.001	0.055 ± 0.045
Average $SumTU_{D.magna}$ (all)	$6.8*10^{-6} \pm 3.9*10^{-6}$	0.0007 ± 0.0004
Average $SumTU_{D.magna}$ (legacy)	$1.3*10^{-7} \pm 1.7*10^{-8}$	0.0006 ± 0.0003
Average $SumTU_{O.mykiss}$ (all)	$1.7*10^{-5} \pm 8.7*10^{-6}$	0.0004 ± 0.0002
Average $SumTU_{O.mykiss}$ (legacy)	$6.3*10^{-7} \pm 6.8*10^{-9}$	$0.0002 \pm 6.3*10^{-5}$
Average $SumTU_{P.subcapitata}$ (all)	0.006 ± 0.003	0.036 ± 0.008
Average $SumTU_{P.subcapitata}$ (legacy)	0.0002 ± 0.00008	0.002 ± 0.002
Storm flow water samples		
Average# compounds (all)	7.7 ± 0.9	21.3 ± 1.4
Average# compounds (legacy)	3.5 ± 0.3	6.9 ± 0.5
Average sum conc. (μg L^{-1}) (all)	0.277 ± 0.088	1.845 ± 0.339
Average sum conc. (μg L^{-1}) (legacy)	0.045 ± 0.015	0.129 ± 0.018
Average $SumTU_{D.magna}$ (all)	0.002 ± 0.001	0.016 ± 0.007
Average $SumTU_{D.magna}$ (legacy)	0.001 ± 0.001	0.003 ± 0.001
Average $SumTU_{O.mykiss}$ (all)	0.004 ± 0.003	0.011 ± 0.003
Average $SumTU_{O.mykiss}$ (legacy)	0.001 ± 0.001	0.001 ± 0.001
Average $SumTU_{P.subcapitata}$ (all)	0.101 ± 0.045	0.892 ± 0.292
Average $SumTU_{P.subcapitata}$ (legacy)	0.004 ± 0.002	0.012 ± 0.005
Sediment samples		
Average# compounds (BS, all)	1.3 ± 0.5	5.9 ± 1.2
Average# compounds (SS, all)	2.1 ± 0.5	6.9 ± 1.1
Average# compounds (BS, legacy)	0.9 ± 0.4	3.8 ± 0.9
Average# compounds (SS, legacy)	1.3 ± 0.4	4.2 ± 0.8
Average sum conc. (μg kg^{-1} DW) (BS, all)	6.0 ± 2.5	65.1 ± 14.2
Average sum conc. (μg kg^{-1} DW) (SS, all)	13.1 ± 3.6	167.6 ± 57.0
Average sum conc. (μg kg^{-1} DW) (BS, legacy)	2.5 ± 1.1	22.7 ± 7.6
Average sum conc. (μg kg^{-1} DW) (SS, legacy)	6.6 ± 2.8	48.4 ± 21.3
Average $SumTU_{C.riparius}$ (BS, all)	0.0003 ± 0.0001	0.141 ± 0.083
Average $SumTU_{C.riparius}$ (SS, all)	0.001 ± 0.001	0.117 ± 0.090
Average $SumTU_{C.riparius}$ (BS, legacy)	$7.8*10^{-5} \pm 2.6*10^{-5}$	0.137 ± 0.082
Average $SumTU_{C.riparius}$ (SS, legacy)	0.001 ± 0.001	0.108 ± 0.090

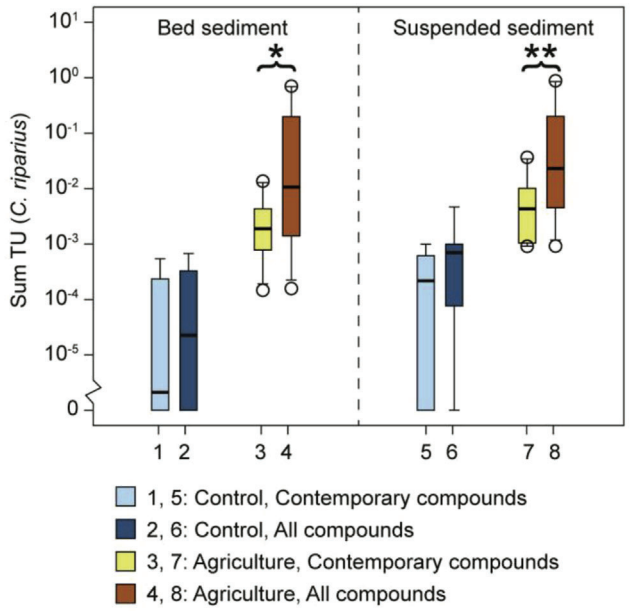

Fig. 3. Average $SumTU_{C.riparius}$ for bed sediment and suspended sediment samples. SumTU is grouped according to stream category (control, n = 9; agricultural, n = 10). Asterisks significant differences at the 10% level (*) and 5% level (**). The boxplots display the median (bold line), first and third quartiles (upper and lower end of box) and the 1.5-fold interquartile range (error bars). Outliers are indicated with open circles.

conservative and broadly applicable model for pesticide mixtures with similar, dissimilar and unknown MOAs and has a relatively small risk of underestimating the effects (Backhaus and Faust, 2012; Nowell et al., 2014). Moreover, the SumTU approach has been shown to strongly correlate with an ecological indicator for pesticide pollution (SPEAR) (Liess and von der Ohe, 2005) and provides as strong a correlation to SPEAR as other models that consider different MOAs of sample constituents, e.g. the msPAF (Schäfer et al., 2013).

3.3. Potential sources of the legacy pesticides

The majority of the legacy pesticides included in this study (e.g. organochlorines and triazines) have the potential to persist for several decades in agricultural soils to which the compounds have been applied in the past (Aliyeva et al., 2013; Manz et al., 2001). In consequence, agricultural soils may still be important sources providing continuous fluxes of legacy pesticides to freshwater ecosystems (Barth et al., 2007; Gilliom, 2007). The detection frequency of legacy pesticides was highest in base-flow water samples and sediment samples; although their concentrations increased 2–15 fold in water during storm flow (Table 2). This could indicate that a dominant source of legacy pesticides was upper soil layers in the catchments, originating from past agricultural applications, where surface runoff occurs (Manz et al., 2001). Re-suspension of contaminated sediment may have altered the partitioning between particle bound and dissolved phases of pesticides and hence could be an additional important source governing the observed increase in legacy pesticide concentrations during storm flow (Quesada

Table 3
Relative contribution of selected groups of pesticides to the SumTU based on *D. magna* for storm flow water samples and *C. riparius* for sediment samples. The values are grouped according to the stream category (control and agriculture). The median SumTU values for the respective samples are given.

	Median SumTU	Storm flow water		Suspended sediment		Bed sediment	
		Control	Agriculture	Control	Agriculture	Control	Agriculture
		<0.001	0.004	<0.001	0.011	<0.001	0.014
Contemporary pesticides	Herbicide	5.9	6.9	70.4	0.9	22.8	2.3
	Fungicide	1.5	9.5	<0.1	<0.1	<0.1	<0.1
	Pyrethroid	24.3	62.6	<0.1	1.5	8.7	5.2
	Other insecticide	<0.1	0.9	<0.1	<0.1	<0.1	<0.1
Legacy pesticides	Herbicide	<0.1	<0.1	4.0	<0.1	<0.1	0.1
	Fungicide	<0.1	<0.1	25.6	<0.1	1.1	<0.1
	Organochlorine	NA	NA	<0.1	0.2	67.4	0.8
	Organophosphate	42.0	15.4	<0.1	97.4	<0.1	91.6
	Pyrethroid	26.3	2.7	<0.1	<0.1	<0.1	<0.1
	Other insecticide	<0.1	2.0	<0.1	<0.1	<0.1	<0.1

et al., 2014). Additional sources of potential importance may include atmospheric deposition (Weber et al., 2010), point sources such as waste dumps (Aliyeva et al., 2013), industrial use and commercial products (Connor et al., 2007), and illegal private use (see McKnight et al. (2015) for a detailed description of potential sources of legacy pesticides in streams).

Since the dominant source of legacy pesticides is likely agricultural soils, we expect the flux of legacy pesticides to streams to be relatively comparable between summer and winter, i.e. peaks associated with storm events in winter would be less strong than peaks associated with the additional application of contemporary pesticides in the summer. Data from the extensive Swedish pesticide monitoring program documents that legacy pesticides are still found in stream water outside the primary crop growing season of Nordic countries (Nanos et al., 2012). Hence, in contrast to contemporary pesticides, the toxic pressure of legacy pesticides in streams is likely relatively constant across seasons, additionally indicating that the relative toxic contribution of legacy pesticides to the SumTU increases outside the primary crop growing seasons.

3.4. Identifying compounds of concern

Among the legacy pesticides, the organophosphate chlorpyrifos and organochlorines such as DDT (and degradation products) and lindane were the strongest drivers of high SumTU for daphnia, fish and sediment dwelling invertebrates, whereas diuron and the triazine herbicides (terbutylazine and simazine) were the strongest drivers of high SumTU for algae (Table S5). Chlorpyrifos is still permitted for agricultural purposes in some EU countries but has been banned in Denmark since 2008. The remaining pesticides mentioned are forbidden for agricultural purposes in the EU (DDT since 1979, lindane since 2001 (but 1994 in Denmark), simazine since 2005, diuron since 2008 and terbutylazine since 2009).

Since the legacy pesticides significantly increased the $SumTU_{D.magna}$ in storm flow water and $SumTU_{C.riparius}$ in sediments we further evaluated the relative contribution of specific groups of pesticides to $SumTU_{D.magna}$ and $SumTU_{C.riparius}$ in storm flow water and sediment samples, respectively. The $SumTU_{D.magna}$ in storm flow water was most strongly influenced by contemporary pyrethroid insecticides (62.6%) and the legacy pesticide chlorpyrifos (15.3%) in agricultural streams, whereas the $SumTU_{D.magna}$ was most strongly influenced by legacy and contemporary pyrethroid insecticides (26.3% and 24.3%, respectively) and chlorpyrifos (42%) in control streams (Table 3). The $SumTU_{C.riparius}$ of suspended sediment and bed sediment samples were almost entirely governed by chlorpyirfos in agricultural streams whereas the $SumTU_{C.riparius}$, especially for bed sediments, was more influenced by organochlorine insecticides in control streams (Table 3). Since the half-life of chlorpyrifos in aquatic sediments is proposed to be 20–180 days (Mackay et al., 2014), which is comparable to the half-lives of pyrethroids, our findings could indicate that this active ingredient is illegally used in Denmark. Alternatively, as pointed out by McKnight et al. (2015), chlorpyrifos is well-known for its ability to undergo long-range transport and/or may still be permitted for use in material protection products (e.g. as a biocide).

3.5. Conclusions

Risk assessment, the identification of pesticides of particular concern and the prioritization of mitigation activities strongly rely on monitoring data from streams, and keeping up with the increasing number of (emerging) active ingredients entering the market remains a serious challenge. However, our results suggest that increasing attention should additionally be directed towards legacy pesticides due to their predicted high impacts on the biota of especially agricultural streams. Neglecting central legacy pesticides in stream monitoring programs may underestimate the predicted toxicity of stream sediments by up to 90%. Future assessment schemes and management strategies should seek to quantify the actual toxicity of sediments containing high concentrations of legacy pesticides, and moreover seek to benchmark ecological entities of streams against more extensive pesticide screening programs, including legacy pesticides, in order to evaluate if the combined measurements of past and current use pesticides increase the explanatory power of correlations between all types of pesticides and their ecological effects. Monitoring programs should continuously re-address the status of legacy pesticides in freshwater systems to register developments in long term exposure profiles. To reduce costs, the frequency and concentration might be related to land-use history which can then be used as a proxy for potential exposure risk. Our understanding of pesticide exposure in streams needs expansion and should progress towards interpreting ecosystem responses in a temporal context where land use history is a key determinant to when and where to sample.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2015.07.021.

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Supporting information for the article: The legacy of pesticide pollution: An overlooked factor in current risk assessments of freshwater systems

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This file contains 10 pages with 3 figures and 4 tables.

Table S1. List of compounds that water samples were analysed for. The list is augmented with information regarding analytical limits of detection (LOD) and quantification (LOQ). Moreover the current usage status for Denmark and EU is indicated for each compound.

Compound	Type	LOD (µg/L)	LOQ (µg/L)	Current legal status in Denmark for agricultural use (and in EU if not allowed in Denmark)
Azoxystrobin	Fungicide	0.001	0.002	Approved
Difenokonazol	Fungicide	0.005	0.01	Approved
Epoxiconazol	Fungicide	0.005	0.01	Approved
Fenpropimorph	Fungicide	0.003	0.01	Not allowed in DK (Approved in other EU countries)
Fluazinam	Fungicide	0.002	0.01	Approved
Fuberidazole	Fungicide	0.001	0.002	Not allowed in DK (Approved in other EU countries)
Imazalil	Fungicide	0.02	0.05	Not allowed in DK (Approved in other EU countries)
Metalaxyl	Fungicide	0.001	0.002	Approved
Metrafenone	Fungicide	0.003	0.01	Approved
Penconazole	Fungicide	0.003	0.01	Not allowed in DK (Approved in other EU countries)
Prochloraz	Fungicide	0.005	0.01	Not allowed in DK (Approved in other EU countries)
Propamocarb	Fungicide	0.001	0.002	Approved
Propiconazole	Fungicide	0.005	0.01	Approved
Prothioconazole-desthio	Fungicide	0.003	0.01	Approved
Pyraclostrobin	Fungicide	0.002	0.01	Approved
2,4-D	Herbicide	0.01	0.05	Approved
Aclonifen	Herbicide	0.008	0.02	Approved
Atrazine	Herbicide	0.001	0.002	Not allowed in DK and EU
Bentazone	Herbicide	0.005	0.01	Approved
Clomazone	Herbicide	0.001	0.002	Approved
Clopyralid	Herbicide	0.005	0.05	Approved
Cycloxydim	Herbicide	0.01	0.05	Approved
Dichlorprop	Herbicide	0.005	0.01	Not allowed in DK and EU*
Diflufenican	Herbicide	0.002	0.004	Approved
Diuron	Herbicide	0.002	0.005	Not allowed in DK (approved in other EU countries)
Ethofumesate	Herbicide	0.003	0.01	Approved
Fenpropidin	Fungicide	0.002	0.01	Approved
Florasulam	Herbicide	0.005	0.01	Approved
Fludioxonil	Herbicide	0.002	0.01	Approved
Fluroxypyr	Herbicide	0.015	0.05	Approved
Hexazinone	Herbicide	0.001	0.002	Not allowed in DK and EU

Iodosulfuron-methyl	Herbicide	0.002	0.01	Approved
Isoproturon	Herbicide	0.001	0.002	Not allowed in DK (Approved in other EU countries)
MCPA	Herbicide	0.005	0.05	Approved
Mecoprop	Herbicide	0.005	0.01	Not allowed in DK (Approved in other EU countries)
Mesosulfuronmetyl	Herbicide	0.005	0.01	Approved
Metamitron	Herbicide	0.003	0.01	Approved
Metribuzin	Herbicide	0.003	0.01	Not allowed in DK (Approved in other EU countries)
Metsulfuronmetyl	Herbicide	0.002	0.01	Approved
Pendimethalin	Herbicide	0.01	0.05	Approved
Phenmedipham	Herbicide	0.001	0.002	Approved
Propyzamide	Herbicide	0.001	0.002	Approved
Prosulfocarb	Herbicide	0.01	0.05	Approved
Quinmerac	Herbicide	0.002	0.002	Not allowed in DK (Approved in other EU countries)
Rimsulfuron	Herbicide	0.002	0.01	Not allowed in DK (Approved in other EU countries)
Simazine	Herbicide	0.002	0.002	Not allowed in DK and EU
Sulfosulfuron	Herbicide	0.002	0.002	Approved
Terbuthylazine	Herbicide	0.001	0.002	Not allowed in DK (Approved in other EU countries)
Thifensulfuron-methyl	Herbicide	0.002	0.01	Approved
Tribenuron-methyl	Herbicide	0.001	0.002	Approved
Triflusulfuron-methyl	Herbicide	0.002	0.002	Approved
Alpha-cypermethrin	Insecticide	0.0005	0.005	Approved
Carbofuran	Insecticide	0.001	0.002	Not allowed in DK and EU
Chlorfenvinphos	Insecticide	0.002	0.002	Not allowed in DK and EU
Chlorpyrifos	Insecticide	0.0001	0.001	Not allowed in DK (Approved in other EU countries)
Cypermethrin	Insecticide	0.001	0.02	Approved
Deltamethrin	Insecticide	0.001	0.02	Not allowed in DK (Approved in other EU countries)
Dimethoate	Insecticide	0.002	0.002	Not allowed in DK (Approved in other EU countries)
Endosulfan-alfa	Insecticide	0.0001	0.001	Not allowed in DK and EU
Endosulfan-beta	Insecticide	0.0001	0.001	Not allowed in DK and EU
Endosulfansulfat	Insecticide	0.0001	0.001	Approved
Esfenvalerate	Insecticide	0.0003	0.003	Approved
Imidacloprid	Insecticide	0.002	0.01	Not allowed in DK (Approved in other EU countries)
Lambda-cyhalothrin	Insecticide	0.0002	0.002	Approved
Lindane	Insecticide	0.0004	0.001	Not allowed in DK and EU
Permethrin	Insecticide	0.006	0.04	Not allowed in DK and EU
Pirimicarb	Insecticide	0.001	0.002	Approved

Tau-fluvalinat	Insecticide	0.002	0.007	Approved
Thiacloprid	Insecticide	0.001	0.002	Approved
BAM	Metabolite	0.002	0.01	
Terbuthylazine-desethyl	Metabolite	0.002	0.01	

* Dichlorprop and mecoprop are still allowed in EU as the stereoisomers dichlorprop-p and mecoprop-p. The chemical analysis of these compounds does not distinguish between the forbidden and allowed stereoisomers.

Table S2. List of compounds that sediment samples were analysed for. The list is augmented with information regarding analytical limits of detection (LOD) and quantification (LOQ). LOQs are only given for the compounds that were detected in the study. Moreover the current usage status for Denmark and EU is indicated for each compound.

Compound	Type	LOD (µg/kg DW)	LOQ (µg/kg DW)	Current legal status in Denmark (and in EU if not allowed in Denmark)
Aclonifen	Herbicide	60		Approved
Alpha-cypermethrin	Insecticide	0.5	10	Approved
Atrazine	Herbicide	10		Not allowed in DK or EU
Azoxystrobin	Fungicide	60		Approved
Cyfluthrin	Insecticide	1		Approved
Cypermethrin	Insecticide	2		Approved
DDE-p,p	Metabolite	3	9	
DDD-p,p	Metabolite	3	9	
DDT-o,p	Insecticide	4	12	Not allowed in DK or EU
DDT-p,p	Insecticide	2	6	Not allowed in DK or EU
Deltamethrin	Insecticide	2		Not allowed in DK (Approved in other EU countries)
Diflufenican	Herbicide	2	7	Approved
Diuron	Herbicide	6	30	Not allowed in DK or EU
Endosulfan-alfa	Insecticide	0.1	0.5	Not allowed in DK or EU
Endosulfan-beta	Insecticide	0.1	0.5	Not allowed in DK or EU
Endosulfansulfat	Metabolite	0.2	0.5	
Esfenvalerat	Insecticide	0.3		Approved
Ethofumesat	Herbicide	3		Approved
Fenpropimorph	Fungicide	50		Not allowed in DK (Approved in other EU countries)
Hexachlorobenzen	Fungicide	0.5	3	Not allowed in DK or EU
Carbofuran	Insecticide	10		Not allowed in DK or EU
HCH-alfa	Metabolite	0.3		
Lindane	Insecticide	0.6	2	Not allowed in DK or EU
Isoproturon	Herbicide	6		Not allowed in DK (Approved in other EU countries)
Chlorfenvinphos	Insecticide	0.2		Not allowed in DK or EU
Chlorpyrifos	Insecticide	0.1	0.5	Not allowed in DK (Approved in other EU countries)
Lambda-cyhalothrin	Insecticide	0.3	5	Approved
Metalaxyl	Fungicide	30		Approved
Pendimethalin	Herbicide	10	50	Approved
Permethrin	Insecticide	10		Not allowed in DK or EU
Pirimicarb	Insecticide	6		Approved
Propiconazole	Fungicide	60		Approved

Propyzamide	Herbicide	10		Approved
Prosulfocarb	Herbicide	10		Approved
Simazine	Herbicide	10		Not allowed in DK or EU
Tau-fluvalinat	Insecticide	2	5	Approved
Terbuthylazine	Herbicide	6		Not allowed in DK (Approved in other EU countries)
Trifluralin	Herbicide	2		Not allowed in DK or EU

Table S3. Number of base-flow and storm flow water samples and suspended sediment and bulk sediment samples. The stream ID is composed of a letter indicating the agricultural intensity in the catchment (L = low agricultural intensity, H = high agricultural intensity), followed by the stream number.

	L1	L2	L3	L4	L5	L6	L7	L8	L9	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
Base-flow	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Storm flow	2	2	2	4	2	3	4	3	5	3	4	3	4	2	5	3	4	5	4
Suspended sediment	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Bulk sediment	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Table S4. Frequency of contemporary pesticides and legacy pesticides having highest SumTU for daphnia, fish, algae and chironomids (sediment samples). The number of times that the compound generated highest TU in a sample is indicated in parenthesis. Only concentrations that exceed a TU of 10^{-5} were included.

Benchmark organism	**Base-flow legacy pesticides**	**Base-flow contemporary pesticides**
D. magna	Chlorpyrifos (2)	Diflufenican (H) (2) Pirimicarb (I) (2)
O. mykiss	Clorpyrifos (I) (2) Dichlorprop* (H) (2)	Diflufenican (H) (4) Pyraclostrobin (F) (1)
R. subcapitata	Terbuthylazine (H) (5) Hexazinone (H) (5) Simazine (H) (1) Diuron (H) (1)	Diflufenican (H) (12)
	Storm flow legacy pesticides	**Storm flow contemporary pesticides**
D. magna	Chlorpyrifos (I) (16) Deltamethrin (I) (3) Carbofuran (I) (1) Dimethoate (I) (1) Diuron (H) (1)	Diflufenican (H) (25) Alpha-cypermethrin (I) (7) Lambda-cyhalothrin (I) (6) Pyraclostrobin (F) (5) Azoxystrobin (F) (4) Cypermethrin (I) (1)
O. mykiss	Dichlorprop* (H) (11) Chlorpyrifos (I) (9) Deltamethrin (I) (6) Lindane (I) (3) Endosulfan (I) (2)	Diflufenican (H) (31) Lambda-cyhalothrin (I) (7) Tau-fluvalinate (I) (6) Pyraclostrobin (F) (5) Esfenvalerate (I) (1)
R. subcapitata	Terbuthylazine (H) (26) Diuron (H) (16) Simazine (H) (5)	Diflufenican (H) (49)
	Sediment legacy pesticides	**Sediment contemporary pesticides**
C. riparius	DDT (I) (9) Chlorpyrifos (I) (8) Lindane (I) (2) Diuron (H) (2)	Diflufenican (H) (19) Alpha-cypermethrin (I) (5) Tau-fluvalinate (I) (2) Lambda-cyhalothrin (I) (1)

* Potentially not a legacy pesticide, since one stereoisomer (dichlorprop-p) is still allowed in EU and the chemical analysis does not distinguish between this and the forbidden isomers.

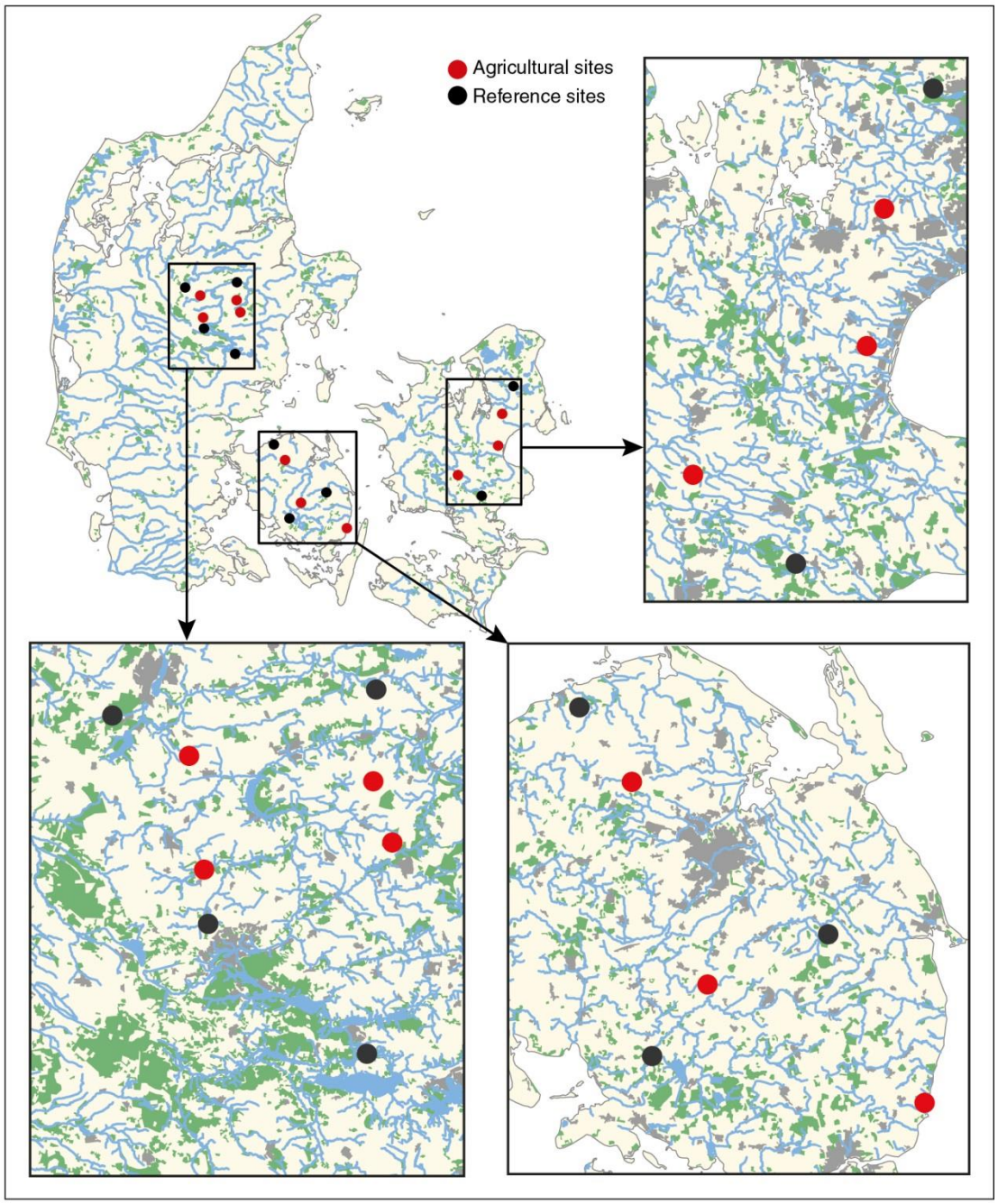

Fig. S1. Scmeatic overview of the Danish study sites.

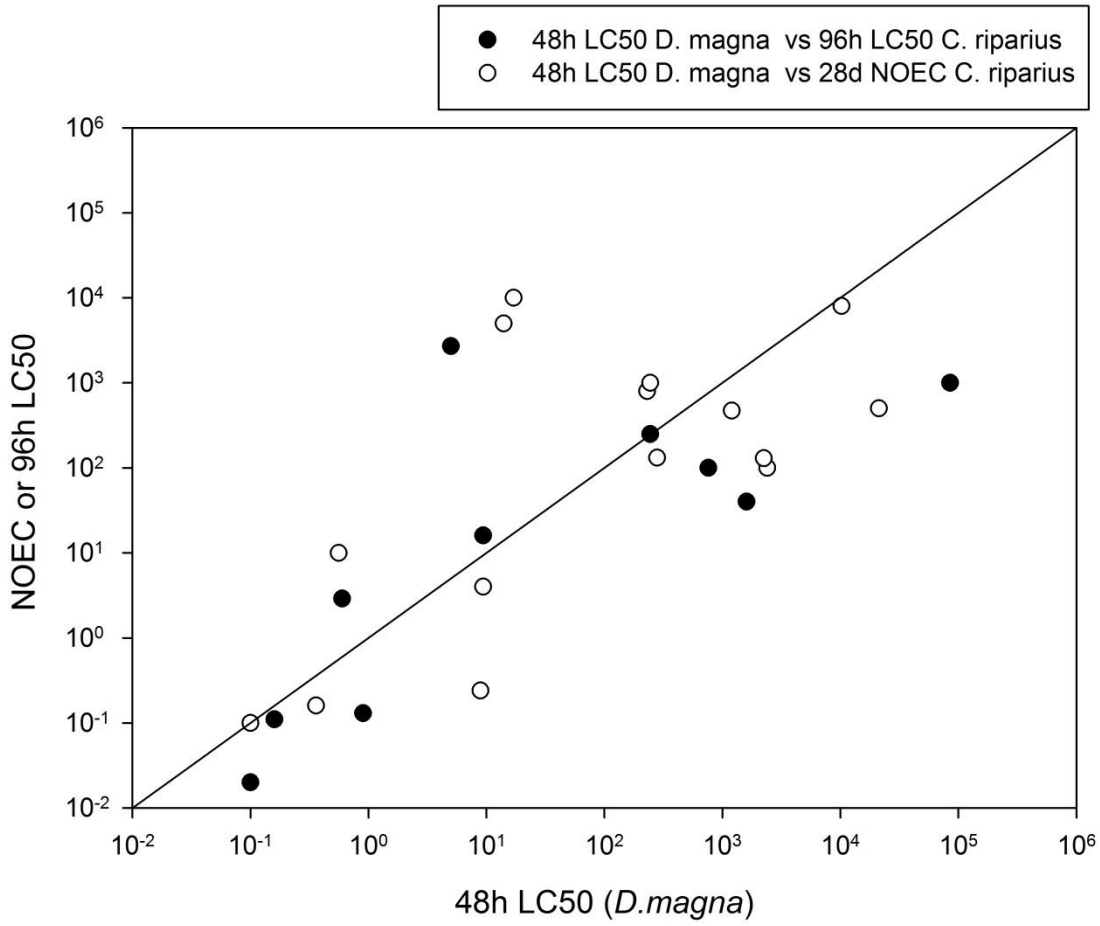

Fig. S2. Acute (filled circles) or chronic (open circles) effect concentrations for *C. riparius* as a function of 48h LC50 for *D. magna*. The diagonal line indicates the 1:1 relationship.

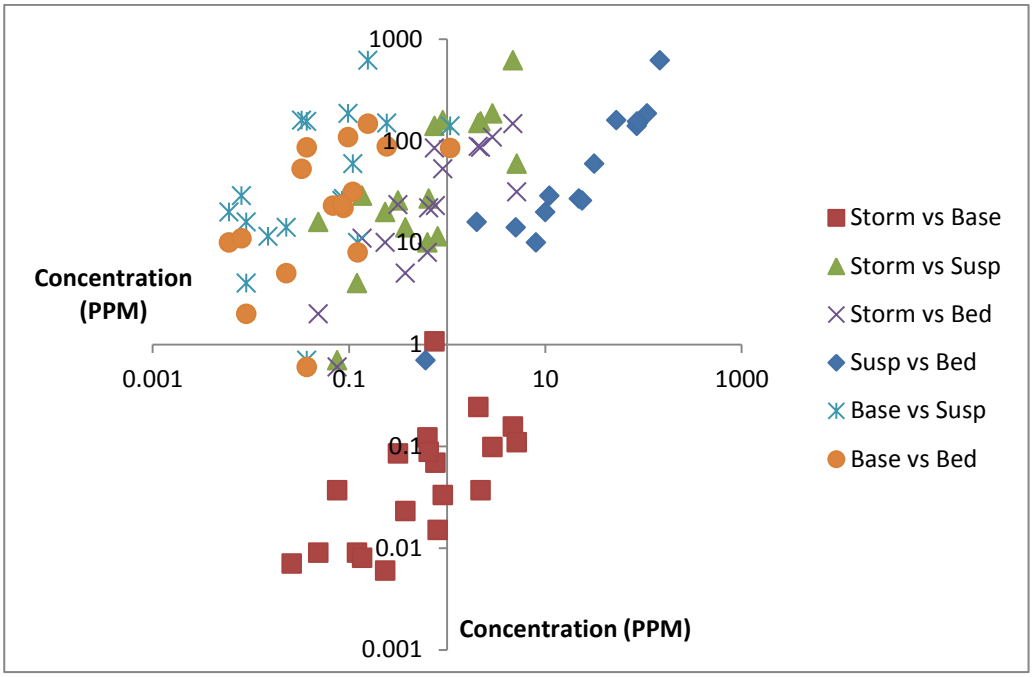

Fig. S3. Correlation between all combinations of summed pesticide concentrations in base-flow and storm flow water samples, and suspended sediment and bed sediment. Each data point for storm flow water samples represents the average concentration of all storm flow water samples in each stream.

Appendix 2. Supplementary Material

TABLE 1. Pesticide concentrations (µg L-1) in stream water in the 19 study streams during baseflow (samples collected in July 2013). Pesticide type refers to Fungicides (F), Herbicides (H), Insecticides (I), and metabolites (Met).

Pesticide	Type	C1	C2	C3	C4	C5	C6	C7	C8	C9	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
Azoxystrobin	F				0.001						0.006	0.008		0.018		0.001				
Difenkonazol	F																			
Epoxiconazol	F															0.012				
Fenpropimorph	F																			
Fluazinam	F																			
Fuberidazol	F															0.011				0.001
Imazalil	F																			
Metalaxyl	F										0.002									
Metrafenone	F																			
Penconazole	F																			
Prochloraz	F																			
Propamokarb	F																			
Propiconazole	F											0.038								
Prothioconazole	F										0.004	0.009						0.003		
Pyraklostrobin	F										0.004									
2,4-D	H																			
Aclonifen	H										0.051									
Atrazine	H					0.001							0.007							
Bentazone	H			0.004											0.032	0.023		0.062		
Clomazone	H																			
Clopyralid	H			0.011	0.018							0.068		0.011		0.01			0.007	
Cycloxydim	H			0.086																
Dichlorprop	H			0.007								0.021				0.02				
Diflufenican	H		0.002		0.007		0.004				0.021	0.012	0.003	0.014	0.009	0.011		0.006	0.003	0.007
Diuron	H											0.041								
Ethofumesate	H																			
Fenpropidin	H																			

Florasulam	H																	
Fludioxinil	H																	
Fluroxypyr	H												0.034					
Hexazinone	H		0.005	0.003	0.002						0.006					0.002	0.005	
Iodsulfuronmethyl	H																	
Isoproturon	H								0.001	0.002								
MCPA	H			0.011	0.042			0.008	0.44	0.022	0.018	0.018	0.14		0.011	0.012	0.013	
Mecoprop	H								0.33									
Mesosulfuronmethyl	H																	
Metamitrone	H																	
Metribuzine	H																	
Metsulfuronmethyl	H																	
Pendimethalin	H							0.012										
Phenmedipham	H																	
Propyzamide	H								0.012				0.001	0.002		0.004	0.002	
Prosulfocarb	H																	
Quinmerac	H																	
Rimsulfuron	H																	
Simazine	H												0.001					
Sulfosulfuron	H																	
Terbutylazine	H		0.001		0.001			0.001	0.002		0.002			0.002				
Terbuthylazinedesethyl	H		0.003		0.003		0.002	0.003	0.005				0.001	0.003	0.001	0.002		
Tifensulfuronmethyl	H																	
Triflusulfuronmethyl	H																	
Tribenuronmehtyl	H																	
Alpha-cypermethrin	I																	
Carbofuran	I																	
Chlorfenvinphos	I																	
Chlorpyrifos	I								0.0002	0.0004								
Cypermethrin	I																	

Deltamethrin	I																	
Dimethoate	I																	
Endosulfan-alfa	I																	
Endosulfan-beta	I																	
Endosulfansulfat	I										0.0002	0.0002						
Esfenvalerate	I																	
Imidacloprid	I									0.019	0.06				0.001			
Lambda-cyhalothrin	I																	
Lindane	I										0.003							
Permethrin	I																	
Pirimicarb	I									0.001			0.001				0.001	
Tau-fluvalinat	I																	
Thiacloprid	I										0.001							
BAM	Met	0.009	0.004	0.014	0.008	0.002	0.006	0.007	0.037	0.024	0.021	0.028	0.034	0.013	0.006	0.023	0.003	0.01

TABLE 2. Pesticide concentrations in stream water in the 19 study streams during stormflow. The date of the storm flow incident is indicated in the table. Pesticide type refers to Fungicides (F), Herbicides (H), Insecticides (I), and metabolites (Met).

Sampling date		24/5	24/6	24/5	24/6	22/5	25/6	22/5	28/5	18/6	25/6	22/5	28/5	16/5	27/5	18/6	16/5	27/5	5/6	24/6
Pesticide	Type	C1	C1	C2	C2	C3	C3	C4	C4	C4	C4	C5	C5	C6	C6	C6	C7	C7	C7	C7
Azoxystrobin	F		0.001		0.002	0.001	0.001	0.002	0.038	0.002	0.001			0.001		0.029			0.001	
Difenkonazol	F																			
Epoxiconazol	F															0.047				
Fenpropimorph	F																			
Fluazinam	F																			
Fuberidazol	F						0.002													
Imazalil	F																			
Metalaxyl	F						0.003													
Metrafenone	F																			
Penconazole	F																			
Prochloraz	F																			
Propamokarb	F																			0.001
Propiconazole	F					0.007														
Prothioconazole	F				0.003	0.006	0.027		0.003	0.004				0.015		0.058	0.018			0.01
Pyraklostrobin	F	0.004																		
2,4-D	H					0.15														
Aclonifen	H																			
Atrazine	H		0.001																	
Bentazone	H			0.004	0.006	0.2	0.068		0.006											
Clomazone	H					0.001		0.001	0.005		0.027									
Clopyralid	H			0.014	0.011	0.071	0.012	0.021	0.35	0.037				0.026			0.021	0.022		
Cycloxydim	H			0.005																
Dichlorprop	H	0.02		0.095	0.018	0.15	0.006	0.012	0.009	0.007										
Diflufenican	H			0.018	0.008	0.008	0.002		0.06	0.005	0.009	0.22	0.007	0.006	0.07	0.22	0.004	0.005	0.002	0.005
Diuron	H					0.19										0.003				
Ethofumesate	H																			
Fenpropidin	H																			

Florasulam	H																	
Fludioxinil	H																	
Fluroxypyr	H					0.099		0.031	0.18					0.039				
Hexazinone	H			0.004	0.005	0.002		0.003		0.003	0.003				0.003	0.003	0.004	
Iodsulfuronmethyl	H																	
Isoproturon	H					0.001									0.004	0.003	0.003	0.003
MCPA	H	0.037		0.054	1.3	0.13	0.034	0.24	1.2	0.11			0.011	0.017		0.12	0.035	0.02
Mecoprop	H							0.01	0.007								0.01	
Mesosulfuronmethyl	H																	
Metamitrone	H			0.007	0.007								0.004		0.005			
Metribuzine	H																	
Metsulfuronmethyl	H																	
Pendimethalin	H																	
Phenmedipham	H																	
Propyzamide	H	0.001		0.003		0.024	0.003	0.002	0.002				0.004	0.008	0.003	0.002	0.001	0.001
Prosulfocarb	H											0.011						
Quinmerac	H					0.005					0.051							
Rimsulfuron	H																	
Simazine	H																	
Sulfosulfuron	H																	
Terbutylazine	H		0.006		0.01	0.002	0.005	0.002		0.016	0.003			0.02		0.001		0.015
Terbuthylazinedesethyl	H		0.007		0.022	0.006	0.036	0.004		0.021	0.01			0.098		0.004		0.19
Tifensulfuronmethyl	H																	
Triflusulfuronmethyl	H					0.002		0.004	0.019									
Tribenuronmehtyl	H																	
Alpha-cypermethrin	I													0.0008				
Carbofuran	I																	
Chlorfenvinphos	I																	
Chlorpyrifos	I											0.002						
Cypermethrin	I																	

Deltamethrin	I												0.006		0.001		
Dimethoate	I																0.003
Endosulfan-alfa	I																
Endosulfan-beta	I																
Endosulfansulfat	I										0.0001						
Esfenvalerate	I												0.006				
Imidacloprid	I	0.007															
Lambda-cyhalothrin	I						0.0002			0.0002		0.0002					
Lindane	I																
Permethrin	I																
Pirimicarb	I																
Tau-fluvalinat	I												0.005				
Thiacloprid	I					0.004	0.026										
BAM	Met	0.006	0.007	0.005	0.004	0.008	0.008	0.011	0.013			0.002	0.003	0.005	0.005	0.006	0.005

TABLE 2 continued

Sampling date		16/5	27/5	5/6	16/5	27/5	10/6	24/6	1/7	24/5	24/6	11/7	21/5	24/5	24/6	12/7	24/5	24/6	12/7	22/5
Pesticide	Type	C8	C8	C8	C9	C9	C9	C9	C9	A1	A1	A1	A2	A2	A2	A2	A3	A3	A3	A4
Azoxystrobin	F									0.02	0.036	0.012	0.006	0.007	0.009	0.008	0.003	0.006	0.002	0.014
Difenkonazol	F																			
Epoxiconazol	F									0.16	0.084	0.027	0.006		0.008		0.007	0.006	0.009	0.1
Fenpropimorph	F																			
Fluazinam	F																			
Fuberidazol	F																			
Imazalil	F																			
Metalaxyl	F									0.003	0.005	0.003								
Metrafenone	F									0.22	0.055	0.006					0.003			0.016
Penconazole	F																			
Prochloraz	F																			
Propamokarb	F											0.001								
Propiconazole	F									0.014	0.039	0.007	0.029	0.038	0.038	0.043	0.013	0.01	0.007	0.023
Prothioconazole	F	0.009							0.008	0.003	0.13	0.096	0.004	0.004	0.016	0.017	0.004	0.015	0.026	0.027
Pyraklostrobin	F									0.44	0.2	0.027	0.003				0.005	0.003		0.027
2,4-D	H					0.017				0.033			0.019	0.039		0.028	0.02	0.018		0.062
Aclonifen	H									0.032		0.12								
Atrazine	H				0.001		0.001						0.001	0.002			0.002	0.001	0.002	0.001
Bentazone	H			0.007									0.005	0.005			0.015	0.025	0.013	0.019
Clomazone	H									0.002	0.002		0.002	0.002			0.001	0.001		0.08
Clopyralid	H	0.021								0.5	0.1	0.011	0.1	0.18	0.077	0.062	0.078	0.044	0.01	0.220
Cycloxydim	H																			0.016
Dichlorprop	H												0.043	0.031	0.017	0.021		0.03		
Diflufenican	H		0.003							0.13	2	0.13	0.009	0.012	0.023	0.019	0.025	0.045		1.3
Diuron	H												0.026	0.023	0.056	0.043				0.1
Ethofumesate	H										0.003									0.026
Fenpropidin	H																			0.004

Florasulam	H																			0.012
Fludioxinil	H																			
Fluroxypyr	H									1.6	0.14		0.021	0.026		0.021	0.039	0.013		0.05
Hexazinone	H																0.002	0.002	0.003	
Iodsulfuronmethyl	H																			0.013
Isoproturon	H												0.008	0.01	0.002	0.003	0.004		0.001	0.003
MCPA	H			0.013		0.12				6.3	0.47	0.036	0.22	0.39	0.25	0.021	0.26	1.2	0.14	2.4
Mecoprop	H					0.027				0.005	0.025	0.006	0.03	0.049	0.026	0.016		0.005		0.011
Mesosulfuronmethyl	H																			
Metamitrone	H	0.004								0.004			0.006	0.008			0.011			0.068
Metribuzine	H																			
Metsulfuronmethyl	H																			
Pendimethalin	H									0.081	0.084	0.013								
Phenmedipham	H																			0.004
Propyzamide	H		0.002			0.002			0.001	0.004	0.002		0.013	0.014	0.022	0.008	0.005	0.002	0.001	0.18
Prosulfocarb	H									0.011	0.01									0.23
Quinmerac	H																			
Rimsulfuron	H																			
Simazine	H				0.001		0.001						0.001	0.002	0.001			0.001		0.005
Sulfosulfuron	H									0.002										
Terbutylazine	H							0.002			0.021	0.005			0.009	0.011		0.01	0.004	0.013
Terbuthylazinedesethyl	H		0.002	0.003		0.004		0.027	0.011		0.14	0.012			0.022	0.037		0.048	0.014	0.019
Tifensulfuronmethyl	H																			
Triflusulfuronmethyl	H										0.002		0.002	0.002			0.003			0.004
Tribenuronmehtyl	H																			
Alpha-cypermethrin	I									0.035	0.025	0.001								
Carbofuran	I																			
Chlorfenvinphos	I																			
Chlorpyrifos	I									0.0001	0.0002		0.0002	0.0003	0.0004	0.0002	0.002	0.001	0.0005	
Cypermethrin	I									0.025	0.008									0.004

Deltamethrin	I																0.001	0.002		
Dimethoate	I									0.053	0.07		0.15	0.055						0.001
Endosulfan-alfa	I																			
Endosulfan-beta	I																			
Endosulfansulfat	I															0.0004			0.0001	
Esfenvalerate	I																			
Imidacloprid	I									0.026	0.01	0.002	0.11	0.079	0.066	0.076	0.005			
Lambda-cyhalothrin	I										0.004	0.0003					0.001	0.0005	0.0004	
Lindane	I									0.0004			0.001	0.001	0.0006	0.0009				0.0004
Permethrin	I																			
Pirimicarb	I									0.003	0.026	0.003		0.001		0.002				0.006
Tau-fluvalinat	I																			
Thiacloprid	I												0.003	0.005	0.002	0.003	0.002		0.002	0.076
BAM	Met	0.005	0.003	0.006	0.034	0.015	0.033	0.035	0.038	0.022	0.016	0.006	0.022	0.015	0.028	0.018	0.015	0.016	0.007	0.01

TABLE 2 continued

Sampling date		28/5	18/6	25/6	28/5	18/6	18/5	22/5	28/5	18/6	25/6	6/5	16/5	27/5	16/5	27/5	5/6	24/6	16/5	27/5
Pesticide	Type	A4	A4	A4	A5	A5	A6	A6	A6	A6	A6	A7	A7	A7	A8	A8	A8	A8	A9	A9
Azoxystrobin	F	0.002	0.014	0.007	0.006	0.024	0.009	0.004	0.003	0.012	0.008	0.003		0.003	0.001		0.003		0.004	0.004
Difenkonazol	F																			
Epoxiconazol	F	0.039	0.026	0.03		0.01	0.061	0.059		0.011	0.064								0.016	0.039
Fenpropimorph	F											0.004								
Fluazinam	F																			
Fuberidazol	F										0.009				0.002	0.002				
Imazalil	F																			
Metalaxyl	F					0.001														
Metrafenone	F	0.02	0.005	0.004			0.054	0.035	0.032	0.007									0.004	0.005
Penconazole	F					0.009														
Prochloraz	F					0.006														
Propamokarb	F		0.001			0.001														
Propiconazole	F	0.009	0.028	0.014	0.008	0.013	0.01	0.008	0.009							0.005	0.005		0.006	0.014
Prothioconazole	F	0.004	0.019	0.032	0.004	0.025	0.055	0.006	0.006	0.011	0.015	0.014			0.009				0.042	0.046
Pyraklostrobin	F	0.003	0.02	0.024		0.004	0.002			0.003	0.006									
2,4-D	H	0.023						0.05	0.018										0.018	0.046
Aclonifen	H																			
Atrazine	H	0.008						0.002	0.002										0.001	0.002
Bentazone	H	0.28	0.016	0.022	0.97	0.28	0.012	0.053	0.35	0.022	0.017			0.006	0.016	0.032	0.52			0.11
Clomazone	H	0.003	0.003	0.004	0.002	0.002	0.16	0.25	0.15	0.003	0.005				0.023				0.008	0.016
Clopyralid	H	0.410	0.1	0.021	0.140	0.046		0.26	0.59	0.097	0.021	0.014		0.042		0.014	0.043		0.33	0.59
Cycloxydim	H																			
Dichlorprop	H							0.11											0.011	
Diflufenican	H	0.13	0.67	1.5	0.05	0.043	0.14		0.29	0.12	0.064		0.007	0.007	0.037	0.052	0.035	0.034	0.07	0.12
Diuron	H	0.01		0.006	0.034	0.017	0.005	0.004			0.006	0.055							0.002	0.003
Ethofumesate	H	0.003	0.052	0.003		0.004	0.004													
Fenpropidin	H																			

Florasulam	H																	
Fludioxinil	H														0.017			
Fluroxypyr	H	0.31	0.038	0.036	0.34	5.6	0.054	0.061	0.26	0.042	0.023	0.01	0.1			0.031		0.1
Hexazinone	H						0.005	0.007	0.005	0.007				0.003		0.002	0.003	0.005
Iodsulfuronmethyl	H					0.024		0.014	0.004			0.004						0.002
Isoproturon	H	0.001					0.007	0.002	0.002	0.002					0.002		0.013	0.036
MCPA	H	0.75	1.1	0.085	1.5	0.58	1	0.46	1.5	0.51	0.12	0.07	0.65	0.041	0.21	0.026	0.11	0.46
Mecoprop	H	0.033	0.01			0.007	0.006		0.016	0.014	0.006							
Mesosulfuronmethyl	H						0.039	0.021	0.018			0.042	0.019				0.009	0.016
Metamitrone	H	0.003	0.059	0.006	0.004	0.008	0.21	0.39	0.16			0.003		0.012	0.025	0.009	0.008	
Metribuzine	H																	
Metsulfuronmethyl	H				0.003				0.004			0.008				0.003	0.002	
Pendimethalin	H							1.1	0.1									
Phenmedipham	H		0.005															
Propyzamide	H	0.019	0.008	0.006	0.016	0.007	0.095	0.17	0.062	0.01	0.012	0.009	0.005	0.001	0.001	0.001	0.59	2.2
Prosulfocarb	H		0.05	0.024			0.029			0.019								0.02
Quinmerac	H						0.007						0.003					
Rimsulfuron	H																	
Simazine	H		0.001		0.044	0.026	0.001	0.001	0.002		0.033			0.003	0.005	0.003	0.25	0.035
Sulfosulfuron	H	0.051			0.004		0.007	0.016	0.007									
Terbutylazine	H		0.046	0.011		0.048	0.044	0.005		0.019	0.007				0.001			0.006
Terbuthylazinedesethyl	H		0.058	0.072		0.096	0.039	0.009		0.033	0.032		0.002		0.004			0.017
Tifensulfuronmethyl	H				0.041													
Triflusulfuronmethyl	H		0.001				0.002	0.002	0.011			0.014	0.018		0.004		0.001	0.02
Tribenuronmehtyl	H																	
Alpha-cypermethrin	I						0.0007											0.0006
Carbofuran	I						0.11					0.001						
Chlorfenvinphos	I																	
Chlorpyrifos	I						0.002		0.001	0.0005	0.0005						0.0001	
Cypermethrin	I																	

Deltamethrin	I						0.002												
Dimethoate	I				0.079	0.014				0.001									0.002
Endosulfan-alfa	I																		
Endosulfan-beta	I						0.0001												
Endosulfansulfat	I						0.0003		0.0002	0.0002									
Esfenvalerate	I																		
Imidacloprid	I	0.002					0.003		0.004						0.006			0.028	
Lambda-cyhalothrin	I						0.0003			0.0006									
Lindane	I						0.0005												
Permethrin	I																		
Pirimicarb	I	0.002	0.012	0.002	0.014	0.016	0.001	0.001		0.001									
Tau-fluvalinat	I										0.005								0.003
Thiacloprid	I	0.004	0.002	0.002			0.011	0.014	0.009	0.001		0.002						0.01	0.004
BAM	Met	0.009	0.016	0.022	0.012	0.015	0.005	0.014	0.01	0.014	0.015	0.008	0.005	0.005	0.006	0.006	0.005	0.005	0.006

TABLE 2 continued

Sampling date		5/6	17/6	24/6	16/5	27/5	5/6	24/6
Pesticide	Type	A9	A9	A9	A10	A10	A10	A10
Azoxystrobin	F		0.021	0.015	0.006	0.001	0.004	0.001
Difenkonazol	F							
Epoxiconazol	F		0.064	0.049	0.007		0.023	
Fenpropimorph	F							
Fluazinam	F							
Fuberidazol	F							
Imazalil	F							
Metalaxyl	F							
Metrafenone	F		0.005		0.004			
Penconazole	F							
Prochloraz	F							
Propamokarb	F							0.001
Propiconazole	F		0.017	0.014	0.009	0.007	0.007	0.006
Prothioconazole	F		0.037	0.037	0.055		0.006	0.017
Pyraklostrobin	F		0.076	0.017			0.01	
2,4-D	H					0.01		
Aclonifen	H							
Atrazine	H						0.002	
Bentazone	H		0.091	0.054		0.006	0.02	
Clomazone	H		0.008	0.009				
Clopyralid	H		0.47	0.2		0.033	0.01	0.011
Cycloxydim	H							
Dichlorprop	H		0.005	0.012			0.006	
Diflufenican	H	0.098	0.098	0.19	0.037	0.082	0.46	0.055
Diuron	H		0.003	0.002	0.19	0.09	0.063	0.006
Ethofumesate	H		0.004	0.003	0.003			
Fenpropidin	H							

Florasulam	H							
Fludioxinil	H							
Fluroxypyr	H		0.47	0.37		0.041	0.091	0.012
Hexazinone	H		0.003	0.003		0.001		0.001
Iodsulfuronmethyl	H		0.01	0.01	0.003	0.003	0.006	
Isoproturon	H		0.031	0.058				
MCPA	H		0.79	0.067		1.3	0.22	0.09
Mecoprop	H							
Mesosulfuronmethyl	H							
Metamitrone	H				0.014			
Metribuzine	H							
Metsulfuronmethyl	H							
Pendimethalin	H							
Phenmedipham	H							
Propyzamide	H		0.83	0.64	0.008	0.005	0.006	0.003
Prosulfocarb	H	0.018	0.018					
Quinmerac	H							
Rimsulfuron	H							
Simazine	H		0.021	0.029			0.24	0.004
Sulfosulfuron	H							
Terbutylazine	H		0.029	0.043				0.018
Terbuthylazinedesethyl	H		0.041	0.27		0.003		0.18
Tifensulfuronmethyl	H		0.009	0.004				
Triflusulfuronmethyl	H		0.017	0.017	0.001			
Tribenuronmehtyl	H							
Alpha-cypermethrin	I	0.0006	0.0006	0.005	0.0005			
Carbofuran	I							
Chlorfenvinphos	I							
Chlorpyrifos	I				0.0002			
Cypermethrin	I							

Deltamethrin	I						0.004	
Dimethoate	I					0.001		
Endosulfan-alfa	I							
Endosulfan-beta	I							
Endosulfansulfat	I						0.0001	
Esfenvalerate	I							
Imidacloprid	I						0.005	
Lambda-cyhalothrin	I							
Lindane	I							
Permethrin	I							
Pirimicarb	I							
Tau-fluvalinat	I	0.003	0.003	0.004		0.004	0.005	
Thiacloprid	I		0.002	0.001	0.003			
BAM	Met		0.008	0.006	0.019	0.022	0.032	0.026

TABLE 3. Pesticide concentrations (µg kg^{-1} DW) in suspended sediment collected with passive samplers during May-August. Pesticide type refers to Fungicides (F), Herbicides (H), Insecticides (I), and metabolites (Met). The sample from A3 was lost.

Pesticide	Type	C1	C2	C3	C4	C5	C6	C7	C8	C9	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
Aclonifen	H										210									
Alpha-cypermethrin	I										16			0.9	2		2		2	
Atrazine	H																			
Azoxystrobin	F																			
Cyfluthrin	I																			
Cypermethrin	I										4									
DDE-p,p	Met				6			11			120	7		6	5	9.1			7	
DDD-p,p	Met										12	4				3				
DDT-o,p	I										6									
DDT-p,p	I		3		4			7.8			46			4	5	7.2			6	
Deltamethrin	I																			
Diflufenican	H		6	6	15	4	19	6			110	29		160	40	79	6	25	48	130
Diuron	H										20	89		10	7					28
Endosulfan-alfa	I											0.2								
Endosulfan-beta	I											0.46								
Endosulfansulfat	Met											0.68								
Esfenvalerat	I																			
Ethofumesate	H																			
Fenpropimorph	F																			
Hexachlorobenzene	F			1	2		1	4		0.7	0.6	2		0.8	0.6	0.7	3.1	1	2	2
Carbofuran	I																			
HCH-alfa	Met																			
Lindane	I	16	2.5								1	7.3								
Isoproturon	H																			
Chlorfenvinphos	I																			
Chlorpyrifos	I											0.92			0.2	11			0.1	
Lambda-cyhalothrin	I			3							1			0.5						

Metalaxyl	F							
Pendimethalin	H			72			30	
Permethrin	I							
Pirimicarb	I							
Propiconazole	F							
Propyzamid	H							
Prosulfocarb	H							
Simazine	H							
Tau-fluvalinat	I				3	9.2	3	90
Terbutylazine	H							
Trifluralin	H							

TABLE 4. Pesticide concentrations (µg kg^{-1} DW) in bed sediment collected in August. Pesticide type refers to Fungicides (F), Herbicides (H), Insecticides (I), and metabolites (Met).

Pesticide	Type	C1	C2	C3	C4	C5	C6	C7	C8	C9	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
Aclonifen	H																			
Alpha-cypermethrin	I										5				1	0.7				
Atrazine	H																			
Azoxystrobin	F																			
Cyfluthrin	I																			
Cypermethrin	I										2									
DDE-p,p	Met			5	3			6			23	4			4	12			5	
DDD-p,p	Met										5	3				3			3	
DDT-o,p	I																			
DDT-p,p	I				3						9.3				2	5			4	
Deltamethrin	I												3							
Diflufenican	H			3	15	10	10	3			67	17	7	88	23	37	4	23	38	42
Diuron	H										25	58		20						10
Endosulfan-alfa	I											0.1				0.2				
Endosulfan-beta	I											0.2				0.1				
Endosulfansulfat	Met											0.3				0.1				
Esfenvalerat	I																			
Ethofumesate	H																			
Fenpropimorph	F																			
Hexachlorobenzene	F	2			1			2		0.6		1	2.8	0.7	0.5	0.7	1	0.6	1	1
Carbofuran	I																			
HCH-alfa	Met																			
Lindane	I											1								
Isoproturon	H																			
Chlorfenvinphos	I																			
Chlorpyrifos	I											0.54	7.2		0.9	8.8				
Lambda-cyhalothrin	I										0.6		3			0.3				
Metalaxyl	F																			

Pendimethalin	H		10	12	
Permethrin	I				
Pirimicarb	I				
Propiconazole	F				
Propyzamid	H				
Prosulfocarb	H				
Simazine	H				
Tau-fluvalinat	I			8	35
Terbutylazine	H				
Trifluralin	H				

[Bagside Overskrift]
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