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Effects of a realistic pesticide spraying sequence for apple crop on stream communities in mesocosms: negligible or notable?

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Abstract

Background Several large-scale studies revealed impacts and risks for aquatic communities of small rural lakes and streams due to pesticides in agricultural landscapes. It appears that pesticide risk assessment based on single products does not offer sufficient protection for non-target organisms, which are exposed repeatedly to pesticide mixtures in the environment. Therefore, a comprehensive stream mesocosm study was conducted in order to investigate the potential effects of a realistic spraying sequence for conventional orchard farmed apples on a stream community using pesticides at their regulatory acceptable concentrations (RACs). Eight 74-m-long stream mesocosms were established with water, sand, sediment, macrophytes, plankton and benthic macroinvertebrates. In total, nine fungicidal, four herbicidal and four insecticidal pesticides were applied in four of the eight stream mesocosms on 19 spraying event days in the period from April to July while the remaining four stream mesocosms served as controls. The community composition, the abundance of benthos, periphyton and macrophytes, the emergence of insects, physico-chemical water parameters, and drift measurements of aquatic invertebrates were measured.

Results The pesticide spraying sequence induced significant effects on invertebrates, periphyton, and macrophytes as well as on the water ion composition especially in the second half of the experiment. It was not possible to relate the observed effects on the community to specific pesticides applied at certain time points and their associated toxic pressure using the toxic unit approach. The most striking result was the statistically significant increase in variation of population response parameters of some taxa in the treated mesocosms compared to the controls. This inter-individual variation can be seen as a general disturbance measure for the ecosystem.

Conclusions The pesticide spraying sequence simulated by using RAC values had notable effects on the aquatic stream community in the conducted mesocosm study. The results indicate that the current risk assessment for pesticides may not ensure a sufficient level of protection to the field communities facing multiple pesticide entries due to spraying sequences and other combined stress. Hence, there is still room for improvement regarding the prospective risk assessment of pesticides to further reduce negative effects on the environment.

Keywords Regulatory acceptable concentration (RAC), Benthic macroinvertebrates, Periphyton, Macrophytes, Pesticides, Toxic effects

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Background

The EU pesticide risk assessment (RA) is based on a tiered approach in order to sufficiently address the risk of plant protection products (PPP) for the environment (pesticide regulation, European Commission No. 1107/2009) [1]. The tier 1 RA is a laboratory-based ecotoxicological assessment, which includes several single species tests. More complex and environmentally realistic evaluations (tier 2 and 3) are usually proposed as refinements if the tier 1 RA indicates that the legally specified level of acceptability is not met [2].

A conceptual key element of the pesticide RA is the derivation of regulatory acceptable concentrations (RAC) of PPPs and/ or their active ingredients (AI). For tier 3, RACs are derived on the basis of negligible population effects or population effects with subsequent ecological recovery within a certain time period [2, 3]. In each tier, the RAC needs to be higher than the calculated predicted environmental concentration (PEC) for edge-of-field surface waters [2] in order to conclude on an acceptable risk. On the exposure side, the pesticide entries into the edge-of-field waterbodies, simulated by the PECs, can be reduced by applying mitigation measures such as, e.g. using drift-reducing spray nozzle technology or implementing no-spray-zones or vegetated buffer-strips [2].

The complexity of the higher tier pesticide regulation process increased over the years with the inclusion of multi-species tests, mesocosm and field studies, as well as effect modelling. Some of these studies are usually proposed to refine the risk by, in theory, introducing more realism into the risk assessment, e.g. by estimating more accurately the sensitivity and vulnerability of complex ecosystems [2, 4]. Also, mixture toxicity is included by considering the interactions between the AIs, safeners, synergists, and co-formulants of a single PPP. However, this implies that only technical mixtures such as PPP with one or more AI are included, while the so-called "coincidental" pesticide mixtures, like inter alia tank mixtures or pesticide spraying sequences during a growing season, are not explicitly considered [1, 5]. The application of a series of distinct PPPs with different modes of action, or the use of tank mixtures, is a common practice in current agriculture, assuring sufficient agricultural yield by avoiding the occurrence of different pests over the growing season [6–8]. Consequently, it is questionable if the risk assessment based on single PPP is adequate to protect non-target organisms from the negative effects of exposure to real-world mixtures and/or spray series of PPPs.

Analysis of field data for pesticides in smaller waterbodies of rural areas including detailed evaluation of large-scale aggregated pesticide data demonstrated increasing evidence that for a relevant number of waterbodies, impacts and risks for aquatic communities due to pesticides are indicated [e.g. 9–17]. Therefore, agricultural land use can be considered as a main input pathway of pesticides into streams [16]. In a comprehensive study, Junghans et al. [18] investigated the risk of pesticide mixtures for aquatic communities in five small agricultural streams in Switzerland. By use of stream water for biotests, a high risk for plants, invertebrates and fish at all investigated sites was confirmed for most of the water samples [18]. Herbicides and fungicides were the two pesticide classes most frequently detected in surface waters, and especially herbicides were widely present in aquatic ecosystems due to their high application rates [19, 20]. The analysis of environmental monitoring data from different countries displayed a mixture of three to five pesticides in median, but also exceeding a mixture of ten different compounds in some cases in surface waters. Herbicides were the dominant pesticide class found followed by fungicides and insecticides [21].

These findings demonstrate that there is a potential discrepancy between the claim and objectives of the plant protection regulation [1] and the actual situation in the field. There are several reasons why pesticide concentrations above RACs occur in edge-of-field surface waters. These reasons include an improper use of PPP, more frequent run-off events than anticipated, lack of or insufficient wetland vegetation at the riparian zones for input reduction, simultaneous PPP application of the same set of AIs on different crop areas within a river catchment, or application of a series of different PPPs within a crop-specific spraying sequence. Especially the environmental risk of spraying sequences of different PPPs has been ignored so far by the EU RA [8].

Therefore, the German Environment Agency (UBA) performed a stream mesocosm study in order to investigate the effects of a realistic spraying sequence, as applied to apple crop in the fruit growing area of Lake Constance in southern Germany, on an aquatic stream community for 150 days. Application data of a spraying sequence originally applied in 2010 were kindly provided by the Julius Kuehn Institute (Braunschweig, Germany), which documents the extent of spray series application of PPPs on conventional crop on the basis of annual surveys [22, 23].

Since pesticide concentrations in edge-of-field surface waters should not exceed the RAC for each AI, RACs were chosen to simulate pesticide entries in stream mesocosms due to apple crop spray treatments. The use of a RAC can be seen as a realistic approach since it was derived applying a safety factor that accounts for uncertainties such as extrapolations to other species or from laboratory test conditions to field situations [24, 25]. However, the occurrence of adverse effects as well as the regular exceedances of RACs in the field does frequently

raise the question whether a series of PPP spraying events leading to concentrations that correspond to the RAC values (i.e. a sequence of RACs in time) would still be protective for stream communities [18, 26, 27]. Therefore, we hypothesised that the successive application of PPP, each at its RAC, within the spraying sequence of apple crop leads to adverse effects on the stream community. This hypothesis is based on the fact that the issue of multiple PPP applications is not explicitly considered in RAC derivation. Thus, there are some uncertainties in terms of the protection level ensured in the field. To test the hypothesis, the conducted mesocosm study focussed on endpoints such as species diversity, abundance of benthos, periphyton, macrophytes, emergence of insects, as well as physico-chemical water parameters, and additionally on behaviour (drift) of aquatic macroinvertebrates.

Methods

Stream mesocosm set-up and biological establishment

The study was conducted in eight indoor stream mesocosms at the field station of the UBA (https://www.umweltbundesamt.de/en) in Berlin (Germany). Eight flow-through circular stream mesocosms (length 74 m, width 1 m, water volume 20.5 m^3, glass-fibre reinforced polyester material), each equipped with riffle sections and four separate pool sections per stream were set up [28]. The riffle sections were established with a sand bottom. The deeper pool sections were filled additionally with a 7-cm middle layer of a sand and natural sediment mixture (3:1) from lake Duckwitz (N 53.593894, O 12.345664; Mecklenburg-Western Pomerania, Germany) covered by a 5-cm top sand layer. The systems were filled with a mixture of treated ground water (de-ionised) and deionised water up to a water depth of 27 cm from the UBA field station´s own waterworks (conductivity approx. 500 μS^{-1} cm). Water flow was adjusted to 0.1 m s^{-1} and water loss due to evaporation was compensated by adding deionised water. For further details, see Mohr et al. [28, 29]. A sketch of the mesocosm set-up is shown in Additional file 1: Fig. S1. The systems were illuminated with fluorescent tubes (OSRAM LF72) providing a mean photosynthetically active radiation of about 50 and 120 µmol m^{-2} s^{-1} on the water surface of the riffles and pools, respectively. The light regime followed monthly the seasonal daylight length.

One year before the start of the spraying sequence, the macrophyte species *Elodea canadensis*, *Potamogeton natans*, *Myriophyllum spicatum*, and *Sparganium erectum* were planted in the four pool sections as structural elements in the streams. Eight weeks before the first PPP application (start of the experiment), equal aliquots of a periphyton suspension obtained from a mesotrophic lake (Britzer Garten, N 52.4332, E 13.4160, Berlin, Germany) were added to each stream for inoculation. To ensure sufficient nutrient supply for the growth of macrophytes and periphyton, trace elements and nutrients were added in equal concentrations to each of the streams before and during the experiment: 1×mixture of trace elements (0.1 mg L^{-1} H_3BO_3, 0.02 mg L^{-1} $MnCl_2$*$4H_2O$, 0.001 mg L^{-1} Na_2MoO_4* $2H_2O$, 0.011 mg L^{-1} $ZnCl_2$, $CuCl_2$* $2H_2O$, 0.084 mg L^{-1} $FeCl_3$*$6H_2O$), 5×phosphate (0.02 mg L^{-1} PO_4-P), 5×nitrate (1 mg L^{-1} NO_3-N), and 9×silicate (4 mg L^{-1} Si). Given values for addition refer to water concentrations in the mesocosms).

In order to stock the stream mesocosms with benthic macroinvertebrates, attraction devices consisting of polyethylene net bags (32 cm×20 cm, mesh opening 4 mm) were filled with 100 g of loose organic triticale straw (Eco-village Brodowin, N 52.5439, E 13.5741, Germany). Bags were exposed in three different reference streams for two weeks and subsequently transferred to the stream mesocosms (with an equal number of bags per stream) one to two weeks prior to the start of the experiment (for details see supplemental information Additional file 1: Table S1). As reference sites, the sub-lacustrine creek Barolder Fließ (N 51.9912, E 14.2209, South-Brandenburg, Germany), the stream Lieberoser Mühlenfließ (N 51.9864, E 14.3522, South-Brandenburg, Germany), and the ditch Lietzengraben (N 52.6551 E 13.4741, North of Berlin, Germany) were chosen in order to guarantee a broad variety of benthic macroinvertebrate species. The ecological condition of the reference streams was characterised as good to moderate [30]. After 2–3 days of acclimatisation in the stream mesocosms, straw bags were opened to allow the loose straw to accumulate into 10-cm-deep cross-drains, which were evenly distributed over the sediment bottom acting as hiding spots and further detrital based food supply. Further details can be found in Mohr et al. [29]. In addition, macroinvertebrates were collected from the creeks Rabener Plane (N 52.0463, E 12.5719, South-Brandenburg, Germany) and Bardenitzer Fließ (N 52.0675, E 12.9320, South-Brandenburg, Germany) by means of two polyethylene net bags (38×65 cm, mesh size 4 mm), which were filled with ca. 300 g of dried leaves of *Alnus glutinosa* and exposed for two weeks. Macroinvertebrates were then separated from leaves using a sieve (10 mm mesh) and divided into nine subsamples. Each stream mesocosm was stocked with one subsample and the ninth subsample was immediately fixed in 96% ethanol as a reference sample for initial stocking. Furthermore, the mesocosms were colonised with different species of Trichoptera larvae and *Asellus aquaticus* collected by dip nets or hand from the stream Fredersdorfer Mühlenfließ (N 52.5675, E 13.7890, Brandenburg, Germany), as well as with *Cloeon dipterum* and different snail taxa (*Theodoxus fluviatilis,*

Potamopyrgus antipodarum, *Gyraulus* sp., *Physella* sp., *Lymnaea stagnalis*) from outdoor stream and pond mesocosms at the UBA field station. Details of stocking dates and number of introduced organisms are given in Additional file 1: Table S1.

Eleven weeks (day 77) after the experimental start with first PPP application on day 0, all stream mesocosms were colonised one more time with eight straw bags per system, which had previously been exposed for two weeks in the Lieberoser Mühlenfließ in order to compensate for emerged insects and to simulate re-colonisation with the next generation of insect larvae. For each stocking event, three bags were immediately fixed in 96% ethanol to determine the initial macroinvertebrate community in the mesocosms (Additional file 1: Table S1).

Spray sequence scenario

The original apple crop application scheme, which was provided by Julius Kuehn Institute, comprised 25 application days in the period from March to September 2010 with a total of 23 different PPPs with 21 AI. Fungicidal AIs were used 39 times, insecticidal AIs 13 times, and herbicidal AIs four times. The spray sequence tested in this study was slightly adjusted by excluding a virus, a pheromone, an antibiotic, and a growth regulating product. In addition, the sequence was shortened due to technical and organisational reasons by considering that all remaining PPPs were applied at least once. Thus, 17 PPPs composed of 17 different AIs (nine fungicidal, four herbicidal and four insecticidal AI) were applied on 19 spraying event days in the period from April to July 2015. Information on all applied PPPs, their AIs, mode of action, the producers, used RACs, and sampling dates are listed in Table 1 and Additional file 1: Table S2. Four stream mesocosms were used as treatments by applying the specific RAC of each PPP and by chronologically following the original application sequence as close as possible. The four remaining stream mesocosms served as controls. Control and treatment stream mesocosms were allocated randomly.

Application of plant protection products

The procedures of stock solution preparation are described in Additional file 1: Sect. 1. For a precise application of low pesticide concentrations, high precision micro-dosing rotary piston pumps (IP65 MCP-CPF with pump head FMI205 QP.Q1.CSC/9003, Ismatec, Switzerland), equipped with ceramic pistons and cylinders, PTFE seals, stainless-steel tubes, and fittings were used. During application, pesticide solutions were permanently stirred to avoid separation or sedimentation inside the flask before entering the pump. For rapid mixing of applied PPP over the whole stream water body, the duration of the application was adjusted to one complete water rotation in the circular streams (about 12 min). The control streams were dosed with the same volume of tap water by use of a watering can.

Sampling and analysis of active ingredients

Water sampling for pesticide analysis in all streams was carried out at the beginning and end of the study, to monitor all applied AIs (except dodine). After each application event, water sampling started at $t = 240$ min using stationary fixed rotary piston pumps automatically filling up 1-L samples of stream water during one water rotation in the circular streams. When necessary, additional buffer solution was added. When a PPP was applied on several application dates, additional sampling shortly before re-application was carried out to assess, if there were any residues from the previous application. All samples were transported directly to the laboratories in dark and cool conditions. The AIs were analysed by Eurofins Sofia GmbH (Berlin, Germany) and two chemical laboratories of the UBA. A detailed description of special PPP solution preparations, sampling, and analysis can be found in Additional file 1: Section 1.

The RACs of the AIs were used as references for both, dosing solutions and stream water, concentrations to calculate chemical recovery (Table 1). In the case of the second application of penconazole, the actual concentration was corrected, based on the measured preload concentration. Specific calculational conversions were made in case of copper-oxychloride, glyphosate-IPA, and MCPA-DMA, because analytes (Cu, glyphosate, and MCPA) differed from the AI in the PPP (e.g. Cu-oxychloride). For dodine, no water analysis was carried out, since rapid adsorption on all organic surfaces in the stream mesocosms was expected, due to its high soil partition coefficient K_d (range 2,200 to 18,000 kg L^{-1}) and no sensitive analytical method was available at the time.

Standard water quality parameters

Standard water parameters namely oxygen, pH, electrical conductivity, and water temperature were measured weekly with a multi-parameter probe system (Multi 3430, probes FDO 925, SensoLyt 900, TetraCon 925; each WTW, Germany). For nutrient and major ion analysis, water samples were taken every two weeks. Water samples were filtered (0.45 µm, cellulose-nitrate filters, Schleicher & Schuell, Germany) for further analysis. The major ions fluoride, chloride, bromide, sulphate, lithium, sodium, potassium, magnesium, and calcium were analysed using standard methods (TitrIC-system, Metrohm, Switzerland) [31–33]. Concentrations of o-phosphate, ammonia, nitrite, nitrate, and silicate were quantified photometrically using continuous flow analysis (San++,

Table 1 Concentrations and recovery of applied pesticides for active ingredients and application dates

AN	Date	ED	PPP	Active ingredient	Class of AI	AI in product [g kg]	RAC of AI [µg L^{-1}]	DF[c] [µg L^{-1}]	RDS Mean ± SD [%]	Water Conc in streams Mean [µg L^{-1}]	Recovery of AI in streams Mean ± SD [%]
1	10.04.2015	0	Funguran®	Copper (oxychloride)	F	756	4.54	1	102.6 ± 0.7	3.5	46.3 ± 92.61
2	14.04.2015	4	Delan® WG	Dithianon	F	742	0.82	1	104.0 ± 5.2	0.04	4.9 ± 0.9
3	23.04.2015	13	Syllit®	Dodine	F	408[b]	0.57	1	90.2 ± 6.8	n.a	n.a
4	29.04.2015	19	Syllit®	Dodine	F	408[b]	0.57	1	105.4 ± 15.4	n.a	n.a
			Calypso®	Thiacloprid	I	486[b]	0.32	1.14	89.1 ± 4.0	0.32	101.4 ± 3.7
5	05.05.2015	25	Malvin® WG	Captan	F	800	4.96	1.4	110.6 ± 7.3	5.61	113.2 ± 1.6
			Flint®	Trifloxystrobin	F	521	0.16	1	93.6 ± 0.8	0.14	88.5 ± 1.4
6	11.05.2015	31	Consist® Plus	Captan	F	600	4.96	1.4	140.3 ± 20.4	5.40	108.4 ± 2.4
				Trifloxystrobin	F	40	n.a[d]	1.4	127.4 ± 6.3	n.a	n.a
			Runner®	Methoxyfenozide	I	247[b]	4.45	1	79.3 ± 6.7	3.90	87.6 ± 10.8
7	13.05.2015	33	Syllit®	Dodine	F	408[b]	0.57	1	69.3 ± 47.8	n.a	n.a
8	19.05.2015	39	Malvin® WG	Captan	F	800	4.96	1.4	141.0 ± 32.0	4.41	88.8 ± 5.8
9	21.05.2015	41	Delan® WG	Dithianon	F	742	0.82	1	59.8 ± 11.5	< LOQ	< LOQ
10	28.05.2015	48	Delan® WG	Dithianon	F	742	0.82	1	90.5 ± 7.5	< LOQ	< LOQ
11	04.06.2015	55	Delan® WG	Dithianon	F	742	0.82	1	56.5 ± 4.1	< LOQ	< LOQ
			Topas®	Penconazole	F	105[b]	3.37	1.2	90.0 ± 3.8	3.44	102.1 ± 4.9
			Insegar®	Fenoxycarb	I	255	0.22	1.4	92.0 ± 6.4	0.26	113.8 ± 8.2
12	08.06.2015	59	Glyfos®	Glyphosate IPA	H	512[b]	95.23	1	81.3 ± 3.5	59.8	84.6 ± 2.1
			U 46® M Fluid	MCPA DMA	H	612[b]	15.17	1	127.5 ± 2.8	15.3	123.3 ± 5.7
13	10.06.2015	61	Delan® WG	Dithianon	F	742	0.82	1	27.1 ± 14.7	< LOQ	< LOQ
			Microthiol® WG	Sulphur	F	801	80.4	1	93.0 ± 3.5	79.6	99.3 ± 3.4
14	15.06.2015	66	Delan® WG	Dithianon	F	742	0.82	1	88.4 ± 11.9	< LOQ	< LOQ
			Kumulus® WG	Sulphur	F	804	80.4	1	97.7 ± 13.0	63.8	79.4 ± 9.4
			Movento®	Spirotetramat	I	156[b]	10.31	1	96.1 ± 4.4	8.60	83.3 ± 2.7
15	23.06.2015	74	Delan® WG	Dithianon	F	742	0.82	1	108.5 ± 23.5	< LOQ	< LOQ
16	29.06.2015	80	Malvin® WG	Captan	F	800	4.96	1.4	n.a.[e]	n.a.[e]	n.a.[e]
			Kumulus® WG	Sulphur	F	804	80.4	1	93.3 ± 8.6	66.1	82.2 ± 10.7
17	02.07.2015	83	Delan® WG	Dithianon	F	742	0.82	1	61.5 ± 13.8	< LOQ	< LOQ
			Kumulus® WG	Sulphur	F	804	80.4	1	103.1 ± 9.4	67.1	83.5 ± 6.0
			Vision®	Pyrimethanil	F	189[b]	2.32	1	94.0 ± 1.3	2.10	90.4 ± 1.5
				Fluquinconazole	F	47[b]	n.a[d]	1	68.0 ± 3.2	0.59	102.1 ± 2.4
18	21.07.2015	102	Stomp® aqua	Pendimethalin	H	479[b]	1.16	1.8	73.7 ± 7.3	0.62	53.1 ± 11.2
			Spectrum®	Dimethenamid-P	H	774[b]	0.66	1.2	97.9 ± 3.3	0.62	93.7 ± 2.4

Table 1 (continued)

AN	Date	ED	PPP	Active ingredient	Class of AI	AI in product [g kg]	RAC of AI [µg L^{-1}]	DF[c] [µg L^{-1}]	RDS Mean ± SD [%]	Water Conc in streams Mean [µg L^{-1}]	Recovery of AI in streams Mean ± SD [%]
19	22.07.2015	103	Malvin® WG	Captan	F	800	4.96	1.4	118.9 ± 1.8	4.76	96.0 ± 3.8
			Kumulus® WG	Sulphur	F	804	80.4	1	91.5 ± 1.4	55.8	69.4 ± 10.5
			Topas®	Penconazole	F	105[b]	3.37	1.2	95.2 ± 3.3	2.95 (4.26)[f]	87.5 + -3.0 (126.4.5 ± 5.3)[f]

Pesticides are classified as fungicides (F), herbicides (H) and insecticides (I). The intended concentrations (nominal concentrations) are equivalent to *RAC* regulatory acceptable concentration (status 2014), *AI* active ingredient, *AN* application number, *DF* dosing factor, *DMS* dimethylammonium, *ED* experimental day, *IPA* isopropylamine, *LOQ* limit of quantification, *na* not analysed, *PPP* plant production product, *RDS* recovery of AI in dosing solution, *WG* dispersible granules,

Water Conc. in streams = mean of measured AI concentration in water of the streams; Recovery of AI in streams = mean of water conc. divided by target concentration (RC)

[a] $RDS = C_{stock} * (RC*DF*V_{water})^{-1}$; V_{water} = Volume of water in the stream at time ED; C_{stock} = measured concentration of dosing solution

[b] Concentrations in g/L for fluids

[c] Dosing factor was used in order to obtain the target concentration in the dosing solution, the dosing factor was applied on the product

[d] For combi-product, RAC derivation is based on only one active substance; RAC by date 2014-07-25

[e] No values due to defect in analyser

[f] Value in brackets is the measured concentration without correction of preload concentration (Application Number 11)

Skalar, Netherlands) [34–37]. Dissolved organic carbon was analysed according to DIN EN 1484 [38] as non-purgeable organic carbon by catalytic combustion at 680 ℃ and NDIR-detection by use of a TOC 5000A with ASI 5000A (Shimadzu, Japan) with 3-point calibration and sample injection.

Periphyton and macrophyte sampling and analysis

For periphyton sampling, each stream was equipped with a special rack consisting of a stainless-steel frame holding 56 microscopic slides (76 × 52 mm, soda-lime glass), which was adjusted in parallel to the water current at 13 cm below water surface. For uniform illumination, eight rows of LED strip lights (neutral white; ~ 122 µmol m^{-2} s^{-1} PhAR at water surface; Co. Nobilé, Germany) were mounted under a stainless-steel flat roof.

For periphyton sampling, four colonised glass slides were taken at each sampling date (11 sampling dates in total). Prior to sampling, the slide positions were chosen randomly and the chosen positions were then taken out equally in all streams. The periphyton on both sides of a slide was scraped and rinsed off with a plastic Japan spatula and tap water. Periphyton of all four slides per stream was merged in one sample representing a total surface area of 237.12 cm^2. The suspension was transferred to a glass bottle and filled with tap water up to a volume of 1 L for further analysis.

Species identification of periphyton samples preserved with Lugol's solution or formalin was done by use of light microscopy with differential interference contrast (BX51, Olympus, Japan) using standard preparation techniques [39] and latest identification keys. Species specific cell or filament volumes were given as size classes based on microscopic size measurements and by use of corresponding geometric formulas. Biovolume was calculated as the product of species number and its specific specimen volume. Development of the macrophyte *P. natans* was monitored once a week by counting all floating leaves in each stream, starting 10 days before and ending 151 days after the first application event.

Macroinvertebrates sampling

Determination of macroinvertebrate species and their abundances in the stream mesocosms was carried out using traps stuffed with different substrates in order to attract diverse taxonomic groups. Straw (7 g dry weight) and small ceramic tubes (Eheim, Germany; 400 g) were filled into two polyethylene net bags (13 cm × 17 cm, 3 × 4 mm mesh size), respectively. For each sampling, two benthos traps were placed at two positions in each stream mesocosm for colonisation. After two weeks, the traps were sampled and rinsed with tap water over a sieve (200 µm mesh size) until all organisms were washed out and then stored in ethanol (80%) for species determination.

Emergence samples were taken at four different locations of each stream as described in Mohr et al. [29]. Each emergence trap covered a water surface area of 1 m^2 (polyethylene, 0.5 mm mesh size). Starting four weeks before the first application of PPP, emergence of merolimnic insects was quantified weekly for a period of 24 weeks by emptying and refilling the plastic beaker with 30 ml of "Renner fixation solution" (mixture of ethanol, distilled water, glycerine, acetic acid, and detergent) on the open top of the roof-like trap.

Macroinvertebrate drift was measured as described by Berghahn et al. [40]. Briefly, 30 min after pesticide dosing, two drift nets (size of the opening: 15 cm × 7.5 cm; mesh size of the funnel: 283 µm; total length: 140 cm) were placed in the middle of the stream bottom above the sediment surface (distance between nets approximately 30 m) for 4 h. Furthermore, night drift was measured employing drifts nets for 14 h (6 pm to 8 am). Drift was measured on three application dates with estimated high toxic pressure for benthic macroinvertebrates: (1) day 55: application of dithianon, penconazole and fenoxycarb; (2) day 66: application of sulphur, dithianon and spirotetramat; and (3) day 83: application of sulphur, dithianon, fluquinconazole and pyrimethanil (Tables 1, 3). Macroinvertebrates caught in the drift nets were preserved in 80% ethanol for further evaluation.

Taxonomic identification of macroinvertebrates (samples of initial stocking, sampling and emergence traps as well as drift net catches) were performed using magnifying glasses and a stereomicroscope (Stemi 2000-C; Zeiss, Germany). All macroinvertebrates were identified to lowest practical taxonomic level (mostly genus or family level, see Additional file 1: Table S1) using the latest identification keys (see Additional file 1: section 2). In the case of samples gained from emergence traps, male chironomids were identified to subfamily level, whereas female chironomids were pooled.

Statistical analysis

All statistical analyses were conducted using the statistical software package R Development Core Team (R version 3.6.1) [41] with the additional packages *vegan* [42] and *mvabund* [43]. Generalized linear models for multivariate data (GLM_{mv}) as described in Szöcs et al. [44] were used to evaluate the effects of multiple exposures of low-level applications of pesticides (control *vs.* treatment) on the temporal development of major ions (i.e. water concentration of measured ions), periphyton community (i.e. biovolume of identified periphyton taxa), macroinvertebrate community (i.e. density of

identified invertebrate taxa), and cumulative emergence of insects (i.e. density of identified taxa of emerging insects). Treatment effects were expressed and visualised as deviations from the control ($y=0$) over time (x-axis). A general treatment effect considering the whole experimental duration was analysed by comparison of the model including the time × treatment interaction to the model with time as sole variable. Significance level was set to $\alpha=0.05$ and level for a tendency to $\alpha=0.1$. Since the GLM_{mv} analyses only potential effects on the multivariate level, also species weights of the GLM_{mv} model (as percentage contribution to the deviance of single taxa, Table 2) were used as tool for the selection of taxa used for further analyses. In order to evaluate potential effects of PPPs into more detail, all taxa/parameter with species weight greater than 5% were used for further univariate analysis. As density data are usually skewed and widespread, log(x + 1) transformation was applied before further analysis [45]. Differences in the development of the selected taxa over time between control and treatment were analysed using generalized linear models (GLM, error distribution = Gaussian). If overdispersion was detected using the function *dispersiontest* within the R package *AER* [46], the error distribution 'quasi-Poisson' was used to overcome overdispersion. In case of a significant treatment effect (control *vs.* treatment) without a significant time × treatment effect, additionally sampling date(s) before the first application of a pesticide were compared. This was done in order to exclude that a significant difference between control and treatment already occurred before the first application of PPP for the respective cases (cf. Table 2). In doing so, a Mann–Whitney test (in case of only one sampling date before first PPP application) or a GLM (in case of more than one sampling date before first PPP application) was used to evaluate if start conditions were comparable between control and treatment replicates. For all relevant cases, no significant difference was found before the first application of PPP ($p > 0.05$). We further analysed the variation of the development of the selected taxa between control and treatment replicates. For each sampling date and taxon, the variation was calculated as the absolute difference between the mean value and single value per replicate of the control and treatment mesocosms (non-transformed data), respectively. Statistical analyses were then carried out as described above for the analyses of the development of the selected taxa over time.

Similarity of drifting invertebrates for each sampling date was analysed by non-metric multidimensional scaling (nMDS). When analysing Bray–Curtis similarities, which compare ranked similarities for differences between defined groups, square root transformation of abundance was used to reduce the effect of dominant taxa [47]. Differences in invertebrate drift were determined using PERMANOVA (permutational analysis of variance), which analyses multivariate data on the basis of distance measurements using permutations [48, 49].

Species weight (as percentage contribution) of single species to the differences on the community level in drifting community between control and treatment was estimated using SIMPER [42]. We selected the three species contributing the most to the differences in drift between control and treatment for further analyses. For these three species and for the development in the number of floating leaves of the macrophyte *Potamogeton natans* the same univariate statistical procedures as described above for standard benthos samples were used.

Toxic units

The toxic units (TU) approach was applied in order to describe the "toxic pressure" emanating from the pesticide concentrations dosed in this study. TU is commonly defined as the ratio between the concentration and a defined effective concentration as determined by ecotoxicity testing of the respective compound (e.g. EC_{50}). Hence, a toxic unit of 1 for a given compound would describe the exposure concentration, at which there is a 50% effect when dealing with e.g. an acute EC_{50} of a certain biological endpoint. The TU approach can be used to calculate the toxicity of mixtures for different organisms [2], assuming concentration–additive behaviour of mixture components as default to obtain a theoretical "mixture toxicity indicator". In this study, the TU approach was used to describe the theoretical toxicity as overall impact of the successive applications of the PPPs on the main biological components (periphyton, benthos) of the overall species community:

$$\mathrm{sumTU} = \sum_{i=1}^{n} \frac{\mathrm{ci}}{\mathrm{ECxi}}.$$

The calculation of TU (TU for each AI) was performed with "ci", which refers to the nominal exposure concentration (RAC for each AI tested) in the stream water at the start of application, corrected by the measured concentration in the dosing solution. For TU, the exposure concentration at the time of application was thus divided by the 48 h LC_{50} from acute *Daphnia* test and the 72 h EC_{50} from algae test (as given in EFSA conclusions and lists of endpoints for the respective AIs; https://www.efsa.europa.eu/en/publications). Both assessments were made in parallel. The sum of toxic units (sumTU) was calculated weekly as a measure for toxic pressure over time, which means that TUs can contain PPPs of several application dates. TU for PPPs containing sulphur were not

Table 2 Statistical analysis of the development of ions, periphyton, benthos, and emerging insects as mean values and variation for single taxa over time between control and treatment

				Mean values (GLM)				Variation (GLM)			
Compartment	**Parameter/taxon**		**Contribution [%] (GLM_{mv})**	**Res.Dev**	**Treatment**	**Time**	**Treatment × time**	**Res.Dev**	**Treatment**	**Time**	**Treatment × time**
Ions	Nitrate	[NO_3^-]	32.2	1015.9	0.85	0.24	0.54	1030.2	0.67	0.98	0.76
	Nitrite	[NO_2^-]	13.8	318	0.81	0.22	0.80	332.82	0.96	0.80	0.88
	Ammonium	[NH_4^+]	13.0	9.3	0.38	**< 0.001**	0.85	13.27	0.21	0.41	0.64
	Silicate	[SO_4^{4-}]	11.4	531.6	0.35	**0.03**	0.16	654.77	0.77	0.84	0.59
	Bromide	[Br^-]	8.3	0.11	0.23	**< 0.001**	0.40	0.10	0.47	**< 0.001**	0.08
	Natrium	[Na^+]	7.6	0.03	0.41	**< 0.001**	0.17	1002.4	0.42	0.15	0.74
	Potassium	[K^+]	6.0	0.17	0.37	**< 0.001**	0.06	76.26	0.62	0.09	0.98
	Phosphate	[PO_4^{3-}]	5.3	222.4	0.97	0.11	0.84	235.80	0.92	0.86	0.70
	Synechococcus spp.		7.6	14.6	0.33	**< 0.001**	0.16	8.8	0.13	**< 0.001**	**< 0.001**
	Navicula radiosa		7.3	5.1	0.62	**< 0.001**	0.34	2.5	0.15	**< 0.001**	0.67
Periphyton	Chaetophorales		6.8	15.2	0.79	**< 0.001**	0.48	8.2	0.58	**< 0.001**	**0.021**
	Diatoma spp.		5.6	14.6	0.27	**< 0.001**	0.39	5.9	0.69	**< 0.001**	0.77
	Nitzschia spp.		5.6	68.9	0.65	**< 0.001**	0.72	84.1	**0.04**	**< 0.001**	0.85
	Fragilaria fasciculata/acus		5.1	3.8	0.96	**< 0.001**	0.92	1.7	**0.03**	**< 0.001**	0.11
	Orthocladiinae		15.1	55.3	0.70	**< 0.001**	0.88	44.0	**0.033**	**0.005**	0.18
	Plecoptera		10.4	24.4	0.71	**< 0.001**	0.83	8.9	0.69	**< 0.001**	0.81
	Tanypodinae		7.6	992.4*	0.75	0.94	0.82	52.6	0.91	0.43	0.92
	Trichoptera		6.7	11.8	0.82	**< 0.001**	0.89	5.2	**0.004**	**0.013**	0.29
Benthos	*Physella* sp.		6.7	63.1	0.98	0.07	0.77	28.9	0.19	0.08	0.24
	Potamopyrgus antipodarum		6.5	61.7	0.95	0.52	0.72	61.7	0.95	0.52	0.72
	Lymnaea stagnalis		6.2	49.0	0.90	**0.001**	0.69	42.5	0.69	**0.023**	0.96
	Baetidae		5.3	23.8	0.12	**< 0.001**	0.14	11.8	0.08	**< 0.001**	0.16
	Gammarus spp.		5.0	20.2	0.62	**< 0.001**	0.13	35.8	0.34	**< 0.001**	< 0.001

Table 2 (continued)

			Mean values (GLM)				Variation (GLM)			
Compartment	**Parameter/taxon**	**Contribution [%] (GLM_{mv})**	**Res.Dev**	**Treatment**	**Time**	**Treatment × time**	**Res.Dev**	**Treatment**	**Time**	**Treatment × time**
Cumulative emergence[a]	Baetidae	14.6	85.8	0.44	**< 0.001**	0.24	90.6	0.37	**< 0.001**	0.25
	Chironomidae (female)	14.0	227.7	0.93	**< 0.001**	0.88	189.0	0.004	**< 0.001**	**0.002**
	Orthocladiinae (male)	10.4	199.6	0.82	**< 0.001**	0.89	144.7	**< 0.001**	**< 0.001**	**0.032**
	Chironominae (male)	8.3	40.2	0.62	**< 0.001**	0.64	40.7	**< 0.001**	0.76	**< 0.001**
	Chaoboridae	7.9	6.6	0.64	**< 0.001**	0.42	3.5	0.79	**0.003**	0.26
	Trichoptera	6.9	23.0	0.31	**< 0.001**	0.12	11.8	0.73	**< 0.001**	0.18
	Zygoptera	6.4	6.8	0.83	**< 0.001**	0.99	2.9	0.29	**< 0.001**	**0.009**
	Simuliidae	5.8	54.6	0.72	**< 0.001**	0.98	51.2	0.52	**< 0.001**	0.17
	Nemoura sp.	5.4	111.9	0.98	**< 0.001**	0.65	67.0	0.72	**< 0.001**	0.88
	Tanypodinae (male)	5.3	107.9	0.76	**< 0.001**	0.59	120.0	**0.012**	**< 0.001**	0.095
	∑ all taxa	-	273.1	0.94	**< 0.001**	0.85	293.9	**0.017**	**< 0.001**	**0.014**

Selected taxa had a contribution of ≥ 5% to the deviance in the respective ion composition/community response based on the multivariate generalized linear models (GLMmv). Bold letters indicate a significant effect based on the α-level of < 0.05; asterisk in column residual deviance (Res.Dev) indicates models where overdispersion was detected. Contribution of each taxa is based on the mvGLM with non-cumulative data

included in the analysis since sulphur is considered toxic to sediment organisms only.

Results

Analytical recovery of active ingredients in stream mesocosm water following the spray sequence scenario for apple crop

Overall, achieving similar and very low AI concentrations corresponding to the RAC (some as low as 0.16 µg L^{-1}) for 17 PPPs with different physico-chemical properties in the water of the four treated stream mesocosms was challenging. Analysis of stream water samples, taken directly after PPP application, disclosed that 22 of 35 analysed AIs were within the recovery range with less than 20% deviation from nominal concentrations. Hence, the desired concentrations (RACs) for these PPPs were reached (Table 1). However, a deviation of more than 20% above or below RAC was found for 13 AI in stream water. This was the case for some hydrolytically unstable AIs (dithianon, captan) and for highly adsorptive AIs (copper oxychloride, sulphur). For these substances, target concentrations refer to the respective dosing concentration (Table 1). In case of dodine, dosing solutions reached the RAC in most cases, but recovery in stream water was not analysable (see method section). For the substance dithianon, it was not possible to achieve concentrations corresponding to the RAC in most of the dosing solutions. Stream water samples were below limit of quantification of dithianon (Table 1).

Before the first PPP application, analytical screening of all organic AIs in the eight stream mesocosms revealed that background concentration levels were always below the limit of quantification. Only three of the organic AIs were found in water samples of the four treatment replicates at the end of the study after 144 days: dimethenamid with 0.15 µg L^{-1}, penconazole with 1.7 µg L^{-1}, and methoxyfenozide with 1.8 µg L^{-1}. Possibly these AIs led to higher chronic stress responses than the more short-lived AIs.

Effects on physico-chemical water parameters

During the experimental period, water temperature increased from 12.7 ℃ (day-28) in April to 18.5 ℃ at the end of May (day 48), and varied between 19 to 24.5 ℃ until the end of August (day 145). Data of physico-chemical water parameters for each sampling date are summarised in Additional file 1: Tables S3, S4; and Additional file 1: Fig. S2. GLM_{mv} analysis of the major ion composition (i.e. concentration of the analysed ions per replicate and sampling date) in the stream water of control and treatment was based on 13 single ions and alkalinity as sum parameter (Fig. 1A). Considering the whole experimental duration, no general treatment effect was found (GLM_{mv}, $p = 0.22$), but significant differences in the treatments were found on sampling date day 88 ($p = 0.024$) and day 101 ($p = 0.013$; Fig. 1A). Calculation of the contribution of single parameters to differences between control and treatment (as proportion of the deviance of a respective ion to the overall deviance) revealed eight ions with a contribution of more than or equal to 5% (nitrate, nitrite, ammonium, silicate, bromide, sodium, potassium, and phosphate), but none of these single parameters showed a significant difference between control and treatment (Table 2, Additional file 1: Fig. S2).

Effects on periphyton and macrophytes

During the experimental period, 34 taxa of primary producers were identified in the periphyton biofilm and then grouped to analyse effects of the applied pesticide spraying sequence on periphyton biovolume. A significant general treatment effect (i.e. biovolume of identified periphyton taxa per replicate [control *vs.* treatment] and sampling date) was found over the whole experimental period (GLM_{mv}, $p = 0.043$, Fig. 1B). Furthermore, GLM_{mv} analysis revealed a tendency for differences on day 75 ($p = 0.078$) and significant differences on day 18, day 89, day 103, and day 117 ($p < 0.05$, Fig. 1B) in periphyton community composition.

Six periphyton species contributed to differences between control and treatment with more than 5% (Table 2). A significantly higher variation in biovolume for the species *Synechococcus* spp. and Chaetophorales was found over time in the treatment mesocosms, compared to the controls (Table 2, Fig. 2A-B). In turn, for *Fragilaria fasciculata/acus* and *Nitzschia* spp., there was a significantly higher variation in the control mesocosms.

Since macrophytes standing stock should not be disturbed, only effects on the non-destructive endpoint leaf development of *P. natans* were analysed. At the start of the experiment in spring, *P. natans* shoots had not yet developed floating leaves (Fig. 3). The number of floating leaves increased over time and was significantly higher in the control compared to the treatment (GLM, treatment: $p = 0.016$, treatment × time interaction: $p < 0.001$), indicating a higher net production rate of control macrophytes.

Overall, the toxic pressure of specific PPP applications expressed as sumTU (Fig. 4) for each application date could not be related to effects on the periphyton community (Fig. 1B) nor to effects on *P. natans* (Fig. 3).

Effects on the macroinvertebrate community

For statistical analysis, the identified invertebrate taxa from benthos traps were grouped to higher taxonomic levels. For the resulting 16 taxa, effects of the pesticide spraying sequence were analysed. No general treatment effect (i.e. densities of identified macroinvertebrate taxa

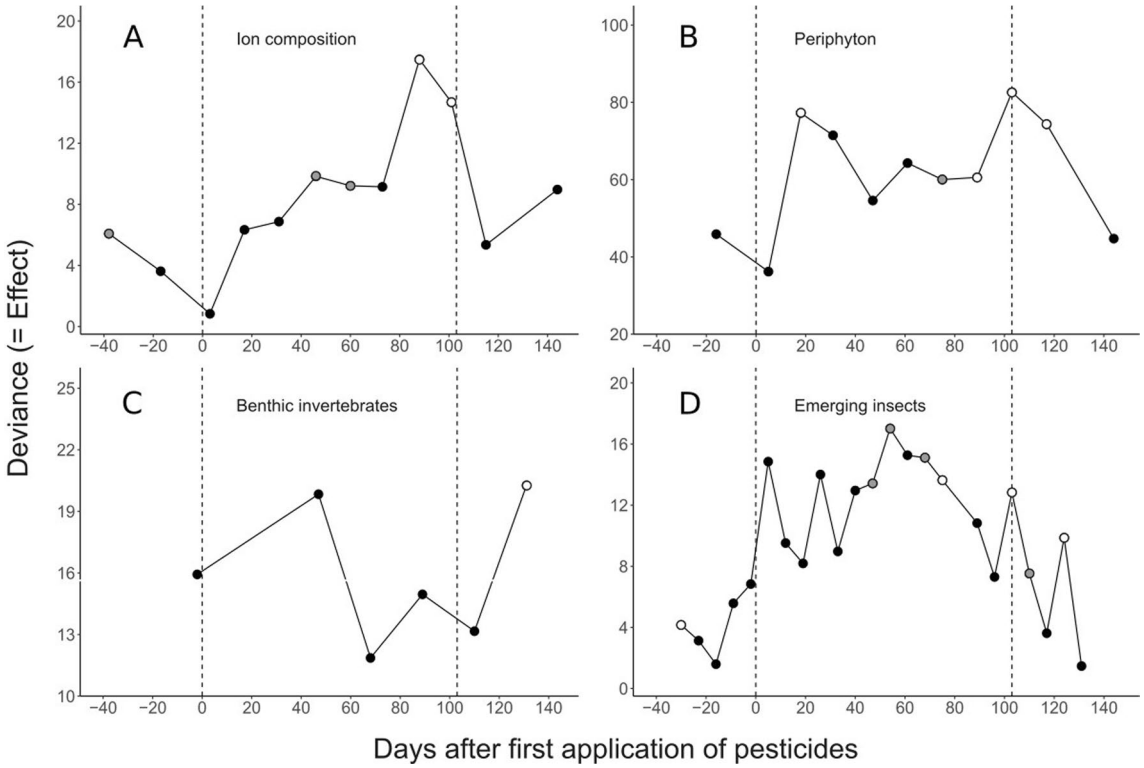

Fig. 1 Visualisation of results from generalized linear models for multivariate data (GLM_{mv}) of the community of **A** ion composition [i.e. water concentration of measured ions]; **B** periphyton [i.e. biovolume of identified periphyton taxa]; **C** benthic invertebrates [i.e. density of identified invertebrate taxa], and **D** emerging insects [i.e. density of identified taxa of emerging insects]. Open circles indicate sampling dates with significant differences ($\alpha = 0.05$) in the treatment mesocosms from the controls. Grey circles indicate a level for a tendency to $\alpha = 0.1$. Day 0 represents the day of the first application of a pesticide. Vertical dashed lines indicate the start (left) and the end (right) of the PPP application period

per replicate [control *vs.* treatment] and sampling date) was found for the whole experimental time (GLM_{mv}, $p = 0.41$, Fig. 1C). Furthermore, GLM_{mv} analysis revealed a significant difference in the community (i.e. densities of identified macroinvertebrate taxa as described above) between the treatment and the control at the end of the study on day 131 ($p = 0.049$, Fig. 1C).

Ten taxa of benthic macroinvertebrates contributed to the differences between controls and treatments with more than or equal to 5% (Table 2). The variation in number of Orthocladiinae, Trichoptera (Table 2), and Turbellaria (Fig. 2C), were higher in the treatment mesocosms, while the variation in the number of *Gammarus* spp. showed a significant increase in the treatment mesocosms over time (Table 2, Fig. 2D).

Drift of invertebrates

Drift of benthic macroinvertebrates was measured after three application dates. A significant treatment effect was detected on the community level only for the pesticide application event on day 83 (sulphur, dithianon, fluquinconazole and pyrimethanil; PERMANOVA, $p = 0.03$, Table 3), but no significant differences in drift of the three taxa Tanypodinae, *Gammarus* spp., and Baetidae, which contributed the most to the community effect, were found (Table 3).

Emergence of aquatic insects

In order to analyse effects of the pesticide spraying sequence on the emergence of aquatic insects, hatched insects were grouped into 18 taxa. No general treatment effect (i.e. densities of identified taxa of emerging insects per replicate [control *vs.* treatment] and sampling date) was found for the whole experimental duration (GLM_{mv}, $p = 0.32$, Fig. 1D). Furthermore, GLM_{mv} analysis revealed significant differences in the community composition of emerging insects at the first sampling date of the experiment before the first pesticide application at day-30 ($p < 0.05$, Fig. 1D) and in the treatment on day 75, day 102, and day 124 ($p < 0.05$). At the first sampling date (day-30) only few individuals were found in the emergence traps indicating stronger random effects.

Ten taxa of emerging insects with a contribution of more than 5% to differences between control and treatment were identified (Table 2). None of the ten taxa revealed a significant difference in the cumulative

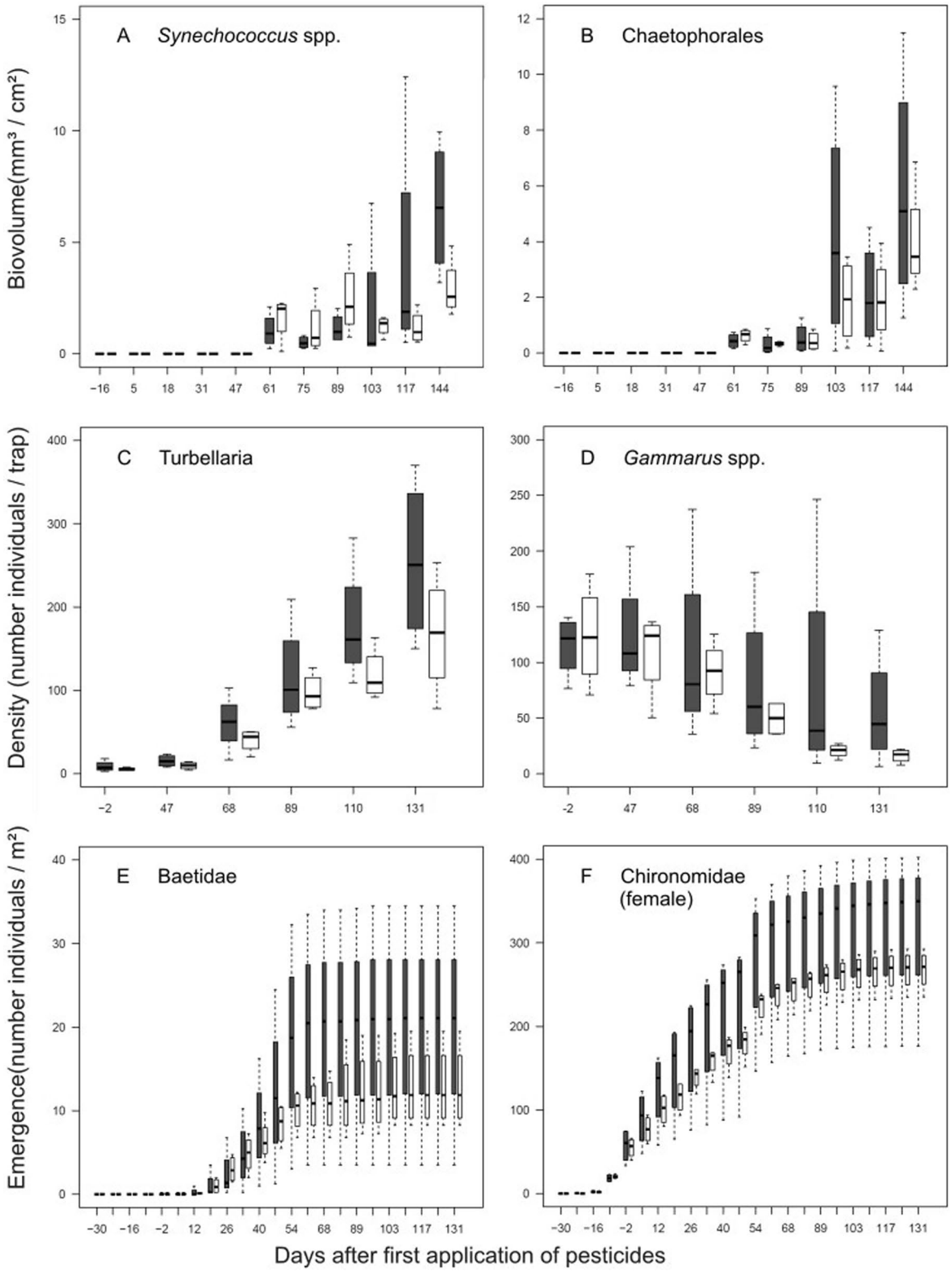

Fig. 2 Development of biovolume of the periphyton taxa **A** *Synechococcus* spp; **B** Chaetophorales; development of abundance of the benthic invertebrate taxa; **C** Turbellaria; **D** *Gammarus* spp; cumulative emergence of the merolimnic insect taxa; **E** Baetidae; **F** female Chironomidae during the experimental period. Grey and white boxplots represent treatment and control, respectively. Day 0 represents the day of the first pesticide application. X-axes are with different scaling for better visual resolution

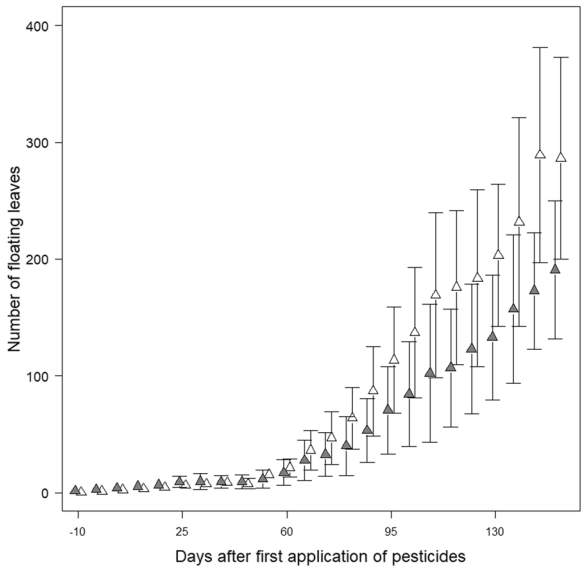

Fig. 3 Development (mean ± SD) of floating leaves of *Potamogeton natans* during the experimental period. Grey and white points represent treatment and control, respectively. Day 0 represents the day of the first pesticide application

number of emerging insects in the treatment over time, when compared to the control. However, variations in the cumulative emergence of some chironomid taxa (i.e. female Chironomidae, male Orthocladiinae, male Chironominae), and cumulative emergence of all taxa (sum) showed increasing variation over time in the treatment (Table 2). Furthermore, cumulative emergence of Baetidae and female Chironomidae displayed increasing variation over time in the treatment (Fig. 2E, F) and the variation in cumulative emergence of male Tanypodinae was significant (Table 2).

Toxic pressure over time

The TU approach showed relatively high toxic pressure on primary producers during the first part of the spraying sequence (weeks 1 to 4; Fig. 4). SumTU exceeded a value of 0.1 (1/10th EC_{50}) during week 1, 3, and 4 (Fig. 4), which corresponds to the time of application of the two fungicides copper and dodine, both being more toxic to primary producers than to invertebrates and delivering rather high sumTUs than the four herbicides applied during the second part of the experiment. In addition, in week 12 and 18, sumTU was also above a value of 0.1.

The TU approach also indicated a toxic pressure on invertebrates in the first part (weeks 1 to 6) of the experiment (Fig. 4). However, the overall toxic pressure exerted on invertebrates was much lower than on primary producers with a maximum sumTU value of 0.035, at no time exceeding a value of 0.1.

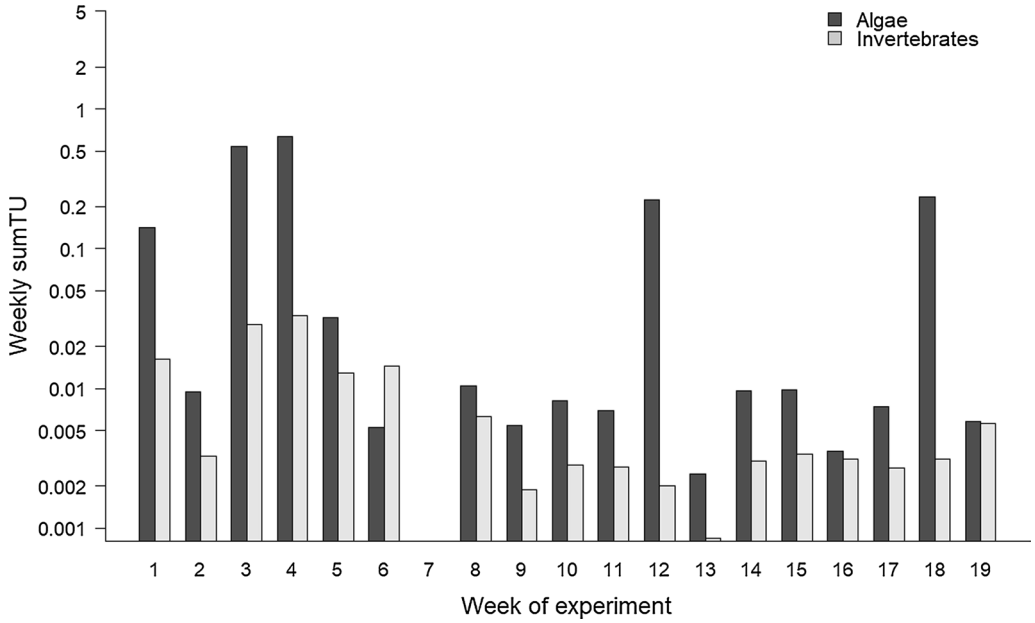

Fig. 4 Sum of toxic units (sumTU) over time for primary producers based on the 72 h EC_{50} from algae test (dark bars) and invertebrates based on the 48 h EC_{50} from acute *Daphnia* test (bright bars). SumTU values are given as weekly average and consider the concentration at the time of PPP application (Note: RAC corrected by recovery of dosing solution; values for sulphur are excluded since they are considered as toxic toward sediment organisms only.)

Table 3 Statistical analysis of drifting invertebrates for day and night drift catches (time) after three different application events

Date	Day of drift catches	Pesticide (class)	PERMANOVA			SIMPER		GLM			
				F value	*P* value	Taxon	Contribution%	Res.Dev	Treatment (t)	Time (d)	*t*×*d*
04-06-2015	55	Dithianon (F) / penconazole (F) / fenoxycarb (I)	Treatment (t)	0.86	0.53	*Gammarus* spp.	16.9	56.7*	0.61	0.56	0.64
			Daytime (d)	4.47	**0.002**	Tanypodinae	13.8	9.1*	0.22	**< 0.001**	0.96
			t × d	0.75	0.63	Orthocladiinae	11.6	54.5	0.41	**0.022**	0.18
15-06-2015	66	Sulphur (F) / dithianon (F) / spirotetramat (I)	Treatment (t)	0.89	0.50	*Gammarus* spp.	19.4	88.0*	0.49	0.24	0.91
			Daytime (d)	2.17	0.06	Orthocladiinae	17.9	51.8*	1.00	0.24	0.49
			t × d	0.09	0.99	Tanypodinae	10.1	14.1*	1.00	0.13	0.50
02-07-2015	83	Sulphur (F) / dithianon (F) / pyrimethanil (F) / fluquinconazole (F)	Treatment (t)	2.76	**0.03**	Tanypodinae	14.7	808.9*	0.22	**0.006**	0.73
			Daytime (d)	4.53	**0.002**	*Gammarus* spp.	12.9	67.4*	0.41	0.27	0.98
			t × d	0.14	0.98	Baetidae	9.4	15.6*	0.25	**0.013**	0.47

For taxon-specific analysis the three taxa with the highest contribution to the difference in the respective drifting community between control and treatment were selected. Bold letters indicate a significant effect (α level = 0.05); asterisk in column residual deviance (Res.Dev) indicate models where overdispersion was detected. Drift was measured on day 55, 66 and 83 of the experiment, day 0 being the day of first pesticide application

Discussion

Effects of the apple crop spraying sequence on the stream mesocosm community

The applied pesticide concentrations on the basis of regulatory acceptable concentrations (RAC values) following an apple crop spraying sequence scenario led to significant differences between control and treatment streams over time, especially towards the end of the study. This was the case for the endpoints: variation in biovolume of selected periphyton species, abundance and variation of abundance of selected benthic macroinvertebrates and insect emergence, as well as the number of floating leaves of *P. natans* (Figs. 1, 2 and 3, Table 2). Changes in physico-chemical parameters can be used as proxy of the sum of all photosynthetic and respiratory activities and therefore act as an indicator for changes in the ratio of respective processes. In our experiment, it was not possible to identify a relationship between specific PPP pulses and their associated toxic pressure exerted over time and significant biological and/ or ecological effects. Indeed, the TU approach relates to exposure concentrations at the time of treatment and acute ecotoxicity (from laboratory testing, i.e. under standardised laboratory conditions, Fig. 4). Thus, its predictive power is limited since it does not consider (i) potential long-term or delayed effects of fast dissipating substances or chronic effects of substances persisting in the water (e.g. copper) nor (ii) effects of species interactions and additional environmental stressors exerted on field communities and uncertainties related to more sensitive species than *Daphnia* and algae. Nevertheless, the community response as shown in Figs. 1–3 indicates that the repeated low-level pesticide applications resulted in measurable cumulative stress for several species and groups, confirming the findings of other studies. In a mesocosm study conducted by Polazzo et al. [50], field-relevant peak exposure concentrations of pesticides resulted in a changed community composition of zooplankton only at the end of the experiment after 50 days. Our results are also in line with the review by Altenburger et al. [6] about mixture effects of substances with different modes of action at concentrations below single species effect thresholds.

The most striking result of the present study was the statistically significant increase in variation of population response parameters of selected taxa in the treatment mesocosms compared to the controls (Table 2, Fig. 2). On the one hand, variation in species responses has always been a big issue in ecotoxicology and species tests [51–53], as it hinders the statistical evaluation of true effects from background noise with regard to mean values. On the other hand, there is increasing evidence that inter-replicate variation of population development parameters as a result of genotype plasticity of individuals, can be an indicative endpoint for chemical pollution [54]. In general, ecotoxicological tests are designed to minimise the variation of possible influencing factors, other than the stressor under assessment [55]. In mesocosm studies with multiple populations of different trophic levels acting together, the likelihood of facing significant variation in species responses is rather high [56]. In the present study, data variation of most endpoints at the beginning of the experiment was low in the control and treatment mesocosms. This indicates that the actions taken to synchronise the community development in the mesocosms by a thorough and homogenous biological establishment were successful. Therefore, the increased variation in abundance of selected species observed in the treatment mesocosms might be considered as an effect of the repeated PPP pulses rather than a general mesocosm variation.

The observed variation in species response to the 19 times repeated low-dosed PPP application might have been induced by genotype plasticity of respective species. The higher the genotype plasticity of species or communities, the more complex the responses will be in terms of physiological adaptations such as detoxification processes or increased escape responses [53]. In addition, small differences in concentrations of some pesticides (see standard deviation of recovery of AI in streams; Table 1) at the start of the experiment may have further promoted the variation in species responses. The slightly different pesticide concentrations in the four treatment mesocosms may have led to different individual stress responses, causing cascadic effects in the populations. This so-called butterfly effect has been described as the sensitive dependence on initial conditions, in which a small change in one state of a system can result in large differences in a later state [57]. In the present study, the concentration differences might have caused indirect or hormesis effects on the species communities indicated by higher abundance values in the treatments (Fig. 2). Especially hormesis effects have been shown to be trigged by very low doses of a range of chemicals and physical agents [58, 59]. In a review by Agathokleous [60], numerous plant and animal species responses to various stressors have been linked to hormesis at low stressor concentrations.

Overall, the increased variation in species response in the treatment mesocosms of the present study should be discussed as a general disturbance measure [53, 61], even if it was not possible to link the results to specific pesticide pulses, to genotype plasticity of the species or the differences in exposure between replicates. When assessing population health, e.g. in the risk assessment of pesticides, the parameter "inter-replicate variation" should also be considered. Studies with high variation should

not per se be considered as unreliable, as this parameter might in fact reflect system resistance and resilience against perturbations or it might serve as an early warning sign of potential disturbances of communities in the field [62–66]. Hence, such results of inter-individual variation can also be seen as valuable source of information for the risk assessment [61].

Relevance of chosen apple spray scenario and implementation for the risk assessment

In Germany, 48.8% of the fruit cultivation area was used for apple production in 2008 [22], and in 2017 about 10 Mio tons of apples were harvested from approx. 473.550 ha [67]. The worldwide production of apples was 83.1 Mio tons in 2017 [68]. Apple orchards are regularly treated with pesticides, often only for a high-quality visual appearance of the fruits to increase the sales value. Fungicides represent the most dominant pesticide group with more than 80% of AI applications per growing season [8, 69, 70] used mainly to prevent apple scab and other fungal infections. Captan and dithianon have been listed as two of the most frequently used fungicides [8, 71] and were also applied several times in the spraying sequence of the present study. In a survey on application of chemical pesticides in apple farming, Roßberg and Harzer [71] reported that the treatment frequency index (TFI), which quantifies the number of registered PPP applied to apple crop, was 28 TFI in 2001 and increased continuously to 33 in 2013. In France, between 2006 and 2008, the mean TFI was 35 for conventional and 26 TFI for organic apple orchards with scab-susceptible apple cultivar [72]. For the shortened apple spraying scenario used in this study, 19 treatments with 17 different AI were applied, which can be considered as a "light to moderate" case scenario for standard practice in Germany [8, 71]. Thus, potential effects on natural communities in streams and rivers in agricultural areas facing multiple stressors in addition to the PPP spraying scenarios such as other chemicals, habitat degradation, and nutrient stress over the years, might be more pronounced [e.g. 73]. Also, the exposure concentrations limited to the RAC values used in this study may not represent a realistic exposure scenario of pesticide entries in rural areas. Liess et al. [27] identified an exceedance of RAC values for at least one pesticide concentration in agricultural streams in Germany for 81% of the water samples tested.

The present study reinforces mounting evidence, that the current environmental risk assessment in the context of the authorisation of PPP may not be safe for ecosystems and their communities, when facing multiple pesticide entries of spraying sequences applied year after year. Indeed, risk assessment is currently restricted since it refers to individual PPP. In other words, it is questionable whether the use of RAC values derived for individual PPPs are enough in view of the typical agricultural practice of applying pesticide tank mixtures and/or spray series. This issue has also been raised by, e.g. Weber et al. [74], Junghans et al. [18], or Covert et al. [75]. Unfortunately, experimental studies investigating effects of realistic spray sequences scenarios are still very rare since it is very difficult to get real application scheme data from farmers. In laboratory studies, Panico et al. [76] discovered strong effects to invertebrates in soils, facing mixtures of pesticides conventionally used on agricultural fields. To our knowledge, until now only three mesocosm studies have assessed complex realistic exposure to pesticides used on bulb, potato, and apple crop, respectively [77–79]. Wijngaarden et al. [77] and Arts et al. [78] revealed significant effects on pond/ditch communities at higher spray application rates, but concluded that risk assessment based on single pesticides seems to sufficiently protect freshwater ecosystems. Both studies had a different experimental approach using pesticide concentrations based on a realistic application and spray drift scenario, but the amount of pesticides and application times were lower than in the present study. Talk et al. [79] used a similar set-up as in this study, but focussed on fungal endpoints in pond mesocosms. The authors could not identify effects on fungal communities or leaf litter decomposition. Overall, more experimental studies applying realistic exposure scenarios are needed, even though implementing them is a challenge and very time consuming. An intensive field study by Schäfers et al. [26] investigating invertebrate communities in differently treated apple orchards in the German region Altes Land revealed that invertebrate communities were significantly affected by pesticide use, depending on exposure pressure. This was confirmed in a larger monitoring study in Germany showing that (i) 83% of agricultural streams did not meet the pesticide-related ecological targets; (ii) agricultural nonpoint source pesticide pollution was the major driving force in the reduction of vulnerable insect populations in aquatic invertebrate communities and (iii) the risk resulting from exposure to pesticide mixtures and to frequent successive pesticide applications needs to be better addressed [17, 27].

Conclusions

The repeated tiny dose makes the poison—meaning that the results of this mesocosm study provide further evidence for notable effects on aquatic stream communities following exposure to repeated low pesticide concentrations simulating a realistic spraying sequence for apple crop. Indeed it induced disturbances for some invertebrates and primary producers. Albeit the observed differences between control and treatment

were rather small, our results support concerns that the current risk assessment for PPPs may not ensure a sufficient level of protection to field communities facing multiple pesticide entries from spraying sequences or other combined pesticide stress. The current framework for single PPP allows risk assessment refinements that claim for more realism. However, it ignores important aspects such as multiple exposure and effects of additional stressors, both natural and anthropogenic, which typically increase the sensitivity of organisms to pesticides [80, 81]. Therefore, it is still very challenging to realistically assess the risk. Obviously, the actual regulatory convention focussing on RAC values derived for individual AIs only, is not sufficient to ensure that no adverse effects on populations might occur in the field in view of multiple chemical (pesticides and other chemicals) exposures as well as further environmental stressors. As a consequence, legally binding protection goals may not be fulfilled. Applying for example an additional mixture assessment factor (MAF) in risk assessment, which has frequently been suggested [82], is one option to better address the issue of mixture exposure occurring in the field. Given the inherent and remaining uncertainties of any (however complex) risk assessment, our findings might support the importance of an ambitioned pesticide reduction strategy as recently brought forward in the European Green deal with the Farm-to-Fork strategy [83, 84], too.

Abbreviations

AI	Active ingredient
EC	Effect concentration
GLM	Generalized linear models
MAF	Mixture assessment factor
PEC	Predicted environmental concentration
PPP	Plant protection product
RA	Risk assessment
RAC	Regulatory acceptable concentration
SI	Supplemental information
TBZ	Tebuconazole
TU	Toxic unit
UBA	German Environment Agency

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12302-023-00739-y.

Additional file 1. **Section S1.** Chemical analysis; **Table S1.** Date, origin and number of stocked macroinvertebrates for each stream mesocosm based on estimates from stored reference samples; **Table S2.** List of active ingredients. International union of pure and applied chemistry name; **Table S3.** Water parameters of control and treatment stream mesocosmsfor each sampling date; **Table S4.** Water-chemical parameters of control and treatment stream mesocosmsfor each sampling date; **Fig. S1.** Sketch of stream mesocosm set-up, **Fig. S2.** Development of the concentration of the nutrients **A**) potassium, **B**) phosphate, **C**) nitrate, and **D**) nitrite during the experiment.

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Author contributions
SM, StM, MF and RS designed and performed the experiment, evaluated data and wrote the original manuscript. SD and TF were involved in designing the experiment and wrote and edited the manuscript. All authors read and approved the final manuscript.

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Declarations

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