Repeated insecticide pulses increase harmful effects on stream macroinvertebrate biodiversity and function

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Abstract

We exposed twelve mesocosm stream channels and four instream channels to one, two, and four pulses of the insecticide lambda-cyhalothrin (0.1 ug L^{-1}) applied at two day intervals, each pulse lasting 90 minutes. Unexposed controls were included. We monitored macroinvertebrate taxonomic composition in the channels and in deployed leaf packs one day before and 29 days after the first exposure. Further, we measured drift in and out of the channels and leaf litter decomposition. Lambda-cyhalothrin exposures induced significantly increased drift in both experiments especially for *Gammarus pulex*, *Amphinemura standfussi*, and *Leuctra* spp. Macroinvertebrate taxonomic composition increasingly changed with increasing number of lambda-cyhalothrin exposures being most pronounced in the mesocosm channels. Further, leaf decomposition significantly decreased with increasing number of exposures in the mesocosm channels. Our study showed that species with predicted highest sensitivity to lambda-cyhalothrin were primary drivers of significant changes in taxonomic composition lasting for at least one month despite continuous recolonization of exposed channels from upstream parts of the natural stream and from the water inlet in the mesocosm channels. The overall results highlight the importance of sequential exposures to insecticides for understanding the full impact of insecticides on macroinvertebrates at the community level in streams.

Key words: Insecticides, sequential exposures, macroinvertebrates, ecological effects, streams

Capsule: Increasing number of sequential exposures with lambda-cyhalothrin increased changes in macroinvertebrate community structure and decreased their ecological function.

1. Introduction

Application of agricultural pesticides often results in unintentional losses to adjacent streams. Primary transport routes include spray drift, surface runoff, and subsurface tile drainage, (Neumann et al., 2002; Kronvang et al., 2004). Pesticide transport via surface runoff and subsurface tile drainage to streams is triggered by major precipitation events resulting in pulses of increased pesticide concentrations (Rasmussen et al., 2015; Boye et al., 2019).

Agricultural insecticides, especially pyrethroids, are important drivers of ecological impairment in streams, especially for macroinvertebrates and loss of their biodiversity (Beketov et al., 2013; Malaj et al., 2014). Pyrethroid insecticides still exceed regulatory threshold levels in streams more frequently than other classes of insecticides, although concentrations rarely reach levels that cause acute mortality (Rasmussen et al., 2015; Wolfram et al., 2018). In fact, pyrethroids exceeded regulatory threshold levels in more than 80% of the agricultural stream water samples in the U.S. (Wolfram et al., 2018). Importantly, pyrethroids are particularly toxic due to their fast mode of action targeting the sodium channel (Vijverberg and van den Bercken, 1990), and short pulses of pyrethroids even below the regulatory acceptable concentrations (RAC) can generate significant and long-lasting effects on macroinvertebrate species (e.g. Liess & Scuhlz, 1996; Rasmussen et al., 2013a). Long-lasting effects are probably produced through a series of sublethal effects (Liess et al., 2013), including increased drift (Schulz and Liess, 1999), reduced pupation success (Schulz, 1997), reduced emergence success (Rasmussen et al., 2017), inhibited mating behavior (Heckmann et al., 2005), and reduced predation success (Rasmussen et al., 2013b).

Importantly, exposure to pyrethroids and other lipophilic insecticides in agricultural streams is not necessarily restricted to one single event coinciding with the time of insecticide application on

63 adjacent agricultural areas. Rasmussen et al. (2015) showed that agricultural streams in Denmark
64 received up to 5 significant pulse exposures during May, June, and July depending mainly on local
65 precipitation patterns. Of further importance, effects of pesticides on stream macroinvertebrate
66 communities generally exceed the effects of similar exposures observed in laboratory and most
67 mesocosm studies (Schäfer et al., 2012; Liess et al., 2016). In part, this might be explained by
68 sequential pesticide exposures occurring in stream ecosystems, as sequential exposure scenarios are
69 not included in standard ecotoxicity testing (Liess et al., 2016). This incongruence between effects
70 observed in the field and in controlled experiments indicates that the current risk assessment of
71 pesticides is not sufficiently protective. Consequently, studies addressing this issue should hold
72 high priority.
73
74 Overall, remarkably few studies address effects of sequential exposures (but see Russo et al., 2018;
75 Bray et al., 2019). Notably, short-term exposure to pyrethroids may result in long-term weakening
76 of individuals, which may render the organisms increasingly susceptible to subsequent exposures
77 (Liess et al., 2016; Ashauer et al., 2017; Russo et al., 2018). Existing studies addressing sequential
78 exposures of insecticides focus on drift in macroinvertebrate assemblages during and after exposure
79 to environmentally unrealistic concentrations of neonicotinoids (Mohr et al., 2012; Berghahn et al.,
80 2012), whereas Russo et al. (2018) collected *Gammarus pulex* from agricultural and forest streams
81 (representing previous exposure regime) and performed standard acute mortality tests to estimate
82 EC_{50} concentrations. Bray et al. (2019) exposed macroinvertebrate communities to two sequential
83 exposures of an organophosphate in combination with additional non-toxic agricultural stressors in
84 a recycling stream mesocosm setup. Bray et al. (2019) observed significant changes in species
85 composition and leaf litter decomposition following addition of the organophosphate, but the
86 exposure continued for multiple days according to the natural degradation of the parent compound

87 in the closed system. Consequently, studies addressing community and ecosystem level effects of
88 sequential insecticide exposure within the range of environmentally realistic exposure scenarios are
89 lacking.
90
91 This study aims to gain insight into reasons behind the observed incongruence between results of
92 studies conducted in the field and in controlled lab- and mesocosm setups, and consequently, to
93 potentially help improve current risk assessment procedures. In this study, we investigate if
94 increasing sequential exposure to a pyrethroid insecticide increases community level effects on
95 macroinvertebrates compared to a single pulse exposure in mesocosm channels and *in situ*
96 (instream) channels to explore ecological responses in experimental setups with different levels of
97 resemblance to natural stream systems. We applied the pyrethroid lambda-cyhalothrin at an
98 environmentally realistic concentration (0.1 µg L^{-1}) (see e.g. Wolfram et al., 2018) and assessed
99 macroinvertebrate drift and changes in taxonomic composition and function following 0, 1, 2, or 4
100 repeated pulse exposures. Leaf litter decomposition was used as measure for ecosystem function.
101 We hypothesized that increased exposure frequency will increase changes in taxonomic
102 composition by reducing densities of especially sensitive taxa who will evade exposed systems or
103 areas through increased drift activity. Since some of the species with highest sensitivity towards
104 pyrethroids are facultative or obligate shredders, these structural changes in the macroinvertebrate
105 assemblages will translate into altered macroinvertebrate mediated leaf litter decomposition.
106
107 2. Material and methods
108 2.1 Mesocosm stream channels
109 We used 12 artificial stream channels (LxWxD = 12x0.3x0.3 m), with steel bottom, sides of acryl
110 plastic, and a slope of 5‰. Each channel was equipped with riffles (n=5) and pools (n=6). Riffles

111 contained stones (diameter >150 mm) and stones/gravel (diameter = 48-96, 24-48, and 12-24 mm,
112 respectively, in a ratio of 1:4:4), whereas pools contained gravel/sand (diameter = 6-12, 3-6, and 1-3
113 mm, respectively, in a ratio of 1:1:0.5). Sediment depth varied from 5-6 cm in pools to 10-20 cm in
114 riffles. An axial pump with water intake from a nearby stream (Lemming stream, Denmark:
115 56°14′49′′N, 9°31′51′′E), continuously fed the channels with stream water. Lemming stream has a
116 high inflow of groundwater and a catchment dominated by forest. Discharge was measured in each
117 stream channel just before each exposure and during background drift measurements (see section
118 2.5) using a 5 L plastic bucket and a stopwatch. Moreover, temperature was measured throughout
119 the experiment in each channel using HOBO TidBit temperature loggers (logging interval 30 min).
120 Macroinvertebrates were introduced to the channels on April 12th, 2010 using the procedure
121 described in Graeber et al. (2017). In brief, macroinvertebrates were collected from Lemming
122 stream using kick sampling along a reach of approximately 500 m. In total, 120 kick samples were
123 transferred to each mesocosm channel. Graeber et al. (2017) showed that this method provided a
124 community structure in the mesocosm channels that was comparable to Lemming stream.
125
126 2.2 Instream channels
127 Instream channels were established in Stavis stream (Denmark: 55°26′25′′N, 10°12′23′′E). This
128 stream has a catchment consisting primarily of forest and contains a highly diverse and pristine
129 macroinvertebrate community. On April 27th, 2010 we created four parallel channels (LxW =
130 25x0.5 m) in the centre of a natural riffle, these channels being separated by 0.5 m high flexible
131 walls made as a double layer of strong plastic folia fixated to the stream bed using an iron chain (2.5
132 kg m^{-1}). The upper parts of the walls were fixed to steel sticks mounted in the streambed and
133 stabilized laterally by 10 mm plastic sticks. Using uranine dye, we confirmed that the walls
134 prevented exchange of water between the channels. Discharge in Stavis stream was measured using

an Ott, Kleinflügel, 30 mm in each channel just before each exposure and during background drift measurements (see section 2.5). Moreover, temperature was measured throughout the experiment in each channel using a HOBO Tidbit temperature logger (logging interval 30 min).

2.3 General experimental approach

We performed experiments in the 12 mesocosm channels and the 4 instream channels following the same experimental protocol. We exposed in-channel macroinvertebrate communities to lambda-cyhalothrin (nominal concentration = 0.1 µg L^{-1}) for 90 minutes, corresponding to approximately ½ of the EC50 based on the 48h acute mortality testing of *Daphnia magna*. Ecotoxicity information for lambda-cyhalothrin was retrieved from the Pesticide Properties Database (Lewis et al., 2016). We used four treatments: Control, 1P (one pulse of lambda-cyhalothrin), 2P (two pulses of lambda-cyhalothrin), and 4P (four pulses of lambda-cyhalothrin), with lambda-cyhalothrin exposure on experimental days 0, 2, 4, and 6 (Day 0 exposure on May 11th and May 4th in the mesocosm and instream channels, respectively). Lambda-cyhalothrin was dissolved in 96% ethanol, and all channels, including controls received an equivalent dose of ethanol. We used a replicated design in the mesocosm channels (n = 3) and a non-replicated design in the instream channels (position in sequence: C–1P–2P–4P). In each channel, we applied lambda-cyhalothrin continuously at the channel inlet using battery driven, adjustable pumps and diffusers (control channels received equivalent doses of ethanol).

We measured exposure concentrations of lambda-cyhalothrin in each channel after 60 minutes of exposure. Water samples for exposure validation were collected at the outlet in the mesocosm channels and at 0, 12.5, and 25 m from the inlet in the instream channels. Samples were collected using 1 L glass bottles.

159

160 The Danish Ministry of Environment granted permission to expose channels in Stavis stream to
161 lambda-cyhalothrin.

162

163 2.4 Quantification of lambda-cyhalothrin exposure concentrations

164 All collected water samples were placed in cooling containers, immediately transported to the
165 laboratory, and analysed within 24 hours. The lambda-cyhalothrin quantification was performed
166 following the methods described in Wiberg-Larsen et al. (2016). For all samples, esfenvalerat was
167 added as internal standard. The extraction of lambda-cyhalothrin was performed on C18-columns
168 (Sep Pak Vac, 6 cc, 1 g, C18 cartridges, Waters). The columns were conditioned with 5 mL
169 methanol and washed with 5 mL mill-Q water. The flow rate of the sample water was 3 mL min^{-1} at
170 20 kPa vacuum. Subsequently, the C18-column was washed with 5 mL mill-Q water and dried for
171 1-2 minutes at 30-40 kPa vacuum. The lambda-cyhalothrin samples were eluted from the columns
172 with 4 mL methanol, evaporated to dryness, and resolved in 0.300 mL 75% methanol.

173

174 Lambda-cyhalothrin concentrations were quantified on a Hewlett Pachard LC-MSD system,
175 comprised of an HP Series 1100 HPLC and G1946A MSD quadrupole mass spectrometer equipped
176 with electrospray ionization (ESI) in positive mode. An HPLC-column (C18, 150x2.1 mm,
177 Phenoemenex from Subware) was used (flow 0.4 mL min^{-1}, injection of 50 µL samples and at
178 25°C). The following LC solvents were used: Eluent A: 10 mM ammoniumacetate: methanol,
179 990:10 (v:v), and Eluent B: 10 mM ammonium acetate: methanol, 10:90 (v:v). The elution gradient
180 was: (time, % Eluent B: (0 min, 75%); (3 min, 100%); (14 min, 100%); (14,1 min, 75%); post run-
181 time: 6 min, 25% Eluent B. Mass spectrometer-settings were: Mode: ESI positive (SIM: *m*/*z* 467 for
182 lambda-cyhalothrin). Drying gas temperature was 350 °C and flow rate was 10 L min^{-1}. Nebulizer

183 pressure was 30 psig and capillary voltage was 3500 V (Fragmentor: 50 V). The standard curve was
184 calculated from internal standard concentrations of 0, 0.7, 3.5, 35.0, 70.0 and 350 ng L^{-1}. The limit
185 of quantification (LOQ) for lambda-cyhalothrin was estimated to 5 ng L^{-1} with recovery rates of 70-
186 110%, and the reported concentrations were corrected according to recovery rates. LOQ was
187 estimated from the lowest point on the calibration curve (ISO/TS 13530).
188
189 2.5 Macroinvertebrates sampling and drift
190 We took Surber samples (area 200 cm^2, mesh size 500 µm) on experimental days 0 (pre-exposure)
191 and 29 from each channel. On each sampling date, 5 and 10 samples were collected per channel in
192 the mesocosm and instream channels, respectively, with sample totals amounting to 120 and 80 in
193 the mesocosm and instream channels, respectively.
194
195 We measured macroinvertebrate drift at the outlet of each mesocosm and instream channel during
196 and two hours after each exposure (total time = 3.5 hours) using drift nets (net opening 507 cm^2,
197 mesh size 200 µm). For the instream channels, drift nets were equipped with lateral aluminium
198 wings to collect almost the entire outflow, whereas all water discharged through the drift nets in the
199 mesocosm channels. As benchmark measure for drift activity, we also measured drift during two
200 hours at mid-day and dusk in all channels in both the mesocosm and instream channels. Baseline
201 drift was measured the day before first exposure (experimental day -1). In addition, we measured
202 influx of new macroinvertebrates to the semi-open mesocosm channels via pumping water from
203 Lemming stream. Drift nets were mounted at the inlet of each stream channel for two hours during
204 mid-day and dusk on experimental days 19/20. For the instream channels, we measured drift
205 activity into the channels at the inlets for two hours during mid-day and dusk.
206

All macroinvertebrate taxa were identified to species/genus level except Oligochaeta, Hydracnidia, and Diptera being identified to family level.

2.6 Leaf packs

We deployed leaves of *Alnus glutinosa* collected on September 29th, 2009 for the leaf package experiments. The leaves were picked from trees just before abscission and stored at -5 ºC until the experiments. We dried the leaves at 60 °C for 48h and prepared 5 and 10 leaf packages for each channel in the mesocosm and instream channels, respectively. Approximately 3 g dw was enclosed in each leaf bag (weighed with a precision of 1 mg). The leaf bags consisted of 10 x 10 cm plastic nets (mesh size 5 mm) enabling macroinvertebrate access to the leaf material. All leaf packs were conditioned in stream water from Stavis stream in small plastic containers for six days before deployment in the channels. The leaf packs were deployed in the mesocosm and instream channels one week before the first exposure (experimental day -7). On experimental days 0 and 29, we sampled 5 and 10 leaf packs from each mesocosm and instream channel, respectively. All macroinvertebrates were removed and stored in 70% ethanol, and the remaining leaf material was dried at 60 °C for 48h and weighed.

2.7 Data treatment and statistics

We checked for potential significant differences in lambda-cyhalothrin exposure concentrations, temperature, and discharge among all exposures and treatments (1, 2, and 4 pulses, unexposed controls not included for lambda-cyhalothrin exposure concentrations) for the mesocosm and instream channels. This was done using a one-way ANOVA in SAS 9.1 for Windows. In case of significant ANOVA tests, we tested all pairwise comparisons using the sequential Holm-Bonferoni corrected Fisher's Least Significant Difference test.

231

232 Differences in drift rate among treatments were only tested for dominant taxa using pairwise t-tests
233 in SAS 9.1 for Windows, as random absence of rare species would potentially introduce type II
234 errors (i.e. false negatives).

235

236 We analysed changes in macroinvertebrate community composition using non-metric
237 multidimensional scaling (nMDS) of Bray-Curtis similarities and 9,999 permutations. Before
238 analyses, data were square root transformed to down-weigh dominant taxa. Significant differences
239 in macroinvertebrate community composition were identified using ANOSIM ($\alpha = 0.05$) followed
240 by multiple pairwise tests using sequential Holm-Bonferoni corrections. We identified potential
241 indicator taxa related to specific treatments using SIMPER. All analyses were performed using
242 PRIMER 6.0 for Windows.

243

244 Leaf weight loss was calculated as the weight difference between days 0 and 29 (see section 2.6).
245 Since the measured water temperatures were not significantly different among treatments in any of
246 the experiments (see Results section), leaf decomposition rates were not standardized according to
247 measured water temperatures. We tested for differences in weight loss among treatments using one-
248 way ANOVA, followed by a sequential Holm-Bonferoni corrected Fisher's Least Significant
249 Difference test in case of significant ANOVAs. Data were $\log_{10}$ transformed in order to meet test
250 assumptions of normal distribution and equal variances. The one-way ANOVA test was performed
251 in SAS 9.1 for Windows.

252

253 # 3 Results

254 ## 3.1 Validation of exposure concentrations

255 The average concentration of lambda-cyhalothrin was 0.067 (± 0.012 SD) µg L^{-1} in the mesocosm
256 channels (n = 21) (Table S1). Average lambda-cyhalothrin concentrations were not significantly
257 different among the exposures and exposure treatments (n = 7) (one-way ANOVA, F = 1.611, P =
258 0.216).
259
260 The average lambda-cyhalothrin concentration was 0.084 (± 0.028 SD) µg L^{-1} in the instream
261 channels (n = 21) (Table S2). Average lambda-cyhalothrin concentrations were significantly
262 different among exposures and exposure treatments (n = 7) (one-way ANOVA, F = 6.492, P =
263 0.002). The lambda-cyhalothrin concentration in the last of the four exposures in the 4-exposure
264 treatment was significantly higher than all other exposures and treatments (average lambda-
265 cyhalothrin concentration = 0.140 µg L^{-1}, $p < 0.05$) except the first of the exposures in the 2-
266 exposure treatment (p = 0.71) (Table S2). No additional significant differences were found. During
267 the second round of exposures, we found trace concentrations of lambda-cyhalothrin in one of the
268 channels where no lambda-cyhalothrin was added (i.e. in the 1-exposure treatment, Table S2).
269
270 3.2 Discharge and temperature
271 Discharge in the instream channels ranged 10.9 – 35.1 L s^{-1} (Table S3), and we found no significant
272 differences in discharge among treatments (one-way ANOVA, $p>0.05$). However, discharge
273 increased in all channels with a factor of approximately 3.5 during the 3rd round of lambda-
274 cyhalothrin exposure due to a rain event (Table S3). Water temperatures ranged 6.4 – 16.4 ºC
275 averaging 11.7 ºC (data not shown). We found no significant differences in average temperature
276 among treatments (one-way ANOVA, $p>0.05$).
277

278 Discharge in the mesocosm channels ranged 1.47 – 3.88 L s-1, and we found no significant
279 differences in average discharge among treatments (one-way ANOVA, $p>0.05$). In general, channel
280 specific discharge was stable throughout the experiment (Table S4). Water temperatures in the
281 mesocosm channels ranged 5.9 – 15.7 ºC averaging 10.4 ºC (data not shown). We found no
282 significant differences in average temperature among the treatments (one-way ANOVA, $p>0.05$).
283
284 3.3 Macroinvertebrate drift
285 3.3.1 Mesocosm channels
286 During and just after the first exposure, drift rates increased significantly for 6 abundant taxa in
287 channels exposed to lambda-cyhalothrin compared to controls (paired t-test, $p<0.025$) (Fig. 1).
288 Furthermore, drift rate also increased significantly for these taxa in response to the second, third,
289 and fourth exposure, although the absolute increase was less pronounced compared to the first
290 exposure (t-test, $p<0.025$) (Fig. 1). Among less abundant taxa, *Oreodytes sanmarkii* (Coleoptera)
291 and Simuliidae (Diptera) showed significantly higher drift in response to lambda-cyhalothrin
292 exposure during the first exposure (t-test, $p<0.05$) (data not shown), while these taxa were almost
293 entirely absent in drift nets during subsequent exposures (n.b. *O. sanmarkii* was also absent in
294 surber samples, Table S6).
295
296 Influx of new macroinvertebrates in the semi-open mesocosm channels included several taxa, but
297 especially individuals of *Gammarus pulex* (Malacostraca), *Leuctra digitata/fusca* (Plecoptera), *A.*
298 *standfussi* (Plecoptera), *Baetis rhodani* (Ephemeroptera), Tanypodinae (Chironomidae, Diptera),
299 and Orthocladiinae (Chironomidae, Diptera) were re-introduced to the channels (Table S5).
300
301 3.3.2 Instream channels

302 During and just after the first lambda-cyhalothrin exposure, drift rate increased significantly for *G.*
303 *pulex*, *B. rhodani*, *Heptagenia sulphurea* (Ephemeroptera), *L. fusca*, *Elmis aenea* (Coleoptera),
304 *Agapetus ochripes* (Trichoptera), and Tanypodinae in channels exposed to lambda-cyhalothrin
305 compared to the untreated control (paired t-test, $p<0.011$) (Fig. 2). The subsequent exposures,
306 additionally, increased drift rate significantly for these species compared to the untreated control
307 (paired t-test, $p<0.05$) (data not shown).
308
309 Similar to the mesocosm channels, influx of new macroinvertebrates into each of the open instream
310 channels was dominated by *G. pulex*, *L. fusca*, *B. rhodani*, Tanypodinae, and Orthocladiinae (data
311 not shown).
312
313 3.4 Taxonomic composition of macroinvertebrates
314 3.4.1 Mesocosm channels
315 Before lambda-cyhalothrin treatments, we found 42 macroinvertebrate taxa and a total abundance
316 of 7076 individuals m^{-2} in the surber samples (all channels included; Table S6), and the taxonomic
317 composition was not significantly different among treatments (Figure 3A; ANOSIM, Global R =
318 0.034, p=0.09, stress value = 0.23).
319
320 The macroinvertebrate taxonomic composition was significantly different among treatments after
321 lambda-cyhalothrin exposure(s) (experimental day 29) (Fig. 3B; ANOSIM, Global R = 0.234, p =
322 0.001, stress value = 0.18). The taxonomic composition of macroinvertebrates in unexposed control
323 channels were significantly different from the 1P (ANOSIM, R = 0.167, p = 0.001), 2P (ANOSIM,
324 R = 0.473, p = 0.001), and 4P (ANOSIM, R = 0.459, p = 0.001) treatments, respectively (Fig. 3B).
325 Further, the taxonomic composition in the 1P treatment was significantly different from those in the

2P (ANOSIM, R = 0.161, p = 0.004) and 4P (ANOSIM, R = 0.171, p = 0.003) treatments, respectively (Fig. 3B). The taxonomic composition in the 2P and 4P treatments were not significantly different (Fig. 3B, ANOSIM, p > 0.05). The strongest drivers for significant differences in the taxonomic composition of macroinvertebrates among treatments were abundances of *G. pulex, A. standfussi,* and *L. digitata/fusca* which decreased with increasing number of lambda-cyhalothrin pulses, whereas abundances of Tanytarsini and especially Orthocladiinae increased with increasing numbers of lambda-cyhalothrin pulses (SIMPER analysis, Table S7).

Before the first lambda-cyhalothrin exposure, the taxonomic composition of macroinvertebrates located in the leaf packs strongly resembled the taxonomic composition in the surber samples (Table S8), although leaf packs in the 4P treatment contained a significantly different taxonomic composition compared to the other treatments (Fig. 3C; Global R = 0.081, p=0.005, stress value = 0.16; ANOSIM, R = 0.163, p = 0.003). After lambda-cyhalothrin exposure(s), the taxonomic composition in leaf packs was significantly different among treatments (Figure 3D, ANOSIM, Global R = 0.149, p = 0.001, stress value = 0.16) with the taxonomic composition in unexposed control channels being significantly different from those in 2P and 4P treatments (Fig. 3D, ANOSIM, R = 0.337, p = 0.001 and R = 0.397, p = 0.001, respectively). No further significant differences between treatments were found. Similar to the surber samples, the significant differences in taxonomic composition of macroinvertebrates in leaf packs were driven mainly by decreasing abundance of *G. pulex, A. standfussi, and L. digitata/fusca* and increasing abundance of Chironomini with increasing number of lambda-cyhalothrin pulses (SIMPER analysis, Table S9).

3.4.2 Instream channels

Prior to the first lambda-cyhalothrin exposure, we found 53 macroinvertebrate taxa and a total abundance of 4370 individuals m^{-2} (all channels included; Table S10), and we found no significant difference in community composition among treatments (Figure S1A, ANOSIM, Global R = 0.048, p=0.12, stress value = 0.20).

The community composition was significantly different among treatments after lambda-cyhalothrin exposure (experimental day 29) (Fig. S1B, ANOSIM, Global R = 0.078, p = 0.025, stress value = 0.19) with the taxonomic composition in the control treatment being significantly different from those in the 2P and 4P treatments (Fig. S1B, ANOSIM, R=0.199, p=0.025 and R=0.25, p=0.006). We found no further significant differences among treatments. Strongest drivers for the observed significant changes in the taxonomic composition of macroinvertebrates were *G. pulex, L. fusca*, *H. sulphurea*, *Limnius volckmari* (Coleoptera), *E. aenea*, and *Dicranota* sp. (Diptera) decreasing in abundance, and Oligochaeta and Chironomidae increasing in abundance with increasing number of sequential exposures (SIMPER analysis, Table S11).

Before the first lambda-cyhalothrin exposure, the macroinvertebrate taxonomic composition in leaf packs strongly resembled the taxonomic composition in the surber samples (Table S12), and we found no significant difference in the macroinvertebrate taxonomic composition among treatments (Figure S1C; Global R = 0.054, p=0.096, stress value = 0.20). After lambda-cyhalothrin exposure, the taxonomic composition in leaf packs was significantly different among treatments (Figure S1D; Global R = 0.091, p = 0.007, stress value = 0.19) with the community composition in the control treatment being significantly different from the 4P treatment (R = 0.187, p = 0.019). We found no further significant differences in community composition among treatments. Similar to the surber

372 samples, the significant changes were primarily driven by decreasing abundances of *G. pulex*, *L.*
373 *fusca*, and *E. aenea* and increasing abundances of Oligochaeta and chironomids
374 (SIMPER analysis, Table S13).
375
376 3.5 Leaf litter decomposition
377 Leaf weight loss 29 days after the first exposure with lambda-cyhalothrin was significantly higher
378 in the control treatment compared to the 2P and 4P treatments in the mesocosm channels (Fig. 4, p
379 = 0.046 and p = 0.001, respectively). We found no further significant differences in weight loss
380 among treatments. Leaf weight loss before the first exposure (experimental day 0) was not
381 significantly different among treatments (one-way ANOVA, $p > 0.05$).
382
383 Leaf weight loss 29 days after the first exposure with lambda-cyhalothrin was not significantly
384 different among treatments before or after lambda-cyhalothrin treatments for the instream channels
385 (one-way ANOVA, $p>0.05$, data not shown).
386
387 4 Discussion
388 4.1 Macroinvertebrate drift
389 Macroinvertebrate drift increased significantly during and just after exposure to 0.1 µg L^{-1} lambda-
390 cyhalothrin in the mesocosm and instream channels. In general, increasing drift activity was most
391 pronounced during the first exposure, but also the 2^{nd}, 3^{rd}, and 4^{th} exposure provoked significantly
392 increased drift activity for multiple species (note that drift rates were recorded for the third and the
393 fourth exposure in the treatment receiving a total of 4 exposures). The initiation of mass drift events
394 due pyrethroid insecticide exposure reflects hyperactivity and has been reported in multiple studies
395 (e.g. Heckmann and Friberg, 2005; Beketov and Liess, 2008; Nørum et al., 2010).

We found a strong overlap in species exerting significant drift responses between the mesocosm and instream channels with strongest responses observed for *G. pulex*, *A. standfussi*, *L. digitata/fusca*, *H. sulphurea*, and *A. ochripes*. Wiberg-Larsen et al. (2016) documented that these species had low LC50 values (< 1 µg L^{-1}) subsequent to a 90 min exposure to lambda-cyhalothrin with the lowest LC50 obtained by *A. ochripes* (0.028 µg L^{-1}). Moreover, Wiberg-Larsen et al. (2016) showed that these species had strong behavioural responses to lambda-cyhalothrin exposure exerting hyperactivity at concentrations approximately 1/100 of the LC50 which corresponds to the observations in our study. Moreover, the very low LC50 for *A. ochripes* suggests that recurring exposures with 0.1 µg L^{-1} lambda-cyhalothrin should prompt pronounced mortality. Indeed, we did observe mass death of *A. ochripes* pupae in the 2P and 4P treatments (own observations). Due to the strong similarity in the drift rate responses to lambda-cyhalothrin exposure between the mesocosm and instream channels, the increased discharge during the rain event occurring just before the third round of exposure in the instream channels did not significantly influence the results.

4.2 Macroinvertebrate community changes

Macroinvertebrate taxonomic composition in the mesocosm channels and instream channels changed significantly in consequence of lambda-cyhalothrin exposure, and the changes progressively increased with increasing number of exposures. The changes consistently occurred in stream channel habitats as well as inside deployed leaf packs and persisted for a minimum of 29 days after the first exposure. Macroinvertebrate taxa identified as main negative indicators (decreasing abundance with increasing number of lambda-cyhalothrin exposures) strongly overlapped with the taxa occurring with highest abundance in drift samples during lambda-cyhalothrin exposure in both mesocosm and instream channels (e.g. *G. pulex, A. standfussi,* and *L.*

digitata/fusca). This suggests that the mass drift events provoked by lambda-cyhalothrin exposure was a key driver for the observed changes in taxonomic composition. Several taxa of especially Ephemeroptera, Plecoptera, and Trichoptera showed low abundances in unexposed control channels and a consistent absence in the 2P and 4P treatments, but due to the low abundances in only untreated control channels, the species indicator analysis (SIMPER) did not identify these as significant indicators for lambda-cyhalothrin exposures. Nonetheless, their occurrence patterns were systematic which corresponds well with previous observations that species belonging to these insect orders are generally considered highly sensitive to insecticide exposure (Wogram and Liess, 2001). Species of the chironomid subfamilies Orthocladiinae and Tanytarsini did not increase drift rates during lambda-cyhalothrin exposure consequently leading to increased post-exposure relative abundances. In general, Chironomidae contains numerous species covering a broad amplitude in insecticide sensitivity (Wiberg-Larsen et al., 2016). Hence, the identification level to subfamily cannot pinpoint a potential species turnover in Chironomidae mediated by lambda-cyhalothrin exposure. However, the dominant species of Orthocladiinae and Tanytarsini produced silky tubes on hard surfaces (Orthocladinae) or in sand (Tanytarsini) which could partly protect them from peak concentrations of lambda-cyhalothrin in the channel water rendering them less prone to suffer comprehensive effects.

The changes in macroinvertebrate taxonomic composition following lambda-cyhalothrin exposure in the instream channels were not as pronounced as in the mesocosm channels as significant differences were only found between channels receiving multiple exposures and the unexposed control channel. Moreover, no significant changes in taxonomic composition were observed inside the leaf packs in the instream channels. This is partly explained by a smaller increase in macroinvertebrate drift activity during lambda-cyhalothrin exposure. Importantly, however, a heavy

444 rain event occurring on experimental day 3-4 governed a substantial increase in discharge in Stavis
445 stream which may have prompted increased macroinvertebrate drift in and out of the channels (e.g.
446 Brittain and Eikeland, 1988) potentially clouding treatment mediated community changes.
447
448 4.3 Leaf decomposition
449 We found that macroinvertebrate induced leaf decomposition significantly decreased with
450 increasing number of lambda-cyhalothrin exposures in the mesocosm channels whereas no
451 significant treatment effects were found in the instream channels. The decreased leaf decomposition
452 in Lemming stream channels probably reflects the observed community changes, especially the
453 reduced abundance of *G. pulex* (see e.g. Aßmann et al., 2010). Similar effects were observed by
454 Rasmussen et al. (2008) who exposed a macroinvertebrate assemblage (including *G. pulex*) to 0.1
455 $\mu g\ L^{-1}$ lambda-cyhalothrin for 90 minutes, although the authors maintained comparable abundance
456 of *G. pulex* among treatments and explained the reduced leaf decomposition with treatment
457 mediated reductions in shredding activity. Hence, our results may well reflect a combination of
458 reduced abundance of key shredders as well as reduced feeding activity. The absence of treatment
459 effects on leaf decomposition in the instream channels can be explained by comparable
460 macroinvertebrate taxonomic composition inside leaf packs among treatments (discussed above).
461 This further suggests that changed taxonomic composition overall was a more important driver of
462 impaired leaf decomposition than reduced feeding activity in the mesocosm channels.
463
464 4.4 Perspectives
465 The seasonal timing of our experiments was optimal, as exposure was performed during the main
466 spraying season for insecticides in Denmark. Secondly, we applied lambda-cyhalothrin in
467 environmentally realistic concentrations and exposure durations (Liess et al., 1999; Rasmussen et

al., 2015). Moreover, Rasmussen et al. (2015) showed that small agricultural streams are often subjected to significant pesticide exposure 2-5 times within the months May, June, and July documenting the relevance of studying ecological effects of sequential exposures. Presence of ecologically significant insecticide concentrations are, however, stochastic depending on timing of precipitation during the spraying season (e.g. Solomon et al., 1996). Whereas the time between exposures was only two days in our study, reflecting a worst-case scenario, all channels in both experiments were subject to continuous recolonization from unexposed parts of streams with a diverse macroinvertebrate community, which increases the environmental relevance on one side but diverges from the worst-case scenario on the other side. In summary, our results most likely do not reflect a worst-case scenario as the absence of recolonization should generate stronger treatment effects. Such worst-case scenarios derive from longer stream reaches receiving simultaneous insecticide input, as is often the case in heavily utilized agricultural landscapes (McKnight et al., 2012), thereby reducing recolonization potential.

An important limitation in our study is the lacking ability to separate effects of sequential exposures from the effects of the total period of exposure, as channels receiving e.g. 1 and 4 pulses of lambda-cyhalothrin had different total exposure durations (90 and 360 minutes of exposure). However, as the main aim of the study was to address the observed stronger ecological effects of pesticides in the field compared to single-exposure controlled laboratory and mesocosm studies, and as streams receiving multiple pulses of pesticide exposure, additionally, experience longer total exposure durations, the study setup naturally fulfils its purpose. Another limitation to our study is that the time since last exposure diverges among treatments with channels receiving more exposures having less time for recovery. However, as the maximum divergence (between treatments receiving 1 and 4 pulse exposures) was 4 days, and as the post-exposure observational period was comparably long

(ranging 23 to 27 days), the maximum divergence of 4 days is inconspicuous when compared to the length of the post-exposure observational period. Consequently, this discrepancy likely did not significantly influence the results.

Studies addressing effects of sequential pesticide exposures on macroinvertebrate communities or single species are scarce (but see Pestana et al., 2009; Dennis et al., 2012; Mohr et al., 2012; Finotello et al., 2017; Russo et al., 2018; Bray et al., 2019), highlighting the relevance of the present study. The numerous potential combinations of active ingredients, time between exposures, and exposure concentrations control types and intensity of ecological effects (e.g. Pestana et al., 2009; Mohr et al., 2012), but due to the scarcity in published studies addressing sequential exposures, this remains largely unknown. Nevertheless, progressing ecotoxicological research towards incorporating sequential exposures may provide key insights that can help explaining field-based observations of pesticide induced effects occurring at surprisingly low exposure concentrations (Liess et al., 2016). Our study further indicates that observed strong effects of pesticide exposure on macroinvertebrate drift rates can be translated into community changes that may persist for months even in the presence of upstream colonizers. However, our study also lends support to the observations that functional measures of stream ecosystems may be less sensitive endpoints compared to structural changes.

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614 Figure captions

615

616 Fig. 1. Macroinvertebrate drift rates in the mesocosm channels. Drift rate represents the average
617 number of individuals caught in drift (number of individuals hour^{-1} ±SE) during (90 min) and
618 immediately after (120 min) exposure to lambda-cyhalothrin. Values are shown for 6 different
619 dominant taxa for treatments receiving no lambda-cyhalothrin, and 1, 2, and 4 pulses of 0.1 µg L^{-1}
620 lambda-cyhalothrin for 90 minutes (n = 3). For the treatment receiving at total of 4 pulses, drift
621 rates are shown for both the third and fourth exposure. For all species and all exposures, the
622 increase in drift rate was significant compared to untreated controls (paired t-test, $p<0.05$). Note the
623 exponential scale on the Y-axis.

624

625 Fig. 2. Macroinvertebrate drift rate in the instream channels. Drift rate represents the sum of
626 individuals (hour^{-1}) caught in drift nets during (90 min) and immediately after (120 min) the first
627 exposure to lambda-cyhalothrin. Drift rates for all taxa presented in the figure were significantly
628 higher in channels exposed to lambda-cyhalothrin compared to untreated controls. Note the
629 exponential scale on the Y-axis.

630

631 Fig. 3. nMDS scaling of Bray-Curtis similarities on macroinvertebrate communities on riffles (A,B)
632 and leaf packages (C,D), respectively, in mesocosm channels. Macroinvertebrate taxonomic
633 composition is depicted before lambda-cyhalothrin exposure on experimental day 0 (A, C) and after
634 exposure on experimental day 29 (B, D). Macroinvertebrate taxonomic composition is shown for
635 control treatments (CON) and treatments receiving 1, 2, and 4 pulses of lambda-cyhalothrin (1P, 2P,
636 and 4P, respectively) with 2 days between exposures.

637

638 Fig. 4. Mean weight loss (g) of leaf material in the mesocosm channels just before (experimental
639 day 0) and 29 days after the first lambda-cyhalothrin exposure. CON = untreated control channels;
640 1P, 2P, and 4P = channels treated with one, two, and four pulses of lambda-cyhalothrin,
641 respectively. Groups that do not have letters in common are significantly different. Asterisks
642 indicate significant differences between days 0 and 29.