1 **Structural changes in a macrozoobenthos** 2 **assemblage following imidacloprid pulses in** 3 **aquatic field-based microcosms**

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14 **Abstract** A field-based microcosm experiment was conducted to investigate the effects of
 15 repeated pulses of the neonicotinoid insecticide imidacloprid on a lentic benthos assemblage. This
 16 specific microcosm method was chosen because it allows for both the test of a wide range of
 17 organisms under natural conditions and at the same time gain insight into intra- and interspecific
 18 interactions. The macrozoobenthos that colonised the microcosms was exposed to three pulses
 19 each one week apart, at nominal concentrations ranging from 0.6 to 40 µg/L. Imidacloprid
 20 underwent a fast aqueous photolysis due to optimal sunlight conditions during the test phase
 21 (DT50 28 h ±8; monitored for 21 days). Nonetheless, a decreased abundance and emergence of
 22 Ephemeroptera and a decreased survival of chironomids species of the subfamilies Tanytopodinae
 23 and Orthocladiinae were observed at time weighted average concentrations of 2.3 µg/L. On the
 24 contrary, the gastropod *Radix* sp. became dominant at high imidacloprid concentrations probably
 25 due to reduced competition for food with sensitive species. This study demonstrates that repeated
 26 short-term contamination of imidacloprid at low concentration levels may affect aquatic
 27 ecosystems, even under optimal conditions for photodegradation. The microcosm approach with
 28 its simple but field-relevant design proved to be a useful tool for assessing the effects of
 29 imidacloprid contaminations.

30

31

32 **Introduction**

33 Imidacloprid is the active component of many commercial pesticides. Compared
34 to other insecticides, its irrefutable advantages are the high insecticidal toxicity at
35 low concentrations and the low toxicity to mammals (Tomlin 2000). Imidacloprid
36 belongs, in fact, to the neonicotinoids, a class of neuroactive insecticides, and it
37 binds to the nicotinic acetylcholine receptors (nAChR) (Buckingham et al. 1997;
38 Goedkoop et al. 1998; Matsuda et al. 2001). Such receptors are present in all
39 animals but they are made of different subunits: those of arthropods are very
40 susceptible to imidacloprid, whereas those in vertebrates are not; this confers to
41 imidacloprid selectivity on specific taxa (Tomizawa et al. 2000). This also indicates
42 that imidacloprid is effective probably against virtually every insect. These
43 properties have made it a successful replacement of many formerly used
44 insecticides in numerous applications (Liu and Casida 1993).

45 Imidacloprid was designed to be an insecticide with very low impact on the
46 environment; it is considered harmless to the non-target aquatic ecosystem
47 (Bayer CropScience AG 2002) because of its rapid and complete degradation
48 accelerated by sunlight. For example, Moza (1998) under laboratory conditions
49 found a half-life (DT_{50}) of 4 h at irradiation of 290 nm. A number of other physico-
50 chemical properties, however, raised the concern that imidacloprid has a high
51 potential to contaminate surface waters. For example, its DT_{50} in soil ranges from
52 104 – 228 days (IUPAC Footprint Database,
53 <http://sitem.herts.ac.uk/aeru/iupac/397.htm>); this property in combination with its
54 moderate water solubility may result in a high contamination potential during

55 runoff (Armbrust and Peeler 2002). Finally, imidacloprid was considered harmless
56 also because of the low toxicity to *Daphnia magna* (LC₅₀ of 85 mg/L).

57 Single species laboratory tests have shown that aquatic organisms are
58 affected by imidacloprid even at low concentrations. For example, the mayfly
59 *Epeorus longimanus* (LC₅₀ at 96 h = 0.65 µg/L) (Alexander et al. 2007), the non-
60 biting midge *Chironomus tentans* (LC₅₀ at 96 h = 5.75 µg/L and 5.40 µg/L for the
61 technical and the commercial formulation Admire; Stoughton et al. 2008). The
62 strengths of laboratory, single species tests are: standardisation - which implies
63 reproducibility- and reliability, which helps to understand the mode of action of a
64 chemical and hence the building of a cause-effect relationship. These tests are
65 often performed under artificial conditions using laboratory reared organisms, in
66 order to restrain abiotic and biotic factors that may influence the bioavailability of
67 chemicals or the fitness of the organisms and hence the outcome of the test. The
68 flaw inherent in this practice is that nature's complexity becomes simplified. The
69 ecological relevance of the laboratory tests is strongly reduced (Forbes and
70 Forbes 1993) and the risk of erroneous conclusions increased. Most of the
71 laboratory tests conducted with imidacloprid have focused on exposure scenarios
72 in which the concentration of the insecticide has been kept constant during the
73 experiment. However, recent investigations focused on the simulation of realistic
74 exposure scenarios. Repeated pulse exposures best reflect pollution events
75 caused by the existence of many pollution sources (e.g. spray drift, stormwater
76 runoff in urban areas, nurseries, orchards, etc.) and this scenario is more relevant
77 for describing the fate of imidacloprid in the environment (CCME 2007; Pestana et
78 al. 2009; Mohr et al. 2012). Several imidacloprid pulses could have the potential to
79 cause cumulative effects as it has been indicated amongst others by Tennekes

(2010); Tennekes and Sanchez-Bayo (2011); Berghahn et al. (2012). However, this is a hypothesis that still needs further validation. Additionally, Pestana et al. (2009) and Mohr et al. (2012) tested the effects of imidacloprid pulses on whole aquatic communities in stream mesocosms and observed that insects' larvae were negatively affected.

So far, there is no published study dealing with the effects of repeated imidacloprid pulses on lentic aquatic macroinvertebrate assemblages. Exposure in lentic and lotic ecosystems are different: in a lotic system a stream section is exposed to a pulse for a short duration due to the flow velocity, in a lentic system on the contrary the pulse will be subject to degradation processes rather than dilution processes due to water flow and turbulence. The use of field-based microcosms (Pettigrove and Hoffmann 2005) provides an ecologically relevant method to evaluate the effects of pesticides on lentic aquatic organisms. Unlike mesocosms, the small size and the low costs of these microcosms allow for a high number of replicates and treatments and also for a randomised distribution in the field. The microcosms are set up in water bodies or at their shore and are let colonised by indigenous, egg-laying insects' species. The assemblage of macroinvertebrates of the study site in the season under investigation is partially represented in the microcosms. Furthermore, a large number of species could be investigated.

In this paper the effects of repeated imidacloprid pulses on an indigenous assemblage of lentic macrozoobenthos organisms was investigated. It was hypothesized that imidacloprid would rapidly dissipate from the water phase of the shallow microcosms due to its fast aqueous photolysis. The aim was to investigate whether imidacloprid would still cause lethal or sublethal effects on

105 aquatic organisms under these conditions. Finally, it was intended to compare the
106 results of this lentic microcosm study with those of the lotic mesocosms study by
107 Mohr et al. (2012). Abundance, number of species, and emergence (as total
108 number of adults) were chosen as endpoints to detect significant structural
109 changes in the macroinvertebrate community which had developed in the
110 microcosms.

111

112 **Materials and Methods**

113 **Field-based microcosms design**

114 Each microcosm consisted of a 20-L polypropylene container (45.5 × 30 × 21 cm,
115 KIS System Box 8605, by ABM Italy Spa). A total of 56 microcosms were used.
116 The microcosms were exposed in the reservoir pond at the field station of the
117 German Federal Environment Agency (UBA) in Berlin, Germany. This location
118 was chosen because it fulfilled the prerequisites for this microcosm method. It was
119 unpolluted and closed to the public, which excluded possible sources of
120 disturbance during the experiment.

121 The setup followed the method described by Pettigrove and Hoffmann
122 (2005) with some modifications. Each microcosm contained 750 mL of fine,
123 homogenized sediment (silt and clay loam with ca. 3% organic matter). The
124 sediment was taken from an uncontaminated lake on the island Ruegen
125 (Germany) and treated as described in Mohr et al. (2007). Approximately 15 L of
126 filtered water from the reservoir pond of the field station were added into each
127 microcosm.

128 The microcosms were fixed in pipe-framed rafts and left to float on the
129 reservoir pond to achieve a homogeneous colonisation. Each raft consisted of
130 eight microcosms distributed in a stratified random design: two microcosms for the
131 controls and one for each of the six treatments. The microcosms were covered
132 with a coarse net (mesh size ca. 2 cm) to prevent colonisation by large predatory
133 macroinvertebrates such as dragonflies, whereas small flying insects were still
134 able to lay eggs inside the microcosms. In order to reduce the risk of heat
135 accumulation, the microcosms were two-thirds submerged in the water. The
136 colonisation phase lasted three weeks (late May-June). During this time,
137 imidacloprid was added in three pulses, each one week apart in order to simulate
138 imidacloprid pulses caused by different pollution events. The third pulse
139 represented the end of the colonization phase and all the microcosms were
140 covered with stockings (company Ja! stockings, XL- XXL champagne colour) that
141 acted as a fine nylon mesh. This mesh was fine enough to retain emerging insects
142 and to prevent new ones from colonizing the microcosms, but was sufficiently
143 coarse to allow for aeration inside the microcosms.

144 The collection of the emerged insects started shortly after the third pulse,
145 lasted seven weeks and was concluded when the emergence rate strongly
146 declined. Adults were removed weekly but at irregular intervals due to logistic
147 constraints using a mechanical, modified battery powered aspirator (Hausherr's
148 Machine Works U.S.). At the end of the experiment, the content of each
149 microcosm was filtered through a 500 μ m sieve to collect the remaining insect
150 larvae. The larvae were stored in 80% ethanol and identified using a stereoscope
151 (Zeiss, Stemi 2000).

152 *In situ* measurements of water temperature, turbidity, conductivity,
153 dissolved oxygen, and pH were recorded once a week during the experiment
154 (Sensor: Cond 340i; Multi 340i; Turbidity: TURB 555IR). The water temperature of
155 the reservoir pond, air temperature, and ultraviolet radiation (UBA meteorology
156 station) were also recorded.

157

158 **Imidacloprid exposure and measurements**

159 The macroinvertebrates were exposed to six nominal concentrations (0.6; 1.4;
160 3.2; 7.5; 17.3 and 40 µg/L) which were applied 3 times as pulse, each one week a
161 part. These concentrations are within the average range measured in
162 contaminated water bodies (Lamers et al. 2011; Starner and Goh 2012).

163 Each test concentration had seven replicates apart from the controls with
164 fourteen replicates. Exposure to imidacloprid was monitored by analyzing
165 overlying water samples from all the treatments. 1L of water was collected 6h, one
166 week, six weeks after each pulse and at the end of the experiment in all
167 treatments. Sacrificial tanks for one treatment (17.3 µg/L nominal concentration)
168 were set up. Water was collected here additionally one day, two days, three days
169 and seven days after each pulse. The whole sediment of these sacrificial
170 microcosms was collected for chemical analysis. Imidacloprid was extracted using
171 a solid phase extraction (SPE) column of modified polystyrene–divinylbenzene
172 resin (ENV + 200 mg/6 mL, IST Biotage). Derivatization of imidacloprid for gas
173 chromatographic-mass spectrometric (GC-MS) analysis was performed with
174 pentafluorobenzoyl chloride (CAS-No. 2251-50-5) instead of hepta-fluorobutyric
175 anhydride and pyridine. For water: detection limit: 4 -5 ng/mL, determination limit:

12 – 15 ng/mL. Equal to: for 500 mL. Water sample: detection limit: 0.01 µg/L;
determination limit: 0.03 µg/L. For sediment: detection limit: Extraction 75 ng/mL.
For 20 g frozen sample: 3.8 ng/g TM = 3,8 µg/kg TM. All the chemical analyses
were carried out on a gas chromatography-mass spectrometry system (GC-MS-
System by HP: Gas-phase chromatograph HP 6890/MSD HP 5973; capillary
column 50 cm. CP Sil 8; carrier gas: helium) (MacDonald and Meyer 1998).

183 **Imidacloprid dissipation analysis**

For calculating the degradation rates, the decline of imidacloprid was displayed as
a function of time (Fig. 1). Under the simplified assumption that the amount of
degraded imidacloprid remains constant and independent of its concentration, the
reaction rate declines proportionally in function of time (single first-order kinetic,
SFO) and follows an exponential decay according to the equation:

$$A(t) = A_0 e^{-kt}$$

where $A(t)$ denotes the residual concentration at time t , A_0 represents the initial
imidacloprid concentration and k , the reaction rate, is the rate constant. The rate
constant k is measured as the slope of the plot of the logarithm of concentration
versus the reaction time and was determined using the Maximum Likelihood
Estimation (MLE) in Origin 8. The rate constant was also used to determine the
half-life (DT_{50}), according to the formula:

$$t_{1/2} = \frac{\ln(2)}{k}$$

where $t_{1/2}$ denotes the time after which half of the chemical has been chemically
transformed.

199 **Data analysis**

200 Total abundance (larvae plus emerged adults), number of species, and
201 emergence (number of adults) of common taxa were monitored as endpoints of
202 the experiment. Statistical analyses and graphs were performed using the
203 statistical program R (R Development Core Team 2008) and SPSS version 19. As
204 the data were non-normally distributed (Kolmogorov–Smirnov Test),
205 nonparametric tests were chosen. The treatments are graphically displayed using
206 parallel boxplots. Median and interquartile ranges (IQR, 25th and 75th quartiles) are
207 used to give an idea of centrality and spread. For comparison among treatments
208 of the total abundance, and the abundances of common species Kruskal–Wallis
209 tests were run. When a difference among treatments was detected, two-tailed
210 Mann-Whitney U tests were used for identifying which pairs of treatments were
211 different. The Jonckheere-Terpstra trend test was used to test whether the
212 diversity, abundance, and emergence of common taxa would gradually decline
213 with increasing imidacloprid concentrations (Jonckheere 1954). Differences
214 between treatments were tested for significance at the 5% level. Power analysis
215 (G-test) was run where “no statistical significance” was found, to assure that the
216 statistical tests had enough power to detect an effect.

217 **Results**

218 **Dissipation of imidacloprid in the water phase**

219 All three pulse applications took place on sunny days, with air temperature
220 average ranging from a minimum of 10°C in the night and 24 °C during the day,
221 and relatively high levels of ultraviolet radiation (UV-B between 6 - 11 µW/cm²).

222 The water of all microcosms was clear during the whole experiment and became
223 temporarily turbid only during rain events (up to 53 NTU – Nephelometric Turbidity
224 Unit) due to dispersed sediment.

225 The physicochemical parameters of the water in the microcosms were
226 similar within the treatments and also similar to those of the reservoir pond. The
227 pH ranged between 8 and 9 and was similar to the pond's pH of 8. The water
228 temperature ranged from 16 to 22°C (pond temperature 17 – 21°C). The
229 conductivity declined over time from 835 $\mu\text{S}/\text{cm}$ at the start to 615 $\mu\text{S}/\text{cm}$ at the
230 end of the experimental period. The conductivity of the pond water was ca. 950
231 $\mu\text{S}/\text{cm}$.

232 The detailed chemical analysis of the water conducted for the 17.3 $\mu\text{g}/\text{L}$
233 nominal concentration treatment showed that the DT_{50} for each pulse were 20 h,
234 36 h and 29 h (monitored for 21 days). After each pulse, a very rapid initial
235 decrease in the insecticide concentration was observed within 6h after application,
236 suggesting that aqueous photolysis was the main breakdown pathway (Fig. 1). At
237 the end of the experiment, 0.13 $\mu\text{g}/\text{Kg}$ and 1.72 $\mu\text{g}/\text{L}$ were the highest
238 imidacloprid concentrations found in the sediment and in the water of the
239 treatment with the highest contamination (see Table 1).

240 The rapid dissipation rates of imidacloprid in the microcosms indicated that
241 in the assessment of the level of toxic response the use of nominal concentrations
242 would be misleading whereas the use of Time-Weighted Average (TWA)
243 concentrations would be more appropriate (see discussion section for more
244 details). The TWA concentrations of the different treatments ranged from 0.2 to 12
245 $\mu\text{g}/\text{L}$ (Table 2). In the following, both the nominal concentrations (nc) and the TWA

246 concentrations will be displayed in the figures while the text will exclusively refer
247 to the TWA concentrations.

248

249 **Colonization success of macroinvertebrate taxa in control microcosms**

250 An average number of 680 individuals per microcosm, ranging from 347 to 1010
251 (IQR spread) was collected during the whole experiment. The macroinvertebrate
252 assemblage was dominated by Chironomidae (Diptera) (65%) from the
253 subfamilies Chironominae, Tanypodinae and Orthoclaadiinae. The second most
254 abundant and frequent family was the Gastropoda (ca. 18 %), represented by the
255 pulmonate snail *Radix* sp. which probably entered the microcosms as a planktonic
256 stage with the water. Other relatively abundant insect families were the
257 Ephemeroptera (*Caenis* sp. and *Cloeon* sp.) whereas Ceratopogonidae.
258 Chaoboridae, Culicidae, other Diptera and Nematoda were present in only a small
259 number of microcosms.

260

261 **Total number of species and common taxa in treatments**

262 A significant decrease in total number of species and abundance of Chironomidae
263 was observed in the two highest treatments of 5.2 and 12 µg/L TWA. Of the 6 (2-8
264 IQR) most common genera found in the controls only 4 (1 – 4 IQR) were found at
265 the highest concentration (Kruskal Wallis Test $p=0.002$; Mann-Whitney U Test
266 $p=0.025$ for control vs. 5.2 µg/L TWA and $p=0.001$ for control vs. highest
267 concentration). The average number of chironomids found in the control was 275
268 (105.7 – 357 IQR), which decreased to 148 (130 – 212 IQR) and further to 51 (0 –
269 109 IQR) at 5.2 µg/L and 12 µg/L TWA, respectively (Kruskal-Wallis Test $p\leq 0.05$;

270 Mann-Whitney-U Test $p = 0.003$ for control vs. highest concentration; Jonckheere-
 271 Terpstra $p \leq 0.05$; Fig. 2). *Paratanytarsus grimmii* was excluded from the statistical
 272 analysis because it reproduces through parthenogenesis and is able to breed in
 273 the microcosms.

274 Three species belonging to the subfamily Orthoclaadiinae were particularly
 275 sensitive to imidacloprid (Fig. 3). Of the four species that colonised the
 276 microcosms, three (*Corynoneura* sp., *Cricotopus* sp., *Eukiefferiella* sp.) were
 277 strongly affected as none of them was present at the highest concentration,
 278 contributing strongly to the general decrease of chironomids species (Kruskal-
 279 Wallis Test $p \leq 0.05$; Mann-Whitney-U Test $p = 0.001$ for control vs. highest
 280 concentration; Jonckheere-Terpstra $p \leq 0.05$). *Psectrocladius* sp. was the only
 281 Orthoclaadiinae species still present at 12 $\mu\text{g/L}$ TWA, although its abundance was
 282 also reduced. The total abundance of Orthoclaadiinae was significantly lower at the
 283 highest concentration (Kruskal Wallis Test $p \leq 0.05$).

284 Among the Tanypodinae, the abundance of *Ablabesmyia* sp. declined
 285 starting from 2.3 $\mu\text{g/L}$ TWA concentration (Kruskal-Wallis Test $p = 0.007$;
 286 Jonckheere- Terpstra Test $p = 0.002$) and was significantly affected at the highest
 287 concentration (Mann-Whitney-U Test $p < 0.001$, Fig. 4).

288 The number of *Radix* sp. (Gastropoda) increased significantly at the
 289 highest imidacloprid concentration. An average of 55 (19.5 – 241.5 IQR) snails
 290 was found in the control compared to 399 (222 – 615 IQR) snails in the treatment
 291 with the highest imidacloprid concentration (Kruskal Wallis Test $p \leq 0.05$; Mann-
 292 Whitney-U Test $p = 0.007$; Fig. 5).

293 Finally, the abundance of Ephemeroptera, represented by only two taxa
 294 (*Caenis* sp. and *Cloeon* sp.) decreased at the highest imidacloprid concentration

295 but not significantly (Kruskal Wallis test $p = 0.2$). At the highest imidacloprid
 296 concentration *Caenis* sp. was present in only two of the seven microcosms (Fig.
 297 6b) whereas the Baetidae species *Cloeon* sp. was completely absent.
 298 Unfortunately, *Cloeon* sp. colonised only few control microcosms and was
 299 therefore too infrequent to run a powerful statistic test (G-Test $p < 0.05$).
 300

301 **Effects of imidacloprid on emergence (as number of adults)**

302 The total number of adults Chironomidae declined with increasing
 303 imidacloprid concentrations but not significantly; the number of *Ablabesmyia* sp.
 304 adults declined significantly (Kruskal- Wallis Test $p = 0.002$; Jonckheere- Terpstra
 305 Test $p = 0.001$) but this decline, as the general decline of Chironomidae adults
 306 reflected reduced survival (hence reduced abundance) rather than delayed
 307 emergence.

308 The number of adults Ephemeroptera appeared to be sensitive to
 309 increased amount of imidacloprid (Jonckheere-Terpstra Test $p = 0.037$). In
 310 particular, *Caenis* sp. adults were absent from microcosms with imidacloprid
 311 concentrations exceeding $0.4 \mu\text{g/L}$ TWA (Kruskal-Wallis Test $p = 0.016$;
 312 Jonckheere-Terpstra Test $p < 0.001$, Fig. 6a).

313 **Discussion**

314 **Fate of imidacloprid in the microcosms**

315 In this study, imidacloprid rapidly dissipated from the water column of the
 316 microcosms after each pulse with a mean DT_{50} of $28 \text{ h} \pm 8$ monitored for 21 days in
 317 the field-based microcosms. This result is similar to those reported by Moring et

al. (cited in CCME 2007), Moza et al. (1998); and Wamhoff and Schneider (1999), who determined a half-life of 30 h in outdoor microcosms. In Moring's study, imidacloprid was added in four pulses, each spaced two weeks apart. In Moring's and the current study the chemical analyses of sediment and pore water did not indicate major residues, which supports the hypothesis of photolysis as main breakdown pathway. During the experiment at hand, the UV radiation levels were very high and the transmission of light in the water column was almost unhindered due to both the absence of turbidity and color substances: The water in the microcosms was also moderately alkaline (pH 8-9), which may also have contributed to the rapid breakdown of imidacloprid (Zheng and Liu 1999). The clear water in the microcosms simulated lentic ecosystems exposed to spray drift events rather than to storm-water runoff.

Once imidacloprid enters aquatic ecosystems, the sediment could also become a source of pollution for benthic organisms. In this study, the absence of high concentrations of imidacloprid in the sediment may be due to the rapid breakdown in its degradation products in the water column. Some hydrolytic metabolites of imidacloprid (e.g. 5-hydroxy imidacloprid and olefin) are equally toxic to insects (Suchail et al. 2001) and may contribute to the overall toxicity.

With regard to the high concentrations found in many surface waters it may also be argued that due to factors like turbidity, water colour, and pH the half-life of imidacloprid in aquatic environment and in the sediment may be considerably higher than expected. In any case, the concentrations in many surface waters are much higher than in the study at hand, which rather simulated a best case scenario.

342 Some studies have suggested that imidacloprid and pesticides in general enter
343 water bodies more frequently than what has been estimated so far (Liess et al.
344 1999; Neumann et al. 2002; Phillips and Bode 2004; Knabel et al. 2012).
345 Analyses of sediments and survey techniques employing passive samplers
346 showed that the actual amount of imidacloprid in water bodies is often
347 underestimated. In two creeks in Germany, the mean concentration of
348 imidacloprid in water detected by chemical analysis was 0.1 µg/L whereas the
349 sediment analyses indicated concentration of 344 µg/Kg (Höcker 2001).

350

351 **On the use of nominal concentrations: are they representative?**

352 This study can be considered a chronic study in which the concentration of the
353 toxicant is not held constant and the whole life cycle of a number of taxa and
354 species was investigated. Due to the rapid photolysis of imidacloprid (Fig.1) the
355 use of nominal concentrations would overestimate imidacloprid concentrations
356 during the experiment. In general, the use of TWA concentrations is
357 recommended for substances that show a relevant decay during the study, so that
358 appropriate reference concentrations for describing hazardous effects can be
359 determined (OECD 2008). In the case of imidacloprid, the use of TWA
360 concentrations for describing the effects in this study is better because of the
361 rapid photolysis and the low potential for bioaccumulation due to its K_{ow} (IUPAC
362 Database; CCME 2007).

363 In this experiment, the Ephemeroptera started being affected at a nominal
364 concentration of 3.2 µg/L, which corresponds to a three time lower TWA
365 concentration of 1 µg/L whereas the rest of the aquatic community was affected at

the nominal concentration of 7.5 µg/L, which corresponds to a TWA concentration of 2.3 µg/L. These TWA values are in agreement with concentrations found in other community studies (Sánchez-Bayo and Goka 2006; Hayasaka et al. 2012).

Imidacloprid effects on the macroinvertebrate assemblage

The macroinvertebrate community that colonized the microcosms was affected by the repeated imidacloprid pulses although imidacloprid rapidly disappeared from the water phase. The assemblage was remarkably affected in the two highest treatment levels of 5.2 and 12 µg/L TWA, respectively. Some adverse effects were also visible at lower concentrations: at 1 µg/L TWA, a concentration which seems to have an effect on Ephemeroptera emergence and at 2.3 µg/L TWA where there is a significant decline in the abundance of *Ablabesmyia* sp. (Tanypodinae).

Chironomidae are pioneer species (Armitage et al. 1995) and hence not surprisingly were the most common insect found in the microcosms. Different species are known to have different ranges of sensitivity towards pollutants in general (Pettigrove and Hoffmann 2005), which was also evident in this study.

The decline in the survival of Tanypodinae and the reduced emergence of *Caenis* sp. have also been observed in a stream-mesocosms study, in which the organisms were exposed to three pulses of 12 µg/L imidacloprid (corresponding to a 0.85 µg/L TWA concentration, Mohr et al. 2012). These similar results although found in two different systems (lentic and lotic) may indicate that low but repeated imidacloprid pulses are likely to have effects on aquatic benthic communities.

389 The findings of reduced adult numbers (emergence success) were also
390 supported by Handy (1994), who postulated that sporadic pollution pulses, if not
391 immediately lethal, may still have long term effects (or sublethal effects) and may
392 become lethal when ulterior stressors are added. In tests where the imidacloprid
393 pulses did not reach lethal concentrations, often sub-lethal effects, such as
394 feeding inhibitions or a reduction of body size occurred (Alexander et al. 2007).
395 In addition, Tennekes and Sanchez-Bayo (2011) demonstrated that one short
396 term exposure to a low imidacloprid concentration - which is expected to cause
397 only sub-lethal effects had the same or even stronger effect as one single
398 exposure to a high concentration. They inferred that imidacloprid has a cumulative
399 effect. The effects observed in our study were probably cumulated effects caused
400 by repeated pulses. However, colonization of the microcosms was still possible
401 until the third pulse. This means that some invertebrates were potentially exposed
402 to only one pulse instead of three. If outcomes caused by the cumulative effect
403 were present at the low concentrations, they may have been masked by the
404 organisms exposed to only one pulse. Nevertheless, our results based on total
405 survival and emergence (as number of adults) are comparable to those obtained
406 in a stream mesocosms study with a similar exposure (Mohr et al. 2012) despite
407 differences in size, system (lentic/lotic), and complexity.

408 The findings of this study are particularly relevant for univoltine organisms
409 that may be frequently exposed to imidacloprid pulses over their life cycle and are
410 therefore very likely to be more strongly affected by short term, sub-lethal
411 contaminations than short-living groups (for instance the chironomids). It should
412 also be considered that under natural conditions, organisms are exposed to a
413 plethora of stressors: mixture of chemicals, predators, changing abiotic and

414 environmental conditions, etc. As a result, cumulative or synergistic effects can
415 happen or the time available for detoxification mechanisms and recovery may not
416 be sufficient (Ashauer et al. 2012).

417 This study also made clear that the identification of organisms only to the
418 family level could become a confounding variable: often different species have
419 different sensitivities and therefore the identification to at least the genus level is
420 recommended. For example, in this study *Procladius* sp. and *Ablabesmyia* sp.
421 although belonging to the same family, the Tanyptodinae, showed a completely
422 different response to imidacloprid. *Ablabesmyia* sp. was very sensitive whereas
423 *Procladius* sp. seemed to be tolerant; interspecific differences in sensitivity to
424 imidacloprid were found also in mayflies (Roessink et al. 2013).

425

426 **Implication for the ecosystem**

427 The microcosm method used in this experiment has the same advantage as
428 mesocosms studies, because it allows for insight in potential intra- and
429 interspecific interactions (indirect effects). A typical indirect effect is the increase
430 in abundance of tolerant species. In this study an increase in the number of the
431 pulmonate gastropod *Radix* sp. was observed with increasing imidacloprid
432 concentrations. Since an increase in population density of Gastropoda has been
433 also observed elsewhere as an indirect effect in insecticide-stressed aquatic
434 ecosystems and community-level studies (CCME 2007) we could hypothesise that
435 this could be an example of an indirect effect caused by imidacloprid. This
436 increase could be explained by both the fact that molluscs in general have a
437 moderate tolerance to the insecticide imidacloprid due to its mode of action and

438 also by the reduction of competition for food and space with other species which
439 were more sensitive. In the microcosms, the snails took over the functional role of
440 the more sensitive grazers such as chironomids and mayflies. This phenomenon
441 is referred to as “functional redundancy” (Tilman et al. 1997). In this respect,
442 functional endpoints may be useful in gaining complementary information but
443 structural endpoints seem to be more adequate to detect a toxicant’s effects as
444 also shown by Pestana et al. (2009).

445 The survival of only the tolerant species has clear consequences for any
446 ecosystem exposed to pollutants. Cucker (1983) observed that the increased
447 density of *Lymnaea* sp. caused a reduction of biomass in all food competitors
448 (free living scrapers and grazers, and tube-dwelling scrapers) and even in mobile
449 predators. According to Cucker (1983) the snails themselves became a stressor:
450 for example, they affected the chironomids by direct contact, causing larval
451 displacement or scrapers and predators causing a temporary disruption of
452 foraging activity. Even for the tube-dwellers chironomids, frequent snail
453 encounters would result in damaged tubes, increased maintenance costs, and
454 simultaneously increased exposure to predators. In this experiment, some of the
455 sensitive species already affected by imidacloprid may have become further
456 stressed by interaction and competition with a greater number of snails and other
457 tolerant species. Indirect effects may have even more far-reaching consequences
458 if tolerant species are vectors for diseases or hosts of parasites, which is often the
459 case in snails.

460 Indirect effects observed in multispecies tests may not reflect what may
461 occur in a natural environment in detail but they are an inevitable and
462 unpredictable consequence of the direct effects and as such worthwhile to be

463 considered. Depending on the endpoints chosen, an increase in abundance of
464 tolerant species may mask the effects of the toxicant, as for example a decline of
465 abundance or emergence of sensitive species.

466

467 **Conclusions**

468 This study demonstrated that repeated short-term pulses of imidacloprid at low
469 concentration levels affected aquatic ecosystems, even under favourable
470 conditions for photolysis of imidacloprid in water. As a consequence, an
471 ecosystem may experience direct and indirect effects, imbalance and cascading
472 effects on many trophic levels, not only restricted to the aquatic food web. The
473 implications of this are not easily foreseen. Considering the increased use of
474 imidacloprid and of pesticides in agriculture and in gardening in general, episodic
475 contaminations (pulses) will not be rare events and represent a realistic and
476 recurrent risk.

477 The proposed microcosms approach, with its field-relevant and simple design
478 proved to be a useful tool for assessing the effects of imidacloprid contaminations.

479

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484

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- 592

593 Tables

594 **Table 1.** Imidacloprid concentrations (in µg/L and µg/Kg for water and sediment, respectively):
 595 water and sediment samples collected at the end of the experiment (7 weeks after 3rd pulse).

Treatments	Control	0.6 µg/L	1.4 µg/L	3.2 µg/L	7.5 µg/L	17.3 µg/L	40 µg/L
Water	0.0	0.0	0.06	0.13	0.37	0.99	1.72
Sediment	0.0	0.0	0.0	0.0	0.02	0.04	0.13

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597

598 **Table 2.** Imidacloprid concentrations: Nominal (NC) and corresponding time weighted average
 599 (TWA) concentrations (in µg/L) after each pulse

Nominal concentrations (µg/L)	TWA for pulse 1	TWA for pulse 2	TWA for pulse 3	Mean TWA (µg/L)
0.6	0.24	0.18	0.11	0.2
1.4	0.57	0.43	0.26	0.4
3.2	1.3	0.98	0.6	1.0
7.5	3.04	2.31	1.4	2.3
17.3	7.01	5.32	3.23	5.2
40	16.22	12.3	7.46	12

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602

603 Figure legend

604 **Fig.1** Degradation of imidacloprid in water after each pulse in the 17.3 µg/L (nominal
605 concentration) treatment. **(a) 1st pulse:** Correlation $R^2 = 0.83$; $K = 0.035$; $DT50 = 20$ h; $TWA = 7.01$
606 µg/L; Mean UVB= 2.35 µW/cm². **(b) 2nd pulse:** Correlation $R^2 = 0.99$; $K = 0.02$; $DT50 = 36$ h; $TWA =$
607 5.32 µg/L; Mean UVB= 2.78 µW/cm². **(c) 3rd pulse:** Correlation $R^2 = 0.71$; $K = 0.023$; $DT50 = 29$ h;
608 $TWA = 3.23$ µg/L; Mean UVB= 2.75 µW/cm². More details in Materials and Method

609 **Fig. 2** Comparison of chironomids diversity and abundance across imidacloprid concentrations..
610 **nc**= nominal concentrations; **TWA**= time weighted concentrations. The asterisks indicate
611 treatments significantly different from the control ($p < 0.05$). Numbers and signs different from
612 asterisks indicate outliers

613 **Fig. 3** Decline of Orthoclaadiinae diversity and total abundance across imidacloprid concentrations..
614 **nc**= nominal concentrations; **TWA**= time weighted concentrations. The asterisks indicate
615 treatments significantly different from the control ($p < 0.05$). Numbers and signs different from
616 asterisks indicate outliers

617 **Fig. 4** Total abundance of *Ablabesmyia* sp. (Tanypodinae) across imidacloprid concentrations.
618 **nc**= nominal concentrations; **TWA**= time weighted concentrations. The asterisks indicate
619 treatments significantly different from the control ($p < 0.05$). Numbers and signs different from
620 asterisks indicate outliers

621 **Fig. 5** Increased abundance of *Radix* sp. (Gastropoda) across imidacloprid concentrations. **nc**=
622 nominal concentrations; **TWA**= time weighted concentrations. The asterisks indicate treatments
623 significantly different from the control ($p < 0.05$). Numbers and signs different from asterisks
624 indicate outliers

625 **Fig. 6** Emergence of adult *Caenis* sp. (Caenidae, Ephemeroptera) and its total abundance (larvae
626 and adults) across imidacloprid concentrations. **nc**= nominal concentrations; **TWA**= time weighted
627 concentrations. The asterisks indicate treatments significantly different from the control ($p < 0.05$).
628 Numbers and signs different from asterisks indicate outliers

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