Effects of neonicotinoid-contaminated leaves on aquatic shredders

Auswirkungen von Neonicotinoid-kontaminierten Blättern auf aquatische Shredder

by

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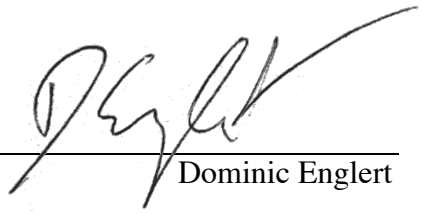
DECLARATION

I hereby declare that I independently conducted the work presented in this thesis entitled "Effects of neonicotinoid-contaminated leaves on aquatic shredders". All used assistances are mentioned and involved contributors are either co-authors of or are acknowledged in the respective publication. In all cases, I designed and planed the studies, conducted the experiments, performed the associated analyses, evaluated the data, and wrote the respective publication – with support of the named persons. This thesis has never been submitted elsewhere for an examination, as a thesis or for evaluation in a similar context to any department of this University or any scientific institution. I am aware that a violation of the aforementioned conditions can have legal consequences.

Landau, July 6, 2018



Place, Date Dominic Englert

The results gained during this thesis, were documented in four manuscripts and submitted to peer-reviewed international journals. Thus far, three articles have been published while one is currently under review.

APPENDIX A.1:

Englert, D., Bakanov, N., Zubrod, J. P., Schulz, R., Bundschuh, M., 2017. Modeling re-mobilization of neonicotinoid residues from tree foliage in streams - a relevant exposure pathway in risk assessment? *Environmental Science & Technology* 51, 1785-1794. https://pubs.acs.org/doi/abs/10.1021/acs.est.6b05213

Idea and study design: D. Englert, R. Schulz and M. Bundschuh; performance of experiment: D. Englert and J. P. Zubrod; insecticide extraction and quantification: D. Englert and N. Bakanov; model development and data analysis: D. Englert and J. P. Zubrod manuscript drafting: D. Englert and M. Bundschuh; manuscript revision: all.

APPENDIX A.2:

Englert, D., Zubrod, J. P., Link, M., Mertins, S., Schulz, R. Bundschuh, M. 2017. Does waterborne exposure explain effects caused by neonicotinoid-contaminated plant material in aquatic systems? *Environmental Science & Technology* 51, 5793-5802. https://pubs.acs.org/doi/abs/10.1021/acs.est.7b00827

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APPENDIX A.4:

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"It ain't about how hard you can hit.

It's about how hard you can get hit and keep moving forward.

How much you can take and keep moving forward."

S. Stallone

I could not have done this thesis without the support of numerous people in my corner, which kept me upright and moving forward.

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1. ABSTRACT

Systemic neonicotinoids are one of the most widely used insecticide classes worldwide. In addition to their use in agriculture, they are increasingly applied on forest trees as a protective measure against insect pests. However, senescent leaves containing neonicotinoids might, *inter alia* during autumn leaf fall, enter nearby streams. There, the hydrophilic neonicotinoids may be remobilized from leaves to water resulting in waterborne exposure of aquatic non-target organisms. Despite the insensitivity of the standard test species *Daphnia magna* (Crustacea, Cladocera) toward neonicotinoids, a potential risk for aquatic organisms is evident as many other aquatic invertebrates (in particular insects and amphipods) display adverse effects when exposed to neonicotinoids in the ng/L- to low µg/L-range. In addition to waterborne exposure, in particular leaf-shredding invertebrates (= shredders) might be adversely affected by the introduction of neonicotinoid-contaminated leaves into the aquatic environment since they heavily rely on leaf litter as food source. However, dietary neonicotinoid exposure of aquatic shredders has hardly received any attention from researchers and is not considered during aquatic environmental risk assessment. The primary aim of this thesis is, therefore, (1) to characterize foliar neonicotinoid residues and exposure pathways relevant for aquatic shredders, (2) to investigate ecotoxicological effects of waterborne and dietary exposure on two model shredders, namely *Gammarus fossarum* (Crustacea, Amphipoda) and *Chaetopteryx villosa* (Insecta, Trichoptera), and (3) to identify biotic and abiotic factors potentially modulating exposure under field conditions.

During the course of this thesis, ecotoxicologically relevant foliar residues of the neonicotinoids imidacloprid, thiacloprid and acetamiprid were quantified in black alder trees treated at field relevant levels. A worst-case model – developed to simulate imidacloprid water concentrations resulting from an input of contaminated leaves into a stream – predicted only low aqueous imidacloprid concentrations (i.e., ng/L-range). However, the model identified dietary uptake as an additional exposure pathway relevant for shredders up to a few days after the leaves' introduction into the stream. When test organisms were simultaneously exposed (= combined exposure) to neonicotinoids leaching from leaves into the water and via the consumption of contaminated leaves, adverse effects exceeded those observed under waterborne exposure alone. When exposure pathways were separated using a flow-through system, dietary exposure towards thiacloprid-contaminated leaves caused similar sublethal adverse effects in *G. fossarum* as observed under waterborne exposure. Moreover, the effect sizes observed under combined exposure were largely predictable using the reference model "independent action", which assumes different molecular target sites to be affected. Dietary toxicity for shredders might, however, be reduced under field conditions since UV-induced photodegradation and leaching decreased imidacloprid residues in leaves and thereby the toxicity for *G. fossarum*. In contrast, both shredders were found unable to actively avoid dietary exposure. This thesis thus recommends considering dietary exposure towards systemic insecticides, such as neonicotinoids, already during their registration to safeguard aquatic shredders, associated ecosystem functions (e.g., leaf litter breakdown) and ultimately ecosystem integrity.

2. ZUSAMMENFASSUNG

Systemische Neonicotinoide gehören zu den weltweit meist genutzten Insektiziden. Neben ihrer Anwendung in der Landwirtschaft werden sie zunehmend zur Bekämpfung von Baumschädlingen in der Forstwirtschaft eingesetzt. Die im Herbst von Laubbäumen fallenden Blätter können allerdings immer noch Neonicotinoide enthalten. Gelangen diese kontaminierten Blätter schließlich in nahegelegene Bäche werden die wasserlöslichen Neonicotinoide wieder mobilisiert und somit potenziell aquatische Nicht-Zielorganismen über die Wasserphase exponiert. Obwohl der Standardtestorganismus *Daphnia magna* (Crustacea; Cladocera) relativ unempfindlich gegenüber Neonicotinoiden ist, sind viele andere aquatische Invertebraten bereits bei einer Exposition im ng/L- bis niedrigem µg/L-Bereich negativ beeinträchtigt. Besonders laubzersetzende Invertebraten (= Shredder) könnten, zusätzlich zu einer Exposition über die Wasserphase, durch den Eintrag von Neonicotinoid-kontaminiertem Laub in ein Fließgewässer negativ beeinträchtigt werden, da Laub für sie eine essentielle Nahrungsquelle darstellt. Jedoch erhielt dieser Expositionspfad im Zusammenhang mit aquatischen Shreddern und Neonicotinoid-kontaminiertem Pflanzenmaterial bisher kaum Aufmerksamkeit seitens der Forschung und findet keine Berücksichtigung in der aquatischen Umweltrisikobewertung. Das Hauptziel dieser Arbeit war daher (1) Neonicotinoidrückstände in Blättern zu quantifizieren sowie für Shredder relevante Expositionswege zu identifizieren, (2) ökotoxikologische Effekte einer Exposition über die Wasserphase sowie über die Nahrung für zwei Modell-Shredder *Gammarus fossarum* (Amphipoda) und *Chaetopteryx villosa* (Insecta) zu untersuchen, und schließlich (3) biotische und abiotische Faktoren zu betrachten, welche eine Exposition unter Feldbedingungen potenziell beeinträchtigen könnten.

Im Rahmen dieser Arbeit konnten Rückstände der Neonicotinoide Imidacloprid, Thiacloprid und Acetamiprid in Blätter behandelter Schwarzerlen quantifiziert werden. Ein entwickeltes „Worst-Case Modell“ prognostizierte niedrige Imidaclopridwasserkonzentrationen für einen Bach in welchen Imidacloprid-kontaminierte Blätter eingetragen werden. Jedoch konnte mit Hilfe des Modells die Aufnahme über die Nahrung als ein für aquatische Shredder relevanter Expositionspfad identifiziert werden. Der Konsum von Neonicotinoid-kontaminierten Blättern führte, bei gleichzeitiger Exposition über die Wasserphase (= kombinierte Exposition), in beiden Testorganismen zu stärkeren Effekten als die alleinige Exposition über die Wasserphase. Des Weiteren gelang es in einem weiteren Laborexperiment die beiden Expositionswege mittels einer Durchflussanlage zu separieren. Hierbei führte die separate Exposition von *G. fossarum* sowohl über die Nahrung (= Konsum von Thiacloprid-kontaminierten Blättern) als auch über die Wasserphase zu vergleichbaren Effektgrößen. Zudem ließen sich die unter einer kombinierten Exposition beobachteten Effektgrößen weitestgehend mit dem Referenzmodell der „Unabhängigen Wirkung“ vorhersagen, was eine Wirkung auf unterschiedliche molekulare Zielorte vermuten lässt. Die durch Imidacloprid ausgelöste toxischen Effekte auf *G. fossarum* konnten schließlich durch eine Behandlung der Blätter mit UV-Strahlung (repräsentativ für Sonnenlicht) sowie durch Leaching in Wasser reduziert werden. Jedoch waren beide Shredder-

Spezies nicht dazu in der Lage aktiv eine Aufnahme von Neonicotinoiden über die Nahrung zu vermeiden. Daher geht aus dieser Arbeit die Empfehlung hervor, bereits während der Registrierung von systemischen Pestiziden, auf nahrungsbedingte Effekte zu testen und dadurch aquatische Shredder als auch assoziierte Ökosystemfunktionen (z.B. Laubabbau) zu schützen.

3. INTRODUCTION

3.1 BACKGROUND

Agrochemicals, in particular insecticides, are routinely applied on arable crops to minimize yield losses caused by insect pests (Oerke, 2005). Neonicotinoids are a relatively novel insecticide class similar to nicotine in terms of their chemical structure and function against the nicotinic acetylcholine receptor of insects (Matsuda et al., 2001; Tomizawa & Casida, 2003). Today, neonicotinoids constitute one of the most widely used insecticide classes worldwide with registrations in more than 120 countries (Elbert et al., 2008; Jeschke et al., 2011). Since the introduction of their first compound, namely imidacloprid, in the early 1990s, neonicotinoids have quickly risen to the top of the insecticide market. In 2008, they held a 24% share of the total agrochemical market while dominating (with a share of 80%) the market for insecticidal seed treatments (Jeschke et al., 2011). Their tremendous success was likewise facilitated by a series of political events leading to the ban or withdrawal of older insecticide classes from the market due to development of resistance or increasing regulatory hurdles (e.g., organophosphates and carbamates; Elbert et al., 2008; Jeschke & Nauen, 2008). Their unique characteristics also allow them to have a relatively low acute toxicity to mammals compared to older insecticides (but see Gibbons et al., 2015), while remaining extremely toxic to most insect pests. Additionally, their physico-chemical properties allow for a broad range of application methods (Elbert et al., 2008; Tomizawa & Casida, 2003). Another important characteristic distinguishing neonicotinoids from most other insecticides is their high systemic nature. Neonicotinoids are rapidly taken up by roots or leaves and distribution in all plant parts ensuring long lasting protection against root-, stem-, and leaf-feeding pests (Jeschke & Nauen, 2008). However, the preemptive use of neonicotinoids as seed coatings as well as their high environmental accumulation in soil and plants (Bonmatin et al., 2015; Giorio et al., 2017) contradicts pivotal principles of integrated

pest management: amongst others, the application of pesticides only when needed and the avoidance of persistent compounds (Matyjaszczyk, 2017; Tookter et al., 2017).

Due to neonicotinoids' suspected contribution to the decline of pollinators (e.g., Pisa et al., 2015; 2017; Sanchez-Bayo, 2014), three compounds, namely imidacloprid, clothianidin and thiamethoxam, have been temporarily banned (since 2013) for certain applications within the European Union (European Commission, 2013). Currently, the European Food Safety Authority is conducting a re-evaluation of the risks these compounds pose to pollinators (EFSA, 2017). In addition to the risks for terrestrial arthropods, their impact on aquatic organisms is under close scrutiny by different environmental authorities (e.g., EFSA, 2014; EPA, 2016). Neonicotinoids' off-site transport is favored by their persistence in soils (e.g., dissipation time 50% for imidacloprid is up to 1,250 days; Bonmatin et al., 2015) as well as their relatively high water solubility (up to 590 g/L for Nitenpyram at pH 7 and 20°C; Lewis et al., 2016). For instance, after crop planting, neonicotinoids might be washed off from coated seeds by rainfall or irrigation water (de Perre et al., 2015; Hladik et al., 2014; Schaafsma et al., 2015). In this way, neonicotinoid residues can accumulate in soils (Bonmatin et al., 2015) and may be subsequently transported to nearby surface waters via overland runoff, tile drain lines (Chretien et al., 2017) and snowmelt (Main et al., 2016). Aquatic ecosystems may be additionally contaminated with neonicotinoids via spray and dust drift as a consequence of planting coated seeds (Greatti et al., 2006; Greatti et al., 2003; Xue et al., 2015) but also through the release of wastewater (Hladik & Kolpin, 2016; Münze et al., 2017). This leads to these insecticides being frequently detected in surface waters during and also outside of the growing season with average concentrations of individual neonicotinoids around 0.08 to 0.73 μg/L (Morrissey et al., 2015; Sánchez-Bayo et al., 2016). Peak concentrations, however, can be considerably higher in streams draining agricultural areas (e.g., up to 320 μg imidacloprid/L

in Dutch agricultural streams; Van Dijk et al., 2013). From an ecotoxicological perspective, these aqueous-phase neonicotinoid concentrations are worrisome as many aquatic insects (e.g., dipterans, ephemeropterans, plecopterans, and trichopterans) but also amphipods (Crustacea) already show negative responses when exposed to neonicotinoid levels in the ng/L- to low μg/L-range (e.g., reviewed in Morrissey et al., 2015; Pisa et al., 2015; 2017; Sánchez-Bayo et al., 2016).

The uptake of the systemic neonicotinoids into plants adds an additional pathway for these insecticides to reach aquatic environments. This is not considered during their environmental risk assessment and only rudimentary covered by scientific literature: the input via neonicotinoid-contaminated plant material. This input is not necessarily limited to arable crops and trees (Bonmatin et al., 2005; Kreutzweiser et al., 2007) that are intentionally treated with neonicotinoids, but also non-treated crops (through cross-contamination; Mörtl et al., 2017), flowers (Botias et al., 2015; Main et al., 2017) and – although not documented thus far – trees growing in the vicinity of agricultural fields which may take up neonicotinoids from field runoff. At the first glance, this path seems particularly relevant for the detritus of seed-treated crops that is left on the field after harvest (cf. Rosi-Marshall et al., 2007; Tank et al., 2010). However, both the low uptake efficiency of seed-applied neonicotinoids (Alford & Krupke, 2017) as well as the dilution of their residual concentrations with increasing plant biomass (Balfour et al., 2016) could minimize the amount of neonicotinoids transported via crop detritus into the aquatic system. In contrast, neonicotinoids have been frequently shown to persist, in the ng/g to μg/g-range, in foliage of coniferous and deciduous trees (e.g., Tattar et al., 1998), which are being increasingly treated with neonicotinoids to manage native and invasive pest insects in urban (e.g., parks; Szczepaniec et al., 2011) and forest areas (Benton, 2016; USDA, 2016). In Tennessee (USA), for instance, over 200,000 eastern hemlock trees received – over 8 years – between one and

eight imidacloprid treatments (>4 tons applied on ~4,500 ha of hemlock forest; Benton et al., 2017). Due to the high persistence of imidacloprid in tree foliage (i.e., up to 8 years; Benton et al., 2016a; Eisenback et al., 2014) a contamination of the surrounding environment could be anticipated when conifers shed their oldest needles during autumn. This pathway seems even more relevant for deciduous trees since they tend to accumulate greater neonicotinoid amounts in their leaves (e.g., Tattar et al., 1998) and lose (in temperate regions) their complete foliage during autumn leaf fall (Kreutzweiser et al., 2007; Mota-Sanchez et al., 2009). Contaminated leaves and needles might subsequently enter nearby streams through vertical fall or lateral movement (Abelho, 2001). Once submerged, the hydrophilic neonicotinoids are largely remobilized in a matter of days into the water through leaching from contaminated leaves (as shown for ash leaves by Kreutzweiser et al., 2007), consequently exposing aquatic organisms via the aqueous phase. Previous studies found no adverse effects on aquatic invertebrate communities in streams or lakes located in forests managed with these systemic insecticides. They did, however, occasionally detect imidacloprid at concentrations (<1 μg/L; Benton et al., 2016b; 2017; Churchel et al., 2011; McAvoy et al., 2005) in the range of currently existing acute and chronic ecological water quality thresholds defining acceptable levels for imidacloprid in surface waters (reviewed in Morrissey et al., 2015). While aqueous neonicotinoid concentrations, such as those remobilized from contaminated leaves, may pose a general threat to aquatic organisms, particularly leaf-consuming invertebrates (= shredders) might additionally be exposed to neonicotinoids when feeding on contaminated leaves recently introduced into streams (Kreutzweiser et al., 2007; 2008b; 2009).

In heterotrophic low-order streams, shredders are regarded as key drivers in the ecosystem function of leaf litter breakdown (Cummins & Klug, 1979). They transform allochthonous organic material (such as leaves) – which constitutes a major share (up to 99%;

Fisher & Likens, 1973) of the annual energy input into these systems – into finer particles (e.g., fecal pellets; Cummins & Klug, 1979). The latter serves as the predominant energy source for collectors of local as well as downstream communities (Cummins & Klug, 1979). Moreover, both shredders and collectors are important prey for many aquatic predators (e.g., fish; Cummins, 1973; MacNeil et al., 1999). Therefore, any adverse effects on shredders, mediated by the input of neonicotinoid-contaminated leaves into streams, may have far reaching consequences for the in-stream energy flow across multiple trophic levels. Until now, however, the risks for shredders associated with the input of neonicotinoid-contaminated plant material into streams remain poorly understood and the few existing studies are exclusively limited to one active ingredient (AI), namely imidacloprid. Those laboratory microcosm studies conducted by Kreutzweiser et al. (2007; 2008a; 2008b; 2009) reported reduced feeding and increased mortality for two leaf-shredding insect species – the stonefly *Pteronarcys dorsata* and the crane fly *Tipula* sp. – exposed to leaves from imidacloprid-treated ash trees. Since both species were shown unable to detect and, thereby, actively avoid imidacloprid-contaminated leaves (Kreutzweiser et al., 2009), a general risk for shredders and the associated leaf litter breakdown could be assumed.

This urges the need for a more systematic assessment of the fate of different neonicotinoids in tree foliage as well as associated ecotoxicological implications of neonicotinoid-contaminated leaves for aquatic organisms. In particular, dietary exposure has been hardly considered for these hydrophilic insecticides. However, this pathway might be, along with waterborne exposure, primarily relevant for shredders (cf. Kreutzweiser et al., 2007). Moreover, knowledge about potential active avoidance strategies (i.e., through sensing neonicotinoids in leaves; cf. Kreutzweiser et al., 2009) of different shredder species as well as of abiotic factors that modulate neonicotinoid residues in leaves and, hence, the leaves' toxicity for shredders would aid the estimation of

neonicotinoids' effects in the field. Regarding the abiotic factors, particularly photolytic degradation of neonicotinoids as well as the remobilization of these highly hydrophilic substances might potentially reduce (dietary) exposure for shredders.

3.2 OBJECTIVES

Given the knowledge gaps identified above, the primary aim of this thesis is to identify and characterize ecotoxicological implications associated with leaves from neonicotinoid-treated trees for aquatic shredders using a set of laboratory bioassays (see chapter 4) to answer three main research questions:

1. Can neonicotinoid insecticides – when applied at field relevant doses – accumulate in foliage of deciduous trees at levels that cause ecotoxicologically relevant aqueous concentrations in streams following an input of contaminated leaves?

2. What is the relative importance of waterborne neonicotinoid exposure – e.g., originating from agricultural runoff or remobilization of the insecticides from contaminated leaves – in comparison to dietary exposure (i.e., feeding on contaminated leaves) for aquatic shredders?

3. Can biotic (i.e., active avoidance by shredders) or abiotic factors (i.e., photolytic degradation and remobilization from leaves) reduce adverse effects for shredders prior to and following the input of neonicotinoid-contaminated leaves into streams?

4. THESIS LAYOUT AND METHOD OVERVIEW

This thesis is subdivided into four experimental phases (Fig. 4.1) to answer the three research questions outlined above.

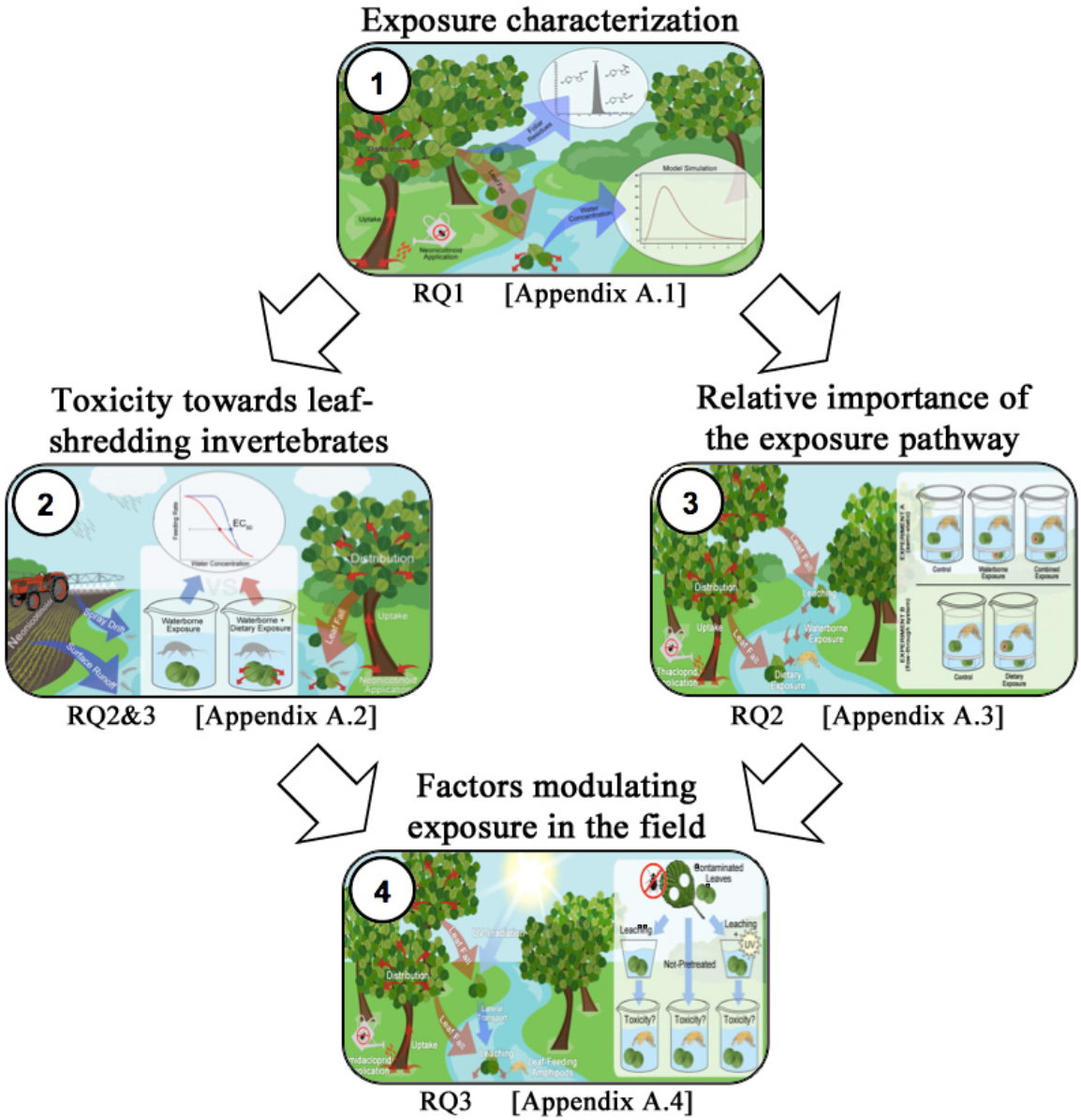

Fig. 4.1. Scheme displaying the four phases (encircled numbers) of this thesis including their main objective (box title), the research questions (RQs; see chapter 3.2) that were addressed and the corresponding publications **[Appendix A.1-A.4]**.

4.1 MODEL NEONICOTINOIDS AND TEST SPECIES

In order to answer the main research questions outlined above in a systematic approach, three neonicotinoids – namely imidacloprid, thiacloprid and acetamiprid – varying broadly in their physico-chemical properties (Table 4.1), were chosen as model neonicotinoids. *Alnus glutinosa* (L.) Gaertn. (black alder), a deciduous tree widely distributed within riparian zones of temperate Europe, was chosen as model tree species since it may be exposed towards neonicotinoids via direct treatment (i.e., some commercially available products are registered for use against, for example, the alder borer *Rosalia funebris*; Bayer CropScience, 2017) or indirectly via runoff from adjacent agricultural fields (as previously shown for flowers; Botias et al., 2015; Main et al., 2017; Mogren & Lundgren, 2016). The trees were purchased from an ecological tree nursery in April 2014 (Baumschule von der Mühlen, Küsten, Germany) and had, according to the provider, never been treated with any kind of pesticides prior to their use in this thesis.

Table 4.1. Information about commercial neonicotinoid products used during this thesis as well as the physico-chemical parameters, leaching properties and environmental persistence of their active ingredient **[Appendix A.1]**.

	imidacloprid	**thiacloprid**	**acetamiprid**
product name	Confidor® WG 70	Calypso®	Mospilan®SG
supplier	Bayer CropScience	Bayer CropScience	Cheminova DE GmbH
concentration of the AI within the product	700 g/kg	480 g/L	200 g/kg
molecular mss (g/mol)[1]	255.66	252.72	222.67
solubility in water at 20°C (mg/L) [1]	610 (high)	184 (moderate)	2950 (high)
octanol/water partition coefficient (log P_{ow}) [1]	0.57	1.26	0.8
GUS leaching potential index[2]	3.76 (high)	1.44 (low)	0.94 (very low)
soil persistence (DT_{50} in days) [3]	100 - 1250	3.4 - >1000	31 - 450
aqueous photolysis (DT_{50} in days) [1]	0.2 (fast)	(stable)	(stable)
water hydrolysis (DT_{50} in days) [1]	>365 (at pH 9; 25°C; stable)	stable (pH 5 to pH 9)	420 (at pH 9; 25°C; stable)

[1]Lewis et al. (2016), [2]Miranda et al. (2011), [3]Bonmatin et al. (2015)

Two shredders were used as test organisms, representing two taxonomic orders that essentially contribute to leaf litter breakdown in European streams (Dangles & Malmqvist, 2004), namely *Gammarus fossarum* Koch (Crustacea: Amphipoda) and *Chaetopteryx villosa* Fabricius (Insecta: Trichoptera). Due to neonicotinoids' selective action against the nicotinic acetylcholine receptors of insects (Tomizawa & Casida, 2003), *C. villosa*

was generally assumed to be more sensitive towards neonicotinoid exposure. Amphipods from the genus *Gammarus* are, due to their well-documented sensitivity against (chemical) stressors, widely used in non-standard toxicity tests (Kunz et al., 2010). Moreover, gammarids have been shown to be equally vulnerable towards neonicotinoid exposure compared to many aquatic insects (Agatz et al., 2014; Englert et al., 2012; Nyman et al., 2013).

4.2 PHASE 1: EXPOSURE CHARACTERIZATION

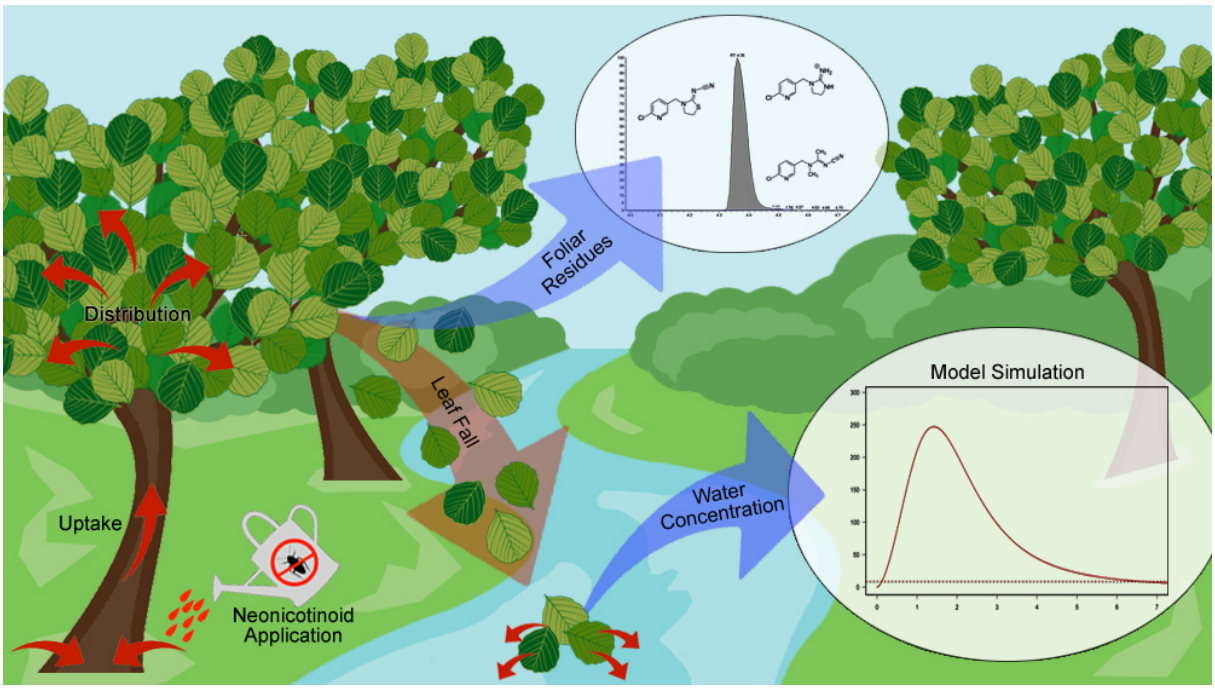

Fig. 4.2. Scheme illustrating the work conducted during the first phase of this thesis. Image adopted from **Appendix A.1**.

The first phase aimed at quantifying residues of the three model neonicotinoid compounds in foliage of deciduous trees to estimate the potential amount of these insecticides that might be remobilized in streams following an input of contaminated leaves. Therefore, black alder trees were soil-drenched in early June 2014 with commercial products containing imidacloprid, thiacloprid or acetamiprid (Table 4.1), each at six concentrations 0, 0.375, 0.15, 0.6, 2.4 and 9.6 g AI per cm trunk diameter at breast height (DBH). Although 0.6 g AI/cm DBH equals the maximum amount of imidacloprid recommended for a single soil application on trees (Bayer CropScience, 2017), two intentionally overdosed treatments – i.e., 2.4 and 9.6 g AI/cm DBH – were used to test foliar residues under elevated application doses. At the

time of leaf fall (i.e., October 2014) and thus four months post application, the foliage was harvested and stored at -20°C until further use. Foliar neonicotinoid residues were extracted by accelerated solvent extraction (Thermo Scientific Dionex, Sunnyvale, CA; USA) using a method developed during this thesis **[Appendix A.1]**. Subsequently, quantification was performed with ultrahigh-performance liquid chromatography-mass spectrometry (Thermo Scientific, Bremen, Germany) and finally compared to environmentally relevant levels found in a literature search (using the online database ISI Web of Science; search string: "neonicotinoid* and tree*"). The foliar neonicotinoid residues measured at the highest field-relevant application rate (i.e., 0.6 g AI/cm DBH; Bayer CropScience, 2017) finally served as input parameter for a model predicting worst-case waterborne exposure in streams following the input of contaminated leaves. In essence, the model simulated the simultaneous input of 600 g foliage/m^2 (~70% of the annual input reported for a first order stream in Germany; Benfield, 1997) containing 80 μg imidacloprid/g (mean residues detected in trees treated at the highest field relevant level; i.e., 0.6 g/cm DBH) into a 1 m wide, 0.3 m deep and 100 m long stream stretch of rectangular cross section (as used during modeling of the European Union's exposure assessment of pesticides; FOCUS, 2015) and a current velocity of 0.3 m/s (= average velocity measured for a second-order stream in southwest Germany, i.e., Triefenbach; Englert et al., 2015). Moreover, the remobilization rate was derived from a non-linear model fitted to the leaching data published by Kreutzweiser et al. (2007) for imidacloprid and ash leaves assuming equal leaching dynamics for black alder. Although the amount of foliage that simultaneously enters the stream represents a worst-case scenario, the model predictions are also markedly influenced by the stream' characteristics, which are considered field relevant. Further details on the calculations and a full list of parameters can be found in **Appendix A.1**. The analytical methods applied during this phase were used to quantify neonicotinoid concentrations in leaf material and water samples in the remainder of this thesis.

4.3 PHASE 2: TOXICITY TOWARDS LEAF SHREDDING INVERTEBRATES

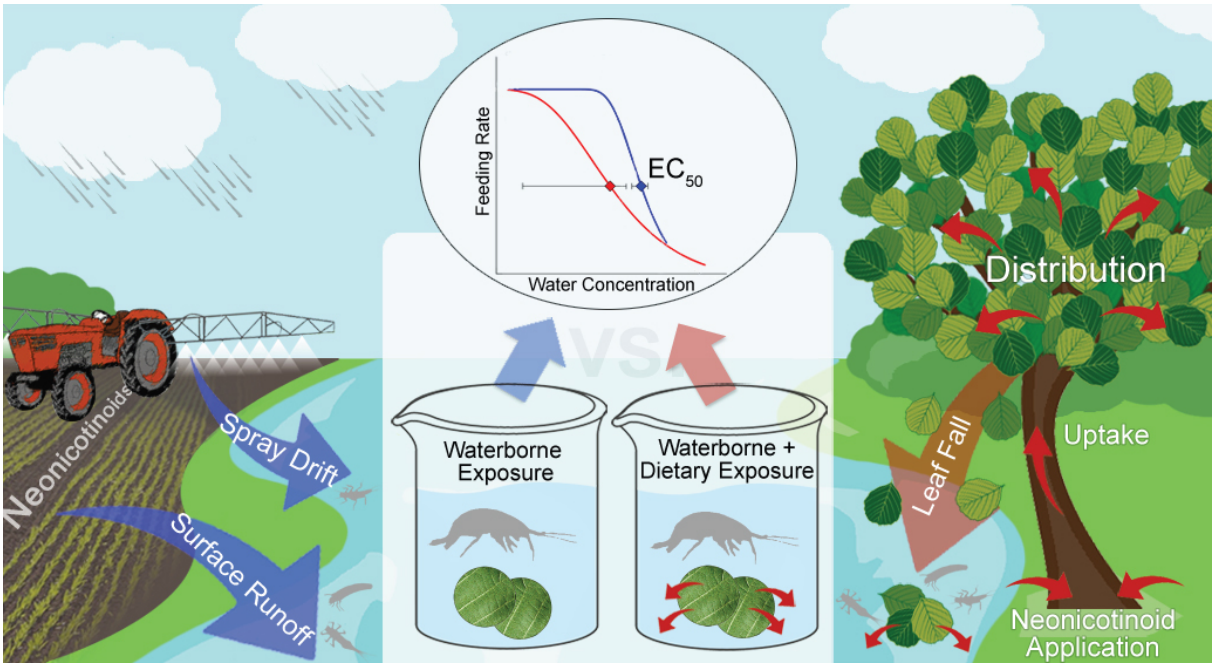

Fig. 4.3. Scheme illustrating the work conducted during the second phase of this thesis. Image adopted from **Appendix A.2**.

Using a range of 7-day feeding activity assays, the second phase investigated potential ecotoxicological differences between a waterborne exposure scenario, representative for neonicotinoid spray drift or surface runoff, and a scenario simulating the input of neonicotinoid-contaminated leaves into a stream (= combined exposure). In the latter scenario, test organisms were simultaneously exposed via both the consumption of contaminated leaves as well as via the water phase (through leaching of neonicotinoids from leaves). Both test organisms, namely *G. fossarum* (Hainbach: 49°14'N; 8°03'E; cryptic linage B; Feckler et al., 2012) and *C. villosa* (Sauerbach: 49°5'N; 7°37'E), originated from relatively pristine streams located in the Palatinate forest upstream of any settlement and agricultural activity. Pre-exposure of test organisms towards pesticides is therefore likely negligible. Further, according to the local forestry office, neonicotinoids have not previously been used in this area.

For the waterborne exposure scenario, the model shredders were individually subjected to the neonicotinoids imidacloprid, thiacloprid or acetamiprid applied directly to the water phase at increasing concentrations (= waterborne exposure; 0 to 24 μg AI/L) while feeding on neonicotinoid-free leaves. In contrast, the combined exposure scenario was realized by offering them leaves collected from trees that had been treated with different doses of one of the three neonicotinoids (i.e., ranging from 0 to 9.6 g AI/cm DBH; see chapter 4.2). Besides organisms' survival, their feeding activity served as a sensitive and ecologically relevant endpoint (Maltby et al., 2002). Finally, 7-day lethal and effective concentrations (LC_x/EC_x) as well as the progressions of concentration-response curves were compared for both exposure scenarios. Those calculations were, in case of the waterborne exposure experiments, conducted with nominal neonicotinoid test concentrations. In contrast, for the combined exposure experiments, neonicotinoid water concentrations measured at the termination of the experiments (i.e., after 7 days) were used. By considering only the final concentrations, the model derived for the combined exposure scenarios likely overestimates the actual exposure due to continued leaching of neonicotinoids from the leaves into the water. Moreover, a range of food-selection assays (in principle following Bundschuh et al., 2009) was performed to determine potential active avoidance behaviors of both shredders. Over 24 h, organisms' feeding rate on a simultaneously offered neonicotinoid-free and neonicotinoid-contaminated alder leaf disc was recorded.

4.4 PHASE 3: RELATIVE IMPORTANCE OF THE EXPOSURE PATHWAY

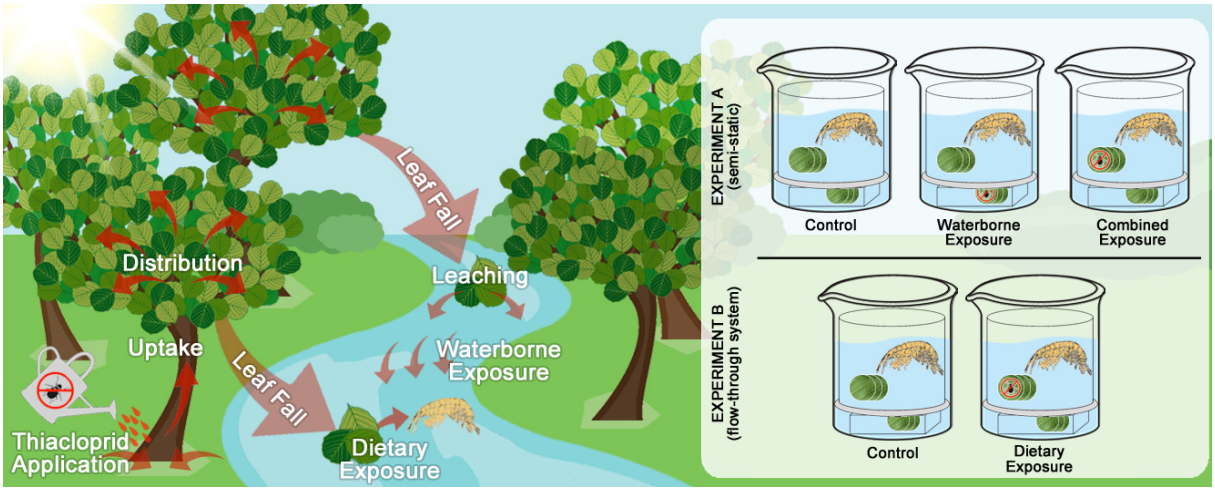

Fig. 4.4. Scheme illustrating the work conducted during the third phase of this thesis. Image modified from **Appendix A.3**.

Since the second phase of this thesis revealed – for both test organisms – a higher toxicity of the combined exposure pathway compared to sole waterborne exposure, the third phase aimed at unraveling the relative importance of the dietary exposure pathway in more detail. Using leaves collected from alder trees treated with the neonicotinoid thiacloprid (at 0.6 g AI/cm DBH), *G. fossarum* was exposed for 21 days either to waterborne, combined (dietary + waterborne) or dietary exposure. The latter scenario was conducted using a flow-through system, which kept thiacloprid water concentrations at negligible levels. Besides gammarids' survival and leaf consumption, their thiacloprid body burden (Inostroza et al., 2016), body weight and lipid content (Van Handel, 1985) were monitored. Effects observed in the dietary exposure treatment were compared to a separate thiacloprid-free control accounting for the water renewal process (see Fig. 4.4). Finally, differences between waterborne, dietary and combined exposure were assessed using the effect sizes relative to the corresponding controls. In case the effects observed under combined exposure turned out to exceed those induced by each individual exposure pathway alone, they were tested (based on confidence intervals (CIs)) for compliance with one of the most commonly used reference models, namely "independent action" (IA; Bliss, 1939).

4.5 PHASE 4: FACTORS MODULATING EXPOSURE IN THE FIELD

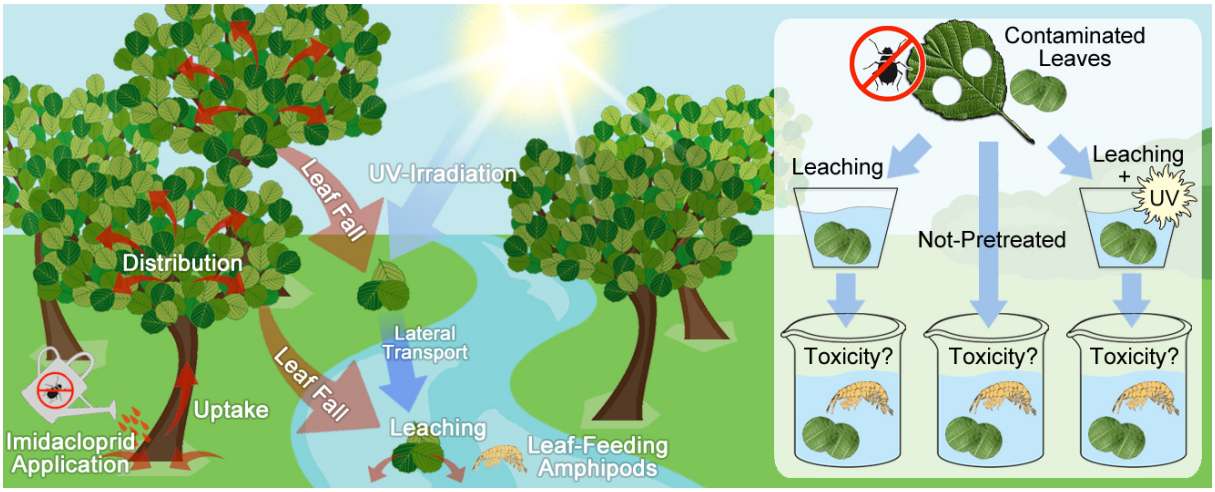

Fig. 4.5. Scheme illustrating the work conducted during the fourth phase of this thesis. Image adopted from **Appendix A.4**.

Finally, the fourth phase of this thesis investigated two abiotic processes that were hypothesized to modulate neonicotinoid residue levels in contaminated leaves prior to and following their input into streams, namely photodegradation and remobilization in water. Imidacloprid was selected as model neonicotinoid since it was one of the most widely used insecticides worldwide for many years and is currently applied to trees in US forest service programs (USDA, 2016). Based on imidacloprid's susceptibility towards photolysis and high water solubility, irradiation with ultraviolet light (UV) – e.g., when leaves are lying on the forest floor – or remobilization of the insecticide from leaves within the aquatic environment were assumed to reduce the leaves' toxicity for shredders. To test this hypothesis, *G. fossarum* was fed over 7 days with imidacloprid-contaminated leaves that had either been submerged in water for 1, 3 and 7 days or UV-irradiated for 1 day at field relevant intensities (i.e., UV-A: 4.15 W/m^2; UV-B: 0.15 W/cm^2; Häder et al., 2001) followed by leaching over 1 day while the test organisms' feeding activity was afterwards monitored over 7 days. Moreover, imidacloprid-contaminated leaves, that received neither UV-irradiation nor leaching treatment, were used as positive control. For each treatment, imidacloprid-free leaves were subjected to the same process and were used as corresponding negative controls. In addition to these feeding experiments, a separate fate experiment was set up using sufficient

amounts of leaf material required for imidacloprid quantification. Imidacloprid residues were quantified in leaves subjected to the maximum leaching duration used in the experiment (i.e., 7 days) as well as in leaves that were UV-irradiated for 1 day (at the same intensities as described above). The latter approach was realized to quantify solely the effect of UV-irradiation (without the addition of leaching in water) on leaves' imidacloprid residues.

5. RESULTS

The results gained during the four experimental phases, which were part of this cumulative thesis, were documented in four manuscripts and submitted to peer-reviewed journals. Thus far, three articles have been published while one is currently under review (Fig. 4.1) **[Appendix A1- A4]**.

5.1 CHARACTERIZATION OF EXPOSURE CONDITIONS AND PATHWAYS

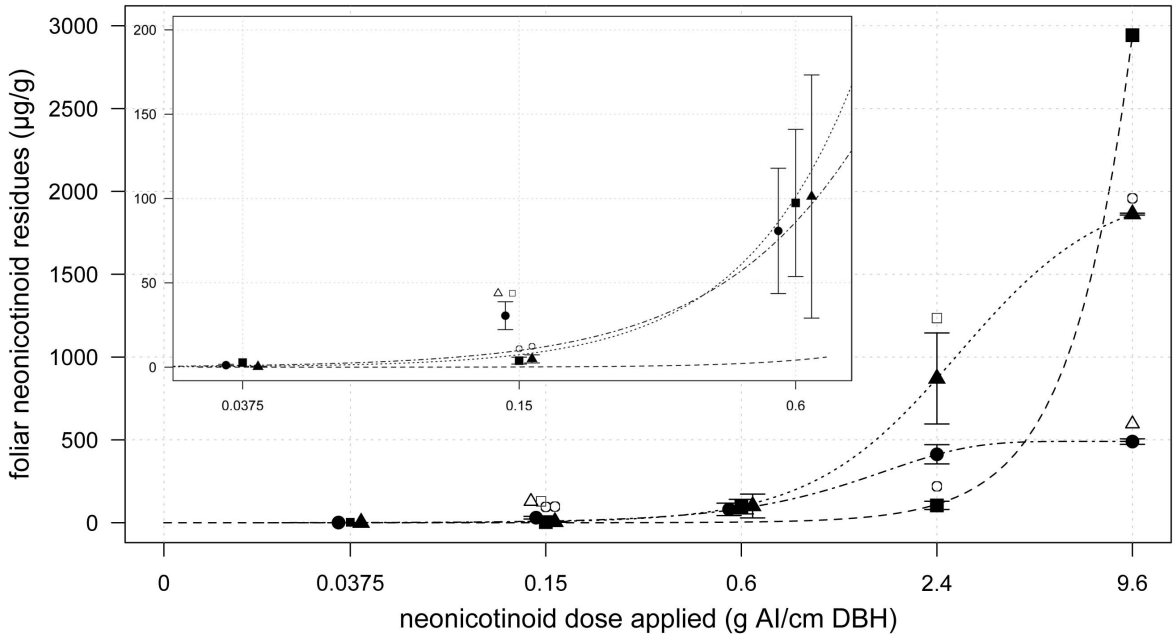

Fig. 5.1. Mean (± standard error (SE)) residues of imidacloprid (●), thiacloprid (▲) and acetamiprid (■) measured in foliage from treated black alder trees as well as the best fitting models (imidacloprid: dot-dashed line; thiacloprid: dotted line; acetamiprid: dashed line). The inset displays the residues measured in the 0 to 200-μg/g range in greater detail. Open symbols above error bars indicate statistically significant differences ($p < 0.05$; n = 2-3) between neonicotinoid compounds (i.e., compared to imidacloprid (❍), thiacloprid (△) or acetamiprid (□), respectively) within the same dose applied. Missing SEs indicate a replication of only one **[Appendix A.1]**.

Neonicotinoid treatments in June resulted in measurable foliar residues four months after application (i.e., October, at the time of leaf fall). In contrast, none of the three neonicotinoids were detected in foliage of control trees (Fig. 5.1). While neonicotinoid residues were mostly

similar for trees treated at field relevant levels of 0.0375 to 0.6 g AI/cm DBH, differences were more distinct for overdose treatments (i.e., 2.4 and 9.6 g AI/cm DBH; Fig. 5.1).

Overall, trees treated at field-relevant levels (i.e., up to 0.6 g AI/cm DBH) displayed foliar neonicotinoid residues ranging from 0.44 to ~110 μg/g dry weight (Fig. 5.1) and were, therefore, in the range of the foliar residues found in the 29 reviewed studies (Fig. 5.2). In essence, the reviewed data indicated higher foliar neonicotinoid residues in deciduous trees treated by trunk application, even though the method uses less neonicotinoids compared to soil application (Fig. 5.3).

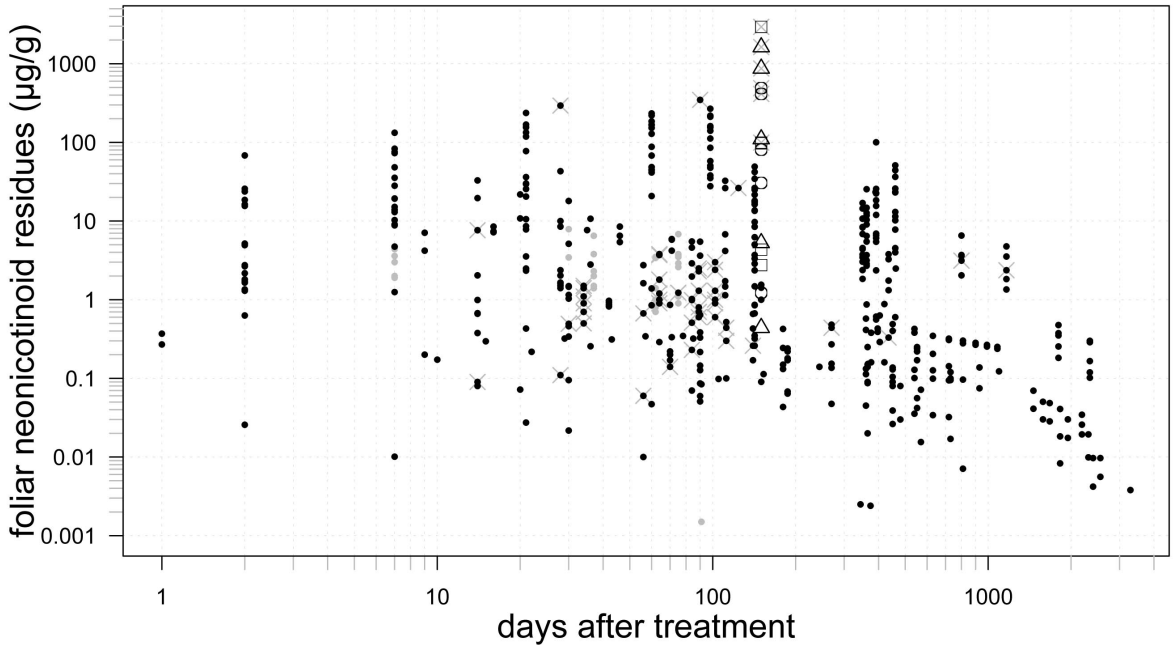

Fig. 5.2. Foliar imidacloprid (black solid circles) and dinotefuran (gray solid circles) residues derived from a literature review of peer-reviewed publications as well as imidacloprid (❍), thiacloprid (△) or acetamiprid (□) residues measured in alder foliage during the present study. A gray cross additionally indicates foliar residues derived from trees treated above the maximum doses recommended for soil application (i.e., 0.6 g imidacloprid and 0.95 g dinotefuran/cm DBH) or trunk injection (i.e., 0.25 g imidacloprid) **[Appendix A.1]**.

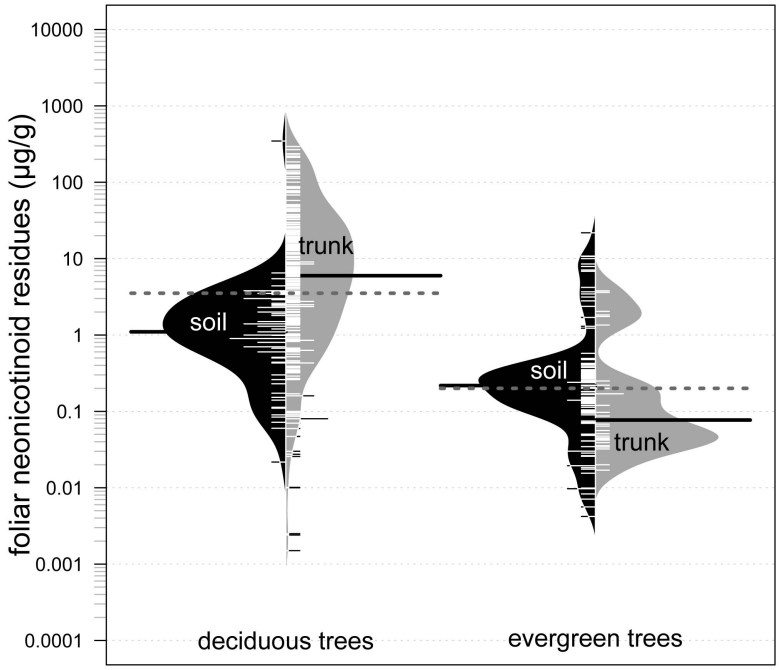

Fig. 5.3. Bean plots displaying the density trace (black and gray area) of the individual neonicotinoid residues (small white lines) derived from a literature review of peer-reviewed publications. Residue data is itemized by deciduous and evergreen trees as well as by soil and trunk application. Black solid lines represent the median of the respective sub-group. Gray dashed lines indicate the overall median for deciduous and evergreen trees, respectively **[Appendix A.1]**.

The leaching model was developed to predict imidacloprid water concentrations in a 1 m wide, 0.3 m deep and 100 m long stream stretch following the input of contaminated foliage. Under the parameterized worst-case conditions (in terms of foliar residues of 80 µg/g and large amounts of foliage entering the stream), the maximum concentration in the first 1-m segment was ~2 ng imidacloprid/L. The water concentration, however, increased due to continued leaching and downstream transport to maximum levels as high as ~250 ng imidacloprid/L 100 m further downstream (reached after ~34 h; Fig. 5.4) and would continue to increase if the stream stretch that receives imidacloprid-contaminated foliage was extended (Fig. S7) **[Appendix A.1]**. Stream water concentrations predicted by the model depended upon foliage-associated parameters as well as on the streams' characteristics. Examples are illustrated in Figs. S7-S11 **[Appendix A.1]**.

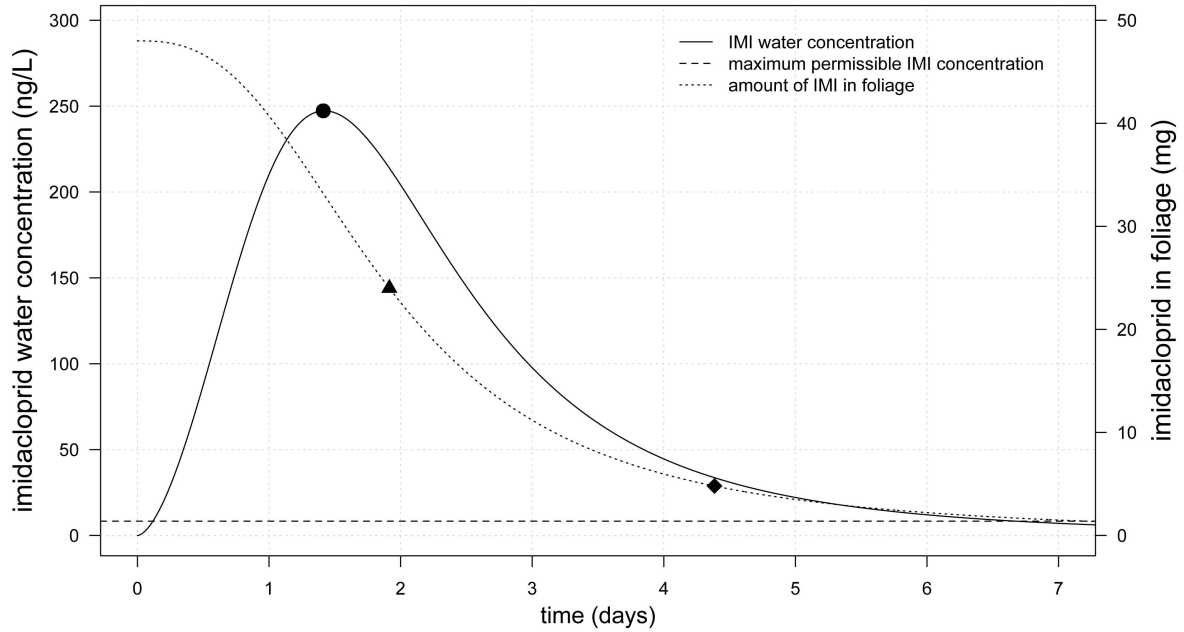

Fig. 5.4. Modeled imidacloprid concentration (solid line) in the water phase of the 100th stream segment including the peak imidacloprid concentration (●) as well as the lowest imidacloprid threshold level of 8.3 ng/L (i.e., maximum permissible concentration; Smit et al., 2015; dashed line). Moreover, the total amount of imidacloprid in foliage (dotted line; within each stream segment; surface area: 1 m^2) following a simulated input of 600 g foliage/m^2 with an initial residue level of 80 μg imidacloprid/g as well as corresponding retention times (RT_{50}: ▲ and RT_{10}: ◆) are also displayed **[Appendix A.1]**.

5.2 TOXICITY OF NEONICOTINOID–CONTAMINATED LEAVES TOWARDS SHREDDERS

In 7-day feeding activity experiments assessing waterborne neonicotinoid exposure, mortality of *C. villosa* and *G. fossarum* remained, irrespective of the tested compound and concentration (up to 24 μg AI/L), below 7 and 23%, respectively (Table. 5.2). The combined exposure scenario, which assessed both dietary neonicotinoid uptake as well as waterborne exposure due to neonicotinoids' remobilization from leaves, caused similar mortalities for *Gammarus*, as observed for the waterborne exposure alone (i.e., ≤20%; Table S3) **[Appendix A.2]**. Only when aqueous neonicotinoid concentrations exceeded the range tested in the waterborne scenario by one order of magnitude, mortalities of 37 and 47% were observed (Table S3) **[Appendix A.2]**. For *Chaetopteryx*, in contrast, 7-day LC_{50} values (i.e., 11.5 μg imidacloprid/L and 21.6 μg thiacloprid/L) derived from the combined exposure scenario were observed at water concentrations that caused no mortality in the waterborne exposure experiments (Table. 5.1).

Table 5.1. 7-d EC_{20} and EC_{50}-values as well as LC_{20} and LC_{50}-values (±95%CIs; in μg/L) of *G. fossarum* and *C. villosa* derived from feeding activity experiments under waterborne (WB) and combined (CO) exposure. While, for the WB exposure, calculations were conducted with nominal neonicotinoid concentrations, those for the CO exposure were based on concentrations measured at the termination of the experiments (i.e., after 7 days). EC_xs printed in bold indicate a statistically significant difference between the WB and CO exposure scenario **[Appendix A.2]**.

		EC_{20} ±95% CIs		EC_{50} ±95% CIs		LC_{20} ±95% CIs		LC_{50} ±95% CIs	
		WB	CO	WB	CO	WB	CO	WB	CO
G. fossarum	imidacloprid	**3.63±1.69**	**0.40±0.75**	**8.26±2.68**	**2.23±2.17**	n.o.	n.o.	n.o.	n.o.
	thiacloprid	**1.66±0.53**	**0.20±0.22**	3.06±0.76	2.37±2.12	n.o.	33.10±30.75	n.o.	n.o.
	acetamiprid	2.28±2.74	0.02±0.02	**8.43±4.88**	**0.31±0.42**	21.34±12.62	328.62±4193	n.o.	n.o.
C. villosa	imidacloprid	**10.45±2.31**	**0.32±0.76**	**19.35±2.65**	**7.05±8.37**	n.o.	3.72±5.27	n.o.	11.45±5.72
	thiacloprid	n.o.	1.20±4.10	n.o.	8.06±8.69	n.o.	10.35±1.93	n.o.	21.60±2.53
	acetamiprid	34.96±28.01	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.

n.o. = not observed within the range of concentrations tested

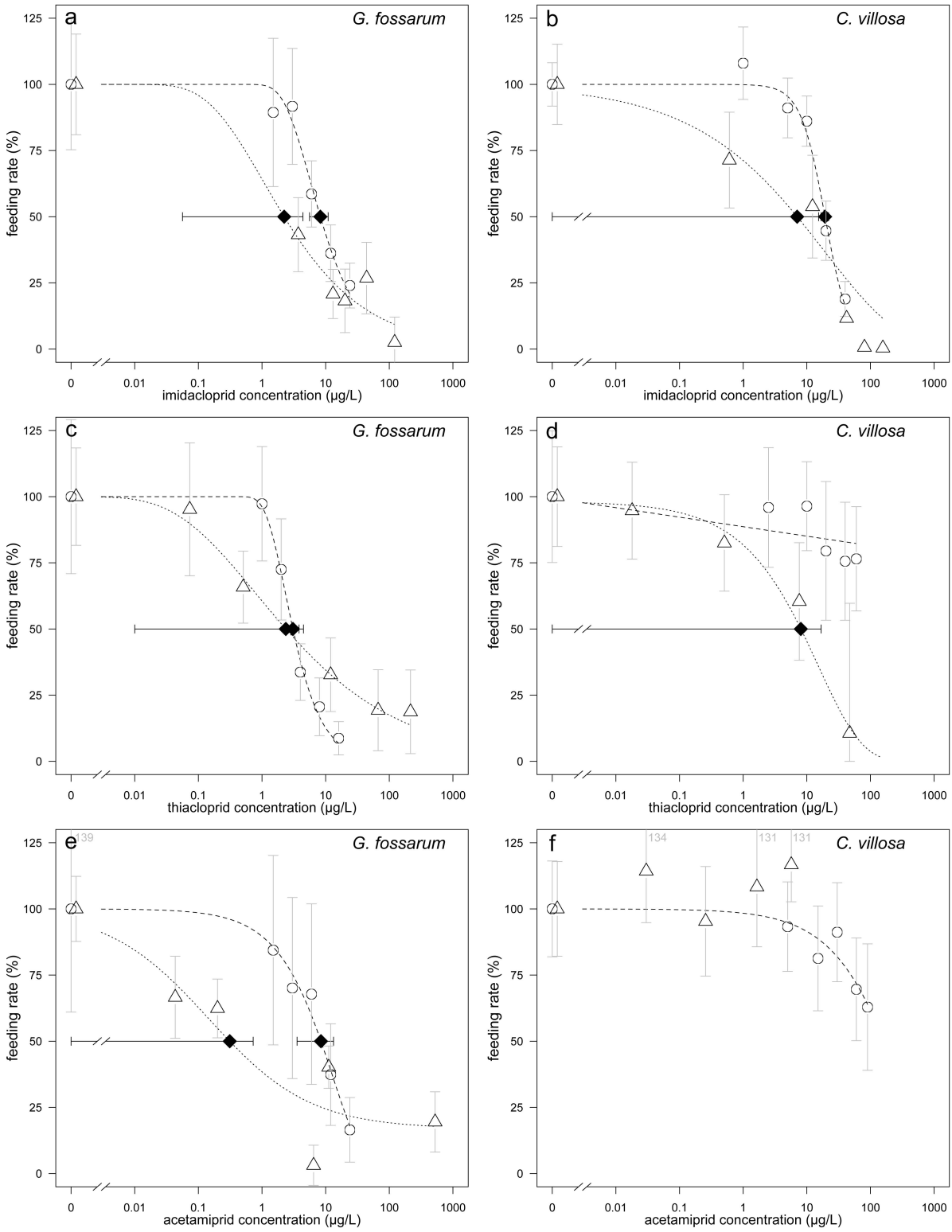

Fig. 5.5. Relative feeding rate (±95%CIs) of *G. fossarum* (a, c, e) and *C. villosa* (b, d, f) subjected to waterborne (circles) or combined (= waterborne + dietary; triangles) exposure towards imidacloprid (a,b), thiacloprid (c,d) and acetamiprid (e,f). The best fitting concentration-response model for waterborne (dashed line) and combined (dotted line; except in f) exposure as well as corresponding EC_{50}-values (±95%CIs; solid diamonds) are displayed **[Appendix A.2]**.

In contrast to the test organisms' mortality, their feeding on leaves was markedly influenced by waterborne as well as combined neonicotinoid exposure. Consequently, complete concentration response curves (and EC_x-values) could be generated for most of the neonicotinoid compounds and exposure scenarios (except for *Chaetopteryx* exposed to acetamiprid and thiacloprid; Fig. 5.5). In essence, comparisons of the concentration-response curves for the test organisms' feeding rate showed a higher toxicity of the combined exposure as the curves of this scenario ran, for both shredders, mostly statistically significantly below those of the waterborne exposure scenario (Fig. 5.5 and Fig. S1) **[Appendix A.2]**. Further, all 7-day EC_{20} and EC_{50} values calculated for the combined exposure scenario were lower, though not in every case statistically significantly, than their counterparts derived from waterborne exposure experiments (Fig. 5.5 and Table 5.1) **[Appendix A.2]**.

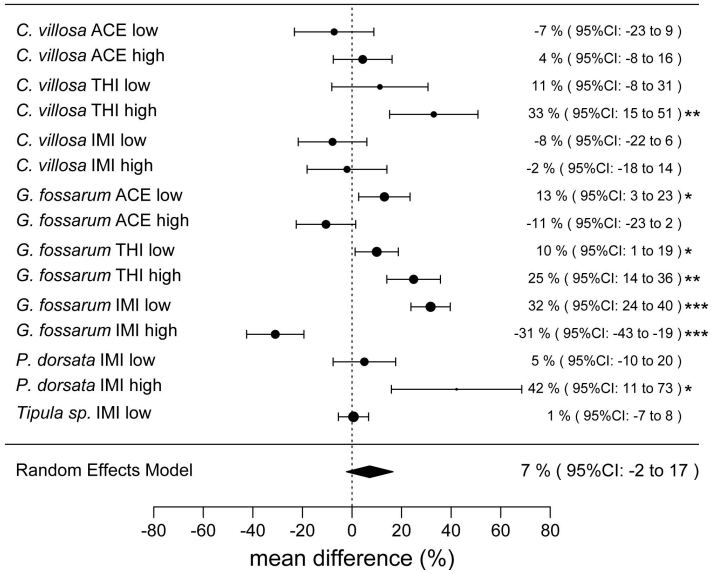

Fig. 5.6. Relative mean difference (±95%CIs) in leaf consumption of *G. fossarum* and *C. villosa* as well as *P. dorsata* and *Tipula* sp. (published by Kreutzweiser et al., 2007) obtained by a random-effects meta-analysis of food selection assays where organisms had the choice between neonicotinoid-free and neonicotinoid-contaminated leaf discs. Means at the right side of the middle line indicate a higher consumption of neonicotinoid-free leaf discs, while means at the left side indicate a higher consumption of contaminated discs. Point sizes indicate the weight (= inverted variance) of the respective experiment to the overall effect. Organisms consumed statistically significantly more of one of the food types if CIs do not include zero (dotted line; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)) **[Appendix A.2]**.

A random-effects meta-analysis conducted with the results of the 12 food-selection assays revealed preferential feeding neither on neonicotinoid-free nor on any neonicotinoid-contaminated leaf discs when pooling data among shredder species and neonicotinoid compounds (Fig. 5.6) **[Appendix A.2]**. Although in some of the food-selection assays a statistically significant difference in the feeding on the neonicotinoid-contaminated and on the control leaf discs was observed, these cases were randomly distributed among neonicotinoids and shredder species **[Appendix A.2]**. However, when the meta-analysis was conducted with the data for *G. fossarum* and *C. villosa* separated by the three neonicotinoid compounds, thiacloprid-free leaves were statistically significantly preferred over thiacloprid-contaminated leaves (Fig. S4) **[Appendix A.2]**.

5.3 RELATIVE IMPORTANCE OF THE EXPOSURE PATHWAY

During the 21-day feeding experiment conducted with *G. fossarum* being exposed to thiacloprid-contaminated black alder leaves, the flow-through system kept aqueous thiacloprid concentrations in the dietary exposure scenario successfully below or only slightly above detectable levels (limit of quantification = 0.01 μg/L). In contrast, in the waterborne and combined exposure scenario concentrations of up to 9 μg thiacloprid/L were detected (Fig. 5.7).

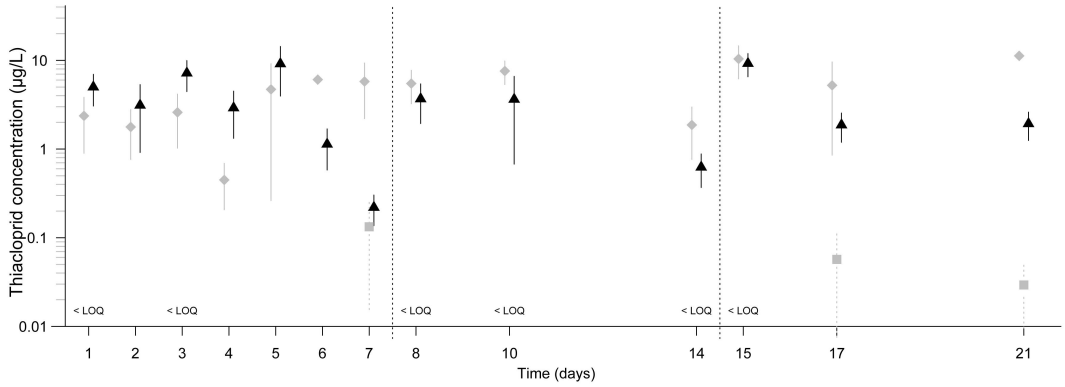

Fig. 5.7. Thiacloprid water concentrations (mean ± SE; n = 3-4) measured during the 21-day feeding experiment. ◆ marks concentrations of the waterborne, ■ the dietary and ▲ the combined exposure treatment, respectively. Except for three cases, thiacloprid water concentrations in the dietary exposure treatment were below the limit of quantification (0.01 μg/L) **[Appendix A.3]**.

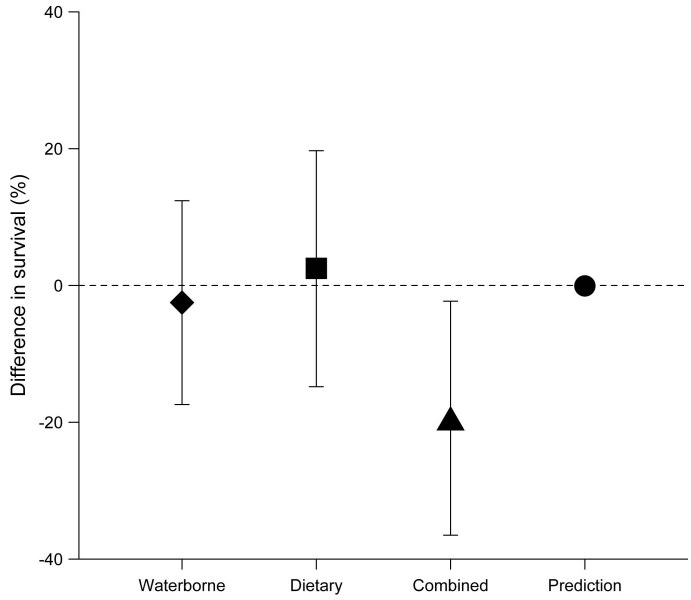

Fig. 5.8. Mean difference in survival (±95% CIs) of *G. fossarum* (after 21 days; n = 40) exposed towards thiacloprid via different pathways relative to the corresponding control (dashed line). The prediction of the IA model is also displayed as a point estimate (●) **[Appendix A.3]**.

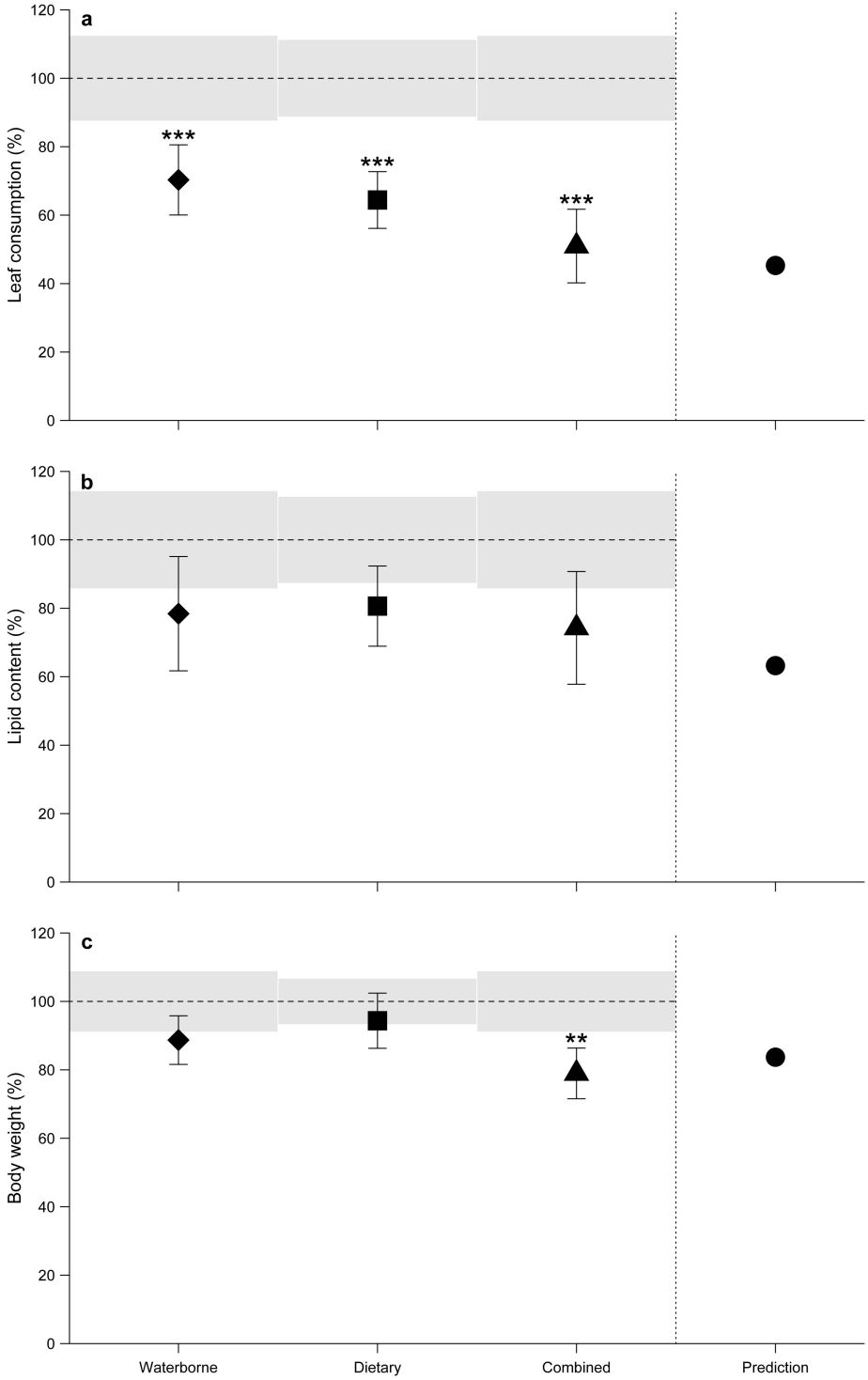

Fig. 5.9. Mean (±95% CIs) a) leaf consumption, b) lipid content and c) body weight of gammarids exposed for 21 days to thiacloprid, relative to the corresponding control (dashed line). Grey areas indicate the 95% CIs of the corresponding control. Please note that the dietary exposure treatment was compared to a separate control due to the flow-through system used. The predictions for the combined exposure derived from IA models are also indicated as point estimate (●). Asterisks denote statistically significant differences compared to the respective control, $p < 0.01$ (**), $p < 0.001$ (***) **[Appendix A.3]**.

After 21 days, survival of *G. fossarum* experiencing waterborne or dietary thiacloprid exposure deviated only marginally from the corresponding control (Fig. 5.8) **[Appendix A.3]**. However, a statistically significant decrease in gammarids' leaf consumption was observed (relative to the corresponding controls) during the 21-day lasting waterborne (by 30%) and dietary (by 36%) thiacloprid exposure (Fig. 5.9a). These reductions in gammarids' leaf consumption went along with non-significant decreases in animals' lipid content (by 22 and 19%, respectively) and body weight (by 11 and 6%; Fig. 5.9b,c). While dietary and waterborne thiacloprid exposure caused comparable effects in gammarids' survival, leaf consumption, lipid content and body weight, those induced by the combined exposure scenario exceeded in their magnitude (with effect sizes of 20, 49, 26 and 21%, respectively) those observed for each of the exposure pathways individually (Fig. 5.8 & 5.9a-c). Only gammarids' thiacloprid body burdens were at comparable levels irrespective of the exposure pathway (except for control animals; Fig. 5.10) **[Appendix A.3]**. Whereas the IA model successfully predicted the additive effects observed for gammarids' leaf consumption, lipid content and body weight (judged based on the 95% CIs ranges; Fig. 5.9), the deviation between the IA model and observed survival of gammarids indicates synergistic effects.

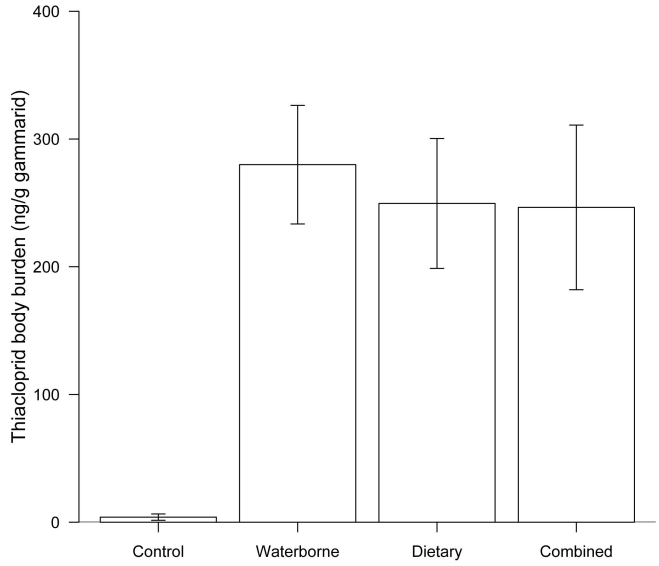

Fig. 5.10. Mean thiacloprid body burden (±SE) in gammarids. Residues were measured (n = 4-5) after 21 days of thiacloprid exposure via water, diet or a combination of both **[Appendix A.3]**.

5.4 ABIOTIC FACTORS MODULATING EXPOSURE AND TOXICITY

During the fate experiment, leaves that were submerged in water for 7 days or UV-irradiated for 1 day displayed a ~45 and ~90% reduction in foliar imidacloprid residues (though only statistically significant for the latter) compared to not pre-treatment imidacloprid-contaminated leaves, respectively (Fig. 5.11).

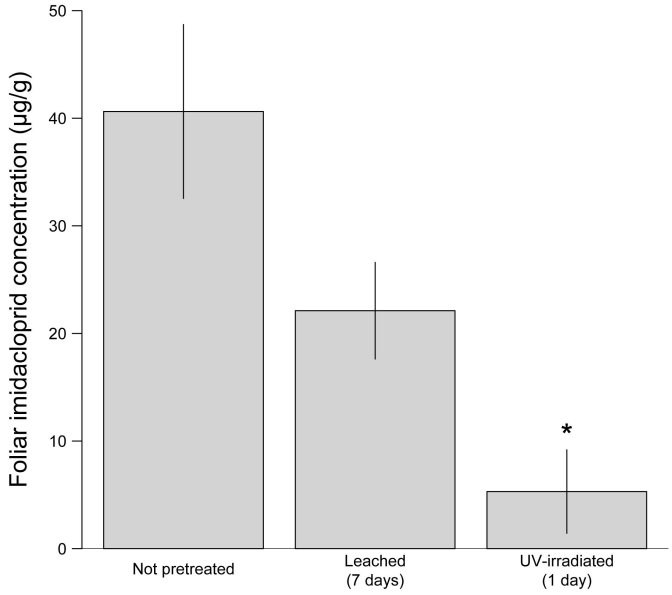

Fig. 5.11. Mean (±SE; n = 3) foliar imidacloprid residues measured in imidacloprid-contaminated leaves that were not pretreated, leached in test medium for 7 days or were UV-irradiated for 1 day prior to quantification. Asterisks denote statistically significant differences compared to non-pretreated imidacloprid-contaminated leaves: $p < 0.05$ (*) **[Appendix A.4]**.

Consistent with observations during the second phase of this thesis, gammarids exposed towards imidacloprid-contaminated black alder leaves displayed an up to 80% lower leaf consumption (Fig. 5.12 & 5.13). However, the leaching of these leaves in water for 1 and 3 days prior to their use in the feeding assay lowered the reductions in gammarids' feeding rate to 40% and 49%, respectively (Fig. 5.12). Moreover, gammarids exposed to imidacloprid-contaminated leaves that were previously submerged in water for 7 days displayed no adverse implication in their feeding rate – gammarids' feeding rate was even ~30% (although non-significantly) higher compared to the corresponding control (Fig. 5.12) **[Appendix A.4]**. Similarly, exposure towards imidacloprid-contaminated leaves that were at first UV-irradiated for 1 day at field relevant intensities and afterwards leached in water for

1 day effectively reduced adverse effects on gammarids' feeding rate to a level comparable to the corresponding control (Fig. 5.13) **[Appendix A.4]**.

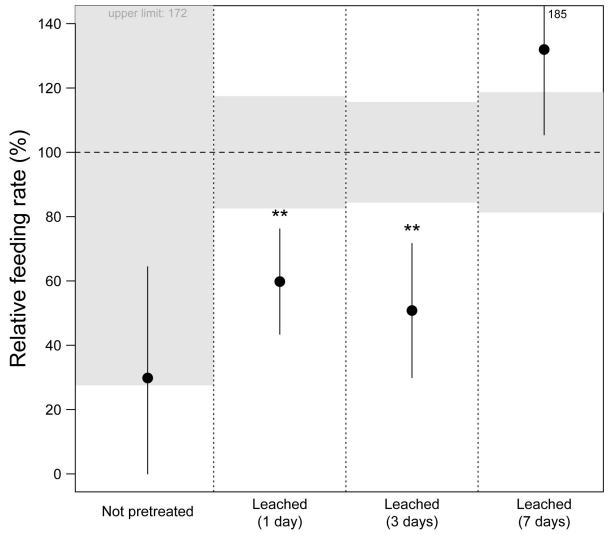

Fig. 5.12. Relative feeding rate (mean ±95% CIs) of *G. fossarum* exposed to imidacloprid-contaminated leaves that were previously leached in test medium for 0, 1, 3 or 7 days. Asterisks denote statistically significant differences compared to the corresponding control (dashed line): $p < 0.01$ (**). Grey areas indicate the 95% CIs of the corresponding control **[Appendix A.4]**.

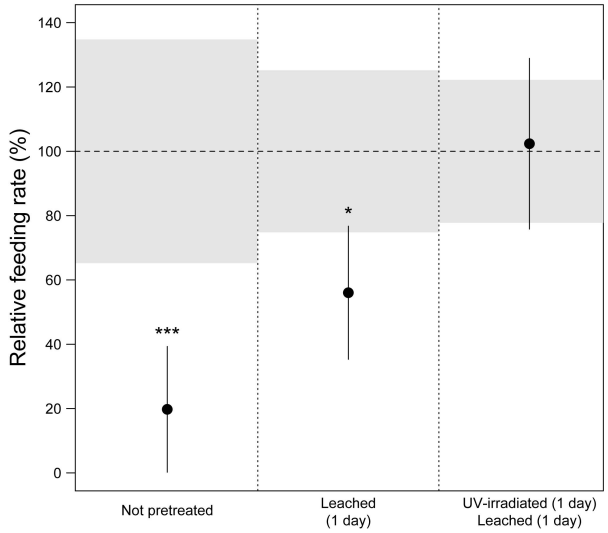

Fig. 5.13. Relative feeding rate (mean ±95% CIs) of *G. fossarum* exposed to imidacloprid-contaminated leaves that were previously leached in test medium for 1 day or UV-irradiated for 1 day before being leached in test medium (for 1 day). Contaminated leaves that were neither leached in water nor UV-irradiated served as positive control. Asterisks denote statistically significant differences compared to the corresponding negative control (dashed line): $p < 0.05$ (*) and $p < 0.001$ (***). Grey areas indicate the 95% CIs of the corresponding control **[Appendix A.4]**.

6. DISCUSSION

6.1 RQ 1: CHARACTERIZATION OF EXPOSURE CONDITIONS AND PATHWAYS

Although the use of systemic neonicotinoid insecticides on trees has been mostly limited to trials in Europe (e.g., Brendler & Bechmann, 2005; Niesar et al.; van-Velzen, 2014), their application has become a well-implemented tool in urban and forest pest management programs of other countries, including the United States (Benton et al., 2017; Szczepaniec et al., 2011; USDA, 2016). The application of these insecticides in forests may, however, contaminate relatively pristine ecosystems, including nearby streams, which are usually spared from organic pollution. In this regard, a few publications have documented aqueous neonicotinoids concentrations in surface waters of forest areas where trees have received imidacloprid soil-application (Benton et al., 2016b; 2017; Churchel et al., 2011; McAvoy et al., 2005). Given the high leaching potential of imidacloprid (Gupta et al., 2002; Miranda et al., 2011) as well as the up to 10-fold greater substance volume required for tree soil application compared to trunk injection (e.g., Tattar et al., 1998; Fig. S6) **[Appendix A.1]**, neonicotinoids may leach from forest soils (particularly for soils with low organic matter content: Liu et al., 2006) into nearby streams where aquatic organisms may become exposed to them via the water phase. Although this input may be avoided if trunk injection is used instead of soil drenching (e.g., as discussed in Kreutzweiser et al., 2008b), both application methods result in an accumulation of these systemic insecticides within tree needles and leaves (Fig. 5.2; reviewed in **Appendix A.1**). Due to the high persistence of neonicotinoids in plants (e.g., up to 8 years in conifers), residues might ultimately end up in the surrounding environment. Given the relatively high foliar residues of imidacloprid, thiacloprid and acetamiprid detected in black alder trees during the present thesis (Fig. 5.2) as well as those reported (mostly for imidacloprid) for various deciduous trees in literature (with median foliar residues of 3.5 μg/g; Fig. 5.3), this scenario seems particularly relevant for deciduous trees

which may lose the majority of their neonicotinoid residues together with their complete foliage during autumn leaf fall (Mota-Sanchez et al., 2009). If these contaminated leaves enter nearby streams via vertical fall or indirectly following lateral movement (Abelho, 2001), leaf-shredding invertebrates might particularly be exposed due to their diet (feeding on contaminated leaves; Cummins & Klug, 1979) or collaterally via the stream water (through leaching of the neonicotinoids from leaves; Kreutzweiser et al., 2007).

The peak imidacloprid water concentration predicted for the stream parameterized in the worst-case model scenario was above several of the currently existing chronic ecological water quality thresholds setting acceptable levels for neonicotinoids (mostly imidacloprid) in surface waters (reviewed in Morrissey et al., 2015) **[Appendix A.1]**. For instance, the recently recommended Maximum Permissible Concentration of 8.3 ng imidacloprid/L (Smit et al., 2015) was exceeded in most of the simulated scenarios (Fig. S7-S11) **[Appendix A.1]**. However, changing the foliage input scenario from a simultaneous input to a continuous input scenario, in which the input is equally distributed throughout autumn, would lead to a lower but chronic (i.e., lasting for several weeks) aqueous imidacloprid exposure. Moreover, the impact of neonicotinoids remobilized from leaves might not be locally limited to the site of their input. The streams' flow may wash away aqueous neonicotinoid residues (and possibly contaminated leaves) ultimately leading to a potential exposure of downstream habitats. However, in stream segments that are less shaded by riparian vegetation, concentrations of photo-labile neonicotinoids (e.g., imidacloprid; Lewis et al., 2016) may, depending on the proportion of sunlight transmitted into water, decline due to photolytic degradation (Lu et al., 2015) as well as by dilution through inflowing streams and ditches.

In addition to aqueous neonicotinoid concentrations, another risk for aquatic organisms may come directly from the neonicotinoid-contaminated foliage. Despite imidacloprid's high water solubility (Lewis et al., 2016), neonicotinoid residues are detectable in submerged

leaves for several days following their input into the water body (i.e., 90% of residues are lost within ~4.5 days; Fig. 5.4; cf. Kreutzweiser et al., 2007; but see Fig. 5.11). Therefore, regardless of the neonicotinoid concentrations predicted for the water phase, aquatic shredders might be exposed to neonicotinoids while feeding on contaminated leaves (cf. Kreutzweiser et al., 2007; 2008b; 2009). The dietary neonicotinoid exposure of shredders was, therefore, further investigated in the remaining part of this thesis.

6.2 RQ 2: WATERBORNE AND DIETARY TOXICITY TOWARDS SHREDDERS

The experiments conducted during the second and third phase of this thesis revealed considerable lethal and sublethal effects for the amphipod *G. fossarum* and larvae of the caddisfly *C. villosa* when exposed towards neonicotinoids, regardless of the AI tested. Whereas scientific literature lacks information regarding neonicotinoid-induced effects on caddisflies from the genus *Chaetopteryx*, 7-day EC_{50}-values observed for *Gammarus* exposed towards neonicotinoids via the water phase were consistent with former publications (Agatz et al., 2014; Beketov & Liess, 2008; Englert et al., 2012; Feckler et al., 2012; Roessink et al., 2013; Zubrod et al., 2017) **[Appendix A.2]**. To date, most research dealing with neonicotinoids' effects on aquatic ecosystems have focused exclusively on waterborne exposure pathways (e.g., see those reviewed in Morrissey et al., 2015, but see Kreutzweiser et al., 2007; 2008b), while dietary exposure is commonly presumed irrelevant for neonicotinoids (and hydrophilic substances in general).

The results of the concentration-response experiments (Fig. 5.5 & Table 5.1) demonstrate, for all three neonicotinoids, that the adverse effects observed for both test species' feeding rate under combined exposure (i.e., dietary uptake and waterborne exposure due to remobilization from leaves) cannot solely be explained by neonicotinoid concentrations measured in the water phase. The presumed relevance of dietary exposure under a combined exposure scenario was further emphasized by *Chaetopteryx's* effect thresholds (i.e., 7-day LC_{50} values; Table 5.1), which were observed at the neonicotinoid water concentrations that caused zero mortality in the waterborne exposure experiments (Table S3) **[Appendix A.2]**. Although the insect larvae and the amphipod may differ greatly in their sensitivity towards neonicotinoids – due to toxicokinetic and toxicodynamic differences (Nyman et al., 2014) – the discrepancy in observed survival between the two species in response to the two exposure scenarios could mainly be explained by the generally (up to 4-fold) higher leaf consumption

of *Chaetopteryx* compared to *Gammarus* (Fig. S2) **[Appendix A.2]**. Accordingly, organisms processing greater amounts of leaves may take up considerably higher neonicotinoid doses via the dietary pathway.

The introduction of neonicotinoid-contaminated leaves into a stream would, as predicted for imidacloprid by the leaching simulation, rather result in low aqueous concentrations (Fig. 5.4; Figs. S7-S11) **[Appendix A.1]**. This questions the transferability of effects observed under the combined exposure to the field situation. In this regard, dietary exposure was separately assessed in a 21-day lasting bioassay using a flow-through system to keep thiacloprid water concentrations leaching from leaves at negligible levels (Fig. 5.9). In essence, sole dietary exposure towards thiacloprid-contaminated leaves collected from trees treated at field relevant levels caused similar reductions in gammarids' leaf consumption and lipid content (Fig. 5.9a,b) as observed for gammarids that were solely exposed via the water phase towards thiacloprid leaching from the same leaves. This reinforces the assumed relevance of the dietary exposure pathway for shredders exposed to neonicotinoid-contaminated leaves under field conditions (i.e., low aqueous neonicotinoid concentrations). Only in streams which do not provide sufficient dilution, as a consequence of low discharge, might waterborne exposure be a more relevant cause for potential additive (Fig. 5.9a,b,c) or synergistic (Fig. 5.8) effects under combined exposure.

6.3 RQ 3: BIOTIC AND ABIOTIC FACTORS MODULATING EXPOSURE AND TOXICITY

Although this thesis uncovered potential risks for leaf-shredding invertebrates when exposed to neonicotinoid-contaminated leaves at the lab-scale, exposure and consequently toxicity originating from contaminated leaves may deviate under field conditions. Imidacloprid residues in leaves have been demonstrated to decline over time (Fig. 5.11), presumably due to photodegradation following irradiation with UV-light, ultimately mitigating adverse effects on *G. fossarum* (Fig. 5.13). This could be particularly relevant for neonicotinoid-contaminated leaves which first lie on the forest floor or stream bank before being transported into the stream (i.e., lateral input). Although this process is assumed to be irrelevant for photo-stable neonicotinoids (e.g., thiacloprid and acetamiprid; Lewis et al., 2016), foliar concentrations of photo-labile compounds (such as imidacloprid; Lewis et al., 2016) might be meaningfully reduced – depending on the amount of sunlight that reaches the forest floor. Mitigation through photodegradation might even be more relevant during autumn leaf fall as canopy opens and increases the amount of sunlight transmitted to the forest floor.

Following the leaves' introduction into the stream, remobilization of neonicotinoids from submerged leaves – facilitated by neonicotinoids' high water solubility – has been demonstrated to reduce foliar residues (Fig. 5.11) and consequently the toxicity for *G. fossarum* (Fig. 5.12). The time until neonicotinoids are completely remobilized from leaves might, on the one hand, vary between neonicotinoids due to varying water solubility (Lewis et al., 2016). On the other hand, remobilization of imidacloprid from ash leaves (Kreutzweiser et al., 2007) into water took considerably less time as observed in the present thesis for black alder (Fig. 5.11). Therefore, it could be assumed that the remobilization of these insecticides from leaves also depends on the respective tree species. Although leaching of imidacloprid from leaves into water would clearly decrease dietary exposure for shredders

under field conditions, the remobilized insecticide might be washed away by the streams' flow consequently exposing downstream communities to aqueous neonicotinoid concentrations (cf. Fig. 5.4).

Besides these abiotic factors, shredders could avoid dietary exposure towards neonicotinoid-contaminated leaves in the field by switching to an alternative non-contaminated food source if they are able to detect neonicotinoids in leaves (e.g., as shown for *G. fossarum* and the fungicide quinoxyfen; Zubrod et al., 2015). A previous publication by Kreutzweiser et al. (2009) detected no such behavior in larvae of the stonefly *P. dorsata* and the cranefly *Tipula* sp. when exposed to imidacloprid-contaminated leaves. Since *Gammarus* and some caddisflies species are known for their selective food choice (e.g., Gonçalves et al., 2014; Rong et al., 1995), differing results seemed conceivable for experiments conducted in the course of this thesis. However, only for thiacloprid a small but statistically significant preference towards uncontaminated leaves (compared to thiacloprid-contaminated leaves) was detected by the meta-analysis across all food-choice experiments (Fig. S4) **[Appendix A.2]**. In contrast, the meta-analysis conducted across all three neonicotinoids and test species (including those of Kreutzweiser et al., 2009) revealed no preferential feeding (Fig. 5.6) indicating the organisms' inability to actively avoid dietary neonicotinoid exposure. Consequently, the risk of dietary neonicotinoid exposure may be assumed if shredders encounter and consume contaminated leaves recently introduced into a stream.

7. CONCLUSION AND OUTLOOK

The consumption of contaminated leaves from trees treated at field-relevant neonicotinoid levels (i.e., 0.6 g AI/cm DHB) triggered sublethal effects in test organisms (e.g., Fig. 5.9), which might eventually impair energy transfer processes in heterotrophic streams. On one hand, a reduced feeding of shredders (Fig. 5.5 & Fig. 5.9a) might lower their contribution to local leaf litter breakdown directly (Bundschuh et al., 2011; Wallace et al., 1997). On the other hand, lipids constitute resources organisms invest into reproduction (Glazier, 2000; Plaistow et al., 2003). Therefore, reductions in shredders' lipid content (Fig. 5.9b) might indirectly impact leaf litter breakdown through lower shredder abundances (Dangles et al., 2004). Both the reduced shredder abundances as well as the reduced leaf consumption may also lower the feces production of shredders thereby restricting the amount of food available for collectors (Cummins & Klug, 1979) as well as for juvenile gammarids (McCahon & Pascoe, 1988). Furthermore, populations of vertebrate and invertebrate predators preying upon shredders and collectors (Cummins, 1973; MacNeil et al., 1999) may also be adversely affected by less abundant prey or through the consumption of neonicotinoid-contaminated prey (Fig. 5.10; cf. Douglas et al., 2015).

In North America, neonicotinoids are already applied across vast forest areas threatened by native and invasive insect pests (Benton, 2016; USDA, 2016). Meanwhile, first trials are being conducted in Europe (Heald, 2015; van-Velzen, 2014). The use of neonicotinoids in forest pest management programs could possibly increase in the future as insect pest numbers are predicted to rise under current climate change scenarios (Ramsfield et al., 2016). Hence, the input of neonicotinoid-contaminated leaves into forest streams, and the consequent dietary exposure to shredders might become even more relevant in the future.

Neither the United States nor the European Union, routinely test for dietary toxicity during their aquatic environmental risk assessment of pesticides (EFSA, 2013; EPA, 2004). For

instance, in the European Union, testing for dietary toxicity is only recommended for substances characterized by extremely high octanol/water partition coefficients (log P_{ow} >6; EFSA, 2013). Therefore, this pathway is considered irrelevant for neonicotinoid insecticides, which are generally characterized by high hydrophilicity and a low log P_{ow}. However, the present thesis underpins the importance of the dietary exposure pathway for neonicotinoids and for systemic insecticides in general, particularly for aquatic shredders. Therefore, updating current protocols for aquatic environmental risk assessment by including dietary exposure during the registration of systemic insecticides is suggested as a safeguard for ecosystem integrity.

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APPENDIX

APPENDIX A.1

Modeling re-mobilization of neonicotinoid residues from tree foliage in streams – a relevant exposure pathway in risk assessment?

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ABSTRACT

Systemic neonicotinoid insecticides are increasingly used as a crop protection measure to suppress insect pests on trees. However, senescent foliage falling from treated trees represents a rarely studied pathway through which neonicotinoids may enter non-target environments, e.g., surface waters. To estimate risk posed by this pathway, neonicotinoid residues were analyzed in foliage from black alder trees treated with one of three neonicotinoid insecticides (imidacloprid, thiacloprid, or acetamiprid) at five concentrations, each ranging from 0.0375 – 9.6 g active ingredient/cm trunk diameter at breast height (n=3). Foliar residues measured at the time of leaf fall were used as input parameters for a model predicting imidacloprid water concentrations over a 100-m-long stream stretch as a consequence of re-mobilization from introduced foliage (input: 600 g foliage/m^2 containing 80 μg imidacloprid/g). The water concentration (up to ~250 ng/L) predicted by the model exceeded the recently proposed Maximum Permissible Concentration of 8.3 ng/L for ~6.5 days. Moreover, dietary uptake was identified as an additional exposure route for aquatic organisms. The alternative pathway (i.e., introduction via leaf fall) and exposure route (i.e., dietary uptake) associated with the systemic nature of neonicotinoids should be accounted for during their registration process in order to safeguard ecosystem integrity.

INTRODUCTION

Neonicotinoid insecticides are registered for use in agriculture, horticulture, forestry, and tree nurseries in more than 120 countries (Jeschke et al., 2011; Simon-Delso et al., 2015). Their success is attributed to their highly selective toxic mode of action in which molecules bind specifically to the nicotinic acetylcholine receptor of insects (Tomizawa & Casida, 2003). Moreover, neonicotinoids have replaced older insecticides to which pests have developed resistance or are in the process of being withdrawn from the market (e.g., organophosphates; Jeschke & Nauen, 2008; Jeschke et al., 2011). All commercially available neonicotinoids

(e.g., imidacloprid (IMI), thiacloprid (THI), acetamiprid (ACE)) act – due to their physicochemical properties – as systemic insecticides which facilitates their rapid uptake and translocation within plant tissues. Given the high persistence of neonicotinoids in some plants (e.g., in eastern hemlock trees >7 years after a single IMI application; Benton et al., 2016a), they provide long lasting protection against herbivorous insects and plant viruses transmitted by these insects (Jeschke et al., 2011).

The extensive and often preemptive use of neonicotinoids (Douglas & Tooker, 2015), together with their high water solubility (up to 2,950 mg/L for ACE; Table 1) and environmental persistence in soils (e.g., dissipation time (DT_{50}) for IMI up to 1,250 days; Bonmatin et al., 2015) renders these compounds susceptible to off-site transport. Throughout the growing season, intense rainfall may wash neonicotinoid residues from coated seeds, agricultural soils or plant surfaces while, in northern latitude environments, snowmelt runoff may re-mobilize residues accumulated in soils (de Perre et al., 2015; Main et al., 2016). Moreover, non-target ecosystems may be contaminated via spray drift or by dust drift during planting of neonicotinoid coated seeds (Greatti et al., 2016). Once transported off-site, neonicotinoids can be taken up by non-target plants, such as flowers, weeds (Botias et al., 2016; David et al., 2016; Krupke et al., 2012) and – although not documented thus far – by trees growing in the vicinity of argicultural fields. At the same time, trees are increasingly being treated – via soil or trunk application – with neonicotinoids in urban areas (i.e., parks) as well as natural and planted forests to manage invasive insects. For instance, over 200,000 eastern hemlock trees received – over a 8 year period – between one and eight IMI treatments (of 0.28 to 0.57 g active ingredient per cm trunk diameter at breast height each; AI/cm DBH) in the Great Smokey Mountains National Park (Benton et al., 2016b).

During autumn when senecent foliage falls from deciduous trees, neonicotinoids taken up by trees can potentially enter surface waterbodies directly or by lateral movement

(Abelho, 2001). Once submerged, the neonicotinoids can be re-mobilized (via leaching; Kreutzweiser et al., 2007) from foliage into their surrounding aquatic environment and may lead to direct (e.g., impaired feeding and development or reduced survival; Alexander et al., 2008; Kreutzweiser et al., 2007; Pestana et al., 2009) and indirect (e.g., altered predator-prey interaction; Englert et al., 2012) ecotoxicological effects in aquatic non-target organisms through both waterborne exposure and the consumption of contaminated foliage. Since most published studies in this context are limited to one neonicotinoid, namely IMI, a more general understanding about the fate of neonicotinoid residues in tree foliage and associated implications on aquatic organisms is not available. Given the predicted increased pressures by native and invasive insect pests that may be combated involving neonicotinoids, this general understanding is urgently needed (Ramsfield et al., 2016).

The aim of the present study was to (i) quantify the foliar residues of three neonicotinoids in foliage of deciduous trees and ultimately (ii) estimate the potential amount of these insecticides that might be re-mobilized in surface waters following an input of contaminated foliage. Therefore, the model tree species *Alnus glutinosa* (L.) GAERTN. (black alder) was treated in early summer by soil drenching with one of three neonicotinoid insecticides – namely IMI, THI and ACE – at different concentrations. At the time of leaf fall (i.e., autumn, four months post treatment), foliar neonicotinoid residues were extracted using accelerated solvent extraction (ASE), analyzed with ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) and finally compared to environmentally relevant levels found in literature. Though the assessment of differences in uptake and distribution of the neonicotinoids directly after application was beyond the scope of the present study, it was hypothesized that neonicotinoid residues in foliage are a function of the applied dose. The residue levels of the tested neonicotinoids may, however, differ for the same amount of applied insecticide as a consequence of differing physicochemical properties. The foliar

residues measured at the highest field-relevant application rate (i.e., 0.6 g AI/cm DBH; using IMI as a model substance) finally served as (iii) input parameter for a model predicting worst-case waterborne exposure in streams following the input of contaminated foliage and subsequent re-mobilization of neonicotinoids from the latter. Therefore, this mostly neglected pathway of neonicotinoid entry into aquatic systems – i.e., re-mobilization through leaching from foliage (Kreutzweiser et al., 2007) – was examined with the ultimate goal to inform environmental risk assessment.

MATERIAL AND METHODS

SOURCE OF PLANT MATERIAL & INSECTICIDE APPLICATION

Black alder trees were selected as model species as they are widely distributed within riparian zones of temperate Europe (Hewitt, 1999) and may receive neonicotinoids from adjacent agricultural fields (as previously shown for flowers; Botias et al., 2016; David et al., 2016; Krupke et al., 2012). Additionally, they might be treated with neonicotinoids directly as some commercially available neonicotinoid products (e.g., Merit®75WP, active ingredient (AI): IMI) are registered for use against insect pests that can infest alder trees, such as the alder borer (*Rosalia funebris*; Bayer CropScience). Black alder trees with a mean (±SE) height of 196±2 cm and a mean (±SE) trunk diameter at breast height (DBH; i.e., 1.3 m above the ground) of 7.5±0.2 mm (n=50), were purchased from an ecological tree nursery (Baumschule von der Mühlen, Küsten, Germany) in April 2014. According to the provider, the trees had never been treated with any kind of pesticides or other chemical stressors prior to their use in the present study, and no foliar IMI, THI and ACE residues were detected by our own analytical measurements. All trees were situated in 3-L pots and watered with an automatic tap water irrigation system throughout the whole experimental period. At the beginning of June, trees were treated by soil drenching with either 500 mL tap water (=control; n=5) or one of the three commercially available neonicotinoid

insecticides (Confidor®WG70 (70% IMI), Calypso® (40% THI) and Mospilan®SG (20% ACE)) dissolved in 500 mL tap water at one of five concentrations (0.0375, 0.15, 0.6, 2.4, 9.6 g AI/cm DBH; *n*=3; see Table 1). This procedure was used despite recent criticism of this diameter-based dosing approach since it still is the manufacturer's recommended application method (Benton et al., 2016a). Although 0.6 g AI/cm DBH equals the maximum amount of IMI recommended for a single soil application on trees (Bayer CropScience), two intentionally overdose treatments – i.e., 2.4 and 9.6 AI/cm DBH) – were used to allow predictions of foliar residues (using non-linear models; see *Calculations and statistics*) under elevated application doses, which may be required in the future to combat the rising number of insect infestations. Stands were placed underneath every pot to prevent unintentional loss of the neonicotinoids. Plastic bags covered the pots of each tree to minimize the impact of precipitation on applied insecticides and mimic shade conditions of forest floors preventing potential photo-degradation on the soil surface (Gupta et al., 2008).

Table 1. Information about commercial products applied to black alder trees as well as the physicochemical parameters, leaching properties and environmental persistence of their active ingredient.

Active ingredient	**Imidacloprid (IMI)**	**Thiacloprid (THI)**	**Acetamiprid (ACE)**
Product name	Confidor® WG 70	Calypso®	Mospilan®SG
Supplier	Bayer CropScience	Bayer CropScience	Cheminova DE GmbH
Concentration of the AI within the product	700 g/kg	480 g/L	200 g/kg
Molecular Mass (g/mol)[1]	255.66	252.72	222.67
Solubility in Water at 20°C (mg/L) [1]	610 (high)	184 (moderate)	2950 (high)
Octanol/Water partition coefficient (log P_{ow}) [1]	0.57	1.26	0.8
GUS Leaching Potential Index[2]	3.76 (high)	1.44 (low)	0.94 (very low)
Soil Persistence (DT_{50} in days) [3]	100 - 1250	3.4 - >1000	31 - 450
Aqueous Photolysis (DT_{50} in days) [1]	0.2 (fast)	(stable)	(stable)
Water Hydrolysis (DT_{50} in days) [1]	>365 (at pH 9; 25°C; stable)	stable (pH 5 to pH 9)	420 (at pH 9; 25°C; stable)

[1]FOOTPRINT, 2016 [2]Miranda et al., 2011 [3]Bonmatin et al., 2015

During the experimental period (early June to early October), environmental temperature ranged from 10.0 to 26.8°C, while daily precipitation remained below 17 mm (except for a single event of ~40 mm; Figure S1). Shortly before leaf fall in October 2014, tree height and trunk DBH were measured again while the complete foliage of each tree was harvested and weighed on a fresh weight basis. Subsequently, all foliage was stored frozen at -20°C in re-sealable zipper storage bags to ensure the stability of the three neonicotinoids until further use

(Kreutzweiser et al., 2008).

SAMPLE PREPARATION AND EXTRACTION

Neonicotinoid insecticides were extracted from foliage using an ASE™ 350 Accelerated Solvent Extractor system (Thermo Scientific™ Dionex™ , Sunnyvale, CA; USA). A cellulose filter (Restek GmbH, Bad Homburg, Germany) was placed in a 34 mL stainless steel extraction cell (Agilent Technologies, Waldbronn, Germany) together with – depending on the neonicotinoid treatment – 0.5 or 3.0 g of previously freeze-dried foliage samples comprised of at least 20 leaves each. The cell was then filled with acid-washed, annealed sea sand (Altmann Analytik GmbH & Co. KG, Germany) and covered by another cellulose filter. Based on preliminary experiments and literature data (Peterson, 2012), a 5:1 (v:v) mixture of Milli-Q water (Millipore, Bedford, MA, USA) and acetonitrile (LC-grade; Merck, Darmstadt, Germany) was chosen as extraction solvent. For survey of optimal extraction conditions, two factors were considered, namely the temperature regime (60, 80, 100, 120, and 140°C) and the number of extraction cycles (one, two and four). Optimization experiments were run with homogenized foliage samples (n=4) harvested from trees treated with 2.4 g AI/cm DBH. Loaded cells were extracted under the following conditions: equilibration period of 5 min followed by one to four static cycles of 10 min at a temperature of 60 to 140°C with a rinse volume of 40% and purge time of 30 sec. After centrifugation (at 3,500 rpm for 12 min) for the removal of coarse particles, an aliquot of the foliage extracts was diluted (1:10 or 1:100; v:v) with Milli-Q water and subsequently analyzed by UHPLC-MS.

For validation of the final extraction method, 0.5 to 3.0 g of freeze-dried blank foliage (n=8) were spiked with 500 μL of a stock containing the three investigated neonicotinoids (analytical standards; Sigma Aldrich, Seelze, Germany) in methanol (LC-grade; Merck, Darmstadt, Germany) at a concentration of 20 ng/μL each. After the methanol was completely evaporated, spiked neonicotinoids were extracted from foliage under the conditions described

above. Recovery rates (± relative standard deviation) were 109.2% (±10.9) for IMI, 105.0% (±17.6) for THI and 106.8% (±14.0) for ACE. A more detailed assessment regarding methods accuracy and precision is still pending but will be conducted together with future method refinements.

CHEMICAL ANALYSES

Separation of neonicotinoid insecticides from foliage extracts was done with an UHPLC-MS equipped with an EQuan MAX system, while for quantification, a single quadrupole mass spectrometer equipped with an electrospray ionization source was used. Since deuterated analytical standards for internal calibration were available only for one of the investigated analytes, external calibration with matrix-matched standards – prepared out of blank foliage extracts – was used to have the same degree of potential matrix effects as in the real samples. Limits of quantification (LOQ) and detection (LOD) were determined for IMI, THI and ACE on a dry weight basis according to DIN standard 32645 and were 0.06, 0.11, 0.12 μg/g and 0.02, 0.03, 0.04 μg/g, respectively. Further details are given in the Supporting Information.

REVIEW OF FOLIAR NEONICOTINOID RESIDUES IN SCIENTIFIC LITERATURE

A literature search (using the online database ISI Web of Science; search string: "neonicotinoid* and tree*") was conducted in order to provide a general overview about relevant neonicotinoid residue levels found in tree foliage to which the present study could be related. References of the retained articles were also inspected for further relevant publications. Only peer-reviewed publications reporting neonicotinoid residues (in leaves or needles/twigs) were selected. From those studies, information about the neonicotinoid compound applied; the respective application dose and method; the studied tree species; the respective days after treatment; as well as the foliar neonicotinoid residue levels (expressed on fresh or dry weight basis) were extracted. Residues reported on fresh weight basis were converted to dry weight basis if conversion factors were available. In situations where the

weight basis (fresh vs. dry weight) could not be clearly identified (121 out of 485 observations), it was assumed – following a conservative approach – that the concentrations were related to dry weight. Hence, residues reported in this literature review might possibly underestimate actual residue levels. Foliar residues reported as AI per leaf area (e.g., $\mu g/cm^2$), were not included in our analysis. If studies reported several foliar residues at a given point in time for a single tree – e.g., to account for differences in neonicotinoids' spatial distribution – the average was calculated and used in our analysis. This approach resulted in a total of 485 individual neonicotinoid residue levels extracted from 29 different studies (Table S1).

SIMULATION OF WATERBRONE NEONICOTINOID CONCENTRATIONS FOLLOWING THE ENTRY OF CONTAMINATED FOLIAGE IN A MODEL STREAM

Waterborne neonicotinoid concentrations resulting from the entry of neonicotinoid-contaminated foliage into a model stream (modeled explicitly for 1-m long segments) were simulated using the following equation:

$$N_{i,water}^{t} = N_{i,water}^{t-1} + N_{leaching}^{t} + N_{i,inflow}^{t} - N_{i,loss}^{t}$$

where $N_{i,water}^{t}$ = amount of the neonicotinoid dissolved in stream segment *i* at time *t*; $N_{leaching}$ = amount of the neonicotinoid leaching from submerged foliage into the water phase between *t-1* and *t*; $N_{i,inflow}$ = amount of the neonicotinoid flowing into the stream section from the adjacent upstream segment (i.e., *i-1*) between *t-1* and *t*; and $N_{i,loss}$ = amount of the neonicotinoid lost due to the stream's discharge between *t-1* and *t*. The resulting value for $N_{i,water}^{t}$ (ng/stream segment) was then converted to a concentration (ng/L) by adjusting for the stream segments' volume (m^3).

The variable $N_{leaching}^{t}$ is calculated by multiplying the initial amount of neonicotinoid within foliage submerged per stream segment (for details see Supporting Information) with the proportional loss of the neonicotinoid (for every second) from foliage into water. For the

present study, this proportional loss was derived from a non-linear model fitted to IMI leaching data published for ash leaves (Figure S2; Kreutzweiser et al., 2007). It was assumed the leaching dynamics from ash leaves equal those from black alder. If the presented model is to be used with neonicotinoids other than IMI, the determination of a substance-specific leaching factor is required (due to the diverging physicochemical parameters; Table 1). Using IMI – the most common neonicotinoid applied to protect trees – as an example, the model was run using the following parameters: a 100-m long stream stretch with a rectangular cross section (as used during modeling of the European Union's exposure assessment of pesticides; width: 1 m, depth: 0.3 m; current velocity: 0.3 m/s; divided into 1-m long segments with a surface area of 1 m^2; FOCUS, 2015) receives an instantaneous input of 600 g foliage/m^2 containing 80 μg IMI/g. The streams' current velocity of 0.3 m/s was prompted by average values measured for a second-order stream in southwest Germany (i.e., Triefenbach; Englert et al., 2015). The amount of foliage used in the present scenario equaled ~70% of the annual input reported for a first order stream in central Germany (Benfield, 1997), thereby accounting for the peak in leaf litter fall during autumn (Abelho, 2001). Moreover, the initial IMI residue level corresponded to the mean of foliar residues measured in black alder trees receiving the highest field-relevant dose of 0.6 g IMI/cm DBH (Figure 1). The amount of foliage was assumed to simultaneously enter the stream, therefore we refer to this scenario as "worst-case" in the remainder of the manuscript. It should be noted, however, that the model predictions are also markedly influenced by other parameters such as the stream characteristics that are considered field relevant. Although, under laboratory conditions, IMI rapidly degrades in water through photolysis, these mechanisms are less effective under field conditions (Sánchez-Bayo & Goka, 2006). The impact of photolysis would clearly be limited in most forest streams shaded by riparian trees and shrubs, particularly under the low sunlight intensity during autumn leading to aqueous concentrations that can persist for several weeks (reviewed in Bonmatin et al., 2015). Therefore, a potential degradation of IMI within the

water phase was not implemented in our model. Further details on the calculations and a full list of parameters used are given in the Supporting Information and Table S2. The R script containing the model simulation is also provided.

CALCULATIONS AND STATISTICS

During the extraction method development, measured neonicotinoid residues were checked for significant correlations with extraction temperature using Spearman's rank correlation. Residue data sets were checked for normality by visual inspection, while homoscedasticity was tested using Levene's test. In order to test for statistically significant differences, one-way analysis of variance (ANOVA) followed by Tukey tests were applied for neonicotinoid concentrations measured both during extraction method development (i.e., number of extraction cycles or temperature as independent variable) as well as from foliage of treated black alder trees (i.e., between compounds for each dose applied). A two-way ANOVA (variables: dose and compound) was additionally applied to the combined foliar residue data set. If assumptions for parametric testing (i.e., normal distribution and homogeneity of variance) were violated, non-parametric Kruskal-Wallis tests were employed followed by Nemenyi-Damico-Wolfe-Dunn tests (referred to as Nemenyi test in the remainder of the manuscript; function: *kruskalmc* implemented in the R package "pgirmess"; Giraudoux, 2015). Moreover, Dunnett's tests were used to compare the physiological parameters (i.e., trunk DBH, tree height and the amount of foliage produced per tree) of neonicotinoid-treated trees with the untreated control. Treatments that had – as consequence of low tree survival – only a replication of one (i.e., 0.0375 and 9.6 g ACE/cm DBH), were excluded from statistical analyses and instead, effect sizes were reported. Student's *t*-tests instead of Tukey tests were consequently applied to compare the remaining residues (i.e., IMI vs. THI) at these treatments.

Non-linear dose-concentration models were fitted to each foliar residue data set (i.e., IMI, THI and ACE) using the R extension package "drc" (Ritz & Streibig, 2005) in order to predict neonicotinoid dose required to achieve a certain residue level within the trees' foliage as well as to compare dose-concentration relationships among the different compounds used. Models fitting the data sets best were selected based on the Akaike's information criterion as well as visual inspection. In the following, the term significant(ly) is exclusively used with reference to statistical significance ($p < 0.05$). For statistics and figures, R version 3.0.0 for Mac was used (R Development Core Team, 2014).

RESULTS AND DISCUSSION

EXTRACTION METHOD DEVELOPMENT & VALIDATION

During the development of the extraction method, only slight differences (Tukey or Nemenyi test; $p \geq 0.14$; n=4) in neonicotinoid residues were observed when foliage from neonicotinoid-treated black alder trees was extracted using one, two, and four extraction cycles of 10 min with a mixture of Milli-Q water and acetonitrile (5:1; v:v; Figure S3). In contrast, extraction temperature had a non-significant (Tukey or Nemenyi test; $p \geq 0.24$; n=4; Figure S4) but consistent effect: in general, measured neonicotinoid residues rose with increasing extraction temperature from 60 up to 100°C (Figure S4A). While THI and ACE residues remained relatively stable at temperatures exceeding 100°C, IMI residues markedly declined at 120 and 140°C (Spearman's rho = -0.71, $p < 0.01$; Figure S4B). A similar IMI decline, supposedly due to thermal degradation, was observed for ASE extraction temperatures exceeding 120°C, while the authors found the optimum temperature to lie between 80 and 100°C (Xiao et al., 2011). Therefore, two 10 min cycles and 100°C were chosen as optimum extraction conditions and confirmed by successful recoveries (i.e., ranging from 105 to 110%; see *Materials and Methods*). Due to the relatively high foliar neonicotinoid residues found in the present study, purification and concentration of extracts

was not necessary. However, a clean-up procedure (e.g., Schöning & Schmuck, 2003), that reduces the amount of co-extracts interfering with the UHPLC-MS analysis, might be advised for samples containing lower neonicotinoid residues.

FOLIAR NEONICOTINOID RESIDUES IN BLACK ALDER

Neonicotinoid treatments in June resulted in measureable foliar residues at the time of leaf fall and thus four months after application (i.e., October), while none of the three neonicotinoids were detected in foliage of control trees. Foliar neonicotinoid residues significantly depended on the dose and compound applied as well as the interaction of these variables (two-factorial ANOVA, $p < 0.001$), while THI residues were also influenced by the trees' physiological parameters (see Supporting Information). Among the neonicotinoid compounds (but within the same dose), no statistically significant difference in foliar neonicotinoid residue levels was found for trees treated within field-relevant ranges (i.e., 0.0375, 0.15 and 0.6 g AI/cm DBH; Tukey test or Student's *t*-test: $p \geq 0.24$; n=2-3), except for the higher IMI residues at 0.15 g AI/cm DBH (compared to THI and ACE; Tukey test: $p \leq 0.03$; n=3). Differences were more distinct for overdose treatments (at 2.4 g AI/cm DBH only statistically significant for THI vs. ACE; Tukey test: $p = 0.04$; n=3; at 9.6 g AI/cm DBH only statistically significant for IMI vs. THI; Student's *t*-test: $p < 0.001$; n=2-3; Figure 1).

Observed differences in IMI, THI and ACE residues may, among other reasons, be determined by their physicochemical parameters. For instance, uptake and distribution of neonicotinoids in trees increase with increasing water solubility (Byrne et al., 2015). The solubility of neonicotinoids used in the current study (i.e., ACE > IMI > THI; Table 1) would thus predict foliar residue concentrations in that same order. However, our data showed the uptake of ACE was only higher than the other compounds at the highest treatment level, and THI was markedly higher than IMI at the top two treatment levels (Figure 1). This result may,

however, be influenced by the fact that only a single tree survived the highest ACE dose until autumn, questioning the reliability of this point estimate. Moreover, foliage biomass produced on this tree was 5-fold lower compared to trees treated with THI and IMI at the same dose (see Supporting Information and Figure S5C). Hence, the total amount of ACE taken up by the trees might have been allocated to a lower biomass of foliage ultimately leading to higher foliar residues. Moreover, trees treated with ACE in the application range of 0.0375 to 2.4 g ACE/cm DBH displayed similar or even lower residue levels compared to IMI and THI at the respective concentration (Figure 1).

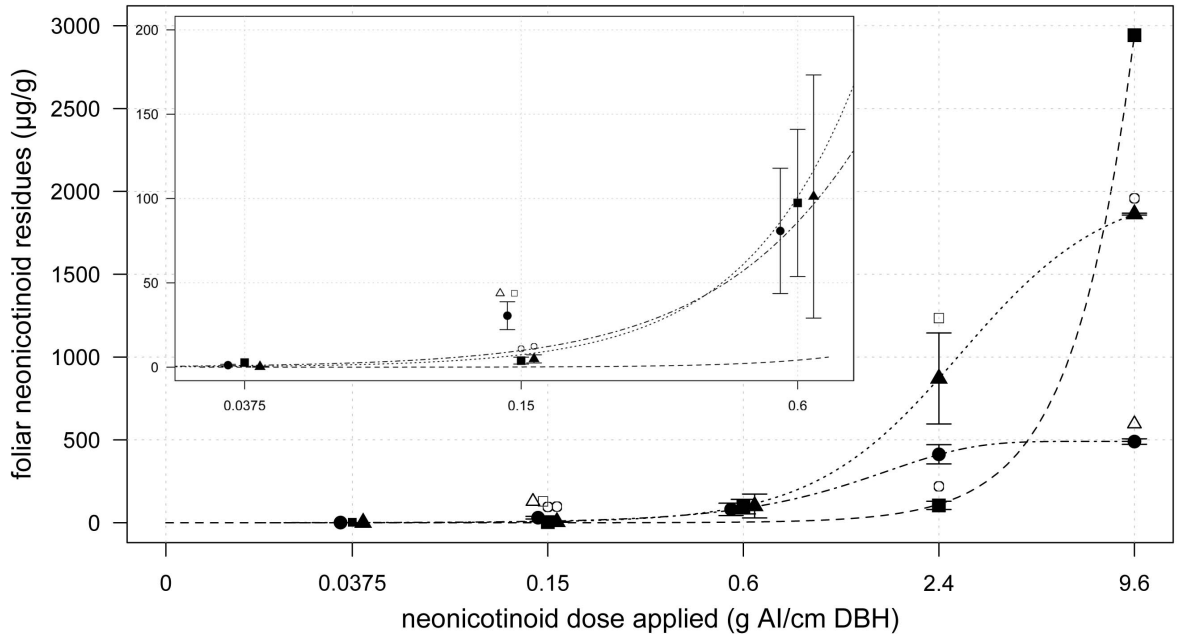

Figure 1. Mean (±SE) residues of IMI (●), THI (▲) and ACE (■) measured in foliage from treated black alder trees as well as the best fitting models (IMI: dot-dashed line; THI: dotted line; ACE: dashed line). The inset displays the residues measured in the 0 to 200-μg/g range in greater detail. Open symbols above error bars indicate significant differences ($p < 0.05$; n=2-3) between neonicotinoid compounds (i.e., compared to IMI (❍), THI (△) or ACE (□), respectively) within the same dose applied. Missing SEs indicate a replication of only one.

Considering the long-time span of four months between application and measurement of foliar residues, it is also possible that observed differences result from a diverging degradation behavior (e.g., depending on the respective half-life times; Table 1) of the parent compounds within the soil and trees. As the trees were irrigated thrice a day, faster degradation of ACE

under wet soil conditions (Gupta & Gajbhiye, 2007) might have degraded ACE into metabolites that can be taken up into the plants along with their parent compound. Such a formation of metabolites (not quantified during the present study) may explain the similar or lower foliar residue levels of ACE compared to IMI and THI (in the application range of 0.0375 to 2.4 g ACE/cm DBH; Figure 1). Additionally the uptake and persistence of neonicotinoids seem to differ between tree species (Tattar et al., 1998), thereby potentially limiting the transferability of dose-concentration relationships to other tree species.

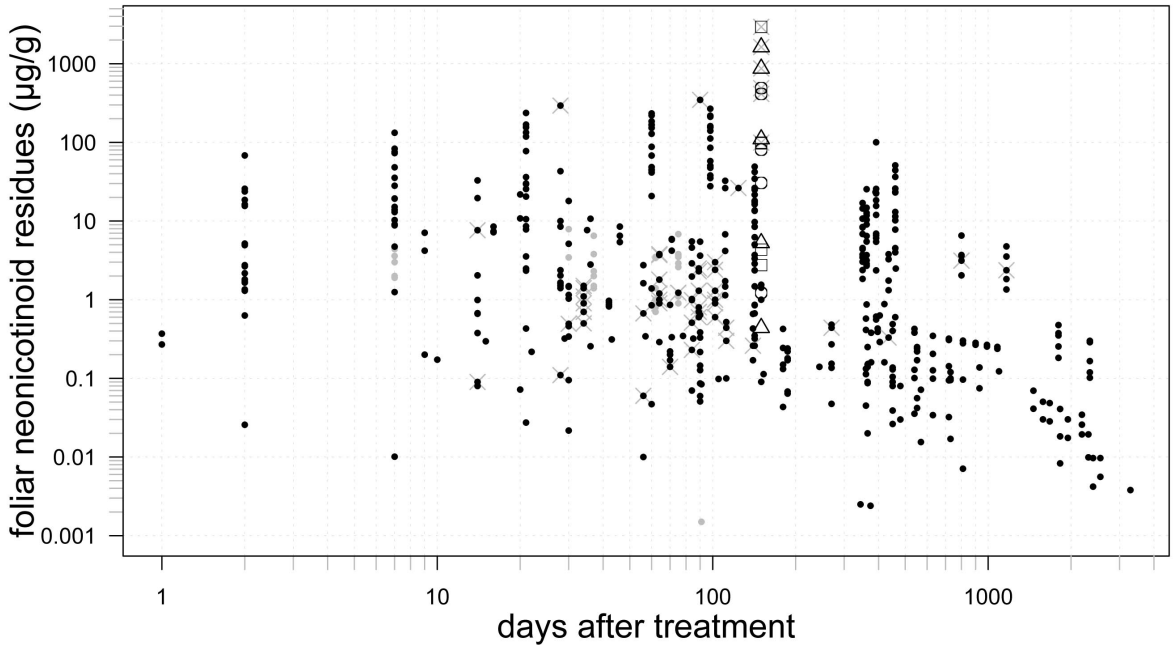

Figure 2. Foliar IMI (black solid circles) and dinotefuran (gray solid circles) residues derived from a literature review of peer-reviewed publications as well as IMI (❍), THI (△) or ACE (□) residues measured in alder foliage during the present study. A gray cross additionally indicates foliar residues derived from trees treated above the maximum dose recommended for soil application (i.e., 0.6 g IMI and 0.95 g dinotefuran/cm DBH) or trunk injection (i.e., 0.25 g IMI).

Overall, black alder trees treated at field-relevant levels of 0.0375 and 0.15 g AI/cm DBH) displayed foliar neonicotinoid residues ranging from 440 ng/g to ~30 μg/g dry weight (Figure 1). Despite relatively high foliar residues (i.e., up to ~110 μg/g) measured in alder trees treated at the maximum recommended dose of 0.6 g AI/cm DBH, they did not exceed the upper residue limit found in the 29 reviewed studies, even if residues from overdosed trees were excluded from these analyses (Figure 2). The reviewed studies, however, focused almost

exclusively on IMI, while only three investigated dinotefuran in addition to IMI (Table S1). Thus, the present study's findings constitute a considerable extension of the currently existing knowledge regarding the fate of these insecticides in tree foliage by providing foliar residue data for THI and ACE at field-relevant, as well as overdose, application levels.

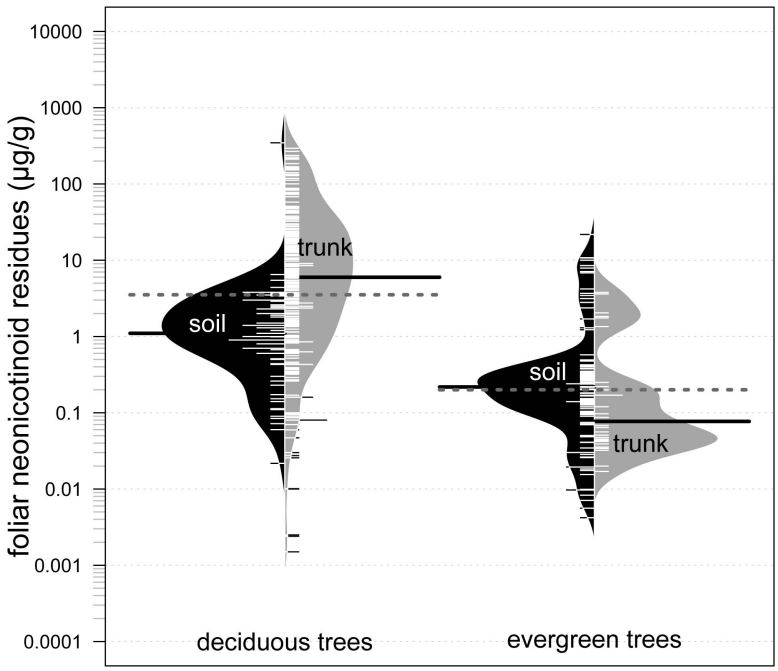

Figure 3. Bean plots displaying the density trace (black and gray area) of the individual neonicotinoid residues (small white lines) derived from a literature review of peer-reviewed publications. Residue data is itemized by deciduous and evergreen trees as well as by soil and trunk application. Black solid lines represent the median of the respective sub-group. Gray dashed lines indicate the overall median for deciduous and evergreen trees respectively.

Moreover, the evaluation of reviewed data indicates higher foliar neonicotinoid residues in deciduous trees treated by trunk application (Figure 3), even though the method uses less substance volume compared to soil application (Figure S6). Comparison of the current study's neonicotinoid residues (shown in Figure 1) to the distribution of residues found in the literature (for deciduous trees; Figure 3) reveals a higher similarity to those residues achieved by trunk rather than by soil application. This might on one hand be explained by the commonly used constant dosage per unit DBH which overestimates the insecticide dose for relatively small trees (as used throughout the present study) and consequently leads to higher

foliar residues (Benton et al., 2016a; Cowles, 2009; Ford et al., 2007). On the other hand, pot stands used to prevent an unintentional contamination (i.e., through leaching) of the adjacent environment following application (Benton et al., 2016b; McAvoy et al., 2005) may have extended the neonicotinoids' residence time in the soil. This might have maximized their uptake into trees, ultimately leading to foliar residue patterns comparable to those usually observed following trunk application.

While neonicotinoid leaching from treated soils following (soil) application may result in a rather imminent exposure of the (terrestrial and aquatic) environment, re-mobilization of neonicotinoids from contaminated foliage might take place several months after their actual application. This pathway is particularly relevant to deciduous trees, since they tend to build up higher foliar residues (median: 3.5 μg/g) compared to evergreen species (median: 0.2 μg/g; Figure 3). Moreover, they lose the majority of their foliage (up to ~70% of the annual leaf fall[15]) during autumn. Downstream transport of neonicotinoids re-mobilized from this foliage might ultimately lead to a potential secondary exposure of downstream habitats during a season of low agricultural neonicotinoid use (e.g., planting of neonicotinoid-coated seeds) and sparse neonicotinoid-occurrences in surface waters arising from the latter (Hladik et al., 2014; Main et al., 2014).

EXPOSURE SIMULATION

Neonicotinoid-contaminated foliage falling from deciduous trees might enter water bodies directly or by lateral movement (Abelho, 2001). Once submerged in the surface water body, this foliage constitutes a potential food source for aquatic macroinvertebrates as well as a source of neonicotinoids leaching into their surrounding environment (Kreutzweiser et al., 2007). Under the conditions of our leaching simulation model (i.e., foliar residues of 80 μg/g and large amounts of foliage entering the stream), the maximum concentration in the first 1-m segment was only ~2 ng IMI/L. The water concentration,

however, increased due to continued leaching and downstream transport to maximum levels as high as ~250 ng IMI/L 100 m further downstream (reached after ~34 h; Figure 4) and would continue to increase if the stream stretch that receives IMI contaminated foliage was extended (Figure S7). Though the IMI concentration slowly declines in the segments where contaminated foliage was introduced during a matter of days (Figure 4), the pesticide is transported to downstream segments, which have not directly received any input of contaminated foliage. In downstream segments that are less shaded by riparian vegetation, IMI concentrations may, depending on the proportion of sunlight transmitted into water, decline due to photolytic degradation (Bonmatin et al., 2015; Pena et al., 2011).

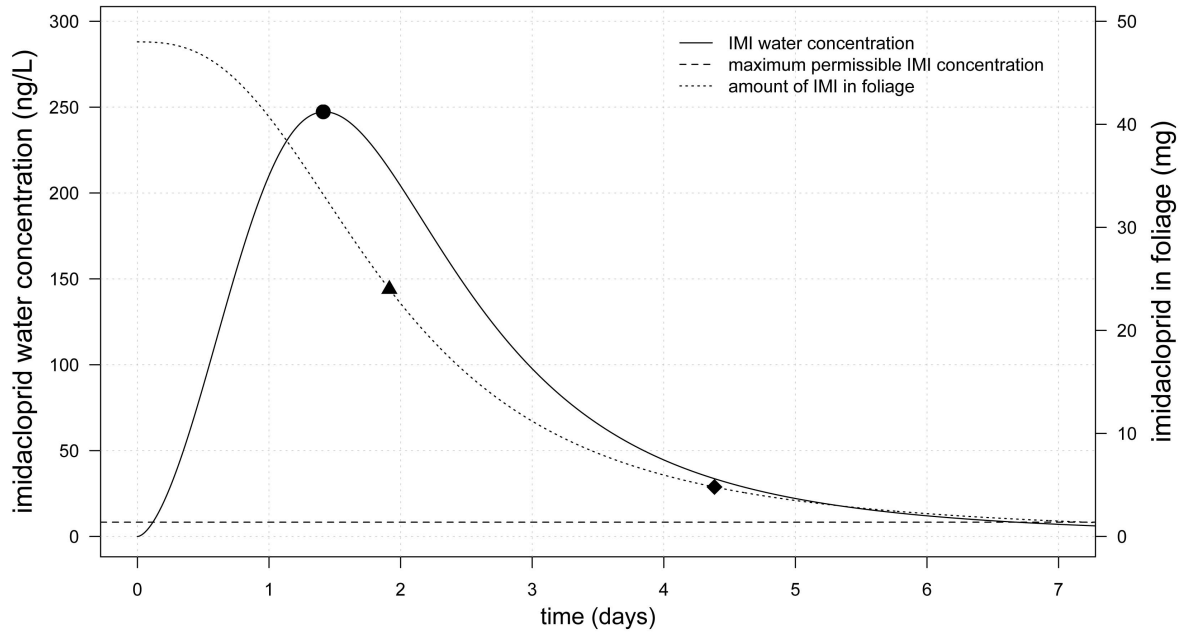

Figure 4. Modeled IMI concentration (solid line) in the water phase of the 100th stream segment including the peak IMI concentration (●) as well as the lowest IMI threshold level of 8.3 ng/L (i.e., maximum permissible concentration; dashed line; Smit et al., 2015). Moreover, the total amount of IMI in foliage (dotted line; within each stream segment; surface area: 1 m^2) following a simulated input of 600 g foliage/m^2 with an initial residue level of 80 μg IMI/g as well as corresponding retention times (RT_{50}: ▲ and RT_{10}: ◆) are also displayed.

The peak IMI water concentration predicted for the stream parameterized in our worst-case model scenario was above several of the currently existing chronic ecological water quality thresholds describing acceptable levels for neonicotinoids (mostly IMI) in surface waters

(reviewed in Morrissey et al., 2015). For instance, the lowest thresholds of 8.3 ng IMI/L, recently recommended as an update of the Maximum Permissible Concentration (Smith et al., 2015) in accordance with the European Water Framework Directive, was exceeded for ~6.5 days (Figure 4). Moreover, even the Maximum Acceptable Concentration of 200 ng IMI/L (Morrissey et al., 2015), set by the European Food Safety Authority as a threshold for short-term or peak exposure, was briefly exceeded for ~1 day (Figure 4).

Stream water concentrations calculated by the model would have been markedly lower under scenarios assuming lower amounts of foliage that (simultaneously) enter the water body (Figure S8) or lower internal neonicotinoid residue levels (Figure S9). Under otherwise identical conditions, foliar residues of 3.5 μg IMI/g, which equals the median foliar residue level found in our literature review for deciduous trees (Figure 3), would have resulted in ~95% lower maximum water concentrations (~11 ng IMI/L) in the 100th stream segment. Changing the foliage input scenario from a simultaneous input to an input equally distributed throughout autumn would likely predict a low but chronic IMI contamination of the stream ecosystem. Besides these foliage-associated parameters, the water concentration calculated by the model developed during this study strongly depends on stream characteristics. For instance, an extension of the stretch receiving contaminated foliage from 100 to 1000 m would result in a 10-times higher concentration (i.e., ~2.500 ng/L in the 1000th stream segment; Figure S7). An increase in stream velocity or depth, on the other hand, would cause proportionally lower maximum water concentrations (see Figures S10&S11) while exposure would be worse for streams characterized by a larger width-to-depth-ratio (i.e., >3.33), which is for instance described for many small streams in Germany (Ohliger & Schulz, 2010; Wogram, 2010).

While most ecotoxicological studies performed with aquatic macroinvertebrates found adverse effects at concentrations exceeding those predicted by our worst-case model scenario

(e.g., reviewed in Pisa et al., 2015), insect nymphs displayed particularly lower effect concentrations (EC_x; i.e., in the ng/L-range). Cavallaro et al. (2016) reported an emergence reduced by 20% (EC_{20}: 60 ng/L) and an altered sex ratio (EC_{20}: 110 ng/L) for the Dipteran *Chironomus dilutus* after a 40-day laboratory life-cycle assay with IMI. Another laboratory study derived 28-day EC_{10}-values (immobility) of 24 and 33 ng IMI/L for the mayflies *Caenis horaria* and *Cloeon dipterum* (Roessink et al., 2013). Moreover, in two artificial stream mesocosm studies, a 12-h neonicotinoid pulse of 100 ng AI/L reduced the body size of mayflies (*Baetis* sp. and *Epeorus* sp. exposed to IMI; Alexander et al., 2008) and led to chronic community changes due to adverse effects on a group of sensitive univoltine insect species (consisting of dipterans, ephemeropterans, plecopterans and trichopterans exposed to THI) which showed no recovery during the 2-year post exposure period (Liess & Beketov, 2011). At higher pulse-concentrations (17.6 μg IMI/L), reductions observed in invertebrate abundance and community diversity were accompanied by a significant reduction in leaf litter decomposition (Pestana et al., 2009). Based on the available literature (e.g., reviewed in Sánchez-Bayo et al., 2016), stream water concentrations >100 ng IMI/L predicted by our model scenario for >2 days could put sensitive aquatic invertebrates at risk.

In addition to neonicotinoid water concentrations predicted by our simulation, another risk for aquatic organisms might arise from the neonicotinoid-contaminated foliage directly (i.e., via dietary exposure). Despite IMI's high water solubility (Table 1), it can be found in submerged foliage for several days following their input into the water body (*cf.* Kreutzweiser et al., 2007) with simulated IMI retention times (RT) of RT_{50}: ~1.9 and RT_{10}: ~4.5 days (Figure 4). Organisms designated to the functional group of shredders heavily depend on allochthonous organic matter (such as the introduced foliage) as a food source (Cummins & Klug, 1979) and are, therefore, likely to face neonicotinoid exposure via their diet. However, information about adverse effects on aquatic organisms following dietary

exposure towards neonicotinoid-contaminated foliage is scarce, except for the work published by Kreutzweiser et al. (2007; 2008; 2009). In these studies, the authors observed reduced feeding and increased mortality in two aquatic insects, the stonefly *Pteronarcys dorsata* and the crane fly *Tipula* sp., when exposed to foliage characterized by IMI-residues between 5.6 and 346 μg/g (Kreutzweiser et al., 2007; 2008). Since these insects were unable to detect and avoid contaminated foliage (Kreutzweiser et al., 2009), detrimental effects for aquatic leaf-feeding invertebrates and ecosystem functioning might be expected.

In conclusion, the present study indicates that neonicotinoid treatment of deciduous trees leads, even at field-relevant application rates, to high foliar residues that can ultimately be released into aquatic environments following autumn leaf fall. Aquatic organisms may display direct (e.g., impaired feeding and development or reduced survival; Alexander et al., 2008; Kreutzweiser et al., 2007; Pestana et al., 2009) and indirect (e.g., altered predator-prey interaction; Englert et al., 2012) ecotoxicological effects if they are exposed to neonicotinoids leaching from foliage into the water phase – as predicted by our model – or via the consumption of contaminated foliage (Kreutzweiser et al., 2007). The relevance of these exposure pathways might become more important in the future as an increasing impact of native and invasive pests, predicted under current climate change scenarios (Ramsfield et al., 2016), will most likely be accompanied by an intensified utilisation of chemical control agents (such as neonicotinoid insecticides) as countermeasures. Thus, it would be prudent to account for these alternative exposure pathways (e.g., aqueous concentrations in water bodies leached from contaminated foliage) and exposure routes (e.g., dietary uptake through consumption of contaminated foliage) during systemic insecticide registration processes to safeguard ecosystem integrity.

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SUPPORTING INFORMATION

ENVIRONMENTAL TEMPERATURE AND PRECIPITATION THROUGHOUT THE EXPERIMENTAL PERIOD

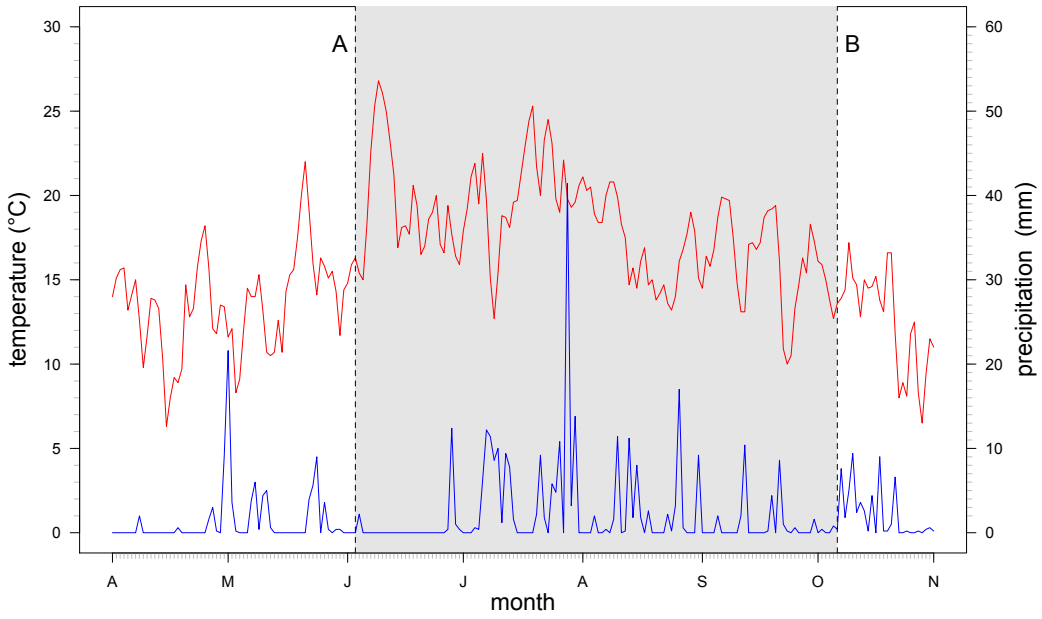

Figure S1. Daily mean temperature (red) and daily precipitation (blue) measured during the experimental period (MUEEF, 2016). The date at which the insecticides were applied (A) as well as the trees' foliage was harvested (B) are also shown.

DETAILS REGARDING CHEMICAL ANALYSES

The separation of neonicotinoid insecticides from foliage extracts was done with an UHPLC-MS (if not mentioned otherwise, compartments were manufactured by Thermo Fisher Scientific, Dreieich, Germany) equipped with an EQuan MAX system, an autosampler (CTC Analytics PAL, Zwingen, Switzerland), two LC pumps, and an analytical column (Thermo Hypersil Gold C18, 50 x 2.1 mm, 1.9 μm). First, an aliquot of the sample (injection volume: 20 μL) was transferred by the loading pump (Surveyor LC pump) onto the pre-concentration column (Thermo Hypersil Gold aQ, 20 x 2.1 mm, 12 μm; Thermo Fisher

Scientific, Dreieich, Germany) for enrichment using Milli-Q water (A) and methanol (B) as eluents both containing 0.1% formic acid and 4 mM ammonium formate (both Sigma Aldrich, Seelze, Germany, p.a. grade). At injection, 98% A and 2% B were used for 2 min at a flow of 1,000 µL/min. Then loaded neonicotinoids were eluted from the pre-concentration column to the analytical column by a back flush for 7 min at 100 µL/min. After the elution step, the gradient program decreased A to 2% and increased B to 98% (over 2 min), delivering the mobile phase at 1,000 µL/min. The gradient program subsequently returned to initial conditions with 98% A and 2% B to equilibrate the pre-concentration column for another 2 min at 1,000 µL/min. Separation of the compounds on the analytical column was done with the same eluents. The gradient program (flow rate: 200 µL/min) was started with 5% B and 95% A at injection, held for the first 2 min. Afterwards B was increased to 100% (0% A) and kept constant for 10 min. Hereafter, the gradient program was returned to initial conditions with 5% B and 95% A for another 3 min.

For quantification of neonicotinoid insecticides, a single quadrupole mass spectrometer (Thermo Orbitrap Exactive) equipped with an electrospray ionization source was used. The mass spectrometry detection was done in the positive ionization mode at a scan range of 100-2,000 m/z. The spray voltage was set at 4.0 kV and the capillary temperature at 280°C. Target compounds were identified using the accurate ion mass $[M+H]^+$ for ACE (m/z = 223.0747), IMI (m/z = 256.0596), and THI (m/z = 253.0309). External calibration with eight matrix-matched standards ranging from 1.0 to 100 µg/L was used to compensate for potential matrix suppression of the target analytes. Matrix-matched standards were prepared out of blank foliage extracts generated by using 0.5 and 3.0 g foliage. The limits of quantification (LOQ) and the limits of detection (LOD) were determined for IMI, THI and ACE on a dry weight basis according to DIN standard 32645 and were 0.06, 0.11, 0.12 µg/g and 0.02, 0.03, 0.04 µg/g, respectively.

DETAILS REGARDING THE REVIEW OF FOLIAR NEONICOTINOID RESIDUES IN SCIENTIFIC LITERATURE

Table S1. Studies used for the literature review of foliar neonicotinoid residues.

Study	Tree species	Active ingredient applied	Application method	Time of measurement (post treatment)
Acimovic et al. 2014 [2]	Apple (*Malus domestica*)	Imidacloprid	Trunk injection	14, 28, and 42 days
Ali and Caldwell 2010 [3]	Royal palm (*Roystonia regia*)	Imidacloprid, Dinotefuran	Soil application	30 and 75 days
Benton et al. 2015 [4]	Eastern hemlock (*Tsuga canadensis*)	Imidacloprid	Basal drench	4, 5, 6, and 7 years
Benton et al. 2016 [5]	Eastern hemlock (*Tsuga canadensis*)	Imidacloprid	Basal drench	4, 5, 6, and 7 years
Coots et al 2013 [6]	Eastern hemlock (*Tsuga canadensis*)	Imidacloprid	Soil tablets, soil injection	3, 6, 9, 12, 15, 18, 21, 24, and 27 months
Cowles et al. 2006 [7]	Eastern hemlock (*Tsuga canadensis*)	Imidacloprid	Soil injection, soil drench, trunk injection	3 months to ~3 years
Dilling et al. 2010 [8]	Eastern hemlock (*Tsuga canadensis*)	Imidacloprid	Soil injection, soil drench, trunk injection	3, 6, 9, 12, 15, 18, 21, and 24 months
Doccola et al. 2009 [9]	Wiliwili (*Erythrina sandwicensis*)	Imidacloprid	Trunk injection	35 days and 13 months
Doccola et al. 2012 [10]	Eastern hemlock (*Tsuga canadensis*)	Imidacloprid	Soil injection, trunk injection, also in combination	70, 435, 800, and 1165 days
Harrell 2006 [11]	Green ash (*Fraxinus pennsylvanica*)	Imidacloprid	Trunk injection	30 and 90 days
Joseph et al. 2011 [12]	Eastern hemlock (*Tsuga canadensis*)	Imidacloprid	Trunk injection	15, 19, 27, and 31 months
Kaakeh 2006 [13]	Date palm	Imidacloprid	Soil drench	10 and 20 days
Kreutzweiser et al. 2007 [14] *	Ash (*Fraxinus* spp.)	Imidacloprid	Soil injection, trunk injection	3 months
Kreutzweiser et al. 2008 [15] *	Sugar maple (*Acer saccharum*)	Imidacloprid	Trunk injection	14 days and ~4 months
Kreutzweiser et al. 2009 [16] *	Sugar maple (*Acer saccharum*)	Imidacloprid	Trunk injection	28 days
Kung et al. 2015 [17]	Eastern hemlock (*Tsuga canadensis*)	Imidacloprid	Soil injection, trunk injection	3 and 9 years
Leiva et al. 2016 [18]	Hamlin orange (*Citrus sinensis*)	Imidacloprid	Soil drench	2, 9, 20, 28, and 36 days
Mayfield et al. 2015 [19]	Eastern hemlock (*Tsuga canadensis*)	Imidacloprid	Soil injection	~4 - 6 years
McCullough et al 2011 [20]	Ash (*Fraxinus* spp.)	Imidacloprid, Dinotefuran	Trunk injection, trunk spray	7, 37, 62, and 75 days
Mota-Sanchez et al. 2008 [21]	Green ash (*Fraxinus pennsylvanica*), white ash (*F. americana*)	Imidacloprid	Trunk injection	2, 7, 21, 60, 105, 150 days and ~1 year
Nix et al. 2013 [22]	Black walnut (*Juglans nigra*)	Imidacloprid, Dinotefuran	Soil pellets, trunk spray	30, 91, 153, 244, and 366 days
Poland et al. 2006 [23]	Elm (*Ulmus pumila*), poplar (*Populus nigra*), willow (*Salix matsudana*)	Imidacloprid	Trunk injection	1 day, 1 and 9 months
Sétamou et al. 2010 [24]	Grapefruit (*Citrus paradisi*)	Imidacloprid	Soil drench	9, 15, 22, 29, 36, 43, 57, 64, 71, 78, and 85 days
Tanis et al. 2012 [25]	Ash (*Fraxinus americana* and *F. pennsylvanica*)	Imidacloprid	Trunk injection	2, 7, 21, 60, 98, 350, 362, and 460 days
Tattar et al. 1998 [26]	Eastern hemlock (*Tsuga canadensis*), white pine (*Pinus strobus*) and pin oak (*Quercus palustris*)	Imidacloprid	Soil injection, trunk injection	1, 2, 4, 8, 12, 16, and 20 weeks
Ugine et al. 2012 [27]	Norway maple (*Acer platanoides*)	Imidacloprid	Trunk injection	~142 days
Ugine et al. 2013 [28]	Norway maple (*Acer platanoides*)	Imidacloprid	Trunk injection	~111 and 142 days
VanWoerkom et al. 2014 [29]	Apple (*Malus domestica*)	Imidacloprid	Trunk injection	2, 7, 14, 30, 60, 90 days and > 1 year
Xu et al. 2015 [30]	Wiliwili (*Erythrina sandwicensis*)	Imidacloprid	Trunk injection	3 weeks and 5 months

* Conversion factors (2.39 and 4.27 for sugar maple and ash, respectively), provided by D. Kreutzweiser via personal communication[32], were used to calculate foliar residues on a dry weight basis.

SIMULATION OF WATERBORNE NEONICOTINOID CONCENTRATIONS FOLLOWING THE ENTRY OF CONTAMINATED FOLIAGE INTO A MODEL STREAM: CALCULATION DETAILS

Waterborne neonicotinoid concentrations resulting from an entry of neonicotinoid-contaminated foliage into a model low-order stream (divided into several 1-m long segments) were simulated using the following equation:

$$N_{i,water}^{t} = N_{i,water}^{t-1} + N_{leaching}^{t} + N_{i,inflow}^{t} - N_{i,loss}^{t}$$

where $N_{i,water}^{t}$ = amount of the neonicotinoid dissolved in stream segment *i* at time *t*; $N_{leaching}$ = amount of the neonicotinoid leaching from submerged foliage into the water phase between *t-1* and *t*; $N_{i,inflow}$ = amount of the neonicotinoid flowing into the stream section under consideration of the respective upstream segment (i.e., *i*-1) between *t-1* and *t*; and $N_{i,loss}$ = amount of the neonicotinoid lost due to the stream's discharge between *t-1* and *t*. The resulting value for $N_{i,water}^{t}$ (ng/stream segment) was then converted to a concentration (ng/L) by adjusting for the stream segments' volume (m^3).

The variable $N_{leaching}^{t}$ is calculated by multiplying the initial amount of neonicotinoid within foliage submerged per stream segment ($N_{foliage}$) with the proportional loss of IMI (for every second) derived from a non-linear model fitted to IMI leaching data published by Kreutzweiser et al. (2007; Figure S2). For this purpose, several non-linear regression models were fitted to the data using the R extension package "drc" (Ritz & Streibig, 2005). The model fitting the data best, (i.e., log-logistic with two parameters: b = 2.6515, e = 165420, lower limit at 0, upper limit at 1), was finally selected based on Akaike's Information Criterion as well as expert judgment.

The initial amount of the neonicotinoid within foliage introduced per stream segment ($N_{foliage}^{t}$) is calculated by multiplying the foliar neonicotinoid concentration (= $concN_{foliage}$; in

ng/g), the total amount of foliage introduced per stream section (= $amount_{foliage}$; in g/m^2) and the stream segment area ($A_{stream\ segment}$; in m^2).

Moreover, the loss of the neonicotinoid ($N_{i,loss}^{t}$) from each stream segment between *t-1* and *t* due to the stream's discharge is calculated as follows:

$$N_{loss}^{t} = N_{i,water}^{t-1} \times Loss_{factor}$$

while the $Loss_{factor}$ (in 1/s) is calculated by dividing the stream's discharge (Q_{stream}; in m^3/s) by the volume of the stream segment (V_{stream}; in m^3)

$$Loss_{factor} = \frac{Q_{stream}}{V_{stream}}$$

Q_{stream} and V_{stream}, in turn, are calculated as follows:

$$Q_{stream} = w_{stream} \times d_{stream} \times v_{stream}$$

$$V_{stream} = w_{stream} \times l_{stream} \times d_{stream}$$

while w_{stream} = the stream width (in m), d_{stream} = the stream depth (in m), v_{stream} = the stream velocity (in m/s) and l_{stream} = the length of the stream segment (in m). Moreover, the amount of the neonicotinoid lost due to the stream's discharge in stream segment *i* is added to the following segment (*i*+1) and designated $N_{i,inflow}$. The input parameters used in the present study's simulation are given in Table S2.

Table S2. Input parameters used in the present study's simulation.

Parameter	Value
Stream width (w_{stream})	1 m
Stream segment length (l_{stream})	1 m
Number of segments (*i*)	100
Stream depth (d_{stream})	0.3 m
Stream velocity (v_{stream})	0.3 m/s
Imidacloprid residues in foliage ($concIMI_{foliage}$):	80 μg/g
Amount of foliage ($amount_{foliage}$)	600 g/m^2
Observation time (t)	14 days

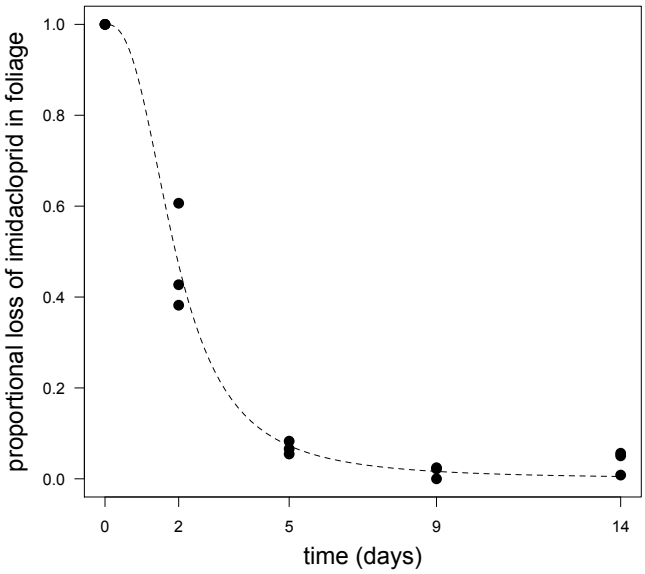

Figure S2. Proportional loss of IMI in ash foliage in water determined in fate microcosms by Kreutzweiser et al. (2007) as well as the non-linear model (dashed line) fitted to the data.

RESULTS OBTAINED DURING THE DEVELOPMENT OF THE EXTRACTION METHOD

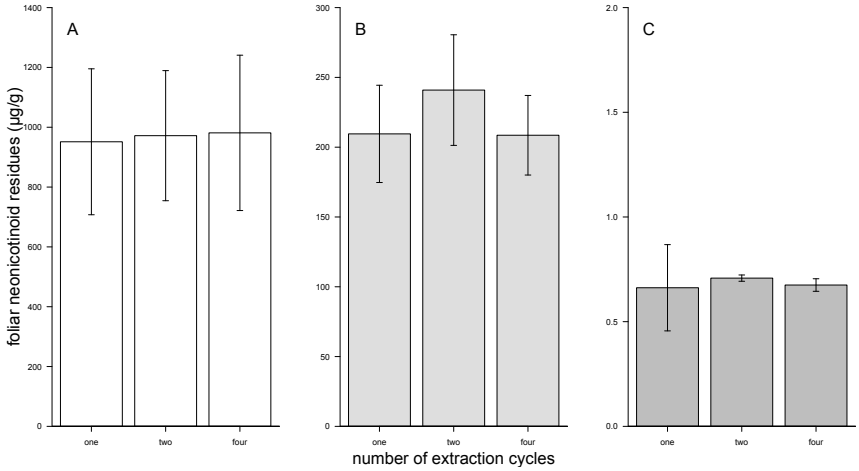

Figure S3. Mean (±95%CIs; *n*=4) residue levels of A) IMI, B) THI, and C) ACE measured in foliage from treated trees after extraction with one, two or four cycles (10 min each) at 100°C and a mixture of Milli-Q water and acetonitrile (5:1; v:v) as extraction solvent.

Table S3. Results of the ANOVAs (F-ratio) and Kruskal-Wallis tests (chi-square) conducted with the neonicotinoid residue data obtained during the development of the extraction method for different numbers of extraction cycles and extraction temperatures.

	Substance	**df**	**F-ratio**	**chi-square**	***p*-value**
Nr. of extraction cycles	Imidacloprid	2,9	0.04		0.961
(1/2/4)	Thiacloprid	2.9	2.862		0.109
	Acetamiprid	2		2.4789	0.290
Extraction Temperature	Imidacloprid	2,9	1.516		0.271
(60/80/100°C)	Thiacloprid	2		5.6923	0.058
	Acetamiprid	2,9	1.633		0.248
Extraction Temperature	Imidacloprid	2		6.5769	0.0373
(100/120/140°C)	Thiacloprid	2		4.7692	0.092
	Acetamiprid	2		3.4774	0.176

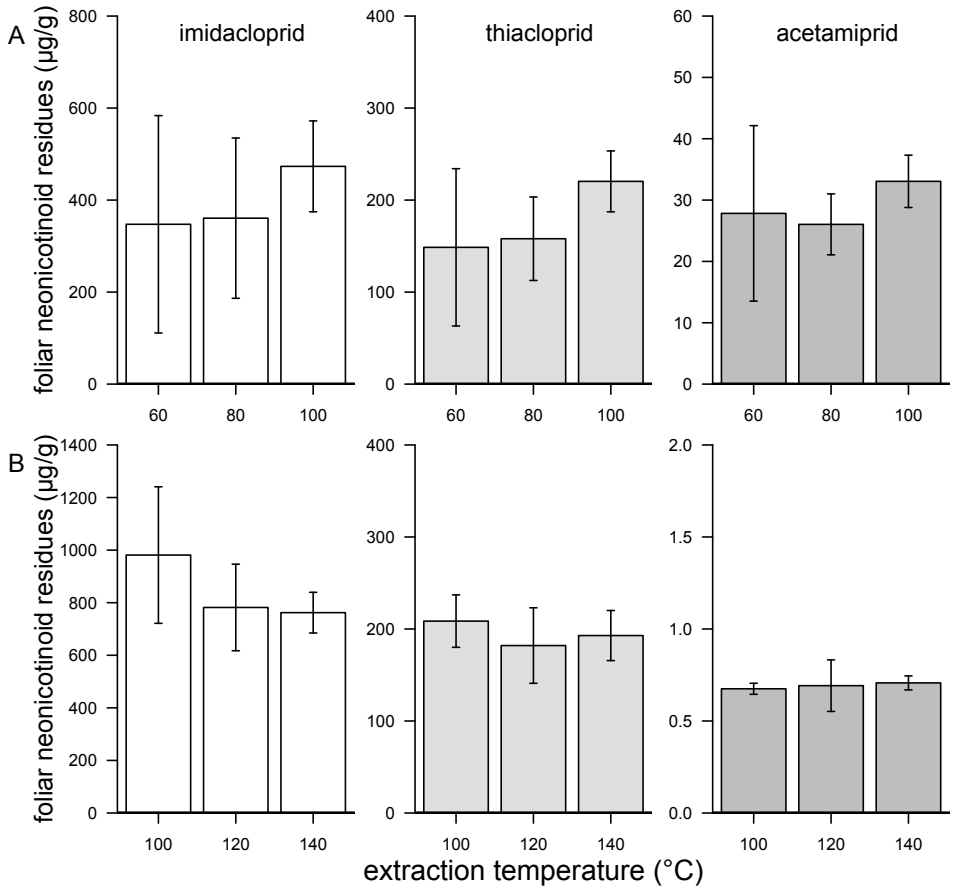

Figure S4. Mean (±95%CIs; *n*=4) residue levels of IMI, THI, and ACE in foliage from treated black alder trees after extraction with either A) 60, 80 and 100°C or B) 100, 120 and 140°C using four 10 min extraction cycles and a mixture of Milli-Q water and acetonitrile (5:1; v:v) as extraction solvent.

PHYSIOLOGICAL PARAMETERS OF BLACK ALDER TREES

Soil drench application of black alder trees with IMI and THI did not significantly affect the tree height, trunk DBH (except at 2.4 g THI/cm DBH; Dunnett's test: $p = 0.04$) and the amount of foliage produced per tree compared to untreated control trees (Figure S5A-C; Dunnett's test: $p \geq 0.16$, n=2-5) measured at the time of leaf fall (i.e., October). In contrast, black alder trees that received ACE displayed a by up to 22% reduced trunk DBH and had produced up to 79% less foliage (in terms of fresh weight) in the overdosed treatments (i.e., 2.4 and 9.6 g ACE/cm DBH; Figure S5B and S5C) indicating potential adverse effects of ACE on tree health. It needs, however, to be mentioned that, some ACE treatments (i.e., 0.0375, 2.4 and 9.6 g ACE/cm DBH) had only a replication of one as consequence of low tree survival. Hence, conclusions regarding tree health should be drawn carefully.

Moreover, analyses of covariance (ANCOVAs) were conducted to determine statistically significant differences between the applied neonicotinoid dose on the respective foliar residues controlling for the measured physiological tree parameters as covariates (i.e., tree height, trunk diameter and the amount of foliage). If a significant effect of a covariate was found, we additionally tested for significant interactions between the applied doses and the respective covariate.

Only for thiacloprid we detected a significant negative relationship between residues and the covariates "amount of foliage" and "trunk diameter" (see ANCOVA tables below; Table S4&5), while this relationship was only observed at high doses when using "amount of foliage" as covariate (see significant interaction terms in Table S4).

Table S4. ANCOVA table displaying the influence of the dose applied on foliar THI residues using the amount of foliage grown per tree as a covariate.

	Estimate	**SE**	***t*-value**	***p*-value**
Intercept of dose 0.0375 g AI/cm DBH	5.44316	199.16069	0.027	0.97991
Difference between intercepts for doses 0.0375 and 0.15 g AI/cm DBH	3.94996	225.51800	0.018	0.98713
Difference between intercepts for doses 0.0375 and 0.6 g AI/cm DBH	1077.92857	216.87188	4.970	**0.01565**
Difference between intercepts for doses 0.0375 and 2.4 g AI/cm DBH	2123.71697	201.40812	10.544	**0.00182**
Difference between intercepts for doses 0.0375 and 9.6 g AI/cm DBH	1810.90143	218.85492	8.274	**0.00370**
Slope of dose 0.0375 g AI/cm DBH	-0.02947	1.13963	-0.026	0.98099
Difference between slopes for doses 0.0375 and 0.15 g AI/cm DBH	0.01213	1.22738	0.010	0.99273
Difference between slopes for doses 0.0375 and 0.6 g AI/cm DBH	-4.36180	1.20187	-3.629	**0.03601**
Difference between slopes for doses 0.0375 and 2.4 g AI/cm DBH	-6.98719	1.15080	-6.072	**0.00897**
Difference between slopes for doses 0.0375 and 9.6 g AI/cm DBH	0.23991	1.21065	0.198	0.85558

R-squared: 0.9996

Table S5. ANCOVA table displaying the influence of the dose applied on foliar THI residues using the trunk diameter at breast height as a covariate (note that interactions turned out to be non-significant and were thus omitted from this model in the course of model simplification).

	Estimate	**SE**	***t*-value**	***p*-value**
Intercept of dose 0.0375 g AI/cm DBH	1675.39	610.27	2.745	**0.02870**
Difference between intercepts for doses 0.0375 and 0.15 g AI/cm DBH	201.68	178.82	1.128	0.29660
Difference between intercepts for doses 0.0375 and 0.6 g AI/cm DBH	314.87	181.34	1.736	0.12610
Difference between intercepts for doses 0.0375 and 2.4 g AI/cm DBH	598.04	191.04	3.130	**0.01660**
Difference between intercepts for doses 0.0375 and 9.6 g AI/cm DBH	1926.64	181.68	10.605	**0.00001**
Slope of trunk diameter at breast height (mm)	-100.30	35.74	-2.807	**0.02630**

R-squared: 0.9383

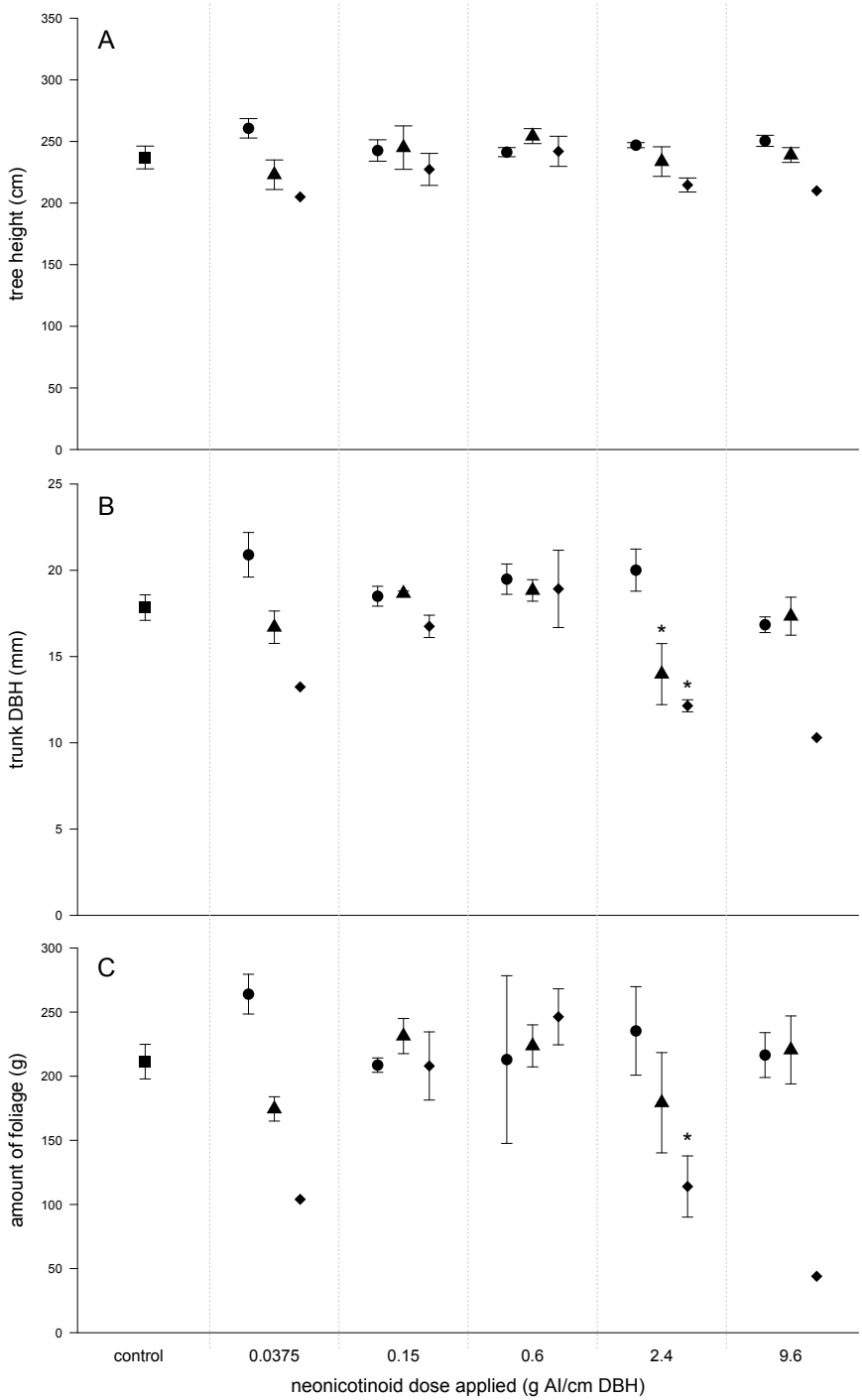

Figure S5. Mean (±SE) A) tree height, B) trunk DBH and C) amount of foliage measured during autumn from trees treated with IMI (●; *n*=2-3), THI (▲; *n*=2-3) and ACE (◆; *n*=1-3) as well as from the untreated control (■; *n*=5). Asterisks denote significant differences compared to the control, $p < 0.05$ (*). Missing SEs indicate a replication of only one.

FURTHER RESULTS DERIVED FROM THE LITERATURE REVIEW OF FOLIAR NEONICOTINOID RESIDUES

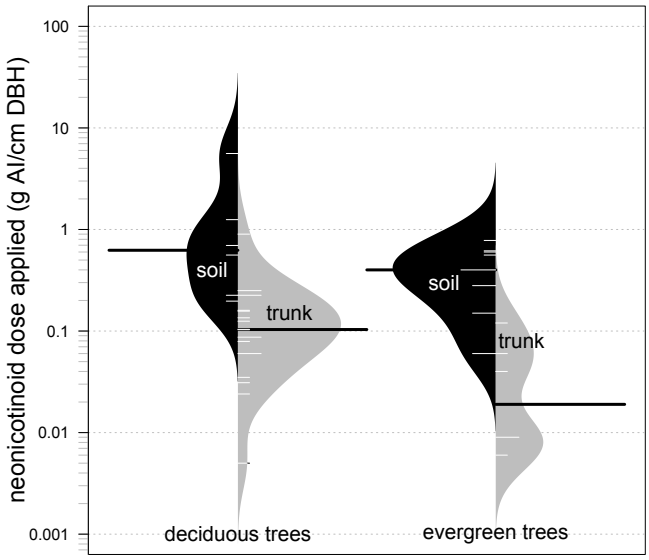

Figure S6. Bean plots displaying the density trace (black and grey area) of the individual neonicotinoid doses (small white lines) that were (trunk and soil) applied on (deciduous and evergreen) trees in the peer-reviewed publications included in the present study's literature review. Black solid lines represent the median of the respective sub-group.

ILLUSTRATION OF SEVERAL MODEL SCENARIOS

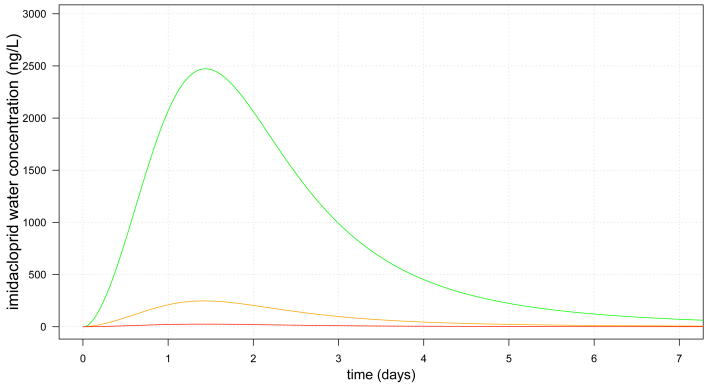

Figure S7. Modeled IMI concentration (solid lines) in the water phase of the 10th (red), 100th (orange) and 1000th (green) stream segment over the course of 7 days assuming a 1000 m-long stream (width: 1 m, depth: 0.3 m, velocity: 0.3 m/s) that receives an input of 600 g foliage/m^2 with an initial residue level of 80 μg IMI/g.

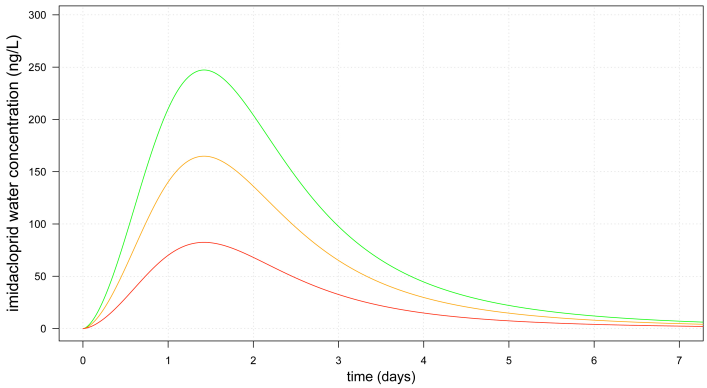

Figure S8. Modeled IMI concentration (solid lines; over the course of 7 days) in the water phase of the 100th stream segment assuming a stream (width: 1 m, depth: 0.3 m, velocity: 0.3 m/s) that receives an input of 200 (red), 400 (orange) or 600 (green) g foliage/m^2 with an initial residue level of 80 μg IMI/g.

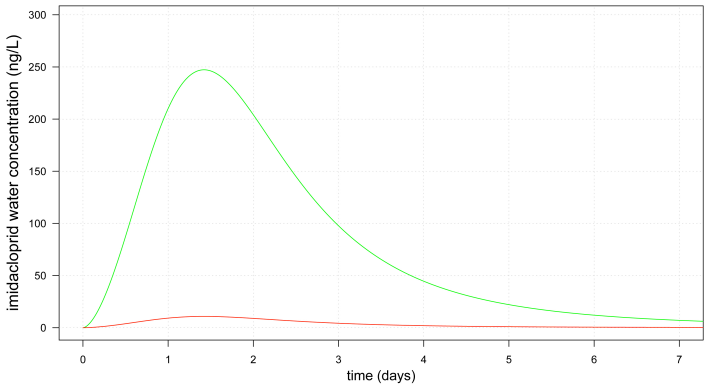

Figure S9. Modeled IMI concentration (solid lines; over the course of 7 days) in the water phase of the 100th stream segment assuming a stream (width: 1 m, depth: 0.3 m, velocity: 0.3 m/s) that receives an input of 600 g foliage/m^2 with an initial IMI residue level of 3.5 (= median neonicotinoid residue level of deciduous trees derived from the literature review; red) or 80 μg/g (= average IMI residue measured in black alder trees that received the highest field-relevant application of 0.6 g AI/cm DBH; green).

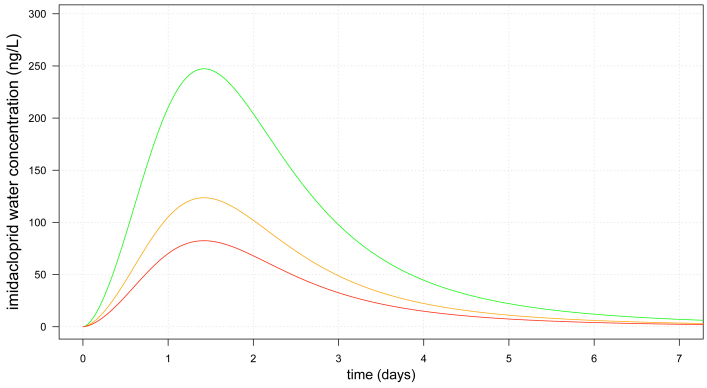

Figure S10. Modeled IMI concentration (solid lines; over the course of 7 days) in the water phase of the 100th stream segment assuming a stream (width: 1 m, depth: 0.3 m) with a velocity of 0.3 (green), 0.6 (orange) or 0.9 m/s (red), respectively, that receives an input of 600 g foliage/m^2 with an initial residue level of 80 μg IMI/g.

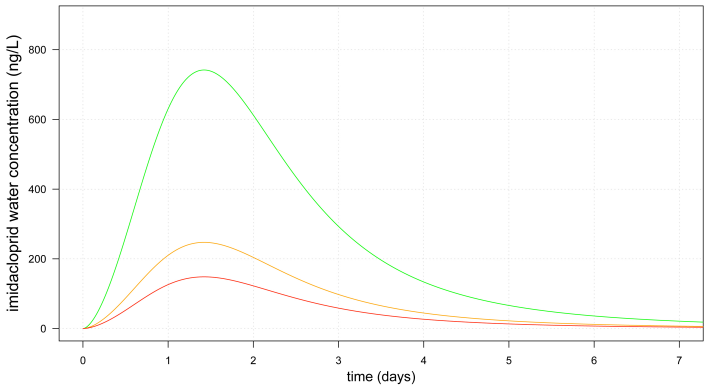

Figure S11. Modeled IMI concentration (solid lines; over the course of 7 days) in the water phase of the 100th stream segment assuming a stream (width: 1 m, velocity: 0.3 m/s) with a depth of 0.1 (green), 0.3 (orange) or 0.5 m (red), respectively, that receives an input of 600 g foliage/m^2 with an initial residue level of 80 μg IMI/g.

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APPENDIX A.2

Does waterborne exposure explain effects caused by neonicotinoid-contaminated plant material in aquatic systems?

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ABSTRACT

Neonicotinoids are increasingly applied on trees as protection measure against insect pests. Consequently, neonicotinoids are inevitably transferred into aquatic environments either via spray drift, surface runoff or – due to neonicotinoids' systemic nature – via senescent leaves. There particularly leaf-shredding invertebrates may be exposed to neonicotinoids through both the water phase and the consumption of contaminated leaves. In 7-d-bioassays (n=30) we examined ecotoxicological differences between these two exposure scenarios for an amphipod and an insect nymph with their feeding rate as the response variable. Organisms either experienced waterborne neonicotinoid (i.e., imidacloprid, thiacloprid and acetamiprid) exposure only or a combined exposure (waterborne and dietary) through both the consumption of contaminated leaves and neonicotinoids leaching from leaves into water. The amphipod (7d-EC_{50}s from 0.3 to 8.4 μg/L) was more sensitive than the insect nymph (7d-EC_{50}s from 7.0 to 19.4 μg/L). Moreover, for both species, concentration-response models derived from water concentrations indicated higher effects under the combined exposure. Together with the observed inability of shredders to avoid neonicotinoid-contaminated leaves, our results emphasize the relevance of dietary exposure (e.g., via leaves) for systemic insecticides. Thus, it would be prudent to consider dietary exposure during the registration of systemic insecticides to safeguard ecosystem integrity.

INTRODUCTION

Since the introduction of imidacloprid (IMI) in 1991, neonicotinoids have become one of the most economically successful insecticide classes (Jeschke et al., 2011). Their tremendous success is attributed to their broad-spectrum insecticidal activity targeting specifically insects' nicotinic acetylcholine receptors (Tomizawa & Casida, 2003). In addition, neonicotinoids benefited from the ban or withdrawal of other insecticides from the market as a consequence of pest resistance or increasing regulatory hurdles (e.g., organophosphates; Jeschke & Nauen,

2008; Jeschke et al., 2011). Additionally, their rapid uptake and distribution in treated plants facilitated by their systemic nature allows for a broad range of application methods, which can – in theory – lessen the total amount of insecticide applied (Jeschke & Nauen, 2008; Jeschke et al., 2011)

Neonicotinoids are typically used as seed coatings, for soil drenching or direct injection (e.g., into tree trunks), but can also be sprayed on crops and trees (Elbert et al., 2004). In addition to spray drift, their physico-chemical properties and environmental persistence in soil and plants (Benton et al., 2016; Bonmatin et al., 2015; FOOTPRINT, 2016) suggest a particular susceptibility of neonicotinoids to an off-site transport into adjacent surface waters via surface runoff. A review of monitoring data indicates average surface water concentrations for individual neonicotinoids in the range of 0.08 to 0.73 μg/L (Morrissey et al., 2015; Sánchez-Bayo et al., 2016), while peak concentrations can be considerably higher in streams draining agricultural areas (e.g. 320 μg IMI/L in Dutch agricultural surface waters; Van Dijk et al., 2013). Several organism groups, particularly insect nymphs and amphipods, show negative responses when exposed to concentrations in the ng to μg/L-range (reviewed in Pisa et al., 2015 and Sánchez-Bayo et al., 2016). In stream mesocosms, for instance, two pulsed contaminations with 0.1 μg/L of the neonicotinoid thiacloprid (THI; lasting ≥9 d) – over a two-year period – caused permanent changes in the macroinvertebrate community due to the loss of sensitive univoltine species (Liess & Beketov, 2011).

The systemic nature of neonicotinoids adds an additional exposure path to those discussed above: the input through plant material that has intentionally (e.g., arable crops and trees; Bonmatin et al., 2005; Kreutzweiser et al., 2007) or unintentionally (e.g., flowers and wetland macrophytes; Botias et al., 2015; Main et al., 2017) been exposed to these insecticides. This path might be particularly relevant for crop detritus left on the field after harvest (*sensu* Rosi-

Marshall et al., 2007; Tank et al., 2010) as well as for senescent leaves falling from neonicotinoid-treated deciduous trees during autumn leaf fall (Kreutzweiser et al., 2007; Englert et al., 2017). This plant debris might end up in nearby surface water bodies through lateral transport or vertical fall (Abelho, 2001). Once submerged, the highly hydrophilic neonicotinoids are largely re-mobilized within days through leaching (Kreutzweiser et al., 2007). This can result in low (i.e., ng/L-range) but several days-lasting waterborne exposures for aquatic organisms (Kreutzweiser et al., 2007; Englert et al., 2017). On the other hand, particularly detritivorous macroinvertebrates (= shredders) may additionally be exposed to neonicotinoids through the consumption of contaminated leaves (Kreutzweiser et al., 2007; 2008). However, a systematic understanding regarding the relevance of these two exposure paths towards neonicotinoids for the likely most susceptible functional group, i.e., shredders, is missing.

Therefore, the present study assessed ecotoxicological differences between a waterborne exposure scenario – representative for neonicotinoid spray drift or surface runoff into streams – and a scenario in which shredders were simultaneously exposed (= combined exposure) via both the consumption of neonicotinoid-contaminated leaves as well as via the water phase (through leaching of neonicotinoids from leaves). Two model shredders representing two taxonomic orders, namely *Gammarus fossarum* (KOCH; Amphipoda) and *Chaetopteryx villosa* (FABRICIUS; Trichoptera), were individually subjected to these exposure scenarios over 7 d using their feeding rate as ecotoxicological response variable. This approach allowed for estimating the contribution of waterborne exposure in the combined exposure scenario. Moreover, a range of food selection assays was provided to determine potential active avoiding strategies of these shredders by sensing neonicotinoids in leaf material. We expected *G. fossarum* and *C. villosa* incapable of avoiding neonicotinoid contaminated leaves (Kreutzweiser et al., 2009) as well as more severe effects on the test

organisms' feeding and survival in the combined exposure scenario compared to waterborne exposure alone (cf. Bundschuh et al., 2013).

MATERIALS AND METHODS

TEST ORGANISMS

All test organisms were kick-sampled at least 7 d prior to the start of each experiment from near-natural streams located in the Palatinate forest upstream of any settlement and agricultural activity. Pre-exposure of test organisms towards neonicotinoids is therefore likely negligible. *Gammarus fossarum* were collected from the Hainbach (49°14'N; 8°03'E) whose population is exclusively composed of the cryptic lineage B (Feckler et al., 2012). In the laboratory, gammarids were divided into different size classes using a passive underwater separation technique (Franke, 1977) and visually checked for macro-parasites (e.g., from the phylum Acanthocephala), which may affect, among others things, the gammarids' feeding behavior (Pascoe et al., 1995). Only adult males (Pöckl, 1992) – identified by their position in precopula pairs – of 6-8 mm body length were used. *Chaetopteryx villosa* (5^{th} instar larvae; determined based on their head capsule widths; Wagner, 1990) were collected from the Sauerbach (49°5'N; 7°37'E). In the laboratory, all animals were kept in aerated stream water from the respective sampling site at 16±1°C, fed *ad libitum* with pre-conditioned black alder leaves and gradually adapted to SAM-S5 medium (= test medium; Borgmann, 1996). For the food selection assays, organisms were starved during the last 4 d prior to the initiation of the bioassay to bring their appetite to a consistent level.

SOURCE OF PLANT MATERIAL & INSECTICIDE APPLICATION

Black alder trees (*Alnus glutinosa* (L.) GAERTN.) were soil drenched with one of three commercially available neonicotinoid insecticides (Confidor®WG70 (70% IMI), Calypso® (40% THI; both Bayer CropScience) and Mospilan®SG (20% Acetamiprid; ACE; Cheminova Deutschland GmbH) at one of five concentrations (0.0375, 0.15, 0.6, 2.4, 9.6 g active

ingredient per cm trunk diameter at breast height (g active ingredient (AI)/cm DBH)) as described in Englert et al. (2017). While the maximum amount of IMI recommended for a single soil application on trees is 0.6 g AI/cm DBH (Bayer CropScience), two intentional overdose treatments – i.e., 2.4 and 9.6 AI/cm DBH – were used to generate leaf material characterized by a wide range of neonicotinoid residues required for concentration-response experiments (i.e., feeding activity experiments). All leaves were collected shortly before defoliation in October 2014 (four months after application) and stored frozen at -20°C to ensure neonicotinoid stability (Kreutzweiser et al., 2008a) until their use in the experiments. Neonicotinoid residues in leaves were quantified prior to the start of the experiments (see section: *Extraction and quantification of neonicotinoids*).

PREPARATION OF LEAF DISCS

For the experiments assessing the waterborne exposure scenario, leaf discs, were prepared as described in Bundschuh et al. (2011). In brief, leaf discs (diameter = 2.0 cm) were cut from leaves collected in October 2013 from black alder trees near Landau, Germany (49°11'N; 8°05'E). These discs were subsequently conditioned in a nutrient medium (Dang et al., 2005) for 10 d together with black alder leaves previously exposed in a near natural stream (Rodenbach, Germany), to establish a microbial community consisting of bacteria and fungi. After conditioning, the leaf discs were dried at 60°C, weighed to the nearest 0.01 mg and re-soaked in test medium 24 h prior to the start of each experiment.

For the experiments assessing the combined exposure scenario as well as for the food selection assays, discs of 2.0 cm diameter were cut from frozen black alder leaves collected either from neonicotinoid-treated trees (with IMI, THI or ACE) or from neonicotinoid-free control trees grown under the same conditions. Leaf discs were not subjected to a microbial conditioning (alder leaves are nutritious food even without conditioning; Graca et al., 2001) in order to prevent the unintended loss of neonicotinoids during this process through leaching

(Kreutzweiser et al., 2007) and allowing for a worst-case assessment. Moreover, leaf discs were freeze-dried instead of oven dried to prevent thermal degradation or vaporization of neonicotinoid residues. Leaf samples used for the quantification of neonicotinoid residues (see section: *Extraction and quantification of neonicotinoids*) were also freeze-dried to account for any possible effects the freeze-drying procedure might have on these compounds. Leaf discs were weighed to the nearest 0.01 mg before being re-soaked in the test vessels (filled with 200 mL of test medium) 24 h prior to the start of each feeding activity experiment. Similarly, leaf discs intended for the food selection assays were re-soaked for 24 h in 100-mL test medium and were subsequently transferred to the food selection assay.

FEEDING ACTIVITY

Independent of the exposure scenario, each feeding activity experiment was comprised of six different neonicotinoid treatments (n=30) including a neonicotinoid-free control (containing uncontaminated test medium as well as untreated leaves) and aimed at obtaining a complete concentration-response curve for the organisms' feeding rate. For the experiments assessing the waterborne exposure scenario, all three neonicotinoids (IMI, THI and ACE) were applied in their commercially available formulations (see above) and serially diluted in test medium to obtain the respective nominal test concentrations (Table 1). Each replicate consisted – irrespective of the shredder species – of one test organism, which was placed together with two preconditioned, neonicotinoid-free leaf discs in a 250-mL glass beaker filled with 200 mL test medium. In contrast, neonicotinoid-contaminated leaf discs, cut from leaves of IMI-, THI- and ACE-treated trees at five concentrations each (see section: *Source of plant material & insecticide application*), were used as neonicotinoid vector for experiments assessing the combined exposure scenario. Thus, shredders' feeding responded to a combination of waterborne exposure (through leaching of neonicotinoids into water) and dietary exposure (i.e., consumption of contaminated leaves). All beakers were aerated during

the 7-d experiments and randomly placed in a climate controlled chamber at 16±1°C. While a 12/12 h day/night rhythm was used for the feeding activity experiments with *C. villosa*, those with *G. fossarum* were performed in total darkness to avoid any negative phototactic response (Holmes, 1901). At the beginning of each experiment, triplicate leaf samples, comprising of at least 20 leaves per treatment, were stored frozen at -20°C until extraction and chemical analyses (see section: *Extraction and quantification of neonicotinoids*).

In each feeding activity experiment, five additional beakers per treatment, without test organisms, accounted for microbial and abiotic leaf mass losses during the experiment. After 7 d of exposure, the test organisms as well as any remaining leaf material were removed, dried and weighed (caddisflies without their cases) as described above. At the termination of the waterborne exposure experiments, triplicate 10 mL-samples were taken from the control treatments and the treatments with lowest and the highest neonicotinoid concentrations tested in order to confirm the desired nominal concentrations via chemical analyses. Likewise, triplicate 10 mL-samples were collected from every treatment of the combined exposure experiments to measure the neonicotinoid water concentration required for concentration-response modeling. All samples were stored frozen at -20°C until chemical analysis. Since the application of neonicotinoids to trees can result in an unequal spatial distribution of the insecticides within tree foliage (Dilling et al., 2010; Tanis et al., 2012), neonicotinoid water concentrations of the combined exposure scenario were expected to show higher within-treatment variation compared to those of the water phase exposure.

FOOD SELECTION ASSAYS

For the food selection assays, one neonicotinoid-free as well as one neonicotinoid-contaminated leaf disc (diameter = 2.0 cm) from trees grown under the same conditions (see section: *Source of plant material & insecticide application*) were simultaneously placed in a 300-mL glass crystallization dish (= feeding arena; n=50). Each feeding arena was filled with

100 mL of test medium and one test organism (placed midway between the two leaf discs) was allowed to feed on these discs for 24 h. For both shredder species, food selection assays were conducted with leaves from trees treated at two different field relevant application levels (i.e., low and high; Table S1) of each neonicotinoid. This resulted in a total of 12 food selection assays (2 species x 3 neonicotinoids x 2 concentrations). All feeding arenas were placed randomized in a climate controlled chamber at 16±1°C in total darkness thereby avoiding any negative phototactic responses of the test organisms. Per experiment, 10 additional replicates without test organisms were set up to quantify the biotic and abiotic leaf mass loss. At the end of the experiments, test organisms as well as any remaining leaf material were removed, dried and weighed as described above. Moreover, triplicate 10 mL-samples were taken at the termination of each experiment and stored frozen at -20°C until chemical analyses.

EXTRACTION AND QUANTIFICATION OF NEONICOTINOIDS

Although concentration-response curves for the organisms feeding were all calculated based on neonicotinoid water concentrations instead of leaves' internal neonicotinoid residues (see section: *Calculations and statistics*), the latter were additionally quantified to illustrate the range of foliar residues used (Table 1). Briefly, IMI, THI and ACE were extracted from freeze-dried alder leaves using an ASE™ 350 Accelerated Solvent Extractor system (Thermo Scientific™ Dionex™ , Sunnyvale, CA; USA; see Englert et al., 2017). The target compounds were identified in leaf extracts and water samples by using an ultrahigh performance liquid chromatography–mass spectrometry system equipped with an EQuan MAX system (Englert et al., 2012; 2017) at the accurate ion mass $[M+H]^+$ for ACE (m/z = 223.0747), IMI (m/z = 256.0596), and THI (m/z = 253.0309). External calibration with matrix-matched standards (prepared out of neonicotinoid-free leaf extracts or test medium, respectively) was used. The limits of quantification (LOQ) and the limits of detection (LOD) for neonicotinoids

(i.e., IMI, THI and ACE) in leaf extracts were 0.06, 0.11, 0.12 µg/g dry weight and 0.02, 0.03, 0.04 µg/g dry weight, respectively (Englert et al., 2017). For the measured neonicotinoid water concentrations, the LOQ was defined as the lowest calibration level (= 0.02 μg/L) due to the absence of signals in blank samples (Turnipseed et al., 2008). As mean neonicotinoid water concentrations measured in the waterborne exposure experiments were within 15% of their nominal concentrations (Table 1), the latter are reported throughout the present study.

CALCULATIONS AND STATISTICS

The feeding rate of the test organisms was calculated in milligram of consumed leaf mass per milligram animal dry weight per day and corrected for the microbial and abiotic leaf mass loss as described in Zubrod et al. (2010; 2015). For the feeding activity experiments, effective and lethal concentrations causing 20 or 50% feeding inhibition or mortality of test organisms (i.e., EC_{20} and EC_{50}-values as well as LC_{20}- and LC_{50}-values), respectively, were determined using several concentration-response models supported by the R extension package "drc" (Ritz & Streibig, 2005). Model calculations were, in the case of the waterborne exposure experiments, conducted with nominal neonicotinoid test concentrations. In contrast, model calculations for the combined exposure experiments were conducted with neonicotinoid water concentrations measured at the termination of the experiments (after 7 d) thereby assuming – due to the continued leaching of neonicotinoids from leaves into the water – worst-case exposure. The model fitting the data best was selected based on Akaike's Information Criterion (i.e., lowest score) as well as visual inspection (for model parameters see Table S2). Despite the relatively high variability that can be associated with the sublethal response variables of such non-standard toxicity tests, earlier studies have demonstrated a high reproducibility with coefficients of variation for EC_{50}-values comparable to the acute *Daphnia* assay (Zubrod et al., 2014). Only EC_X and LC_X-values within the range of measured neonicotinoid water concentrations are reported during the present study. If these values could

be obtained for both exposure scenarios (for the same neonicotinoid and shredder) they were checked for significant differences using the function "comped" implemented in the R extension package "drc" (Ritz & Streibig, 2005). Moreover, concentration-response curves modeled for the organisms' feeding rate were tested for statistically significant differences using the R-function "comped" (Ritz & Streibig, 2005) and the method described by Wheeler et al. (2006; see Supporting Information; Figure S1).

Organisms' feeding rate on neonicotinoid-free and neonicotinoid-contaminated leaf discs was compared individually for every food selection assay using Student's *t*-test or – if the normality assumption was violated – Wilcoxon rank sum tests. In order to test for a consistent feeding preference across all neonicotinoids and different shredder species, a random-effects meta-analysis was conducted (using the R extension package "metafor"; Viechtbauer, 2010). The meta-analysis included results of the present study's food selection assays as well as data from Kreutzweiser et al. (2009) who conducted similar selection experiments with stonefly (*Pteronarcys dorsata)* and crane fly (*Tipula* sp.) larvae. The random effects model was chosen as the results were expected to differ depending on the test species and neonicotinoid compound investigated. Neither *a priori* nor *a posteriori* power analyses were conducted.

Depending on the data, 95% confidence intervals (CIs) for group means or medians (feeding rate) as well as proportions of dead animals were calculated (Altman et al., 2000). The term significant(ly) is exclusively used in reference to statistical significance ($p < 0.05$) throughout the present study. For all statistics and figures, R version 3.1.1 for Mac was used.

RESULTS AND DISCUSSION

MORTALITY

Under waterborne neonicotinoid exposure mortality of *C. villosa* and *G. fossarum* remained, irrespective of the tested concentration and neonicotinoid compound, below 7 and 23%, respectively (Table S3). Whereas scientific literature lacks information regarding

neonicotinoid induced mortality on caddisflies from the genus *Chaetopteryx*, results for *G. fossarum* seem plausible in the light of former publications: the neonicotinoid concentrations tested here (≤ 24 µg/L) were considerably lower than respective 96-h LC_{50}-values reported for *G. pulex* by Beketov & Liess (2008) and Roessink et al. (2013; see also Table 1). Only the highest ACE test concentration of 24 µg/L was relatively close to the 96-h LC_{50} of *G. pulex* (i.e., 50 µg/L) and is, therefore, reflected by the 23% *Gammarus* mortality observed in this treatment (Table S3).

The combined exposure scenario – which assessed both dietary neonicotinoid uptake as well as waterborne exposure due to leaching from leaves – caused similar mortalities for *Gammarus* as observed for the waterborne exposure alone (i.e., ≤20%; Table S3). Only in situations in which water phase concentrations exceeded the tested range of the waterborne scenario by one order of magnitude (i.e., the highest THI and ACE treatments), mortalities of 37 and 47%, respectively, were observed (Table S3). *Chaetopteryx*, in contrast, seem to be more susceptible towards the combined exposure pathway. The 7-d LC_{50}-values (i.e., 11.5 µg IMI/L and 21.6 µg THI/L; Table 2) calculated for the latter (based on measured water concentrations) were observed at concentrations that caused no mortality in the waterborne exposure experiments (Table S3). The discrepancy in mortality between the two species in response to the two exposure scenarios might be explained by the neonicotinoid exposure via the ingestion of contaminated leaves: as *Chaetopteryx* displayed up to four-fold higher leaf consumption compared to *Gammarus* (Figure S2), a higher dietary exposure of *Chaetopteryx* can be anticipated. Besides uptake, other toxicokinetic (e.g., internal distribution, biotransformation) and toxicodynamic differences (e.g., the presence of target receptors) between the two test organisms may determine their sensitivity towards neonicotinoids (cf. Nyman et al., 2014).

FEEDING ACTIVITY

In addition to organisms' survival, their feeding on leaves was markedly influenced by neonicotinoid exposure and – in case of *Gammarus* – similar to values seen in other studies (Agatz et al., 2014; Englert et al., 2012; Feckler et al., 2012; see also Table 1). In case of THI, for instance, the calculated 7-d EC_{50} deviated only marginally from that observed in one of our recent studies (Zubrod et al., 2017; see Table 1), confirming the reported repeatability of the feeding assay (Zubrod et al., 2014). Furthermore, in contrast to the mortality data, complete concentration-response curves for the test organisms' feeding rate could be obtained for most of the neonicotinoids and exposure scenarios (except for *Chaetopteryx* exposed to ACE and THI) allowing for a direct comparison of the EC_x-values (Table 2) as well as the progression of the concentration-response curves (Figure 1). The comparison of the latter showed a higher toxicity of the combined exposure as concentration-response curves of this scenario ran – for both shredders – mostly (and partly significantly) below those of the solely waterborne exposure scenario (Figure 1 and S1). Hence, effects observed under combined exposure cannot solely be explained by neonicotinoid water concentrations. Further, all 7-d EC_{20} and EC_{50}-values calculated for the combined exposure scenario were lower – although not in every case statistically significantly – than their counterparts derived from waterborne exposure experiments (Figure 1; Table 2). Although we assume that this enhanced toxicity was mainly due to the additional route of exposure (i.e., dietary neonicotinoid uptake), neonicotinoid metabolites – of which some can be even more toxic than their parent compounds (Simon-Delso et al., 2015) – formed within plants, may have also influenced the results.

It has, however, to be noted that conditions under which concentration-response curves (and consequently EC_x-values) were derived differed between the two exposure scenarios. Since in the waterborne exposure scenario, neonicotinoid water concentrations were

confirmed to be stable during the 7-d lasting experiments (Table 1), the nominal concentrations were used for concentration-response modeling. In contrast, considering only final concentrations (measured after 7 d) for models of the combined scenarios overestimates the actual exposure due to continued leaching of neonicotinoids from the leaves into the water. Therefore, the use of time-weighted average concentrations (Brock et al., 2009) – accounting for the gradual leaching of neonicotinoids into water (only available for IMI; Kreutzweiser et al., 2007) – would be better for calculation of EC_xs. Accordingly, differences in IMI EC_x-values were more pronounced (for comparison see Table S4) when time-weighted water concentrations were used instead of the maximum concentrations (measured at the end of the 7-d lasting experiment) for the model calculations (see Supporting Information, Figure S3).

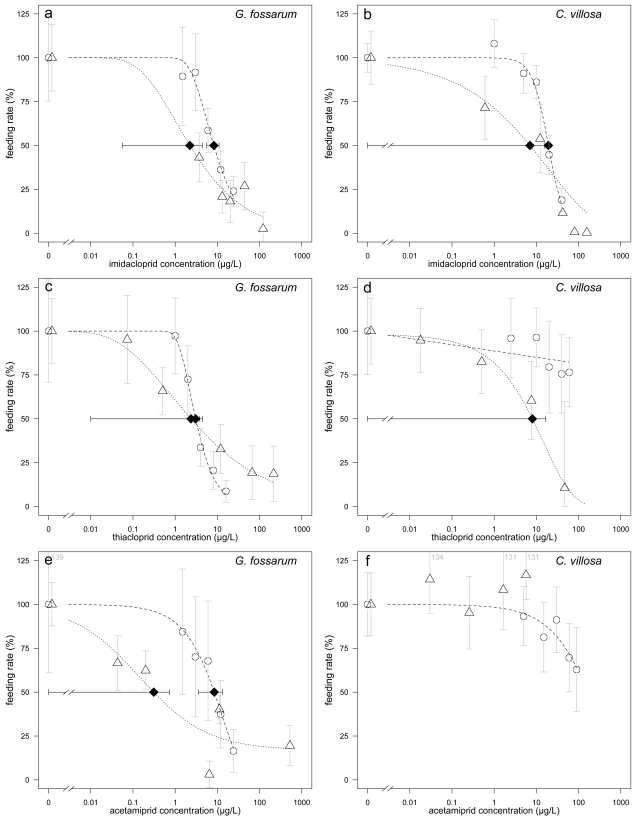

Figure 1. Relative feeding rate (±95%CIs) of *G. fossarum* (a, c, e) and *C. villosa* (b, d, f) subjected to waterborne (circles) or combined (= waterborne + dietary; triangles) exposure towards IMI (a,b), THI (c,d) and ACE (e,f). The best fitting concentration-response model for waterborne (dashed line) and combined (dotted line; except in f) exposure as well as corresponding EC_{50}-values (±95%CIs; solid diamonds) are displayed.

Table 1. Nominal and measured (after 7 d; ±SE; *n*=3) neonicotinoid water concentrations for feeding activity experiments assessing waterborne exposure as well as residues measured in leaves (prior to the experiments; ±SE; *n*=3) and water concentrations (after 7 d; ±SE; *n*=3) for feeding activity experiments assessing the combined exposure pathway. Moreover, literature values regarding effects of neonicotinoids on feeding and mortality of *Gammarus* are displayed.

	waterborne exposure				combined exposure				literature data		
	G. fossarum		*C. villosa*				*G. fossarum*	*C. villosa*			
	nominal concentration (µg/L)	measured concentration (µg/L)	nominal concentration (µg/L)	measured concentration (µg/L)	dose applied to trees (g AI/cm DBH)	foliar residues (µg/g)	measured concentration (µg/L)	measured concentration (µg/L)	species	mortality (LC_x) or feeding (EC_x)	reference
imidacloprid	0	<LOD	0	<LOD	0	<LOD	<LOD	<LOD	*G. pulex*	96 h LC_{50}: 270 µg/L	46
	1.5	1.30 ± 0.88	1	1.10 ± 0.16	0.0375	1.67 ± 0.16	3.71 ± 0.04	0.61 ± 0.20	*G. pulex*	96 h LC_{50}: 263 µg/L	47
	3	n.a.[b]	5	n.a.	0.15	38.73 ± 8.58	13.04 ± 4.95	12.29 ± 5.78	*G. pulex*	96 h LC_{10}: 99 µg/L	47
	6	n.a.	10	n.a.	0.6	148.86 ± 16.55	20.13 ± 5.96	42.20 ± 8.71	*G. pulex*	96 h EC_{50}: 5.34 µg/L	49
	12	n.a.	20	n.a.	2.4	297.14 ± 98.38	44.04 ± 0.71	80.37 ± 8.93			
	24	23.67 ± 0.86	40	44.61 ± 7.15	9.6	473.46 ± 55.80	121.49 ± 11.36	156.74 ± 20.36			
thiacloprid	0	<LOD	0	<LOD	0	<LOD	<LOD	<LOD	*G. pulex*	96 h LC_{50}: 350 µg/L	46
	1	1.03 ± 0.07	2.5	2.89 ± 0.22	0.0375	0.44 ± 0.14	0.07 ± 0.01	0.02 ± 0.02	*G. fossarum*	>50% inhibition at ≤5 µg/L (96 h)	37
	2	n.a.	10	n.a.	0.15	3.00 ± 1.51	0.51 ± 0.43	0.51 ± 0.43	*G. fossarum*	>50% inhibition at ≤5 µg/L (7 d)	24
	4	n.a.	20	n.a.	0.6	29.23 ± 14.69	11.97 ± 5.67	7.12 ± 5.43	*G. fossarum*	7 days EC_{50}: 3.02–3.65 µg/L	50
	8	n.a.	40	n.a.	2.4	1056.79 ± 293.20	66.76 ± 23.63	47.11 ± 4.57			
	16	16.33 ± 0.71	60	n.a.	9.6	1868.32 ± 547.94	215.62 ± 18.41	162.51 ± 50.10			
acetamiprid	0	<LOD	0	<LOD	0	<LOD	<LOD	<LOD	*G. pulex*	96 h LC_{50}: 50 µg/L	46
	1.5	1.37 ± 0.07	5	5.53 ± 0.20	0.0375	2.76 ± 0.66	0.20 ± 0.19	0.25 ± 0.25			
	3	n.a.	15	n.a.	0.15	4.80 ± 1.86	0.04 ± 0.01	0.03 ± 0.02			
	6	n.a.	30	n.a.	0.6	140.98 ± 35.70	11.18 ± 2.13	5.70 ± 1.01			
	12	n.a.	60	n.a.	2.4	121.95 ± 16.29	6.46 ± 1.13	1.64 ± 0.62			
	24	24.63 ± 0.68	90	85.14 ± 4.90	9.6	2943.06 ± 263.93	524.34 ± 67.89	n.t.[c]			

[a]Moreover, literature values regarding effects of neonicotinoids on feeding and mortality of *Gammarus* are displayed. [b]n.a.: not analyzed. [c]n.t.: not tested.

Table 2. 7-d EC_{20} and EC_{50}-values as well as LC_{20} and LC_{50}-values (±95%CIs; in μg/L) of *G. fossarum* and *C. villosa* derived from feeding activity experiments under waterborne and combined exposure. EC_xs printed in bold indicate a statistically significant difference between the waterborne and combined exposure scenario.

		EC_{20} ± 95% CIs		EC_{50} ± 95% CIs		LC_{20} ± 95% CIs		LC_{50} ± 95% CIs	
		waterborne	combined	waterborne	combined	waterborne	combined	waterborne	combined
G. fossarum	imidacloprid	**3.63 ± 1.69**	**0.40 ± 0.75**	**8.26 ± 2.68**	**2.23 ± 2.17**	n.o.	n.o.	n.o.	n.o.
	thiacloprid	**1.66 ± 0.53**	**0.20 ± 0.22**	3.06 ± 0.76	2.37 ± 2.12	n.o.	33.10 ± 30.75	n.o.	n.o.
	acetamiprid	2.28 ± 2.74	0.02 ± 0.02	8.43 ± 4.88	**0.31 ± 0.42**	21.34 ± 12.62	328.62 ± 4192.77	n.o.	n.o.
C. villosa	imidacloprid	**10.45 ± 2.31**	**0.32 ± 0.76**	**19.35 ± 2.65**	**7.05 ± 8.37**	n.o.	3.72 ± 5.27	n.o.	11.45 ± 5.72
	thiacloprid	n.o.	1.20 ± 4.10	n.o.	8.06 ± 8.69	n.o.	10.35 ± 1.93	n.o.	21.60 ± 2.53
	acetamiprid	34.96 ± 28.01	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.

[a] EC_xs printed in bold indicate a statistically significant difference between the waterborne and combined exposure scenario. [b] n.o. = not observed within the range of concentrations tested.

FOOD SELECTION ASSAYS

The meta-analysis conducted with the food selection assays' data revealed neither preferential feeding on neonicotinoid-free nor on any neonicotinoid-contaminated leaf discs when pooling data among shredder species and neonicotinoids (mean difference in effect size: ~7%; $n = 15$; $p = 0.14$; Figure 2). These observations indicate an inability of shredders from different taxonomic groups to avoid dietary neonicotinoid exposure. Although a statistically significantly difference in the feeding on the neonicotinoid-contaminated and on the control leaf discs was observed in some of the food selection assays, the statistically significant cases are randomly distributed across neonicotinoids and shredder species (Figure 2). However, when the data was separated by the three neonicotinoid compounds, a small but consistent significant tendency towards THI-free leaf discs was detected across all experiments with *G. fossarum* and *C. villosa* (mean difference in effect size: ~19%; $n = 4$; $p < 0.001$; Figure S4). Whether this indicates an active avoidance of the insecticide or only of additives contained in the commercial product applied needs to be examined in future studies.

Overall, the food selection assays were in line with the findings of Kreutzweiser et al. (2009) who detected no preferential feeding on IMI-contaminated leaves for larvae of the stonefly *P. dorsata* or the crane fly *Tipula* sp. over 14 days. Though in contrast to their study, waterborne or dietary neonicotinoid exposure most likely played only a

minor role in the outcome of the present study considering the relatively short exposure period (24 h) during which organisms were allowed to feed upon leaf discs as well as the relatively low neonicotinoid water concentrations (Table S1), which were well below 7-d EC_{50}s derived from the feeding activity experiments (Figure 1; Table 2).

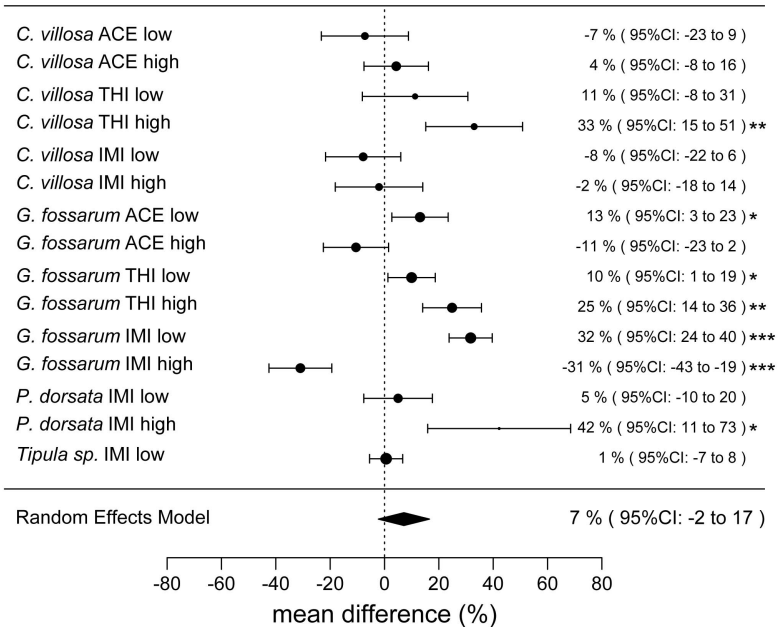

Figure 2. Relative mean difference (±95%CIs) in leaf consumption of *G. fossarum* and *C. villosa* as well as *P. dorsata* and *Tipula* sp. (published by Kreutzweiser et al., 2009) obtained by a random-effects meta-analysis of food selection assays where organisms had the choice between neonicotinoid-free and neonicotinoid-contaminated leaf discs. Means at the right side of the middle line indicate a higher consumption of neonicotinoid-free leaf discs, while means at the left side indicate a higher consumption of contaminated discs. Point sizes indicate the weight (= inverted variance) of the respective experiment to the overall effect. Organisms consumed statistically significantly more of one of the food types if CIs do not include zero (dotted line).

ECOLOGICAL CONSEQUECES

The inability of shredders to avoid neonicotinoid-contaminated leaves implies the possibility of dietary neonicotinoid exposure if organisms encounter and consume contaminated leaves recently introduced into surface waters, for instance, during autumn leaf fall (cf. Kreutzweiser et al., 2009). As indicated by our feeding activity experiments, dietary uptake may – in addition to waterborne exposure (Agatz et al., 2014; Kreutzweiser et al., 2008b) – hamper energy acquisition of shredders with potential

consequences for their population development (Baird et al., 2007) and ultimately their contribution to the leaf litter breakdown process (Bundschuh et al., 2011). The reduced energy intake observed – together with other possible adverse effects of neonicotinoids on shredders' physiology (i.e., on their energy reserves; Nyman et al., 2013) or inter-specific interaction (e.g., predator-prey relationships; Englert et al., 2012) – may induce shifts in vertical interaction within food webs. Moreover, reduced leaf processing may limit the provisioning of feces, thereby indirectly restricting the food supply for collecting invertebrates (Cummins & Klug, 1979) of local and downstream communities.

The results of our experimental approach (i.e., comparing waterborne with combined exposure) support the presumed relevance of the often neglected dietary exposure pathway for hydrophilic substances such as neonicotinoids (but see for hydrophobic substances e.g., Pristed et al., 2016). To uncover the relative importance of dietary neonicotinoid exposure in further detail, future experiments may make use of flow-through systems simulating more field-relevant conditions, namely a continued downstream transport of neonicotinoids leaching from contaminated leaves while shredders still can ingest contaminated leaves. The input of neonicotinoid-contaminated plant material into surface waters, and thus their relevance as a food source for shredders, might become even more relevant in the future. In particular, the rising impact of native and invasive pests, predicted under climate change scenarios (Ramsfield et al., 2016) may be accompanied by an intensified application of chemical control agents (such as neonicotinoid insecticides) as countermeasure. Therefore, including dietary exposure during the registration of systemic insecticides would be a sensible step forward in safeguarding ecosystem integrity.

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SUPPORTING INFORMATION

NEONICOTINOID WATER CONCENTRATIONS IN FOOD SELECTION ASSAYS

Table S1. Neonicotinoid water concentrations (in μg/L; ±SE; n=3) measured at the end of the food selection assays.

	Imidacloprid		Thiacloprid		Acetamiprid	
Treatment	Low	High	Low	High	Low	High
Dose applied to trees (g AI/cm DBH)	0.0375	0.15	0.15	0.6	0.15	0.6
G. fossarum	0.01±0.01	1.74±1.13	< LOD	< LOD	0.05±0.02	1.55±0.98
C. villosa	0.02±0.01	0.25±0.1	< LOD	1.27±0.65	0.01±0.01	0.37±0.24

CONCENTRATION-RESPONSE MODEL PARAMETERS

Table S2. Parameters of concentration-response models fitted to the feeding rate and mortality data obtained in feeding activity experiments with *G. fossarum* and *C. villosa*.

		Neonicotinoid	Endpoint	Model	Parameters
Waterborne exposure	*G. fossarum*	Imidacloprid	feeding	Weibull (type 2 with 2 parameters; upper limit = 1)	b=-1.02401; e=5.77745
			mortality	n.a.	n.a.
		Thiacloprid	feeding	Weibull (type 2 with 2 parameters; upper limit = 1)	b=-1.38174; e=2.34840
			mortality	n.a.	n.a.
		Acetamiprid	feeding	Weibull (type 1 with 2 parameters; upper limit = 1)	b=0.86572; e=12.88601
			mortality	Log-normal (2 parameters; upper limit = 1)	b=0.70349; e=70.60085
	C. villosa	Imidacloprid	feeding	Log-logistic (2 parameters; upper limit = 1)	b=2.24934; e=19.34962
			mortality	n.a.	n.a.
		Thiacloprid	feeding	Weibull (type 1 with 3 parameters)	b=0.016583; d=2.558709; e=0.029837
			mortality	n.a.	n.a.
		Acetamiprid	feeding	Weibull (type 1 with 2 parameters; upper limit = 1)	b=0.73415; e=269.67946
			mortality	n.a.	n.a.
Exposure to contaminated leaves	*G. fossarum*	Imidacloprid	feeding	Weibull (type 2 with 2 parameters; upper limit = 1)	b=-0.49048; e=1.05657
			mortality	n.a.	n.a.
		Thiacloprid	feeding	Weibull (type 2 with 2 parameters; upper limit = 1)	b=-0.343004; e=0.812934
			mortality	Log-logistic (2 parameters; upper limit = 1)	b=-0.418685; e=907.243901
		Acetamiprid	feeding	Log-logistic (3 parameters; upper limit = 1)	b=0.546210; c=0.169134; e=0.146112
			mortality	Log-logistic (3 parameters; upper limit = 1)	b=-3.048729; c=0.045999; e=564.159335
	C. villosa	Imidacloprid	feeding	Weibull (type 1 with 2 parameters; upper limit = 1)	b=0.36601; e=19.17975
			mortality	Weibull (type 2 with 2 parameters; upper limit = 1)	b=1.00897; e=16.46242
		Thiacloprid	feeding	Weibull (type 1 with 3 parameters)	b=0.626131; d=0.981464; e=15.110958
			mortality	Weibull (type 2 with 2 parameters; upper limit = 1)	b=1.540097; e=27.408515
		Acetamiprid	feeding	n.a.	n.a.
			mortality	n.a.	n.a.

n.a.: not assessed

DIFFERENCES IN CONCENTRATION-RESPONSE CURVE PROGRESSION BETWEEN EXPOSURE PATHWAYS

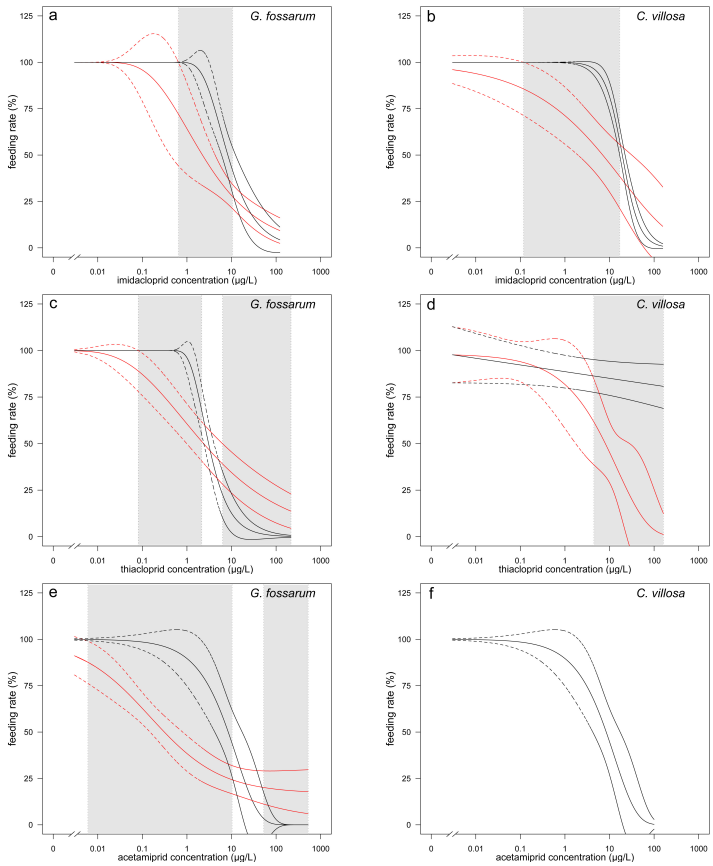

Figure S1. Modeled feeding rate (in percent relative to the control) of *G. fossarum* (a, c, e) and *C. villosa* (b, d, f) subjected to waterborne (black solid line) or combined (red solid line) exposure towards IMI (a, b), THI (c, d) and ACE (e, f). Hatched lines indicate 95% CIs. Gray areas denote concentration ranges where curves differ statistically significantly ($p < 0.05$). Since no concentrations-response curve could be derived for the feeding rate of *C. villosa* during the ACE combined exposure experiment, a comparison was obsolete.

The concentration-response curves obtained for the test organisms' feeding rate under waterborne or combined neonicotinoid exposure were compared for statistically significant differences ($p < 0.05$) by using the variability associated with the underlying concentration-response models. These were compared in 1 ng/L-steps using the "comped"-function available in the R-package "drc" (Ritz & Streibig, 2005) based on the method described by Wheeler et al. (2006). The results are visualized in Figure S1.

LEAF CONSUMPTION OF TEST ORGANISMS IN CONTROL TREATMENTS

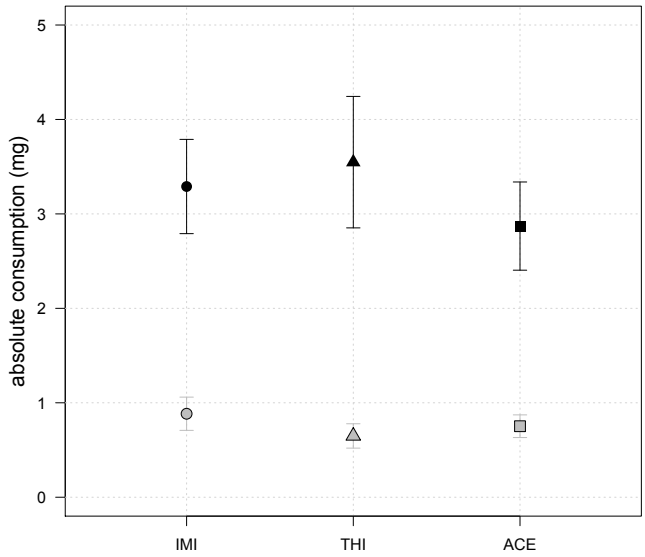

Figure S2. Mean absolute consumption (±95%CIs) and mean feeding rate (±95%CIs) of *G. fossarum* (gray) and *C. villosa* (black) on control leaves observed in the combined exposure experiments with IMI (●), THI (▲) and ACE (■).

MORTALITY OF *G. FOSSARUM* AND *C. VILLOSA* OBSERVED IN FEEDING ACTIVITY EXPERIMENTS

Table S3. Mortality (in %) of *G. fossarum* and *C. villosa* observed in feeding activity experiments under waterborne or combined exposure towards IMI, THI and ACE.

	Waterborne exposure				**Combined exposure**		
	G. fossarum		*C. villosa*			*G. fossarum*	*C. villosa*
	Nominal concentration (µg/L)	*Mortality (%)*	Nominal concentration (µg/L)	*Mortality (%)*	Dose applied to trees (g AI/cm DBH)	*Mortality (%)*	*Mortality (%)*
Imidacloprid	0	3	0	0	0	3	0
	1.5	3	1	0	0.0375	0	10
	3	0	5	0	0.15	0	50
	6	0	10	0	0.6	3	97
	12	0	20	0	2.4	10	93
	24	3	40	0	9.6	7	97
Thiacloprid	0	10	0	0	0	0	0
	1	0	2,5	0	0.0375	0	3
	2	0	10	0	0.15	7	0
	4	13	20	0	0.6	17	13
	8	17	40	3	2.4	20	90
	16	3	60	7	9.6	37	100
Acetamiprid	0	3	0	0	0	7	0
	1.5	0	5	0	0.0375	3	0
	3	10	15	0	0.15	3	3
	6	0	30	0	0.6	3	0
	12	10	60	0	2.4	7	0
	24	23	90	0	9.6	47	n.t.

n.t.: not tested

CALCULATION OF EFFECT CONCENTRATIONS BASED ON TIME-WEIGHTED AVERAGE WATER CONCENTRATIONS

The leaching dynamics published for IMI-contaminated ash leaves (Kreutzweiser et al., 2007) as well as IMI water concentrations (measured at the end of the 7-d lasting feeding activity experiment) were used to predict IMI water concentrations over the course of IMI feeding activity experiments assessing the combined exposure scenario (Figure S2). Time weighted average concentrations were calculated for each treatment and used to fit alternative concentration-response models (using the R extension package "drc"; Ritz & Streibig, 2005) to the feeding data. The model fitting the data best was selected based on Akaike's Information Criterion as well as visual inspection (*Gammarus*: Weibull type 2 with 2 parameters; b=-0.49048; e=0.73318; *Chaetopteryx*: Weibull type 1 with 2 parameters; b=0.36601; e=13.30933). EC_{50}-values were finally calculated for *Gammarus* and *Chaetopteryx* as displayed in Table S4.

Table S4. 7-d EC_{50}-values (±95%CIs; in µg/L) of *G. fossarum* and *C. villosa* derived from feeding activity experiments under waterborne and combined IMI exposure. For the combined exposure scenario, 7-d EC_{50}-values were calculated based on maximum IMI concentrations (measured after 7 d) and time weighted average concentrations, respectively.

EC_{50} ±95% CIs based on:		
Waterborne exposure experiment:	*G. fossarum*	*C. villosa*
Nominal water concentrations	8.26±2.68	19.35±2.65
Combined exposure experiment:		
Maximum water concentrations (after 7 d)	2.23±2.17	7.05±8.37
Time weighted average water concentrations	1.55±1.51	4.89±5.74

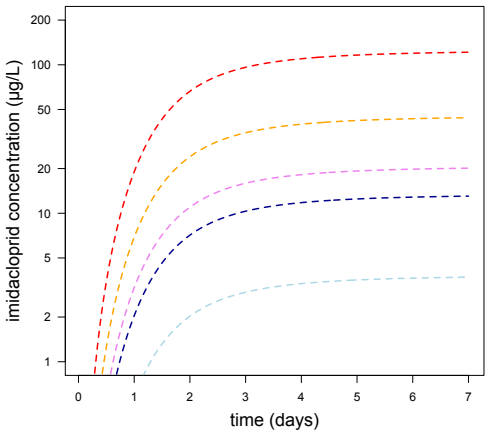

Figure S3. IMI water concentrations (leaching from contaminated alder leaves) predicted for the different treatments of the feeding activity experiment assessing the combined exposure for *Gammarus*: light blue: 0.0375; dark blue: 0.15; violette: 0.6; orange: 2.4 and red: 9.6 g AI/cm DBH.

META-ANALYSIS OUTPUT FOR FOOD SELECTION ASSAYS WITH THI

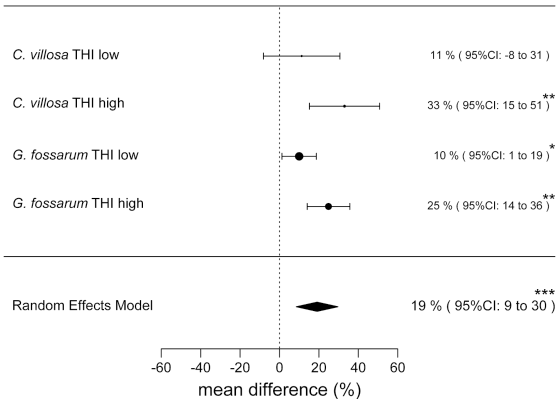

Figure S4. Relative mean difference (±95%CIs) in leaf consumption of *G. fossarum* and *C. villosa* obtained by a random-effects meta-analysis of food selection assays where organisms had the choice between THI-free and THI-contaminated leaf discs. Means at the right side of the middle line indicate a higher consumption of THI-free leaf discs, while means at the left side indicate a higher consumption of contaminated discs. Point sizes indicate the weight (= inverted variance) of the respective experiment to the overall effect. Organisms consumed statistically significantly more of one of the food types if CIs do not include zero (dotted line).

LITERATURE CITED IN THE SUPPORTING INFORMATION

Kreutzweiser, D.; Good, K.; Chartrand, D.; Scarr, T.; Thompson, D., Non-target effects on aquatic decomposer organisms of imidacloprid as a systemic insecticide to control emerald ash borer in riparian trees. *Ecotoxicol. Environ. Saf.* **2007**, *68*, 315-325.

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APPENDIX A.3

Relative importance of dietary uptake and waterborne exposure for a leaf-shredding amphipod exposed to thiacloprid-contaminated leaves

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ABSTRACT

Systemic neonicotinoids are commonly used in forest pest management programs. Senescent leaves containing neonicotinoids may, however, fall from treated trees into nearby streams. There, leaf-shredding invertebrates are particularly exposed due to their diet (feeding on neonicotinoid-contaminated leaves) or collaterally via the water phase (leaching of a neonicotinoid from leaves) – a fact not considered during aquatic environmental risk assessment. To unravel the relevance of these pathways we used leaves from trees treated with the neonicotinoid thiacloprid to subject the amphipod shredder *Gammarus fossarum* for 21 days (n=40) either to dietary, waterborne or a combined (dietary + waterborne) exposure. Dietary exposure caused – relative to the control – similar reductions in gammarids' leaf consumption (~35%) and lipid content (~20%) as observed for the waterborne exposure pathway (30 and 22%). The effect sizes observed under combined exposure suggested additivity of effects being largely predictable using the reference model "independent action". Since gammarids accumulated – independent of the exposure pathway – up to 280 ng thiacloprid/g, dietary exposure may also be relevant for predators which prey on *Gammarus*. Consequently, neglecting dietary exposure might underestimate the environmental risk systemic insecticides pose for ecosystem integrity calling for its consideration during the evaluation and registration of chemical stressors.

INTRODUCTION

Neonicotinoids are one of the most widely used insecticides class worldwide (Jeschke et al., 2011). Their tremendous success is due to multiple factors: Firstly, their systemic action facilitates a rapid uptake and distribution in plants and allows for a broad range of application methods thereby reducing the total amount of insecticide needed to be applied (Jeschke & Nauen, 2008; Jeschke et al., 2011). Moreover, their broad-spectrum insecticidal properties specifically target insects' nicotinic acetylcholine receptors while being

considerably less toxic for vertebrates (Tomizawa & Casida, 2003). In addition, neonicotinoids replaced insecticides (e.g., organophosphates) that were banned or withdrawn from the market as a result of pest resistance management or increasing regulatory hurdles (Jeschke et al., 2011). Neonicotinoids have, however, raised concerns in the past due to their impact on non-target organisms, in particular pollinators (Blacquiere et al., 2012; Pisa et al., 2015; Sanchez-Bayo, 2014). As a consequence, three neonicotinoids (imidacloprid, clothianidin, thiamethoxam) were temporarily banned for certain applications within the European Union (European Commission, 2013). Currently, the European Food Safety Authority is conducting a re-evaluation of the risks these compounds pose for pollinators (EFSA, 2017). Furthermore, the impact of the first neonicotinoid insecticide, i.e., imidacloprid, on aquatic systems has recently been evaluated in the European Union (EFSA, 2014), the United States (USEPA, 2016) and Canada (Health Canada, 2016), since neonicotinoids' frequent use, environmental persistence and physico-chemical properties favour their off-site transport via spray drift, surface runoff (de Perre et al., 2015) and wastewater discharge (Hladik & Kolpin, 2016; Münze et al., 2017). Aquatic invertebrate populations and communities are particularly at risk from short-term (Beketov & Liess, 2008a; Liess & Beketov, 2011) or chronic (Nyman et al., 2013; Roessink et al., 2013) neonicotinoid exposure at environmentally relevant levels in surface waters.

Although waterborne exposures are considered the most relevant for neonicotinoids, their systemic nature facilitates a second path that has received little attention in scientific literature and seems ignored during their aquatic environmental risk assessment so far: Dietary exposure through the consumption of neonicotinoid-contaminated plant material. This material may consist of crop post-harvest detritus left on fields (Rosi-Marshall et al., 2007; Tank et al., 2010) as well as senescent leaves falling from neonicotinoid-treated deciduous trees (Englert et al., 2017a; Kreutzweiser et al., 2007) entering adjacent water bodies through

lateral movement or vertical fall (Abelho, 2001). In particular leaf-shredding invertebrates (= shredders) – which heavily rely on leaf litter as food source (Cummins & Klug, 1979) – might be vulnerable through this pathway. Such dietary exposure might further coincide with exposure via the water phase driven, for instance, by neonicotinoid re-mobilization (i.e., leaching) from leaves into water (Englert et al., 2017a; Kreutzweiser et al., 2007). The relative importance of these exposure pathways (dietary vs. waterborne) has, however, not yet been disentangled, though the significance of dietary exposure under a combined exposure scenario has been suggested in an earlier publication (Englert et al., 2017b).

Therefore, the present study aimed to unravel the relevance of these pathways by subjecting the shredder *Gammarus fossarum* (KOCH) – an amphipod frequently used in non-standard aquatic toxicity studies (Kunz et al., 2010) – for 21 days to leaves from neonicotinoid-treated black alder trees, using thiacloprid (THI) as a model substance. These leaves served as the only neonicotinoid source: gammarids either faced dietary exposure – i.e., feeding on THI-contaminated leaves while a flow-through system prevented THI from accumulating in the water phase – waterborne exposure (through leaching of THI from leaves) or combined exposure (i.e., dietary + waterborne). Besides the test organisms' survival, leaf consumption and THI body burden, gammarids' body weight and lipid content were determined as a proxy for their energy reserves and physiological fitness (Koop et al., 2008). Based on our previous work (Englert et al., 2017b), we expected the most severe effects in the combined exposure scenario, while dietary exposure alone was hypothesized to be less or equally important as waterborne exposure. In case the effects induced by combined exposure turned out to exceed the effect sizes induced by each individual exposure pathway, we further assumed that they could be predicted – although consisting of two exposure pathways instead of a mixture of different chemicals – by one of the most commonly used reference models, namely "independent action" (IA; Bliss, 1939).

Thereby, the present work assessed the relevance of a pathway – i.e., dietary exposure – that is not well reflected in current aquatic risk assessment of neonicotinoids or systemic pesticides in general.

METHODS

SOURCE OF PLANT MATERIAL, NEONICOTINOID APPLICATION AND PREPARATION OF LEAF DISCS

The procedure used to generate THI-free and THI-contaminated black alder leaves for the present study is described in detail in Englert et al. (2017a). Briefly, black alder trees were soil drenched in June 2014 with either 500 mL tap water or 500 mL tap water containing the neonicotinoid product Calypso® (40% THI; Bayer CropScience GmbH, Langenfeld, Germany; dose: 0.6 g THI/cm trunk diameter at breast height). Leaves were collected in October 2014, shortly before leaf fall, and stored frozen at -20°C until further use. Leaf discs (diameter = 1.0 cm) were cut from leaves using a cork borer, freeze-dried and subsequently weighed to the nearest 0.01 mg to determine their initial dry weight.

TEST ORGANISMS

As described in Bundschuh et al. (2011a), adult *G. fossarum* of 6-8 mm body length and visibly free of macro-parasites were collected one week prior to the start of the experiments from the stream Hainbach (49°14'N; 8°03'E). The stream is located in the Palatinate forest upstream of any settlement and agricultural activity and the surrounding forest has no history of neonicotinoid use. Pre-exposure of gammarids towards neonicotinoids is therefore likely negligible. Since the present study was conducted during gammarids' reproductive rest (October to November; Pöckl et al., 2003), organisms could not be separated by sex. Consequently, both male and female gammarids were used. This procedure might increase the variability in the endpoints investigated but also the study's relevance for effects at the population level. Seven days prior to the start of the bioassay, organisms were kept in aerated

stream water at 16±1°C, fed *ad libitum* with black alder leaves and gradually adapted to SAM-S5 medium (= test medium; Borgmann, 1996).

BIOASSAY DESIGN

Each replicate consisted of a 250 mL glass beaker filled with 200 mL test medium. Each beaker was equipped with two cages made of stainless steel mesh (mesh size: 0.5 mm) – a cuboid shape cage at the bottom (4.0 cm x 4.0 cm x 0.5 cm) and a cylindrical shape cage above (height: 8.0 cm, diameter: 5.5 cm) – that were separated by a watch glass (diameter: 6.0 cm; Fig. 1a; cf. Zubrod et al., 2011). The bottom cage contained three leaf discs that were protected from organisms' feeding and allowed controlling for abiotic and microbial leaf mass loss. The upper cage contained one *G. fossarum* and three discs cut from black alder leaves as food. The different exposure scenarios were realized by manipulating the position of THI-free and THI-contaminated leaf discs in these cages as follows: For the control treatment, THI-free leaf discs were placed in both the upper and the bottom cage. Gammarids in the waterborne exposure treatment were allowed to feed on the THI-free leaf discs placed in the upper cage, while THI gradually leached from THI-contaminated leaf discs, which were placed in the bottom cage. For the combined exposure treatment, in contrast, THI-free leaf discs were placed in the bottom cage while in the upper cage THI-contaminated leaf discs served as food for *Gammarus*. As Kreutzweiser et al. (2007) reported only a marginal accumulation of another neonicotinoid (i.e. imidacloprid) with similar physico-chemical properties as THI in leaf material when applied via the water phase, the diet-related uptake of THI during the waterborne exposure treatment is considered negligible in the present study. The dietary exposure treatment was conducted – with THI-contaminated discs in the upper cage and THI-free discs in the bottom cage – using a flow-through system, which continuously (~45 times/d) renewed the test medium and kept THI water concentrations at negligible levels (i.e., below the LOQ; Fig. 2). A separate control treatment, accounting for any potential

effects of the water renewal process itself, was also set up. Each of these five treatments was replicated 40 times. A scheme of the experimental design is illustrated in Fig. 1b.

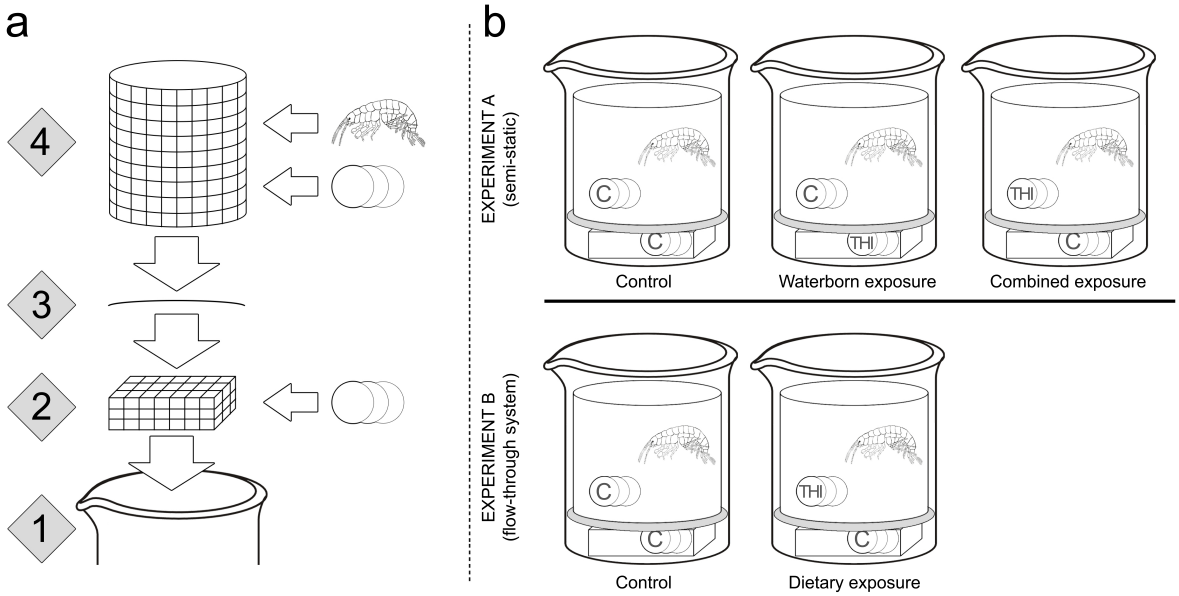

Figure 1. Scheme illustrating the setup of the bioassay. a) At the bottom of a (1) 250-mL glass beaker filled with 200 mL test medium a (2) cuboid shape cage, containing three leaf discs, was situated. A (3) watch glass placed on top of the cuboid cage separated the latter from a cylindrical shape cage containing three leaf discs as well as one *G. fossarum*. b) The different exposure scenarios were realized by manipulating the position of THI-free (C) and THI-contaminated (THI) leaf discs in these cages. While the waterborne and combined exposure scenario used a semi-static regime, the dietary exposure scenario was realized using a flow-through system that kept THI water concentrations at negligible levels.

Beakers were placed in a climate controlled chamber at 16±1°C in total darkness and aerated throughout the 21 day study duration. At weekly intervals, leaf discs in every treatment were renewed together with the test medium to maintain a continuous THI exposure. Thereby, cages allowed a gentle transfer of the test organisms to new vessels containing fresh test medium and leaf discs. Remaining leaf discs and any leaf tissue shredded off were removed from glass beakers, freeze-dried separately and weighed to the nearest 0.01 mg. At the same time, survival of gammarids was monitored. Gammarids were considered dead if no movement was observed after several gentle touches with the tip of a glass pipette. 10-mL water samples were taken daily (n = 4) during the first week as well as on the first, third and last day (n = 3) during following two weeks. Samples were stored at -20°C until further use.

At the termination of the experiment, gammarids were transferred to fresh test medium for 1 h to remove THI residues possibly adsorbed to their bodies' surface. Subsequently, test organisms were carefully blotted dry with a clean tissue, shock frozen in liquid nitrogen and stored in individual glass tubes at -75°C until further use.

THIACLOPRID QUANTIFICATION IN LEAVES, WATER AND GAMMARIDS

THI was extracted from alder leaves using an ASE™ 350 Accelerated Solvent Extractor system (Thermo Scientific™ Dionex™, Sunnyvale, CA; USA; Englert et al., 2017a). Separation of THI from leaf extracts and water samples was done with an ultrahigh performance liquid chromatography–mass spectrometry system equipped with an EQuan MAX system, while for quantification, a single quadrupole mass spectrometer equipped with an electrospray ionization source was used (Englert et al., 2017a). THI was identified at the accurate ion mass of m/z = 253.0309. External calibration with matrix-matched standards (prepared out of blank leaf extracts or test medium) was used. The limits of quantification (LOQ) and the limits of detection (LOD) for THI in leaf samples were 0.11 and 0.03 μg/g. For THI measured in water samples, the LOQ was defined as the lowest calibration level (i.e., 0.01 μg/L) due to the absence of signals in matrix-matched blank samples (Turnipseed et al., 2008).

THI was extracted from gammarids using the method of Inostroza et al. (2016). In brief, frozen gammarids were transferred into a 10 mL centrifuge tube, 1 mL of LC-MS grade acetonitrile, 1 mL of LC-MS grade water and 0.5 mL of LC grade hexane were added and the sample was homogenized using an UltraTurrax. Phase separation between water and acetonitrile was induced by addition of 400 mg of $MgSO_4$ and 100 mg of NaCl. The hexane phase was removed and the acetonitrile phase transferred into a 2 mL glass vial, evaporated to dryness and reconstituted in 500 μL of methanol:water (70:30). THI was analysed using liquid chromatography-high resolution mass spectrometry (Ultimate 2000 LC system coupled

to a QExactive Plus MS via a heated electrospray ionisation source, all from Thermo Scientific™). Separation was conducted using a methanol:water gradient (both eluents containing 0.1% of formic acid) on a Kinetex C18 EVO column (50x2.1 mm, 2.6 μm particle size, Phenomenex). THI was measured in full scan mode and quantified using the accurate mass of m/z = 253.0309. Method matched calibration was employed (i.e., spiked solvents were processed the same way as the gammarid samples) using imidacloprid-d4 as internal standard. The LOQ was 1 ng/g wet weight.

QUANTIFICATION OF GAMMARIDS' BODY WEIGHT AND LIPID CONTENT

For the determination of gammarids' body weight, animals were freeze-dried (for 24 h) and weighed to the nearest 0.01 mg. Subsequently, the lipid content of gammarids (n = 21-25) was analysed as described by Van Handel (1985) and modified by Zubrod et al. (2011) for use with a microplate reader (Tecan Infinite M200, Tecan Group, Crailsheim, Germany). After extraction with 1:1 chloroform:methanol (v:v), lipids reacted with sulfuric acid and vanillin-phosphoric acid reagent. For quantification of the lipid content, absorbance at 490 nm was measured and read against a standard curve prepared from commercially available soybean oil (Sojola Soja-Öl, Vandemoortele, Herford, Germany). Lipid content was finally normalized to gammarid dry weight (μg/mg gammarid).

CALCULATIONS AND STATISTICS

Gammarids' consumption of alder leaf discs (in mg leaf/animal/day; corrected for the abiotic and microbial leaf mass loss as determined from the bottom cages) was calculated for each week as described in Bundschuh et al. (2011a), neglecting the animals dry weight, as this was only measured at the termination of the experiment and would thus induce additional uncertainties. Moreover, the cumulative consumption (in mg leaf/animal/day) was calculated over the entire study duration (i.e., 21 day). In the remainder of this publication the term "leaf consumption" refers – if not indicated otherwise – exclusively to cumulative

consumption. Replicates in which gammarids managed to escape their cage or died were discarded from statistical analyses (except for survival).

Gammarids' cumulative leaf consumption, lipid content and body weight (as dry weight) were checked for normality by visual inspection and Shapiro-Wilk's test, while homoscedasticity was tested using Bartlett's test. To test for statistically significant differences relative to the corresponding control, Student's *t*-tests or, if assumptions for parametric testing were violated, non-parametric Wilcoxon rank-sum tests were conducted. Although our experimental design consisted of two separate experiments, formally rendering any alpha level adjustment for the control vs. dietary comparison unnecessary, alpha-corrections for three comparisons were applied in all cases using Bonferroni-Holm-adjustment. Since the interpretation of our data is mainly driven by effect sizes, this rather conservative approach further reduced type I errors, however, this hardly affected any drawn conclusions. Gammarids' survival was compared to the corresponding control using Chi-squared tests.

Moreover, results observed in the combined exposure treatment were tested for compliance with the reference model "independent action" (IA; Bliss, 1939). IA was calculated by multiplying the average mortality, leaf consumption, body weight or lipid content (as proportion of untreated controls) observed in the single exposure treatments. Although IA was originally designed for binominal responses (e.g., alive/dead) and probabilities, it can be used with gradual data that do not meet the theoretical assumptions of IA (Cedergreen et al., 2008).

Gammarids' THI body burdens were checked for normality and homoscedasticity as described above while analyses of variance followed by Tukey-test was used to test for statistically significant differences between the waterborne, dietary and combined exposure treatment. All null hypothesis significance tests were supplemented by 95% CIs (Altman et al., 2000) and are given in Supplementary Table S1. For all statistics and figures,

R version 3.1.1 for Mac was used. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

RESULTS

THI CONCENTRATIONS IN LEAVES AND WATER

THI residues in leaves quantified prior to the feeding experiment were 245.3±6.9 μg/g (mean ±standard error (SE); n = 3). During the first, second and third week, leaching of THI from leaves into water resulted in mean water concentrations of 3.4, 5.0 and 9.0 μg THI/L in the waterborne exposure scenario. Similarly, weekly mean water concentrations of 4.1, 2.7 and 4.3 μg THI/L were measured in the combined exposure scenario (Fig. 2). In contrast, THI water concentrations in the dietary exposure scenario barely exceeded the limit of quantification (LOQ = 0.01 μg/L; Fig. 2).

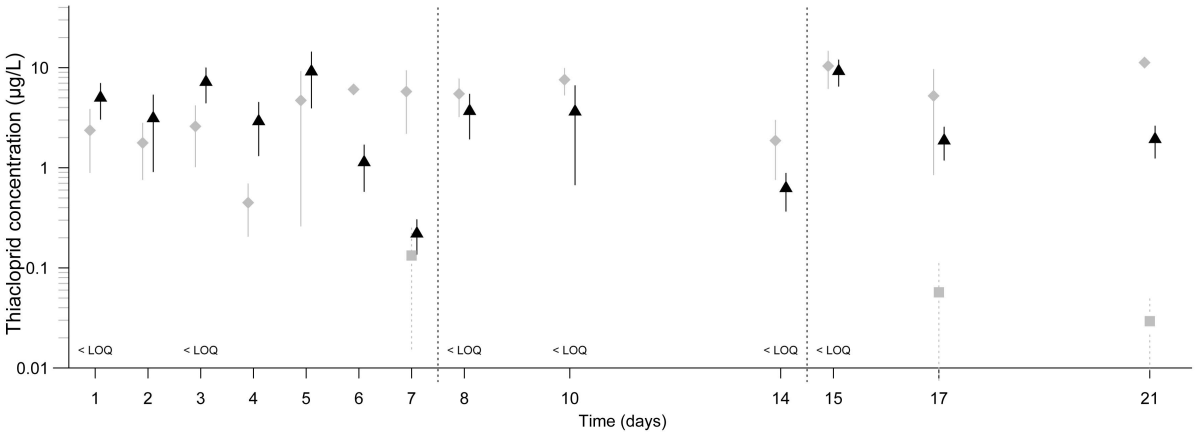

Figure 2. THI water concentrations (mean ± SE; n = 3-4) measured during the 21-day feeding experiment. ◆ marks concentrations of the waterborne, ■ the dietary and ▲ the combined exposure treatment, respectively. Except for three cases, THI water concentrations in the dietary exposure treatment were below the LOQ (0.01 μg/L)

SURVIVAL OF *G. FOSSARUM*

After 21 days, survival of *G. fossarum* experiencing waterborne (difference in proportions: -2.5%; Chi-squared test: p = 1) or dietary THI exposure (difference in proportions: 2.5%; Chi-squared test: p = 1; Fig. 3) deviated only marginally from the corresponding control. In the combined exposure scenario, a 20% reduced survival (compared to the corresponding control;

Chi-squared test: p = 0.15; Fig. 3) was observed, which deviated significantly from the IA model prediction, which suggested an effect size of approximately zero.

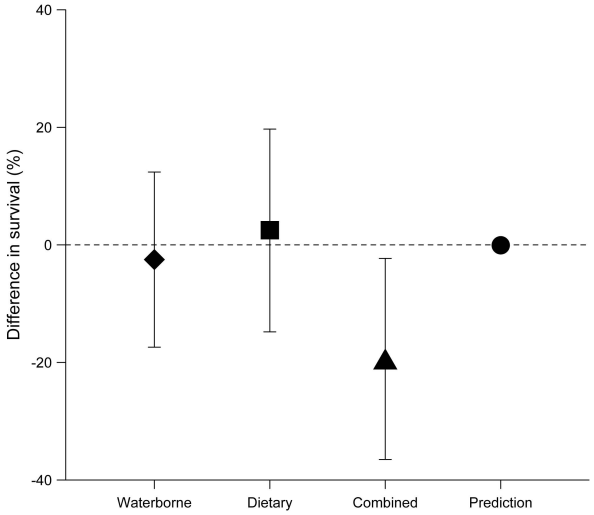

Figure 3. Mean difference in survival (±95% CIs) of *G. fossarum* (after 21 days; n = 40) exposed towards THI via different pathways. The prediction of the IA model is also displayed as a point estimate (●).

GAMMARIDS' LEAF CONSUMPTION

The cumulative leaf consumption of *G. fossarum* was significantly reduced by waterborne (by 30%; *t*-test: p < 0.001; n = 33/34) and dietary (by 36%; *t*-test: p < 0.001; n = 32/33; Fig. 4a) THI exposure relative to the corresponding control. Based on these results, the IA model predicted a 55% reduction in leaf consumption for the combined exposure, which corresponded well with the observed and statistically significant reduction of 49% (*t*-test: p < 0.001; n = 26/34; Fig. 4a).

GAMMARIDS' LIPID CONTENT & BODY WEIGHT

For gammarids exposed via the water phase, a non-significant decrease in lipid content and body weight (by 22 and 11%, respectively; *t*-test: p = 0.054/0.098; n = 21-22/29-30; Fig. 4b,c) was observed when compared to the control. The dietary exposure pathway decreased *G. fossarum*'s lipid content near-significantly by 19% (*t*-test: p = 0.054; n = 24/25), whereas animals' body weight was only slightly and non-significantly reduced (effect size: 6%; Wilcoxon rank-sum test: p = 0.263; n = 28/32; Fig. 4b,c). The by 26 and 21% reduced lipid

content and body weight (only statistically significant for the latter; *t*-test: p = 0.054/0.003; n = 21-22/22-30; Fig. 4b,c) of gammarids in the combined exposure treatment deviated slightly from the effect sizes predicted by the IA model (37 and 16%) but were still within the 95% confidence interval (CI) range.

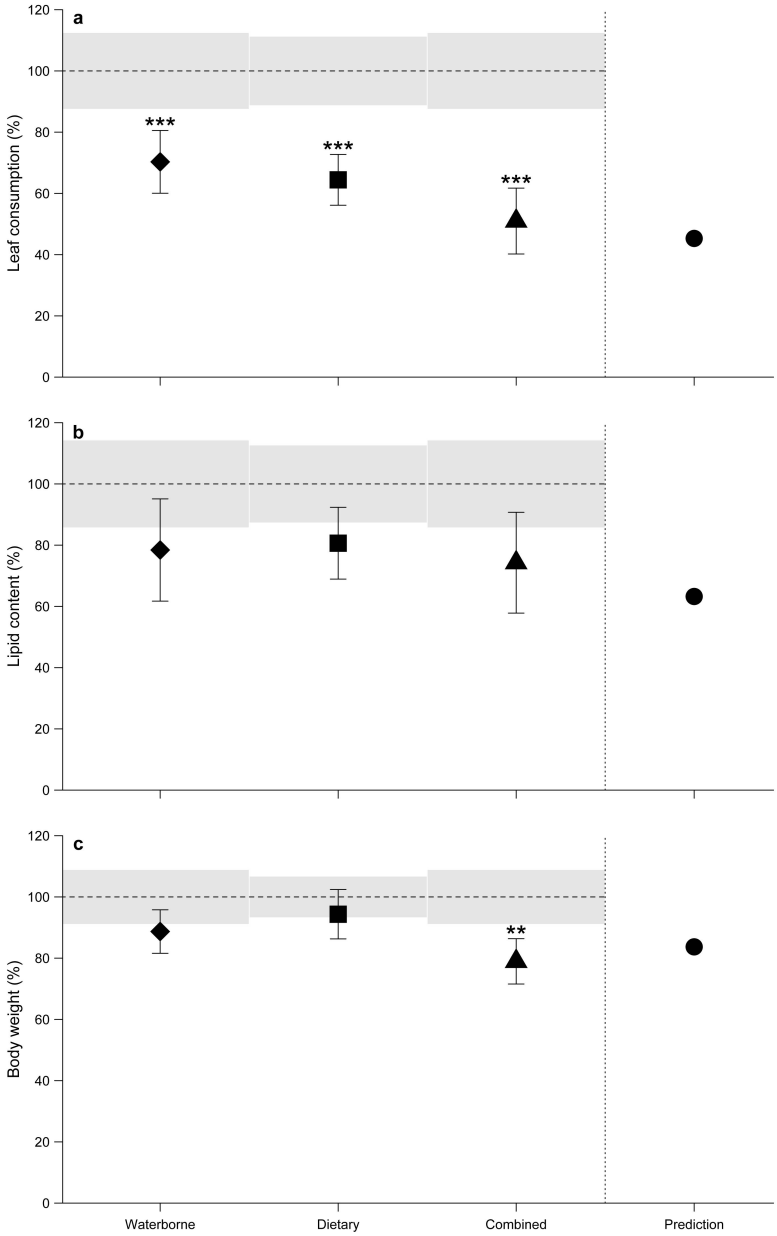

Figure 4. Mean (±95% CIs) gammarids' a) leaf consumption, d) lipid content and c) body weight relative to the corresponding control (dashed line) after 21 days exposure to THI. Grey areas indicate the 95% CIs of the corresponding control. Please note that the dietary exposure treatment was compared to a separate control due to the flow-through system used (see *Methods* section). The predictions derived from IA models are also indicated as point estimate (●). Asterisks denote significant differences compared to the respective control, $p < 0.01$ (**), $p < 0.001$ (***).

GAMMARIDS' THI BODY BURDEN

THI was detected in two out of five gammarids in the control treatment at levels marginally above the LOQ (1 ng/g), namely 9 and 11 ng THI/g wet weight. In contrast, animals exposed to THI for 21 days via the water phase, their diet or both displayed body burdens that exceeded the maximum residue found in gammarids of the control group by up to 25-fold, namely 279.9±46.4, 249.6±50.8 and 246.5±64.5 ng THI/g gammarid. Residue levels did not differ significantly between the three exposure scenarios (Tukey-test: $p \geq 0.90$; n = 4-5; Fig. 5).

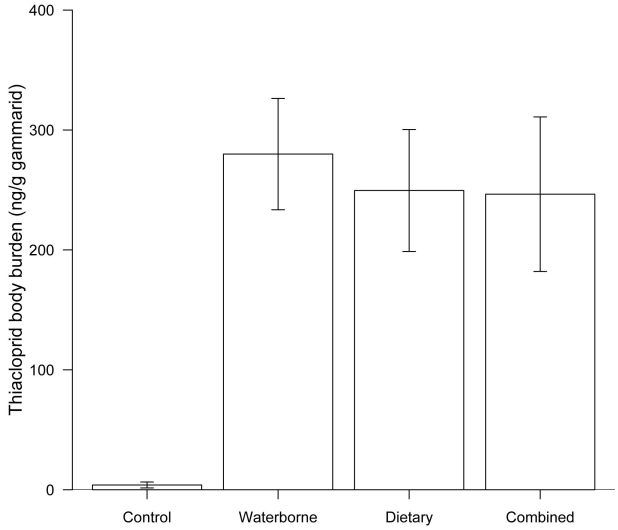

Figure 5. Mean THI body burden (±SE) in gammarids. Residues were measured (n = 4-5) after 21 days of THI exposure via water, diet or a combination of both.

DISCUSSION

The dietary pathway significantly reduced the leaf consumption (by 36%) and, though non-significant, the lipid content (by 19%) of *G. fossarum* to an extent comparable to effects observed under waterborne exposure (Fig. 4a,b) while gammarids' body weight was only marginally altered (Fig. 4c). As most research regarding neonicotinoids' effects on aquatic systems focused exclusively on waterborne exposure pathways (e.g., see those reviewed in Morrissey et al., 2015), studies solely examining dietary and thus excluding waterborne pathways are lacking. Therefore, the present study is, to the best of our knowledge, the first addressing this challenge by means of a flow-through system, which kept THI water

concentrations in the dietary exposure scenario below or only slightly above detectable levels (i.e., LOQ: 0.01 µg/L; Fig. 2). While the leaves' THI concentrations presumably declined throughout each 7-day exposure period (Kreutzweiser et al, 2007), aqueous THI concentrations might have temporarily been above the LOQ, particularly near the leaves' surface. The measured THI water concentrations, nonetheless, strongly suggest that the major share of the effects observed in the dietary exposure scenario was delivered via the consumption of THI-contaminated leaves.

Neither the waterborne exposure nor the dietary uptake of THI-contaminated leaves did, however, affect the survival of *G. fossarum* (Fig. 3). Whereas scientific literature lacks information regarding dietary neonicotinoid exposure on gammarids' survival, results observed in the waterborne exposure scenario of the present study (Fig. 3) are in accordance with former publications. Firstly, the weekly average THI water concentrations (Fig. 2) were, although highly variable presumably due to unequal spatial distribution of neonicotinoids in tree foliage (Dilling et al., 2010; Tanis et al., 2012), up to 95-fold below the 96-h median lethal concentration (i.e., LC_{50}: 320 µg THI/L) reported for *G. pulex* (Beketov & Liess, 2008b). Moreover, in one of our previous studies we observed no THI related mortality in *G. fossarum* after 7 days of waterborne exposure towards 16 µg/L as well as an 7-day LC_{20} of 33 µg THI/L under a combined exposure szenario (Englert et al., 2017b).

In the present study, both the dietary and waterborne exposure pathway induced a reduction in gammarids' leaf consumption (by 36 and 30%, respectively; Fig. 4a), a sublethal response regularly reported in literature as a consequence of exposure towards THI (Englert et al., 2012; 2017b; Feckler et al., 2012; Zubrod et al., 2017) or other neonicotinoids (Agatz et al., 2014; Englert et al., 2017b; Nyman et al., 2013). This reduced energy uptake (i.e., in the form of leaves; Fig. 4a) eventually caused the observed reduction (up to 22%) in *G. fossarums'* lipid reserves (Fig. 4b), a pattern also reported for *G. pulex* in response to a 21-

day imidacloprid exposure (Nyman et al., 2013). Additionally, the allocation of energy to detoxification processes (Gerami, 2013) or cellular repair processes (i.e., to counteract lipid peroxidation due to neonicotinoid induced oxidative stress; e.g., as shown for *G. fossarum*; Malev et al., 2012) might have contributed to the reduction in gammarids' lipid reserves (Fig. 4b).

While dietary as well as waterborne THI exposure caused comparable effects in *G. fossarum* (i.e., regarding survival, leaf consumption, lipid content and body weight; Fig. 2 & 3a-c), those induced by the combined exposure scenario exceeded in their magnitude – independent of the variable – those observed for each of the exposure pathways individually (Fig. 3 & 3a-c). Only gammarids' THI body burdens were at comparable levels irrespective of the exposure pathway (except for control animals; Fig. 5). Since THI body burdens in another amphipod species from a pesticide-impacted river, namely *Dikerogammarus* sp., did not exceed 0.39 ng THI/g (Inostroza et al., 2016), the concentrations found in two of our control animals (i.e., 9 and 11 ng THI/g) are likely attributed to minor laboratory cross-contamination after the termination of the experiment and not field-exposure preceding our study. Although neonicotinoids – as is expected based on their log P_{ow} (between -0.66 and 1.26; FOOTPRINT, 2017) – supposedly do not accumulate in organisms' tissues except for the nervous system (due to the insecticides' affinity for the nicotinic receptors), they are taken up to an increasing extent with rising aqueous neonicotinoid concentration and exposure time (Iturburu et al., 2017). After a few days of exposure, however, the uptake of THI into *G. fossarum* might plateau – as reported for imidacloprid in the mayfly nymph *Isonychia bicolor* after ~5 days (Camp & Buchwalter, 2016) – which likely explains the absence of any differences in gammarids' THI residue levels at the termination of our 21-day study (Fig. 5). Despite the comparable THI residue levels detected in gammarids exposed via the water phase, their diet or both (Fig. 5), the location where the neonicotinoid is

accumulated and, therefore, possibly the affected target site might differ depending on the exposure pathway. This may not only be relevant for neonicotinoids' trophic transfer to predators engulfing or merely piercing their prey (Brooks et al., 2009) but could also explain the mostly additive effects observed under combined exposure (Fig. 3 & 3a-c). The latter have largely been predictable in their magnitude by the IA model (for the variables leaf consumption, lipid content and body weight; see Fig. 4a-c), which assumes different molecular target sites to be affected (Kortenkamp & Altenburger, 2010). The only exception to this good conformity between predictions and observations was gammarids' survival, which was substantially underestimated by the model (Fig. 3). This deviation indicates a synergistic effect on gammarids' survival triggered by the combined exposure via both pathways, though the underlying mechanism remains unclear. Given the potential for additive or synergistic effects under a combined exposure scenario, as well as the adverse effects we observed in gammarids under dietary exposure alone (Fig. 4a,b), the risk posed by neonicotinoid-contaminated leaves falling into streams needs further consideration.

The vast number of studies reporting the introduction of neonicotinoids to surface waters via wastewater discharge (Münze et al., 2017), agricultural spray drift and surface runoff from crops (de Perre et al., 2015), as well as the numerous exceedances of regulatory acceptable concentrations (Szöcs et al., 2017) emphasize the relevance of waterborne neonicotinoid exposure for aquatic organisms. But also relatively pristine streams may receive neonicotinoids following application as part of forest pest management programs (Benton et al., 2017). Although the injection of neonicotinoids directly into the tree trunk may, in contrast to their soil application, limit the initial distribution of the insecticide within the environment (Kreutzweiser et al., 2008), leaves from neonicotinoid-treated trees might still, regardless of the pesticides' application method, be transported into nearby streams. There the relatively fast re-mobilization of leaf-associated neonicotinoids into water

(Kreutzweiser et al., 2007) may presumably limit the relevance of the dietary exposure to a few days after the leaves' introduction into the stream (Englert et al., 2017a). Moreover, depending on the neonicotinoid compound and the shredders' ability to detect contaminated food, dietary exposure might be avoided if an uncontaminated alternative is available (Englert et al., 2017b). Although autumn leaf fall might represent a peak input event for neonicotinoid-contaminated leaves, only low aqueous concentrations (and hence low waterborne exposure) might be expected in streams providing sufficient dilution (Englert et al., 2017a). However, when the stream receiving contaminated leaves fails to provide sufficient dilution as a consequence of low discharge, organisms might be adversely affected through exposure via the water phase and their diet at the same time (cf. Fig. 4a,b).

Irrespective of the pathway investigated in the present study, neonicotinoid exposure at environmentally relevant levels (regarding both water and leaves; Englert et al., 2017a; Morrissey et al., 2015; Süß et al., 2006) triggered adverse effects in *G. fossarum* (Fig. 4a,b) that may impair leaf litter breakdown and eventually energy transfer processes in heterotrophic streams (Bundschuh et al., 2011b; Wallace et al., 1997). Although the susceptibility of *G. fossarum* towards THI may be even higher in other populations (Feckler et al., 2012), reduced leaf consumption may lower their feces production, restricting the amount of food available for collectors including juvenile gammarids (Cummins & Klug, 1979; McCahon & Pascoe, 1988). Furthermore, it could be hypothesized that the consumption of contaminated leaves by shredders would result in the excretion of feces similarly contaminated representing a potential concern for collecting and filtering invertebrates of local or downstream communities (Bundschuh & McKie, 2016).

In addition, as lipids and body weight are indicative for recourses organisms can invest into reproduction, there may be implications for gammarids' population development (Glazier, 2000; Plaistow et al., 2003). Reduced reproduction would eventually result in a

lower abundance of this keystone species (Dangles et al., 2004) and in turn further reduce their contribution to local leaf litter breakdown. Vertebrate and invertebrate predators populations, which frequently feed upon *Gammarus* (MacNeil et al., 1999), may also be adversely affected by the lowered prey abundance or through the consumption of neonicotinoid-contaminated prey (Douglas et al., 2015).

In the European Union, testing for dietary effects is only recommended for substance characterized by extremely high octanol/water partition coefficients (log P_{ow} >6; EFSA, 2013), while for systemic insecticides (normally characterized by high hydrophilicity and low log P_{ow}) this pathway is considered irrelevant. Results of the present study, however, underpin the relevance of dietary exposure pathways for this group of insecticides (as well as for other pollutants; Zubrod et al., 2015) – for aquatic shredders in particular. Considering that the control of native and invasive pests in forests becomes – under global climate change predictions – increasingly relevant (Ramsfield et al., 2016), the input of neonicotinoid-contaminated leaves into surface waters (e.g., during autumn leaf fall) should not be ignored as an exposure pathway during their aquatic environmental risk assessment.

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SUPPLEMENTARY MATERIALS

Supplementary Table S1. Means and corresponding 95% confidence intervals (CIs) of *G. fossarums'* survival, leaf consumption, lipid content and body weight observed in the respective treatments.

Endpoint	**Treatment**	**Mean**	**95% CIs**
Survival	Control	90.0%	76.9 to 96.0
	Waterborne	87.5%	73.9 to 94.5
	Combined	70.0%	54.6 to 81.9
	Control	80.0%	65.2 to 89.5
	Dietary	82.5%	68.1 to 91.3
Leaf consumption	Control	0.42 mg/animal/d	0.37 to 0.47
	Waterborne	0.30 mg/animal/d	0.25 to 0.34
	Combined	0.21 mg/animal/d	0.17 to 0.26
	Control	0.51 mg/animal/d	0.45 to 0.57
	Dietary	0.33 mg/animal/d	0.29 to 0.37
Lipid content	Control	119.86 μg/mg	102.67 to 137.05
	Waterborne	94.00 μg/mg	73.99 to 114.01
	Combined	89.02 μg/mg	69.29 to 108.76
	Control	131.62 μg/mg	114.84 to 148.39
	Dietary	106.14 μg/mg	90.72 to 121.57
Body weight	Control	3.55 mg	3.32 to 3.86
	Waterborne	3.14 mg	2.89 to 3.40
	Combined	2.80 mg	2.54 to 3.06
	Control	3.68 mg	3.43 to 3.93
	Dietary	3.47 mg	3.18 to 3.77

APPENDIX A.4

UV-irradiation and leaching in water reduce the toxicity of imidacloprid-contaminated leaves to the aquatic leaf-shredding amphipod *Gammarus fossarum*

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ABSTRACT

Systemic neonicotinoid insecticides such as imidacloprid are increasingly applied against insect pest infestations on forest trees. However, leaves falling from treated trees may reach nearby surface waters and potentially represent a neonicotinoid exposure source for aquatic invertebrates. Given imidacloprid's susceptibility towards photolysis and high water solubility, it was hypothesized that the leaves' toxicity might be modulated by UV-irradiation during decay on the forest floor, or by leaching and re-mobilization of the insecticide from leaves within the aquatic ecosystem. To test these hypotheses, the amphipod shredder *Gammarus fossarum* was fed (over 7 d; n = 30) with imidacloprid-contaminated black alder (*Alnus glutinosa*) leaves that had either been pre-treated (i.e., leached) in water for up to 7 d or UV-irradiated for 1 d (at intensities relevant during autumn in Central Europe) followed by a leaching duration of 1 d. Gammarids' feeding rate, serving as sublethal response variable, was reduced by up to 80% when consuming non-pretreated imidacloprid-contaminated leaves compared to imidacloprid-free leaves. Moreover, both leaching of imidacloprid from leaves (for 7 d) as well as UV-irradiation reduced the leaves' imidacloprid load (by 46 and 90%) thereby mitigating the effects on gammarids' feeding rate to levels comparable to the respective imidacloprid-free controls. Therefore, natural processes, such as UV-irradiation and re-mobilization of foliar insecticide residues in water, might be considered when evaluating the risks systemic insecticide applications in forests might pose for aquatic organisms in nearby streams.

INTRODUCTION

Neonicotinoids currently constitute the most widely used class of insecticides worldwide (Simon-Delso et al., 2015). In contrast to traditional, broad-spectrum insecticides (such as carbamates and organophosphates) neonicotinoids are virtually non-toxic to mammals. Still, they exhibit high acute toxicity towards insects by selectively binding to their nicotinic

acetylcholine receptors (Tomizawa and Casida, 2003). Due to their physico-chemical properties, neonicotinoids are rapidly taken up by roots or leaves and distributed in all plant parts ensuring protection against to herbivorous insects (mainly sap feeders; Jeschke et al., 2011). Depending on the plant species being treated and the neonicotinoid compound used, this period of protection may last from weeks to month for agricultural crops (Alford and Krupke, 2017; Donnarumma et al., 2011; Laurent and Rathahao, 2003) and deciduous trees (Mota-Sanchez et al., 2009; Poland et al., 2006; Tattar et al., 1998), and up to several years for conifers (Benton et al., 2016a; Eisenback et al., 2014).

In recent years, neonicotinoids have attracted public attention due to their suspected role in the decline of pollinators (Forster, 2009). This incident resulted in a re-evaluation of neonicotinoids by the European Food Safety Authority and ultimately led to a European Union wide temporary ban of three neonicotinoids (imidacloprid (IMI), clothianidin and thiamethoxam) and their application on pollinator-attracting crops (European Commission, 2013). Following this sanction, several countries including the United States and Canada also re-evaluated the risks of these insecticides to pollinators and the aquatic environment (USEPA, 2017; Health Canada, 2016). These reviews covered a variety of scenarios mostly concerning the effects of neonicotinoids when applied on crops via seed treatment and foliar sprays. Although the application of neonicotinoids (primarily of IMI) to deciduous and coniferous forest trees has increased in recent years (Benton et al., 2016b; Eisenback et al., 2010), the potential exposure of non-target invertebrate species due to such use has received little attention. When trees are treated via soil drenching, neonicotinoid concentrations can leach to nearby streams (Cowles, 2009). Moreover, leaves have been shown to accumulate vast amounts of the systemic insecticides regardless of the application method (i.e., soil or trunk application; e.g., Tattar et al. 1998). During autumn leaf fall, such neonicotinoid containing leaves can enter non-target ecosystems (Kreutzweiser et al., 2007), a

factor rarely considered in neonicotinoid risk assessment. Consequently, forest floor dwelling and aquatic invertebrates that consume fallen leaf matter may be indirectly exposed to neonicotinoids in treated forest ecosystems (cf. Kreutzweiser et al., 2008).

While numerous studies have assessed the effectiveness of neonicotinoid application on trees to suppress insect pests (e.g., Coleman et al., 2017; Cowles et al., 2006) as well as indirect effects on their predators (e.g., Eisenback et al., 2010; Szczepaniec et al., 2011), only a few studies have investigated the implications of neonicotinoids on non-target terrestrial and aquatic decomposers exposed to these compounds through the consumption of contaminated leaves (Englert et al., 2017b; Kreutzweiser et al., 2007, 2008, 2009). Further, little to no research has been completed regarding the fate of neonicotinoids in fallen leaves during decay under ambient environmental conditions. For instance, neonicotinoids in leaf tissues may undergo photolytic degradation during sunlight exposure (i.e., ultraviolet (UV) irradiation) on the forest floor. Moreover, within stream ecosystems, the high water solubility of neonicotinoids may result in their re-mobilization from fallen leaves (Kreutzweiser et al., 2007) thereby reducing their potential toxicity to aquatic decomposers. In this context, the present study aimed at assessing the hypothesized consequences of UV-irradiation and leaching duration on the toxicity of IMI-contaminated black alder leaves (*Alnus glutinosa*) to the key shredder *Gammarus fossarum* (KOCH; Dangles et al., 2004) – an amphipod frequently used in non-standard aquatic toxicity studies (Kunz et al., 2010). Therefore, IMI-contaminated leaves, submerged in water for different durations of time or irradiated with UV, were offered as food to *G. fossarum* during laboratory experiments while its feeding rate served as response variable.

MATERIALS & METHODS

TEST ORGANISMS

Gammarus fossarum was chosen as the test organism for the present study as this species is widely distributed in European headwater streams (Westram et al. 2011) and is also an important prey resource for many fish and invertebrate predators (e.g., MacNeil et al. 1999). Moreover, due to their high abundances and efficiency in shredding coarse particulate organic matter (such as leaves), gammarids are considered a key species in nutrient recycling as associated with their breakdown of leaf litter (Dangles et al., 2004) and are frequently used in non-standard toxicity tests (Kunz et al. 2010).

One week prior to the start of each experiment, *G. fossarum* were kick-sampled from the Hainbach stream (49°14' N; 8°03' E) located in the Palatinate Forest. This *G. fossarum* population is exclusively composed of cryptic lineage B (Feckler et al., 2012). As the sampling site was located upstream of any settlement and agricultural activity, and neonicotinoids compounds are not applied to trees by the local forestry office, previous exposure of test organisms to neonicotinoids was unlikely. Adult male gammarids of 6-8 mm body length and visibly free of macro-parasites were selected as per Bundschuh et al. (2011). Prior to testing, organisms were acclimated for 7 d in laboratory aquaria containing well-aerated stream water collected from the sampling site and maintained at 16 ± 1°C followed by a gradual transition to SAM-S5 medium (i.e., test medium; Borgmann, 1996). During this time, organisms were fed *ad libitum* with black alder leaves that had been conditioned with a near-natural microbial community consisting of fungi and bacteria as described in Bundschuh et al. (2011).

SOURCE OF PLANT MATERIAL, IMIDACLOPRID APPLICATION AND PREPARATION OF LEAF DISCS

For the present study, IMI-free and IMI-contaminated black alder leaves were prepared as described in detail in Englert et al. (2017a). In brief, black alder trees were soil drenched once in June 2014 with either 500 mL tap water or with 500 mL of tap water spiked with the neonicotinoid formulation ConfidorWG70 (70% IMI, Bayer CropScience; dose: 0.15 g IMI/cm trunk diameter at breast height (DBH)). Due to the relatively small trees size (mean DBH: 7.5 ± 0.2 mm; Englert et al., 2017a), soil drenching instead of trunk injection was used as application method. The amount of IMI applied to trees represented 25% of the highest dose recommended for soil application (e.g., for the product Merit75WP; Bayer). Shortly before leaf fall in October 2014, all leaves were collected from trees and stored at minus 20°C until further use. To minimize the variation of IMI residues in leaves that can occur among treated trees (e.g., Englert et al. 2017a), only leaves collected from a single tree were used for this study. Leaf discs (diameter = 2.0 cm) were cut from leaves using a cork borer, freeze-dried (for 24 h) and subsequently weighed to the nearest 0.01 mg to determine their initial dry weight.

To simulate leaching of the IMI dose from treated leaves, pre-weighed IMI treated leaf discs were placed into plastic beakers (two discs per beaker) filled with 150 mL of the test medium and allowed to stand for 1, 3, or 7 d prior to the start of exposures. During this period, the test medium was renewed daily to remove any IMI that may have accumulated in the test medium. IMI-contaminated leaf discs that were not subjected to simulated leaching were used as positive control. IMI-free leaf discs were subjected to the same leaching process (i.e., for 0, 1, 3 and 7 d) and used in the corresponding controls to account for any changes in leaf condition as associated with the different leaching times.

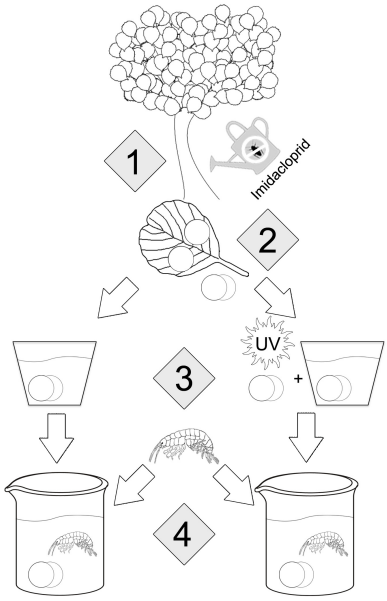

Fig. 1. Scheme illustrating the experimental procedure followed during the bioassay. In June 2014, black alder trees were (1) soil drenched with the neonicotinoid IMI at 0.15 g IMI/cm DBH. (2) Leaf disc were cut from IMI-contaminated leaves harvested in October 2014 and either (3) leached in plastic beakers filled with test medium (for 1, 3 or 7 d) or UV-irradiated (for 0 or 1 d) before being leached (for 1 d). Finally, (4) leaf discs were placed together with one *G. fossarum* in a 250 mL glass beaker filled with uncontaminated test medium and gammarids' feeding rate was assessed over 7 d. IMI-contaminated leaf discs, that received neither UV-irradiation nor leaching treatment, were used as positive control in both experiments. For each treatment containing IMI-contaminated leaf discs, a corresponding control group with uncontaminated leaf discs (i.e., black alder trees soil drenched with 0 g IMI/cm DBH) was set up.

For the experiment assessing the influence of UV-irradiation on the toxicity of IMI-contaminated leaves, IMI-contaminated leaf discs were UV-irradiated for 1 d using a UV fluorescent lamp (Magic Sun 20/160R; Heraeus Holding GmbH; Hanau, Germany) at an intensity (mean ± standard error (SE)) of 4.15±0.09 and 0.15±0.01 W/m^2 for UV-A and UV-B, respectively (measured with a RM12 radiometer; Dr. Gröbel UV-Elektronik GmbH, Ettlingen Germany). This is ~90% below peak intensities measured for UV-A and UV-B, respectively, under clear skies during summer in Central Europe (Häder et al., 2007). The dose generated during the 24 h UV exposure period (UV-A: ~360; UV-B: 13 kJ/m^2) represents the approximate cumulative dose measured over 1.5 and 4 d for UV-A and UV-B, respectively, during October in Central Germany (Häder et al., 2001). Irradiated leaf discs were placed into

plastic beakers filled with 150 mL test medium and allowed to stand for an additional 1 d to simulate their deposition in a natural stream. IMI-contaminated leaf discs that were not subjected to UV-irradiation or leaching simulation were used as positive control and thus directly introduced into the bioassay. In addition, IMI-contaminated leaf discs that were leached in water for 1 d without prior UV-irradiation were used to account for the effects of leaching and to distinguish between UV-irradiation and leaching effects. IMI-free leaf discs were subjected to the same UV-irradiation exposures and leaching periods and used as corresponding negative controls.

BIOASSAY DESIGN

For both the leaching and UV-irradiation experiments, one *G. fossarum* was placed into a glass beaker containing 200 mL of fresh test medium with either two pretreated IMI-contaminated leaf discs or two equally pretreated IMI-free leaf discs serving as corresponding controls (for a schematic overview see Fig. 1). This experimental setup resulted in eight and six treatments (n = 30) for the leaching and UV-irradiation experiments, respectively (see also the caption of Fig. 1). An additional five beakers containing only two leaf discs that had been subjected to the same UV-irradiation and leaching treatments were added to the design to account for microbial decomposition and abiotic losses in leaf mass (Maltby et al., 2000). Bioassays were conducted over a 7 d period, during which all beakers were aerated and randomly allocated to shelves in a climate controlled chamber set at 16±1 °C in total darkness. The latter condition was established to avoid any negative phototactic response of *G. fossarum* (Holmes, 1901).

At the termination of both feeding experiments, water samples (10 mL) were collected in triplicate from each IMI treatment and stored at -20 °C until chemical analysis. Three additional water samples were also randomly collected from the negative controls and stored similarly until analysis. After 7 d of exposure, remaining gammarids, leaf discs and associated

leaf materials were removed from beakers. Prior to weighing, gammarids were oven dried at 60 °C while leaf discs were freeze-dried. Gammarid feeding rates were calculated in milligram of consumed leaf mass per milligram dry weight of the respective individual per day and were corrected for any losses in leaf mass associated with microbial and abiotic decomposition (Table S1; Maltby et al., 2000). Mortality of *G. fossarum* in the leaching and UV-irradiation experiments were < 3 and 23%, respectively (Table S1). Replicates in which gammarids died were not included in statistical analyses.

FATE EXPERIMENT

In addition to the leaching- and UV-irradiation experiments, a separate fate experiment was set up using sufficient amounts of leaf material required for IMI quantification (Englert et al., 2017a). In this experiment, 1.5 g freeze-dried IMI-contaminated black alder leaves (n = 3) were added to 2.5 L glass jars filled with test medium and held for 7 d to quantify IMI residues remaining in leaves following the maximum leaching period. In order to quantify the effect of UV-irradiation on leaf IMI concentrations, an additional 1.5 g of freeze-dried IMI-contaminated leaf tissue (n = 3) was UV-irradiated for 1 d (without additional leaching in water) at the same intensities as described above. Subsequently, IMI residues were quantified in not- pretreated leaves and also in leached and UV-irradiated samples.

IMIDACLOPRID ANALYSIS IN LEAVES AND WATER

IMI was extracted form freeze-dried black alder leaves using an ASE 350 Accelerated Solvent Extractor system (Thermo Scientific Dionex, Sunnyvale, CA) as described in Englert et al. (2017a). Separation of IMI from leaf extracts and water samples was performed using ultrahigh performance liquid chromatography–mass spectrometry equipped with an EQuan MAX system, while for quantification, a single quadrupole mass spectrometer equipped with an electrospray ionization source was used (Englert et al., 2017a,b). IMI was identified at the accurate ion mass of m/z = 256.0596 while matrix-matched standards,

prepared out of blank leaf extracts or test medium, were used for external calibration. The limits of quantification (LOQ) and detection for IMI in leaf extracts were 0.06 and 0.02 μg/g of dry weight, respectively. For the IMI water concentrations, the lowest calibration level (i.e., 0.01 μg/L) was defined as the LOQ due to the absence of a signal in sample blanks (Turnipseed et al., 2008).

CALCULATIONS AND STATISTICS

Gammarid feeding rates were checked for normality by visual inspection of normal probability plots and Shapiro Wilk's tests, with F-tests used to assess homoscedasticity. Student's *t*-tests were used to evaluate for statistically significant differences in gammarid feeding rates between IMI-exposure experiments and corresponding negative controls. Depending on the data properties (i.e., normality and homoscedasticity), IMI residues in leaves that were leached or UV-irradiated were compared to IMI residues of non-pretreated IMI-contaminated leaves using either Student's *t*-tests or Wilcoxon rank-sum tests. Subsequently, *p*-values obtained from the leaching-, UV-irradiation- and fate experiment were adjusted for four, three and two comparisons, respectively, using the Holm-adjustment method. Given the shortcomings of null hypothesis significant testing (Newman, 2008), our interpretation of the data was additionally based on relative effect sizes. Therefore, 95% confidence intervals (CIs; Altman et al., 2000) were calculated for the relative differences found in gammarids' feeding rates. All statistical analyses and figure composition were completed using the software R (version 3.1.1 for Mac computers).

Table 1. Mean (±SE; n = 3) IMI water concentrations measured in feeding experiments after 7 days.

Treatment	Leached (days)	UV-irradiated (days)	Measured concentration (μg/L)
Leaching experiment			
Control	-	-	< LOQ
Imidacloprid	-	-	5.7±2.35
Imidacloprid	1	-	1.65±0.54
Imidacloprid	3	-	0.44±0.11
Imidacloprid	7	-	0.25±0.10
UV-irradiation experiment			
Control			< LOQ
Imidacloprid	-	-	n.a.
Imidacloprid	1	-	n.a.
Imidacloprid	1	1	0.92±0.30

n.a.: not analyzed

RESULTS & DISCUSSION

INFLUENCE OF LEACHING ON TOXICITY

During both the leaching experiment and the UV-irradiation experiment, gammarids feeding on non-pretreated IMI-contaminated leaves consumed 70% (t-test: p = 0.10; n = 25/29; Fig. 2) and 80% (t-test: $p < 0.001$; n = 23/24; Fig. 3) less black alder leaf material relative to the corresponding IMI-free control. However, the reduction was only statistically significant for the UV-irradiation experiment (Fig. 3). In the case of the leaching experiment, the reduction was not statically significant due to an unexpectedly high variation (Fig. 2). Nevertheless, both positive controls reduced gammarid feeding rates by ≥ 70% which agrees well with our previous investigation. There 7 d of exposure to leaves from black alder trees treated with the same amount of IMI (i.e., 0.15 g IMI/cm DBH) resulted in a 79% decrease in gammarid feeding rates (Englert et al., 2017b).

As hypothesized, 1 and 3 d leaching periods for IMI-contaminated black alder leaves prior to their use in the feeding trials resulted in a generally reduced impact on gammarid feeding rates. Specifically, in comparison to the highly reduced feeding rates observed for the positive

controls, 1 and 3 d leaching treatments resulted in only 40% (*t*-test: $p = 0.003$; $n = 30$) and 49% (*t*-test: $p = 0.001$; $n = 29/30$; Fig. 2) reductions in gammarid feeding rates, respectively. Although the uptake of IMI into plants may vary depending on application by soil- or trunk exposure (cf. Tattar et al., 1998), it is expected that the latter method would have resulted in the same response patterns as observed in the current study. The observed reductions in toxicity following leaching treatments are most likely explained by IMI's high water solubility (610 mg/L at 20 °C; Lewis et al., 2016), which results in its rapid re-mobilization from leaf tissues during the pre-experimental leaching phase. Consequently, the extent of IMI exposure experienced by gammarids during the feeding assay was reduced. This assumption is supported by the 70 and 90% lower IMI water concentrations measured in these treatments relative to the concentrations determined for not pre-treated positive controls at the end of the feeding experiment (Table 1). While Kreutzweiser et al. (2007) reported a ≥90% re-mobilization of IMI from ash (*Fraxinus pennsylvanica*) leaves following a 5 d leaching treatment, our fate experiment demonstrated a ~45% non-significant (*t*-test: $p = 0.20$; $n = 3$) reduction of IMI content in black alder leaves following a 7 d leaching treatment relative to non-pretreated IMI-contaminated leaves (Fig. 4). This points towards different leaching dynamics among tree species, an assumption that needs further empirical verification. Moreover, these aqueous IMI concentrations indicate that gammarids feeding on leaf discs leached in water for 1 and 3 d were likely exposed to slightly higher dietary IMI concentrations (compared to gammarids feeding on leaves leached for 7 d) potentially explaining the observed effect on their feeding rates (Englert et al., 2017b). However, the 9% difference observed in the leaf consumption of *G. fossarum* feeding on leaves leached for 1 compared to 3 d was smaller than anticipated and possibly due to differences in the extent of IMI contamination that can occur among individual leaves (cf. Coots et al., 2013; Tanis et al., 2012).

In contrast, gammarids exposed to IMI-contaminated leaves that were previously subjected to 7 d of leaching displayed no significant reductions in feeding rate. In fact, gammarid feeding rates for the 7 d leaching treatment were ~30% higher relative to the corresponding control (*t*-test: $p = 0.10$, $n = 29/30$; Fig. 2). This increase in feeding rate may represent a compensatory response of gammarids (cf. Agatz, 2013; Baudy et al., 2017; Zubrod et al., 2017), to increase energy intake during exposure to sub-lethal concentrations (Maltby, 1999) of IMI in the water and diet (Table 1; Fig. 4). However, the specific mechanisms responsible for this behavior need to be examined in future studies (cf. Zubrod et al., 2017). In addition to the negative relationship observed here between feeding response and leaching duration, stream flow may also wash away insecticide residues re-mobilized from leaf tissues thereby reducing local exposure. However, such redistribution may pose an exposure risk to downstream communities (cf. Englert et al., 2017a).

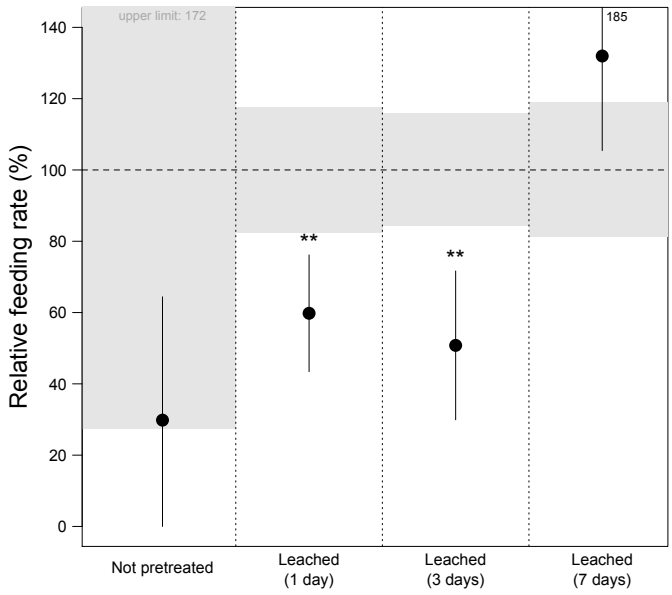

Fig. 2. Relative feeding rate (mean ±95% CIs) of *G. fossarum* exposed to IMI-contaminated leaves that were not pretreated or leached in test medium for 1, 3 or 7 days. Asterisks denote significant differences compared to the corresponding control (dashed line): $p < 0.01$ (**). Grey areas indicate the 95% CIs of the corresponding control.

INFLUENCE OF UV AND LEACHING ON TOXICITY

Rapid degradation of IMI (dissipation time 50%: 0.7 – 1.4 d) has been demonstrated on the surface of plants when the insecticide is applied as foliar spray (Scholz and Reinhard, 1999). Although these conditions might not directly be comparable to IMI bound within plant tissues, we hypothesized that UV-irradiation could reduce (e.g., through photolytic degradation) IMI concentrations in fallen leaves and thereby mitigate the exposure of aquatic organisms to IMI following the deposition of contaminated leaves into streams. Indeed, exposure of gammarids to IMI-contaminated leaves that were UV-irradiated for 1 d at field relevant intensities followed by a 1 d leaching period effectively mitigated adverse effects on gammarid feeding rates (*t*-test: $p = 0.88$, $n = 27/21$; Fig. 3) to a level comparable to the corresponding negative control. As leaching for 1 d (without UV-irradiation) reduced the impact on gammarid feeding rates from 80% (positive control) to ~45% (both relative to the corresponding negative control; *t*-test: $p = 0.02$, $n = 30/26$; Fig. 3), the majority of the reduction in toxicity seems to be due to the UV-irradiation. The 1-d UV-pretreatment also resulted in lower IMI water concentrations in the UV-experiment compared to the concentrations measured in positive control of the leaching experiment (IMI concentrations were not quantified for the positive control of UV-experiment; Table 1). The effectiveness of the UV-pretreatment is further emphasized by the significantly lower concentrations of IMI quantified in UV-irradiated leaves relative to IMI-contaminated leaves that were not pre-treated with UV (by ~90%; Wilcoxon test: $p = 0.03$; $n = 3$; Fig. 4).

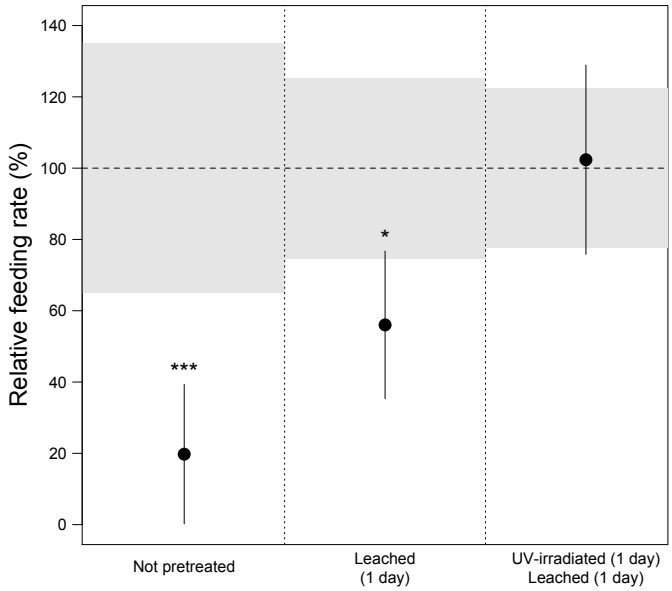

Fig. 3. Relative feeding rate (mean ±95% CIs) of *G. fossarum* exposed to IMI-contaminated leaves that were not pretreated, leached in test medium for 1 day or were UV-irradiated for 1 day before being leached in test medium (for 1 day). Asterisks denote significant differences compared to the corresponding control (dashed line): $p < 0.05$ (*) and $p < 0.001$ (***). Grey areas indicate the 95% CIs of the corresponding control.

Field studies investigating the temporal distribution and dissipation of IMI in foliage still attached to trees demonstrated temporal declines in IMI concentrations that persist over weeks to month (e.g., Coots et al., 2013; McCullough et al., 2011). Thus, it appears likely that the impact of UV-irradiation on leaf IMI content might differ depending on whether the leaves are still alive and attached to the living tree branch or senescing on the forest floor. In healthy leaves, natural phenolic compounds (*inter alia* flavonoids) may minimize photo-oxidative effects by preventing UV-irradiation from entering leaf tissues as well as through their function as effective antioxidants (Agati and Tattin, 2010; Treutter, 2006). These phenolic compounds have, however, been shown to decrease in senescent leaves (Gallet and Lebreton, 1995; Paaso et al., 2017). Thus, detached leaves may no longer be able to maintain the natural UV-protection mechanisms that help permit the photolytic degradation of IMI. Although such photolytic degradation may provide a protective mechanism that mitigates the risks associated with neonicotinoids-contaminated leaves, this mechanism could be diminished on the forest floor and in stream ecosystems where shading can reduce UV

penetration and intensity. Future efforts to investigate neonicotinoid degradation in contaminated leaves should therefore be conducted under field conditions to gain a more complete understanding of neonicotinoid behavior in plant tissues and the potential risks posed to non-target organisms.

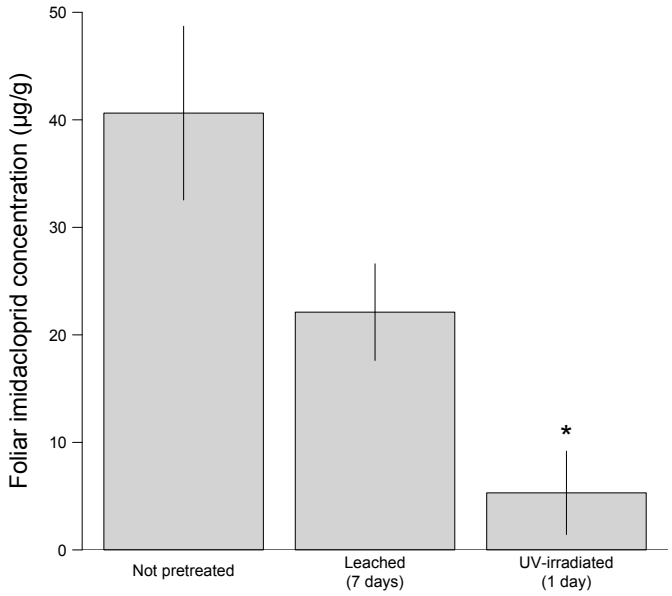

Fig. 4. Mean (±SE; n = 3) foliar IMI residues measured in IMI-contaminated leaves that were not pretreated, leached in test medium for 7 days or were UV-irradiated for 1 day prior to quantification. Asterisks denote significant differences compared to non-pretreated IMI-contaminated leaves: $p < 0.05$ (*).

CONCLUSIONS

The increasing number of native and non-native insect pests that are predicted to threaten forest health under current climate change scenarios (Ramsfield et al., 2016) may lead to increased use of systemic insecticides and their subsequent release into forest floor habitats via leaf senescence. Neonicotinoids associated with dropped leaves from soil- or trunk-treated trees have been demonstrated to induce sublethal exposure effects in non-target shredder species thereby reducing their contribution to the leaf litter breakdown process (cf. Englert et al., 2017b; 2017c; Kreutzweiser et al., 2007; 2008; 2009). Although we demonstrated that leaching time can reduce the toxicity of IMI in leaf tissues to a gammarid species, such risk reduction is likely afforded by the high degree of water solubility that is

characteristic of neonicotinoids. This hydrophilicity may increase the risk of chronic exposure for invertebrate communities downstream of treated forests that deposit contaminated leaf litter stream ecosystems (cf. Englert et al., 2017a). On the other hand, IMI and some other neonicotinoids are vulnerable to photolysis (Lewis et al., 2016). Mitigation of neonicotinoid toxicity through photo degradation may be even more relevant during autumn leaf senescence and deposition when the canopy opens and the extent of sunlight penetration to the forest floor increases. Therefore, natural processes including UV-irradiation and pesticide re-mobilization in water need to be considered when evaluating the risks for freshwater organisms in forest areas managed with systemic insecticides.

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SUPPLEMENTARY MATERIALS

Table S1. Mortality (in %) of *G. fossarum* and leaf mass correction factors, which were used to correct the feeding rate of *G. fossarum* for microbial and abiotic leaf mass losses, observed in the leaching experiment and the UV-irradiation experiment. IMI-free and IMI-contaminated leaves were either not pretreated, leached in water (for 1, 3 or 7 d) or UV-irradiated (for 1 d, followed by a leaching duration of 1 d) prior to their use in the respective experiments.

Treatment	Leached (d)	UV-irradiated (d)	Mortality (%)	Leaf mass correction factor
Leaching experiment				
Control	-	-	0	0.67
Imidacloprid	-	-	3	0.66
Control	1	-	0	0.69
Imidacloprid	1	-	0	0.66
Control	3	-	0	0.67
Imidacloprid	3	-	3	0.63
Control	7	-	0	0.64
Imidacloprid	7	-	3	0.62
UV-irradiation experiment				
Control	-	-	23	0.70
Imidacloprid	-	-	20	0.67
Control	1	-	0	0.70
Imidacloprid	1	-	13	0.66
Control	1	1	10	0.70
Imidacloprid	1	1	23	0.68

APPENDIX A.5

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PEER-REVIEWED ARTICLES

2018

Newton, K., Zubrod, J. P., **Englert, D.**, Lüderwald, S., Schell, T., Baudy, P., Konschak, M., Feckler, A., Schulz, R., Bundschuh, M. 2018. The evil within? Increased quality of plant material treated with systemic fungicides for an aquatic decomposer-detritivore system. *Environmental Pollution* 241, 549-556.

Englert, D., Zubrod, J. P., Neubauer, C., Schulz, R., Bundschuh, R. 2018. Leaching and UV-irradiation reduce the toxicity of imidacloprid-contaminated leaves to the aquatic leaf-shredding amphipod *Gammarus fossarum*. *Environmental Pollution 236*, 119-125.

2017

Englert, D., Zubrod, J. P., Pietz, S., Stefani, S., Krauss, M., Schulz, R., Bundschuh, M., 2017. Relative importance of dietary uptake und waterborne exposure for a leaf-shredding amphipod exposed to thiacloprid-contaminated leaves. *Scientific Reports* 7, 16182.

Zubrod, P. Jochen, Bundschuh, R., **Englert, D.**, Rohrberg, M., Wieczorek, M., Bakanov, N., Schulz, R., Bundschuh, M. 2017. Transient effects following peak exposures towards pesticides – an explanation for the unresponsiveness of *in situ* measured functional variables. *Environmental Pollution* 231, 1393-1397.

Zubrod, J. P., **Englert, D.**, Lüderwald, S., Poganiuch, S., Schulz, R., Bundschuh, M. 2017. History matters: pre-exposure to wastewater enhances pesticide toxicity in invertebrates. *Environmental Science & Technology* 51, 9280-9287.

Englert, D., Zubrod, J. P., Link, M., Mertins, S., Schulz, R. Bundschuh, M. 2017. Does waterborne exposure explain effects caused by neonicotinoid-contaminated plant material in aquatic systems? *Environmental Science & Technology* 51, 5793-5802.

Zubrod, J. P., **Englert, D.**, Wolfram, J., Rosenfeldt, R. R., Feckler, A., Bundschuh, R., Seitz, F., Konschak, M., Baudy, P., Lüderwald, S., Fink, P., Lorke, A., Schulz, R., Bundschuh, M. 2017. Long-term effects of fungicides on leaf-associated microorganisms and shredder populations—an artificial stream study. *Environmental Toxicology and Chemistry* 36, 2178-2189.

Englert, D., Bakanov, N., Zubrod, J. P., Schulz, R., Bundschuh, M., 2017. Modeling re-mobilization of neonicotinoid residues from tree foliage in streams - a relevant exposure pathway in risk assessment? *Environmental Science & Technology* 51, 1785-1794.

2015

Zubrod, J. P., **Englert, D.**, Wolfram, J., Wallace, D., Schnetzer, N., Baudy, P., Konschack, M., Schulz, R., Bundschuh, M. 2015. Waterborne toxicity and diet-related effects of fungicides in the key leaf shredder *Gammarus fossarum* (Crustacea: Amphipoda). *Aquatic Toxicology* 169, 105-112.

Englert, D., Zubrod, J. P., Schulz, R., Bundschuh, M., 2015. Variability in ecosystem structure and functioning in a low order stream: Implications of land use and season. *Science of the Total Environment* 538, 341-349.

Zubrod, J. P., **Englert, D.**, Rosenfeldt, R. R., Wolfram, J., Lüderwald, S., Wallace, D., Schnetzer, N., Schulz, R., Bundschuh, M., 2015. The relative importance of diet-related and waterborne effects of copper for a leaf-shredding invertebrate. *Environmental Pollution* 205, 16-22.

Zubrod, J.P., Feckler, A., **Englert, D.**, Koksharova, N., Schulz, R., Bundschuh M. 2015: Inorganic fungicides as routinely applied in organic and conventional agriculture can increase palatability but reduce microbial decomposition of leaf litter. *Journal of Applied Ecology* 52, 310-322.

Zubrod, J.P., **Englert, D.**, Feckler, A., Koksharova, N., Konschak, M., Bundschuh, R., Schnetzer, N., Englert, K., Schulz, R., Bundschuh M. 2015: Does the current fungicide risk assessment provide sufficient protection for key drivers in aquatic ecosystem functioning? *Environmental Science & Technology* 49, 1173–1181.

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Kalčíková, G., **Englert, D.**, Rosenfeldt, R.R., Seitz, F., Schulz, R., Bundschuh, M. 2014: Combined effect of UV-irradiation and TiO_2-nanoparticles on the predator-prey interaction of gammarids and mayfly nymphs. *Environmental Pollution* 186**,** 136-140.

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Englert, D., Zubrod, J.P., Schulz, R., Bundschuh, M., 2013. Effects of municipal wastewater on aquatic ecosystem structure and function in the receiving stream. *Science of the Total Environment* 454-455, 401-410.

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Englert, D., Bundschuh, M., Schulz, R., 2012. Thiacloprid affects trophic interaction between gammarids and mayflies. *Environmental Pollution* 167, 41-46.

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Bundschuh, M., Zubrod, J. P., **Englert, D.**, Seitz, F., Rosenfeldt, R. R., Schulz, R., 2011. Effects of nano-TiO_2 in combination with ambient UV-irradiation on a leaf shredding amphipod. *Chemosphere* 85, 1563-1567.

Zubrod, J. P., Bundschuh, M., Feckler, A., **Englert, D.**, Schulz, R., 2011. Ecotoxicological impact of the fungicide tebuconazole on an aquatic decomposer-detritivore system. *Environmental Toxicology and Chemistry* 30, 2718-2724.

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Zubrod, J. P., Jevtić, D., Melato, **Englert, D.**, Weil, M., Brockmeier, E., Floehr, T., Knežević, V., Agatz, A., Brinkmann, M., 2013. News from the SETAC Europe Student Advisory Council (April 2013) - the 3rd Young Environmental Scientists (YES) Meeting at the Jagiellonian University, Poland. *Environmental Sciences Europe* 25, 16.

Zubrod, J. P., Jevtić, D., Melato, A., Agatz, A., **Englert, D.**, Floehr, T., Knežević, V., Weil, M., Brinkmann, M., 2012. News from the SETAC Europe Student Advisory Council - September 2012. *Environmental Sciences Europe* 24, 27.

CONFERENCE CONTRIBUTIONS

2018

Roeder, N., Baudy, P., Zubrod, J.P., **Englert, D.**, Konschak, M., Baschine, C., Schulz, R., Bundschuh, M. Does fungicide exposure alter interspecific relationships of aquatic fungi during leaf decomposition? A case study using species-specific qPCR assays. SETAC Europe 28th Annual Meeting 2018 Rome, Italy.

Newton, K., Zubrod, J. P., **Englert, D.**, Lüderwald, S., Schell, T., Baudy, P., Konschak, M., Schulz, R., Bundschuh, M. Leaf litter originating from trees treated with systemic fungicides – a new exposure pathway for aquatic decomposer detritivore systems. SETAC Europe 28th Annual Meeting 2018 Rome, Italy.

2017

Konschack, M., Zubrod, J.P., Baudy, P., Herrmann, B., **Englert, D.**, Schulz, R. Bundschuh, M. Sind wasserexpositionsbezogene und nahrungsbedingte Fungizideffekte unabhängig von der Shredderspezies? 22. Jahrestagung der SETAC GLB, Neustadt, Germany.

Englert, D., Zubrod, J.P., Schulz, R., Bundschuh, M. Neonicotinoids in tree foliage: A risk for aquatic organisms? SETAC North America 38th Annual Meeting 2017 Minneapolis, USA.

Englert, D., Pietz, S., Krauss, M., Stefani, S., Zubrod, J. P., Schulz, R., Bundschuh, M. 2017. Relative importance of waterborne vs. dietary exposure towards the neonicotinoid thiacloprid for leaf-shredders. SETAC Europe 27th Annual Meeting 2017 Brussels, Belgium.

Zubrod, J. P., **Englert, D.**, Poganiuch, S., Lüderwald, S., Schulze, T., Bundschuh, M. 2017. Pesticide toxicity is enhanced by pre-exposure to wastewater. SETAC Europe 27th Annual Meeting 2017 Brussels, Belgium.

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Englert, D., Neubauer, C., Zubrod, J. P., Schulz, R., Bundschuh, M. 2016. UV-irradiation and leaching reduce adverse effects of imidacloprid contaminated leaves for the shredder *Gammarus fossarum*. SETAC Europe 26th Annual Meeting 2016, Nantes, France.

Newton, K., **Englert, D.**, Baudy, P. T., Konschak, M., Lüderwald, S., Schulz, R., Bundschuh, M. 2016. Dietary uptake of systemic fungicide contaminated plant material affects energy processing and growth of a leaf-shredder. SETAC Europe 26th Annual Meeting 2016, Nantes, France.

Konschak, M., Zubrod, J. P., Baudy, P. T., **Englert, D.**, Schulz, R., Bundschuh, M. 2016. Are waterborne and diet-related effects of fungicides transferable among leaf-shredders? SETAC Europe 26th Annual Meeting 2016, Nantes, France.

Zubrod, J. P., **Englert, D.**, Fuß, B., Lüderwald, S., Schulz, R., Bundschuh, M. 2016. Do stable isotope signatures and elemental stoichiometry mirror toxicant-induced changes in predation success? SETAC Europe 26th Annual Meeting 2016, Nantes, France.

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Zubrod, J. P., Wolfram, J., **Englert, D.**, Bundschuh, R., Rosenfeldt, R.R., Seitz, F., Schulz, R. Bundschuh, M. 2015. Long-term effects of inorganic and synthetic fungicides on leaf-associated microorganisms and leaf-shredding macroinvertebrates – an artificial stream study. SETAC Europe 25th Annual Meeting 2015 Barcelona, Spain.

Englert, D., Link, M., Zubrod, J. P., Schulz, R., Bundschuh, M. 2015. Are neonicotinoids a threat for leaf litter breakdown? A laboratory approach with leaf-shredding invertebrates. SETAC Europe 25th Annual Meeting 2015 Barcelona, Spain.

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Englert, D., Zubrod, J. P., Schulz, R., Bundschuh, M. 2014. Seasonal variation in effects of different land-uses on stream ecosystem structure and function. SETAC Europe 24th Annual Meeting 2014 Basel, Switzerland.

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Englert, D., Zubrod, J. P., Schulz, R., Bundschuh, M. 2013. Varying wastewater dilution in receiving streams - implications for stream ecosystem structure and function. 3rd SETAC Young Environmental Scientists Meeting, Krakow, Poland.

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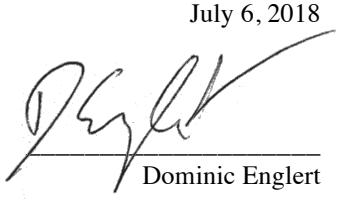

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