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Indication of pesticide effects and recolonization in streams

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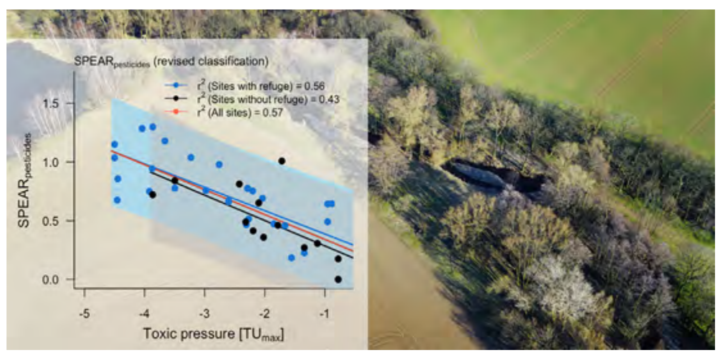
HIGHLIGHTS

- Refuge taxa are identified depending on the presence of nearby refuge areas, independently of the level of pesticide pressure.
- Stressor specificity of $SPEAR_{pesticides}$ is increased by including information on refuge taxa.
- Bio-indicator $SPEAR_{refuge}$ is derived to assess the level of general recolonization in a given stream section.

GRAPHICAL ABSTRACT



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ABSTRACT

The agricultural use of pesticides leads to environmentally relevant pesticide concentrations that cause adverse effects on stream ecosystems. These effects on invertebrate community composition can be identified by the bio-indicator $SPEAR_{pesticides}$. However, refuge areas have been found to partly confound the indicator. On the basis of three monitoring campaigns of 41 sites in Central Germany, we identified 11 refuge taxa. The refuge taxa, mainly characterized by dispersal-based resilience, were observed only nearby uncontaminated stream sections and independent of the level of pesticide pressure. Through incorporation of this information into the revised $SPEAR_{pesticides}$ indicator, the community structure specifically identified the toxic pressure and no longer depended on the presence of refuge areas. With regard to ecosystem functions, leaf litter degradation was predicted by the revised $SPEAR_{pesticides}$ and the median water temperature at a site ($R^2 = 0.38$, $P = 0.003$). Furthermore, we designed the bio-indicator $SPEAR_{refuge}$ to quantify the magnitude of general recolonization at a given stream site. We conclude that the taxonomic composition of aquatic invertebrate communities enables a specific indication of anthropogenic stressors and resilience of ecosystems.

1. Introduction

The intensive use of pesticides worldwide results in high pesticide concentrations in streams (Malaj et al., 2014; Stehle and Schulz, 2015) and causes negative effects on the structure (Liess and von der Ohe, 2005; Münze et al., 2017), ecological functions (Schäfer et al., 2012) and biodiversity (Beketov et al., 2013) of freshwater communities. This widespread degradation of aquatic ecosystems is further accelerated by climate change, increasing exposure to agricultural pesticides (Kattwinkel et al., 2011) and increasing pesticide vulnerability of populations by unfavorable temperatures (Dinh et al., 2016).

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The use of effect-based indicators is a powerful tool for identifying the toxic pressure of pesticides. The invertebrate-based indicator $SPEAR_{pesticides}$ uses trait information of taxa to identify pesticide pressure and the ecological effects in streams. $SPEAR_{pesticides}$ was developed by Liess and von der Ohe (2005) and successfully applied to indicate pesticide pressure in streams of different geographical regions worldwide including Europe (Schäfer et al., 2007; Liess et al., 2008; Orlinskiy et al., 2015; Münze et al., 2015; Münze et al., 2017), Russia (Beketov and Liess, 2008), Australia (Schäfer et al., 2011), USA (Chiu et al., 2016) and South America (Hunt et al., 2017). However, as described in several studies (Orlinskiy et al., 2015; Liess and von der Ohe, 2005), the indicator value of $SPEAR_{pesticides}$ not only is related to the toxic pressure but also is partly confounded by the presence of uncontaminated stream sections. Such stream sections serve as refuge areas for those invertebrates that recolonize affected stream sections mainly by downstream drift. The influence of refuge areas on toxicant effects has been observed particularly under weak to medium pesticide pressure (Orlinskiy et al., 2015; Liess and von der Ohe, 2005). In contrast, high pesticide pressure impeded the successful recolonization of refuge taxa. Consequently, the presence of refuge areas was taken into account to accurately predict the toxic pressure of pesticides with the $SPEAR_{pesticides}$ indicator system.

The influence of external sources of recolonizing organisms on disturbed communities has also been recognized in experimental freshwater studies. For example, in a mesocosm experiment, Caquet et al. (2007) have shown that isolation from external populations highly delays the recovery of sensitive insects from pesticide exposure. Trekels et al. (2011) have concluded from a semi-field study with the insecticide endosulfan that external recovery via adult dispersal is particularly important for univoltine compared with multivoltine aquatic invertebrates. Despite the knowledge on the general effects of refuge areas, it is unknown which taxa in freshwater streams are strongly linked to the presence of refuge areas under pesticide exposure and which traits mainly characterize those taxa. However, this knowledge is crucial to increase the stressor specificity and spatial independence of indicators such as $SPEAR_{pesticides}$.

The aim of the present study was to improve the predictive quality of the $SPEAR_{pesticides}$ indicator by disentangling ecological pesticide effects from the compensatory influence of refuges. For this, we compiled data from three different sampling campaigns (41 sampling sites) between 1998 and 2013 in Central Germany, including data on pesticide exposure and macroinvertebrate community composition. The compiled field data are based on already published field campaigns (Liess and von der Ohe, 2005; Münze et al., 2017) and were complemented with so far unpublished field data collected in 2013. In addition, we aimed at understanding the influence of refuge areas on functional endpoints and to design an indicator, $SPEAR_{refuge}$, to assess the magnitude of general recolonization from nearby refuge areas.

2. Methods

2.1. Study area and data basis

The field data on pesticide exposure and macroinvertebrate communities were derived from three different sampling campaigns covering 41 field sites in the region of Braunschweig, Central Germany (Fig. 1). The first campaign comprised the monitoring of 18 field sites, which were investigated between 1998 and 2000 (Liess and von der Ohe, 2005). This study provides information over 35 sampling years, as some sites were monitored repeatedly up to three years. The second (Münze et al., 2017) and third field campaign comprised 7 and 16 sites, respectively. The sites from the second and third campaign were monitored in 2013. During the second field campaign sites upstream and downstream of waste water treatment plants were monitored. However, we only considered the upstream sites for the present study.

2.2. Pesticide monitoring

In the first field campaign pesticide exposure was assessed by applying event-driven samplers (EDS) to capture peak concentrations (Liess et al., 1999; Liess and von der Ohe, 2005). For the field campaigns in 2013, exposure concentrations were quantified by EDS and passive samplers. Passive samplers yield time-weighted average concentrations, which were converted to similar results of peak exposure by using correction factors (Münze et al., 2015; Münze et al., 2017). The detailed sampling with EDS and passive samplers for all field samplings has been described by Liess and von der Ohe (2005) and Münze et al. (2017), respectively. In brief, EDS consisted of two 1 L glass bottles fixed on a stainless steel rod with an opening at 5 cm of the lower bottle and approximately 10–15 cm of the second bottle above the water level, depending on the normal expected rise in water level, which was determined empirically at each sampling site. The bottles filled with water as the water level rose after rainfall events of at least 10 mm/day. Bottles from EDS were checked weekly (first field campaign) or collected within 48 h after each rainfall event (second and third field campaign). The Chemcatcher® passive samplers consisted of an Empore SDB-RPS (styrene-divinylbenzene reversed-phase sulfonate) extraction disk from 3 M (St. Paul, MN, USA) as the receiving phase and a polyethersulfone PES (pore size of 0.2 μm) from Pall (Dreieich, Germany) as a diffusion-limiting membrane (Münze et al., 2015; Shaw et al., 2009). This configuration has previously been used to successfully capture polar and semi-polar pesticides (Münze et al., 2015; Schäfer et al., 2008a, 2008b). The deployment of the Chemcatchers® lasted 1 to 2 weeks, and the devices were collected after strong rainfall events. The present data comprises only data on pesticide concentrations in the water phase. Therefore, we could not analyse the influence of particle bound pesticides. However, previous studies showed a good relation of peak water concentrations with ecological effects compared to particle bound concentrations (Schäfer et al., 2008c).

2.3. Pesticide analysis and quantification of exposure

The compiled field campaigns include information on exposure and effect from different regions and years. For each study the authors considered the pesticide most relevant for the location and time. This information was obtained from the respective plant protection agencies (see also Münze et al., 2017; Liess and von der Ohe, 2005) and complemented with compounds, which are frequently found in agricultural water samples (J. Kreuger, personal correspondence 2013). The list of targeted and detected compounds is presented in Table S2. The field campaigns screened water samples for a total of 21 (Liess and von der Ohe, 2005) or 88 pesticide compounds (Münze et al., 2017) comprising also legacy pesticides and degradation products (Table S2).

Compounds from the first field campaign (Liess and von der Ohe, 2005) were determined with a gas chromatograph (GC)/electron capture detector (GC NP 5990, Series II; Hewlett-Packard, Avondale, PA, USA) and subsequently confirmed by GC/mass spectrometry (negative chemical ionization, a Varian 3400 GC [Walnut Creek, CA, USA]). To measure the polar and semi-polar compounds ($K_{ow} < 4.5$) sampled during the field campaigns in 2013, aliquots of untreated water samples and Chemcatcher® extracts (Münze et al., 2017) were sent to the Swedish University of Agricultural Sciences (Uppsala, Sweden), where they were analysed by online solid phase extraction coupled with HPLC-MS/MS. The analysis was performed according to the method reported by Jansson and Kreuger (2010), with slight modifications. For the determination of non-polar pyrethroids in water, water samples with a volume of approximately 1 L were filtered through a fibre microfilter (Whatman GF/F, 142 mm) and extracted using a column processor (JT Baker SPE-12G, PA USA) via solid phase extraction (SPE) with Chromabond® C18 SPE cartridges (Macherey-Nagel, Düren, Germany). The cartridges were then frozen at −18 °C for transportation. The analysis was performed on a 7890A GC equipped with a multimode inlet

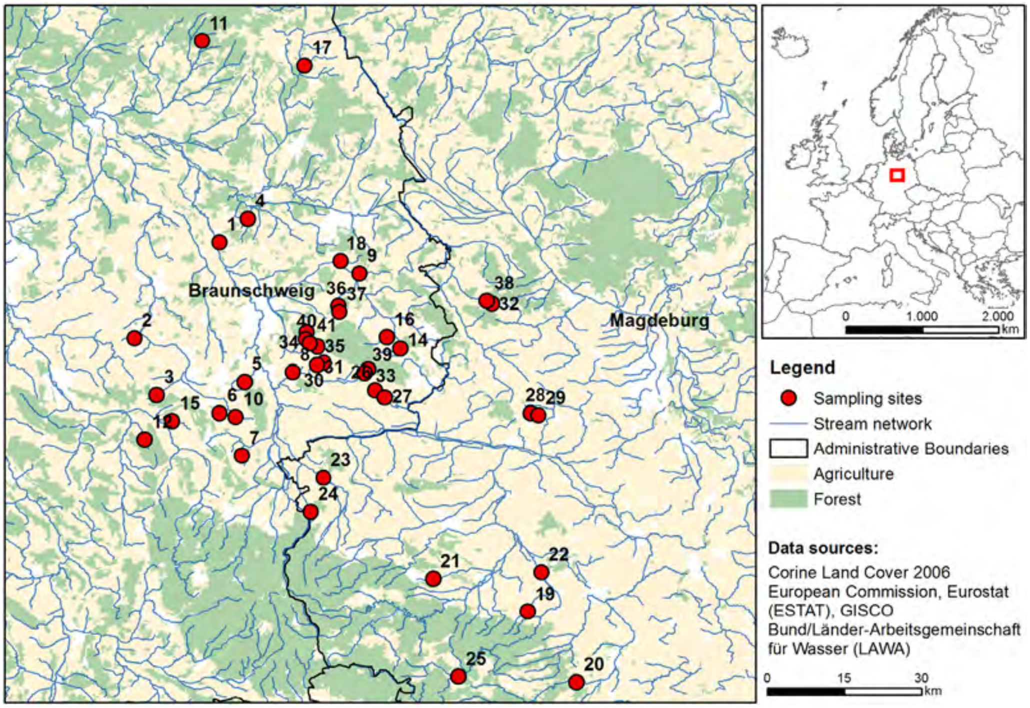

Fig. 1. Location of the 41 monitoring sites between 1998 and 2013 in Central Germany (see also, Table S1).

operated in solvent vent mode and coupled to a 5975C mass detector (Agilent Technologies, Palo Alto, CA, USA). Details regarding extraction and analysis have been described by Münze et al. (2017). Subsequently, all pesticide concentrations from EDS and passive samplers were converted to Toxic Units (TU; Sprague, 1970). To quantify the pesticide toxicity per site and sampling season, we determined TU_{max} (Liess and von der Ohe, 2005, see also Table S1). We further determined TU_{sum} to account for additive effects of the whole mixture per site and season, which yielded very similar results to TU_{max} (Fig. S5). Therefore, we focussed in the following analyses on TU_{max}.

$$TU_{\max} = \max_{i=1}^{n}\left[\log\left(\frac{c_i}{LC_{50i}}\right)\right] \quad (1)$$

where TU_{max} is the highest value of n detected pesticides per sampling site and season, c_i is the peak concentration of pesticide i, and LC_{50i} is the pesticide's corresponding acute lethal concentration (48h) for the most sensitive standard test organism, which was either *Daphnia magna* or *Chironomus sp.* (see also Münze et al., 2017 and Table S3).

2.4. Physicochemical and hydromorphological parameters

We considered those physico-chemical parameters that were monitored regularly throughout all three field campaigns, namely temperature, pH, total dissolved oxygen (TDO) and nutrient levels (PO_4, NO_3, NO_2, NH_4). Morphological parameters comprised the width of the streambed, water depth and hydromorphological degradation based on the structural quality classes according to the guideline of the German Working Group on water issues (LAWA, 2000). The respective classes reflected the deviation from the potential natural state, on the basis of a seven-point scale including parameters such as course development, longitudinal profile, cross profile, bed structure, bank structure, and area surrounding the water body. It has to be noted that some environmental parameters were not available for all sites (e.g. TDO, streambed width or water depth). Details on physicochemical and hydromorphological parameters for all stream sites during or shortly after pesticide contamination as well as missing data are given in Table S4.

2.5. Identification of refuge areas

Prior to the assessment of taxonomic differences of the communities between sites, we grouped all study sites according to the presence of refuge areas. Refuge areas were defined as (i) forested or grassland stream sections with presumably little or no influence of pesticides, (ii) with minimum dimensions of 100 m in width and 300 m in length, (iii) that were at least twice as long as any agricultural stream section in the further upstream and (iv) exhibited a maximal distance to the sampling site of 10 km upstream. The maximal upstream distance of 10 km reflects the tendency of aquatic stages of macroinvertebrates to drift in the downstream direction (Bailey, 1966; Elliott, 1971a). For the identification of refuge areas, we assessed the presence of at least one refuge area in the main stream or any of the nearby tributaries. We identified 28 sites with refuge areas and 13 without upstream refuge areas. At one site (Site ID = 21, Table S1) we detected no clear upstream refuge area, but we observed one refuge area downstream within a distance of only 1.5 km. Therefore, this site was also assigned as a site with refuge area. All other sites were not characterized by such a close located refuge area only downstream of the sampling site. Refuge areas from nearby parallel streams were not considered, since previous studies (Orlinskiy et al., 2015; Liess and von der Ohe, 2005) concluded that only refuge areas with an aquatic connection had a significant influence on $SPEAR_{pesticides}$. The distances between the sampling site and the nearest refuge area were determined in ArcGIS 10.1 using aerial base maps and shape files from the ATKIS database (scale 1:25,000) that were provided by the German Federal Agency for Cartography and Geodesy (Bundesamt für Kartographie und Geodäsie, Leipzig, Germany).

2.6. Invertebrate sampling and structural endpoints

Stream sites were investigated in monthly intervals from April to July between 1998 and 2000 (first field campaign, Liess and von der

Ohe, 2005), from May to July in 2013 (second field campaign, Münze et al., 2017) and from March to August in 2013 (third field campaign).

Taxa from the orders Ephemeroptera, Plecoptera and Trichoptera (EPT) and Amphipoda were identified at the species level for the first and third field campaign. The remaining invertebrates and all taxa sampled during the second field campaign were identified at the family level. Organisms from the family Goeridae and Limnephilidae were re-identified for the present study to the species level in the second field campaign (Münze et al., 2017), because some genera of these families were identified as refuge taxa considering the two campaigns with sampling data identified up to the species level.

Following the classification of stream sites into sites with and without refuge areas, we investigated the invertebrate abundance data for taxa that were only present at sites with or without refuge areas between March and August. We defined refuge taxa with (i) predominant and frequent presence at sites with refuge areas (>5 sites with observations, a maximum of two sites with observations without refuge areas or significantly lower abundances at sites without refuge areas) and (ii) presence at sites with high levels of toxic pressure (minimum two sites, $TU_{max} > -2.5$). The effects of pesticides on macroinvertebrates were quantified using the bio-indicator $SPEAR_{pesticides}$ by Liess and von der Ohe (2005). $SPEAR_{pesticides}$ provides the relative abundance of vulnerable species within a community and is normalized to indicator values under reference conditions ($TU_{max} \leq -4.5$). $SPEAR_{pesticides}$ is calculated by using the following equations:

$$SPEAR_{pesticides} = \frac{\sum_{i=1}^{n} \log(4x_i + 1) \cdot y}{\sum_{i=1}^{n} \log(4x_i + 1)} \quad (2)$$

where n is the total number of taxa in a sample, x_i is the abundance of taxon i (given as individuals per m^{-2}), and y is set to 1 if taxon i is classified as “at risk” (Liess and von der Ohe, 2005), i.e., vulnerable to pesticides under regular exposure events and set to 0 otherwise. Abundance data were $\log(4\times + 1)$-transformed to decrease the influence of populations with mass developments.

$$SPEAR_{pesticides} = \frac{SPEAR_i}{SPEAR_{reference}} \quad (3)$$

where $SPEAR_i$ represents the indicator value of a macroinvertebrate community at a specific site i and time point and $SPEAR_{reference}$ represents the mean $SPEAR_{pesticides}$ under reference conditions regarding toxic pressure.

In the first step, we calculated $SPEAR_{pesticides}$ with the default species classification (software “SPEAR Calculator”, http://www.systemecology.eu/spearcalc/index.en.html, version 0.10.0). Hence, indicator values were calculated without considering the identified refuge taxa and are referred to as “$SPEAR_{pesticides}$ – old classification”. In the second step, we recalculated $SPEAR_{pesticides}$ by classifying all refuge taxa as invulnerable to pesticides, referred to as “$SPEAR_{pesticides}$ – revised classification”.

We further determined $SPEAR_{refuge}$ as an indicator of the recolonization potential of a macroinvertebrate community at a given site. $SPEAR_{refuge}$ was calculated similarly to $SPEAR_{pesticides}$ and represents the ratio of the abundance of refuge taxa versus the total abundance:

$$SPEAR_{refuge} = \frac{\sum_{i=1}^{n} \log(4x_i + 1) \cdot y_{refuge}}{\sum_{i=1}^{n} \log(4x_i + 1)} \quad (4)$$

where x_i is the abundance of taxon i and y_{refuge} is 1 if the taxon is classified as a refuge taxon; otherwise y_{refuge} is 0. As for $SPEAR_{pesticides}$, $SPEAR_{refuge}$ was normalized between 0 and 1 on the basis of reference conditions, i.e., $SPEAR_{refuge}$ for macroinvertebrate communities that were directly sampled in refuge areas.

The performance of $SPEAR_{pesticides}$ in disentangling the effects of pesticides from those of other environmental factors was analysed by comparison with three other common ecological bio-indicators. These bio-indicators comprised the German saprobic index (SI) (Kolkwitz and Marsson, 1909; Pantle and Buck, 1955; Rolauffs et al., 2003), the Shannon diversity index (H′) and the ecological metric %EPT representing the relative abundance of Ephemeroptera, Plecoptera and Trichoptera to the total macroinvertebrate abundance. SI has been developed to indicate oxygen depletion in streams based on the macroinvertebrate communities. For this aim, single taxa are assigned with saprobic values according to their oxygen demand in order to calculate the weighted average of saprobic values at a given site. In comparison, H′ and %EPT are applied to assess the general ecological quality of streams. %EPT and H′ were determined for all three campaigns with taxa aggregated at the family level to make it most comparable to the indicator $SPEAR_{pesticides}$. SI was calculated for only the two field campaigns that included taxa identification up to the species level because saprobic values are not assigned for levels higher than the species level. Indicator values for % EPT, SI and H′ are given in Table S4.

2.7. Functional endpoints

To understand the link between refuge areas, toxic pressure and functional endpoints, we also incorporated leaf litter degradation into the analyses. Leaf litter degradation by macroinvertebrates was assessed in the second and third field campaign by using leaf litter bags with 3 g of dried birch leaves that were deployed over a period of three weeks at each stream site (for details, see Münze et al., 2017). Leaf litter bags included cases with a fine mesh size (75 μm, nylon) and cases with a coarse mesh size (3 mm, polyethylene). Both cases were deployed in triplicate per site to assess leaf litter degradation induced by microorganisms and macro-invertebrates (for further details, see also Münze et al., 2017). Leaf litter degradation by macro-invertebrates was analysed in July (second field campaign) or June (third field campaign, for details, see Table S4). The leaf litter degradation is given as the breakdown rate k per day, which represents the loss of dry mass before and after the deployment of the bags in the streams.

2.8. Statistics

We used multiple linear regressions to determine the influence of refuge areas, toxic pressure and additional environmental variables on $SPEAR_{pesticides}$. To preselect relevant environmental parameters, we first performed single linear regression analyses between $SPEAR_{pesticides}$ and all environmental parameters measured in the field campaigns during or shortly after the main pesticide exposures (temperature, pH, TDO, nutrient levels, streambed width, water depth and hydromorphological degradation). This pre-selection was done separately for the two versions of $SPEAR_{pesticides}$ with and without considering refuge taxa (old or revised classification), SI, %EPT and H′. All environmental parameters that showed a significant relationship with one of the indicators were included in a subsequent multiple regression analysis. Model selection was performed for each indicator using backward and forward model selection with Akaike's Information Criterion (AIC). On the basis of the multiple regression, we further determined the contribution of each significant model term using the metric “first”, which represents the explanatory power of each predictor when included first (Grömping, 2006, R-package *relaimpo*). To analyse the influence of environmental variables on the bio-indicator $SPEAR_{refuge}$ and the funtional endpoint leaf litter degradation, a similar procedure was performed. Regarding the analysis of influential factors for $SPEAR_{refuge}$, we further included the distance to the nearest refuge area as an explaining variable. To this end, we set sites without refuges areas to a distance of 7000 m because at this distance $SPEAR_{refuge}$ was predicted to be 0 (no refuge taxa), considering only sites with upstream refuge areas.

An overview map of the sampling sites of the three field campaigns was generated in ArcGIS 10.1. All other graphs and statistical analyses were generated with R, version 3.3.3 (R Core Team, 2017).

3. Results

3.1. Identification of refuge taxa

In three sampling campaigns comprising 41 stream sites, we detected 87 macro-invertebrate families or higher taxonomic levels. The mean number of detected families per site and sampling time point was significantly higher at sites with refuge areas (mean = 18 families per site) than at the sites without refuge areas (mean = 15 families, *t*-test: $t = 3.18$, $P = 0.002$.).

We identified 11 refuge taxa (Table 1, Fig. S1) that were almost exclusively present at sites with nearby refuge areas and independently of the toxic pressure (for definition, see Methods). The identified refuge taxa corresponded to the orders Ephemeroptera, Plecoptera, Trichoptera, Diptera and Seriata. The refuge taxa were characterized by a weak, good or even high dispersal potential including the ability to cover several km of distance through passive drift, active movements (swimming, crawling), and/or by flying up- and downstream as adult insects (Table 1). Furthermore, several refuge taxa were characterized by resistance traits, which refer to traits that enable survival during periods of non-optimal environmental conditions. For example, refuge taxa such as *Sericostoma* spp., *Ironoquia dubia*, *Serratella ignita*, *Paraleptophlebia submarginata* and *Habrophlebia* spp. are also known to persist in temporary waters. Corresponding resistance traits included asynchronous development, persistent larval/egg stages or imaginal diapause. Table 1 provides a detailed list of ecological traits.

3.2. Improvement of $SPEAR_{pesticides}$

As defined, refuge taxa were observed only at sites with nearby refuge sections, independently of the level of toxic pressure (Fig. S1). In order to decrease the influence of these taxa on the indicative power of $SPEAR_{pesticides}$, we re-classified all refuge taxa as invulnerable taxa, thereby changing the $SPEAR_{pesticides}$ classification for 11 taxa (see Table 1, taxa marked by [a]).

Applying this approach, the best correlations between the toxic pressure $SPEAR_{pesticides}$ were identified in June independent of the re-classification of refuge taxa. In May we also detected similar significant relations between the pesticide pressure and $SPEAR_{pesticides}$, but explanatory power of TU_{max} was considerably lower than in June including all sites (old classification: $r^2 = 0.28$, $P < 0.001$; revised classification: $r^2 = 0.18$, $P < 0.004$, $n = 41$). Therefore, we compared the performance of $SPEAR_{pesticides}$ in June based on the old and the revised classification of refuge taxa by multiple linear regression (Fig. 2, Table 2). In the analyses, we assessed both the indicative power of $SPEAR_{pesticides}$ to identify the toxicity at a sampling site and the confounding effect of other environmental parameters on the indication results. Regarding the old classification of refuge taxa, $SPEAR_{pesticides}$ was mainly explained by TU_{max} (explained variance = 49.12%, $P < 0.001$), followed by the presence of refuge areas (23.39%, $P < 0.001$), streambed width (37.56%, $P < 0.001$) and hydromorphological degradation (27.92%, $P = 0.002$) (Table 2). The presence of refuge areas and increasing streambed width showed a positive influence on $SPEAR_{pesticides}$. Hydromorphological degradation was negatively related to $SPEAR_{pesticides}$. By contrast, when applying the revised classification of refuge taxa, we found that $SPEAR_{pesticides}$ was significantly explained by only TU_{max} (54.83%, $P < 0.001$) and hydromorphological degradation (14.83%, $P = 0.015$). Hence, applying the revised classification, $SPEAR_{pesticides}$ is generally more specific to toxic pressure (Fig. 2).

For the comparative performance of $SPEAR_{pesticides}$, we analysed the relationships between TU_{max} and the bio-indicators %EPT, SI and H′. The explained variance of TU_{max} on %EPT (38.27%, $P < 0.001$, Table 2) and TU_{max} on SI (30.93%, $P < 0.001$.) were lower than those observed for the $SPEAR_{pesticides}$ indicators (49.12% and 54.83%, Table 2). The cross-sensitivity of the bio-indicators to other environmental variables was also analysed and showed that increasing streambed width was related

Table 1
Identified refuge taxa including traits potentially responsible for the respective classification.

Refuge taxon	Identified species in dataset	Order	Resilience traits (dispersal)	Synchronism of life cycle	Resistance stages	Reference
Anabolia nervosa[a] (Fam. Limnephilidae)	*Anabolia nervosa*	Trichoptera	Drift in autumn, good crawler, Good aerial dispersal	Short emergence period, synchronised	Larval diapause	Pitsch, 2002, Elliott, 1971b, Hall, 2002, Graf et al., 2008
Dendrocoelidae	*Dendrocoeleum lacteum*	Seriata	To be identified	To be identified	To be identified	NA
Ephemerellidae[a]	*Serratella ignita*	Ephemeroptera	Good drifter and swimmer/crawler, good aerial dispersal	Poorly synchronised	Egg diapause	Buffagni et al., 2009, Tachet et al., 2010
Ephemeridae[a]	*Ephemera danica*	Ephemeroptera	Burrower, crawler, good aerial dispersal	Poorly synchronised	None	Tachet et al., 2010, Svensson, 1977
Ironoquia dubia[a] (Fam. Limnephilidae)	*Ironoquia dubia*	Trichoptera	Crawler, low aerial dispersal	Short emergence period	Imaginal diapause	Graf et al., 2008, Tachet et al., 2010
Leptophlebiidae[a]	*Habrophlebia lauta*, *Habrophlebia fusca*, *Leptophlebia marginata*, *Leptophlebia vespertina*, *Paraleptophlebia submarginata*	Ephemeroptera	Weak - good swimmer/crawler, common drifter	Poorly synchronised,	Egg or larval diapause	Poff et al., 2006, Buffagni et al., 2009
Leptoceridae[a]	*Athripsodes aterrimus*, *Athripsodes cinereus*, *Mystacides longicornis*	Trichoptera	Active sprawling/walking, low - common drifter, good aerial dispersal	Poorly synchronised	None	Graf et al., 2008, Skuja, 2010, Tachet et al., 2010, Pitsch, 2002
Nemouridae[a]	*Amphinemura sulcicollis*, *Nemoura cinerea*	Plecoptera	Common drifter, sprawling, good aerial dispersal	Poorly synchronised	Egg diapause	Graf et al., 2009, Tachet et al., 2010
Sericostomatidae[a]	*Sericostoma flavicorne/personatum*, *Notidobia ciliaris*	Trichoptera	Good aerial dispersal, good crawling, common drifter	Poorly synchronised	Diapause/quiesence or not known (Notidobia sp.)	Masters et al., 2007, Elliott, 1969, Tachet et al., 2010, Graf et al., 2008, Hall, 2002
Silo spp.[a] (Fam. Goeridae)	*Silo nigricornis*, *Silo pallipes* *Silo piceus*	Trichoptera	Low dispersal (air and water)	Poorly synchronised	None	Graf et al., 2008, Sode and Wiberg-Larsen, 1993
Tabanidae	Not identified	Diptera	Strong flight capacity, high female dispersal	To be identified	Diapause or quiesence	Tachet et al., 2010, Datry et al., 2014

[a] Taxon level that was re-classified as pesticide invulnerable in the SPEAR calculator.

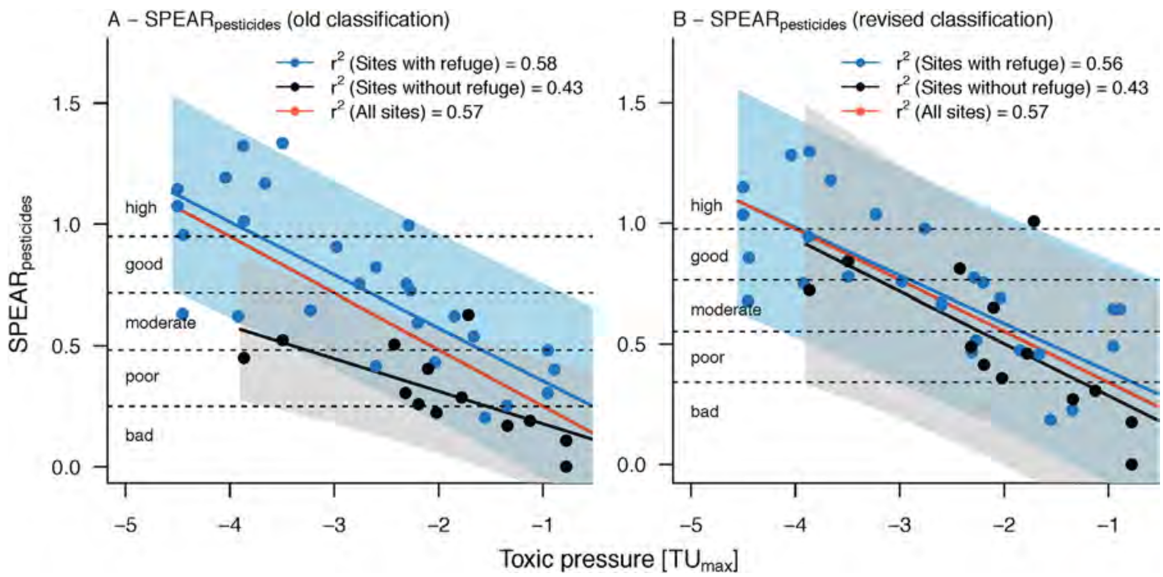

Fig. 2. SPEAR$_{pesticides}$ in relation to the pesticide pressure TU$_{max}$ in June. A: SPEAR$_{pesticides}$ applying the old classification of refuge taxa. (B): SPEAR$_{pesticides}$ applying the revised classification of refuge taxa. Linear regressions are indicated by the regression line and the regression coefficient r^2. Blue and grey areas delimit the 90% confidence interval for sites with and without refuge areas, respectively. In addition, we assigned for the SPEAR$_{pesticides}$ five classes reflecting the ecological status according to the EU Water Framework Directive (WFD, EC, 2000). Boundaries for the classes are based on the regression line of all sites and TU$_{max}$ (≤−4, >−4 and ≤−3, >−3 and ≤−2, >−2 and ≤−1, >−1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to an increase in %EPT (28.99%, $P = 0.001$) and decrease in SI (23.84%, $P < 0.001$). There was also a positive relationship with pH and an increase in %EPT (4.21%, $P = 0.05$). Regarding the index H′ for biodiversity, we detected no significant influence of the toxic pressure. In comparison, streambed width was positively related to H′ (16.06%, $P = 0.015$).

3.3. Functional endpoints

SPEAR$_{pesticides}$ with revised classification and the median water temperature during deployment of the leaf litter bags were significantly related to the breakdown rate k indicating an increase of k with increasing SPEAR$_{pesticides}$ and median temperature (multiple linear regression, $R^2 = 0.38$, $P = 0.003$, $n = 23$). SPEAR$_{pesticides}$ alone explained 10.78% ($P = 0.007$, metric = "first") and the median temperature 17.73% ($P = 0.02$, metric = "first") of the variance in k. The contamination level TU$_{max}$ could not be significantly linked to k.

3.4. SPEAR$_{refuge}$

We developed the bio-indicator SPEAR$_{refuge}$ describing the magnitude of recolonization from uncontaminated upstream regions. Hence, the bio-indicator SPEAR$_{refuge}$ represents the ratio of the refuge taxa in terms of abundance to the total sum of macroinvertebrate abundance.

The mean of normalized SPEAR$_{refuge}$ was 0.77 at sites with refuge areas versus 0.02 at sites without refuge areas. SPEAR$_{refuge}$ decreased significantly with increasing distance to the nearest upstream refuge (linear regression, $r^2 = 0.56$, $P < 0.001$, $n = 41$ Fig. 3). The distance varied between 0 (= refuge area) and 4720m for sites with refuge areas. According to the multiple regression analysis distance and streambed width were significantly related to the median SPEAR$_{refuge}$ ($R^2 = 0.60$, $P < 0.001$, $n = 34$). SPEAR$_{refuge}$ increased with increasing streambed width (explained variance = 30.99%, $P = 0.02$, metric =" first"). Regarding links between SPEAR$_{refuge}$ and general bio-indicators %EPT and H′, we identified a positive significant relationship between %EPT

Table 2
The performance of SPEAR$_{pesticides}$ based on the old and revised classification of refuge taxa in comparison to the indicators %EPT, SI and H′. The link between SPEAR$_{pesticides}$, pesticide toxicity and environmental parameters was assessed by multiple linear regression and analysis of relative importance (metric "first", see Methods). The statistics of the overall regression model is presented in each column heading.

Environmental variable[a]	SPEAR$_{pesticides}$– old classification ($R^2 = 0.75$, $F = 34.52$, $P < 0.001$, $n = 33$)		SPEAR$_{pesticides}$ – revised classification ($R^2 = 0.60$, $F = 27.85$, $P < 0.001$, $n = 37$)		%EPT ($R^2 = 0.57$, $F = 15.38$, $P < 0.001$, $n = 34$)		SI ($R^2 = 0.59$, $F = 20.22$, $P < 0.001$, $n = 27$)		H′ ($R^2 = 0.20$, $F = 5.06$, $P = 0.013$, $n = 34$)	
	Explained variance	P-value	Explained variance	P-value	Explained variance	P-value	Explained variance	P-value	Explained variance	P-value
TU$_{max}$	49.12%	<0.001	54.83%	<0.001	38.27%	<0.001	30.93%	<0.001	*n.s.*	*n.s.*
Refuge area [yes/no]	23.39%	<0.001	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Hydromorphological degradation	27.92%	0.002	14.83%	0.015	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*	*n.s.*
Streambed width [cm]	37.56%	<0.001	*n.s.*	*n.s.*	28.99%	0.001	23.84	<0.001	16.06%	0.015
pH	*n.s.*	*n.s.*	*n.s.*	*n.s.*	4.21%	0.05	*n.s.*	*n.s*	*n.s.*	*n.s.*

n.s. = not significant.
[a] Values slightly differ from values presented in Fig. 2 due to few missing data in the environmental variables (see also Table S4).

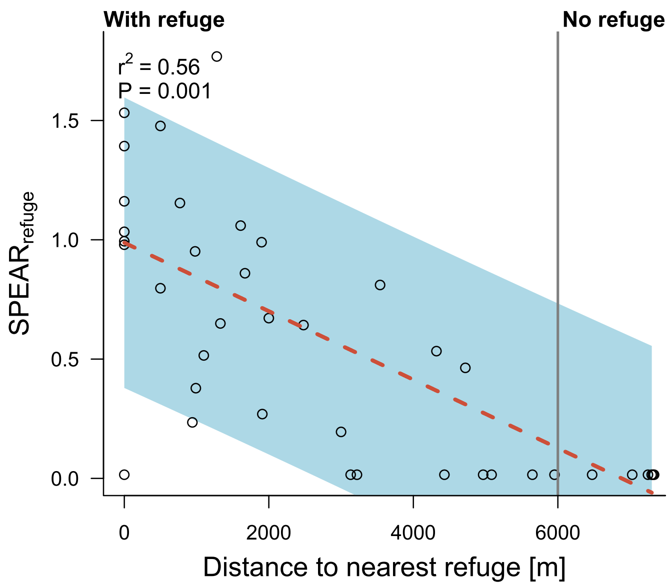

Fig. 3. The relation between $SPEAR_{refuge}$ and the distance to the nearest refuge area for sites with and without refuge areas representing the median of all sampling time points between March and August per site. The vertical line separates sites with and without refuge areas. The sites without refuge areas were set to a distance of 7000 m (for details, see Methods) and added random variation to display all points by using the function "jitter" in R. The relationship between $SPEAR_{refuge}$ and the distance is presented with the regression line, regression coefficient and *P*-value. The blue area delimits the 90% confidence interval. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and $SPEAR_{refuge}$ ($r^2 = 0.23$, $P = 0.001$, $n = 41$ Fig. S4). These results imply that $SPEAR_{refuge}$ is also linked to a limited extent to the general ecological quality at a given site.

4. Discussion

4.1. Re-classification of refuge taxa increases the indicative power of SPEARpesticides

In previous studies, $SPEAR_{pesticides}$ has been found to be not only related to the toxic pressure of pesticides (TU_{max}), but also significantly influenced by the presence of refuge areas (Liess and von der Ohe, 2005; Schäfer et al. 2012; Orlinskiy et al., 2015, Bunzel et al., 2014). In the present revision of the bio-indicator $SPEAR_{pesticides}$, this drawback was reduced by identifying taxa that predominantly depend on refuge areas but not on the toxic pressure in the monitoring region. Accordingly, the $SPEAR_{pesticides}$ with a revised classification of refuge taxa provides a measure for (i) the toxic pressure of pesticides and (ii) the integrity of vulnerable invertebrate species that require uncontaminated streams sections.

The new revision of $SPEAR_{pesticides}$ is mainly related to the toxic pressure of pesticides ($R^2 = 0.57$, $P < 0.001$). While the Shannon diversity index (H′) was not related to the toxic pressure of pesticides, we observed that also the indicator for general ecological degradation, % EPT, and the saprobic index (SI), a measure of oxygen deficiency (Kolkwitz and Marsson, 1909), were related to toxic pressure (Table 2). Diversity indices such as H′ have been described to respond less sensitive to specific stress compared to biotic indicators considering the stress-sensitivity of single taxa (Metcalfe, 1989; Lydy et al., 2000; Schäfer et al., 2011) or other sensitive endpoints including genetic adaptation of individuals (Becker and Liess, 2017) and impaired ecosystem functions (Schäfer et al., 2012). The significant relationship between pesticide exposure and %EPT or SI in the present study can be explained by the common co-occurrence of pesticide exposure and other stressors, such as hydromorphological degradation or oxygen deficiency, in streams with surrounding agricultural land use (Münze et al., 2017; Rasmussen et al., 2012; Frede and Bach, 1993). In addition, most invertebrate taxa are vulnerable to more than one stressor, as demonstrated, for example, for pesticides and low-oxygen conditions (Bunzel et al., 2013) or a decline in structural quality (Rasmussen et al., 2012). Despite the presence of multiple stressors, our results indicate a strong specificity of $SPEAR_{pesticides}$ for pesticide exposure in small agricultural catchments. Similar findings have been reported for streams in agricultural landscapes from different regions including Europe (Schäfer et al., 2007), Australia (Schäfer et al., 2011) and South America (Hunt et al., 2017). Other stressors that are known to have major impacts on the structure of aquatic communities, such as for example, regular drying (Boulton and Lake, 2008), acidification (Lepori et al., 2003, Guerold et al., 2000) or salinization (Cañedo-Argüelles et al., 2013) were not present at our field sites. However, future investigations should further focus on the relevance of strong environmental stressors on pesticide related impacts in aquatic ecosystems. This seems to be highly relevant as a review of multiple stressor studies in controlled conditions revealed that environmental stressors greatly increase the effects of pesticides on populations (Liess et al., 2016).

We also addressed the link between $SPEAR_{pesticides}$ and the ecosystem function leaf litter degradation, which plays a crucial role in the energy cycle of freshwater streams (Wallace and Webster, 1996). In agreement with previous findings regarding the old $SPEAR_{pesticides}$ indicator (Münze et al., 2017; Schäfer et al., 2007), we identified a significant relation between leaf litter breakdown rate, water temperature and the revised $SPEAR_{pesticides}$ ($R^2 = 0.38$, $P = 0.003$, $n = 23$). Hence, the re-classification of refuge taxa did not affect the predictive power of $SPEAR_{pesticides}$ for ecosystem functions such as leaf litter degradation. This outcome indicates that the identified refuge taxa do not dominate the effect on overall leaf litter degradation at a stream site. Nevertheless, more data is necessary to confirm these findings on a larger scale.

4.2. $SPEAR_{refuge}$ reflects the level of recolonization over a short distance

Refuge areas are crucial to ensure high ecological quality for invertebrate communities by providing a source of recolonization for taxa affected by various stressors, such as toxicant exposure or hydrological stress (Bunzel et al., 2014; Trekels et al., 2011; Fritz and Dodds, 2004). In the present study, $SPEAR_{refuge}$ was developed to describe the share of taxa within a macroinvertebrate community originating from nearby refuge areas and, hence, the general recolonization potential at a given stream site.

In the present study, we identified 11 refuge taxa that were almost exclusively observed at sites with nearby refuge areas and did not disappear even at high levels of toxic pressure. Most of these refuge taxa are generally expected to be vulnerable to pesticide exposure because of (i) relatively high physiological pesticide sensitivity, (ii) a long generation time (>1 year) and (iii) presence in the water during the main period of pesticide application (Liess and von der Ohe, 2005). The presence of taxa with high ecological requirements in contaminated or disturbed streams can be linked to dispersal-based resilience, including the sufficient dispersal ability of taxa from uncontaminated stream sections (Fritz and Dodds, 2004; Leigh et al., 2016). The refuge taxa identified in the present study are generally able to move along or between streams via drift and adult dispersal. However, the strength of dispersal appears to be limited to a few kilometres along the stream and differs between refuge taxa. For example, mayflies of the family Ephemeridae have been reported to actively migrate and frequently drift in the water (Tachet et al., 2010; Poff et al., 2006). However, the taxa of the genus *Silo* sp. (family Goeridae) have been described as weak drifters (Tachet et al., 2010) with a low adult dispersal (Sode and Wiberg-Larsen, 1993). In comparison, other taxa known to exhibit high dispersal rates, such as *Baetis* sp. or *Limnephilus lunatus* (Schmedtje and Colling, 1996; Graf et al., 2008), were frequently observed at sites with and without refuge areas (Fig. S2 and S3). Therefore, we conclude that refuge taxa possess the ability to disperse at least over a limited distance, thus enabling them to recolonize in nearby instream sections.

In addition to a sufficient dispersal ability, we detected for most of the refuge taxa asynchronous life cycles or resistant life stages in and out of the water. Such traits of stress resistance are particularly known for taxa persisting in streams with unstable discharge conditions (Diaz et al., 2008; Leigh et al., 2016). Fritz and Dodds (2004) have further concluded from a field study in Kansas that resistance traits are less crucial under prolonged or strong disturbance than recolonization from nearby refuge areas. Similar findings on the importance of recolonization versus resistant traits have been reported by Stanley et al. (1994), who have investigated mechanisms of invertebrate persistence in intermittent streams. Hence, we conclude that mainly the ability to recolonize downstream sections supported by resistant life cycle traits enable taxa with high ecological requirements to persist at least temporarily in streams under high levels of toxic pressure.

Regarding recolonization potential, $SPEAR_{refuge}$ significantly depends on the distance to the nearest refuge area along the stream and to a minor extent also on the streambed width ($R^2 = 0.60$, $P < 0.001$, $n = 34$). Similar results in terms of distance to the nearest refuge have been reported in previous studies. For example, Trekels et al. (2011) have detected a prolonged recovery via aerial dispersal of water bugs from pesticide exposure with an increase in distance from 70 m to 1000 m to the source pond. Moreover, Fritz and Dodds (2004) have investigated the effect of drying and flood events on stream invertebrates and have observed larger increases in taxa richness after the disturbance with decreasing distance to upstream refuge areas. Apart from the influence of distance on $SPEAR_{refuge}$, streambed width was only additionally related to $SPEAR_{refuge}$. Therefore, $SPEAR_{refuge}$ is a promising bio-indicator for assessing the general recolonization of macroinvertebrate communities in agricultural streams from instream refuge areas. Further analyses on $SPEAR_{refuge}$, including not only the distance but also the quality of refuge areas might contribute to a deeper understanding of the ecological interconnectedness of landscape patterns.

5. Conclusion

Stressor-specific ecological indicators are essential for the monitoring of ecosystems, because they reveal ecological effects and assess the potential of single and combined stressors to affect ecosystem structure and function. The revised $SPEAR_{pesticides}$ is applicable as a highly specific indicator for revealing the toxic pressure of pesticides and corresponding effects on aquatic invertebrates.

Author contributions

S.K. performed data analyses and wrote the first draft of the paper. P.O. conducted the third field campaign and sample preparation for chemical analysis, identified invertebrates and contributed to the description of methods. O.K. assisted the third field campaign and performed a quality control of monitoring data K.F. calculated environmental indicators (SI, H′ and %EPT) and contributed to the writing. M.L. developed together with S.K. $SPEAR_{refuge}$, the revision of $SPEAR_{pesticides}$, interpreted the results and contributed to the writing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.02.056.

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