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RESEARCH ARTICLE

Metabarcoding of canopy arthropods reveals negative impacts of forestry insecticides on community structure across multiple taxa

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Abstract

1. Insecticides used to combat outbreaks of forest defoliators can adversely affect non-target arthropods. Forestry insecticides typically suppress Lepidoptera larvae which are the cornerstone of the canopy community of deciduous oak forests. The abrupt removal of this dominant component of the food web could have far-reaching implications for forest ecosystems, yet it is rarely investigated in practice owing to several methodological shortcomings. The taxonomic impediment and the biased nature of arthropod sampling techniques particularly impede the assessment of insecticide impacts on diverse communities.
2. To tackle this issue, we propose an experimental approach combining sampling by pyrethrum knockdown and species determination via DNA metabarcoding, using community subsampling to derive estimates of species abundances. We applied this protocol to investigate the short-term effects of the insecticides diflubenzuron (DFB) and *Bacillus thuringiensis* var. *kurstaki* (BTK) on canopy-dwelling arthropod communities in German oak woodlands.
3. Our approach allowed us to detect most of the diversity and integrate species abundances in our analyses. By classifying arthropod species into assemblages based on their expected sensitivity rather than coarse taxonomic groupings, we could unveil substantial effects of DFB across multiple taxa 5 weeks after application.
4. Although strong effects on single species appear related to direct toxicity, substantial impacts of DFB on parasitoids and xylophagous beetles suggest that

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anti-defoliator treatments can have previously unsuspected indirect effects on some components of forest arthropod communities. The impacts of BTK on community structure were consistent with that of DFB though much weaker.

5. *Synthesis and applications*. Comparing diversity patterns in the arthropod communities of sprayed and unsprayed oak canopies, our results show that selective insecticides can alter species diversity in presumably non-sensitive taxa. Even though the ecological significance of these impacts has yet to be assessed in an operational setting, their existence calls for increased regulatory scrutiny on indirect effects. As community approaches become more attainable with the rapid development of DNA metabarcoding, we suggest the inclusion of community-level end points as regulatory requirements for the approval of forest-use insecticides.

KEYWORDS
Bacillus thuringiensis, canopy arthropods, community analysis, diflubenzuron, diversity patterns, DNA barcoding, indirect effects, taxonomic impediment

1 | INTRODUCTION

Insect outbreaks pose a major challenge in the management and development of land resources. In cropping systems, these eruptions are frequent as the relatively low diversity and transient nature of agroecosystems hinders trophic regulation of herbivores population (Letourneau, 2012). In contrast, insect outbreaks in forests are characterised mainly by sporadic and often spatially synchronous eruptions, resulting from a combination of abiotic and biotic processes whose relative importance is still poorly understood (Letourneau, 2012; Liebhold et al., 2012). Although relatively infrequent, outbreaks of forest insects can substantially impede timber production (MacLean, 2016). Lepidopteran folivores, such as the gypsy moth *Lymantria dispar* L. (Lepidoptera: Erebidae), are notably responsible for growth reduction and increased tree mortality in temperate forests (Lobinger, 1999; MacLean, 2016; Twery, 1991) and are routinely controlled by aerial applications of insecticides. By contrast with the systematic preventative approach that predominates in cropping systems, these treatments are applied curatively to suppress outbreaking populations before defoliation becomes significant (Thompson, 2011). However, despite the occasional nature of these operations and the relative selectivity of the approved substances, the use of insecticides in forest management has raised concerns about its side effects on the arthropod fauna, particularly non-target Lepidoptera (Schweitzer, 2004; Severns, 2002).

In Central European deciduous oak woodlands, insecticide treatments are generally applied from late-April to mid-May against spring feeding defoliators (Gößwein & Lobinger, 2014). During this period, Lepidoptera larvae make up most of the insect biomass in tree crowns and are as such a cornerstone of the food web (Southwood et al., 2004). By suppressing these populations, insecticide treatments can alter species interactions such as competition (Leroy, Gossner, et al., 2021) and predation (Sample et al., 1993), potentially triggering a complex chain of effects and feedback loops across entire communities (Fleeger, 2020). However, while the direct impacts of forestry insecticides are generally well-understood, indirect effects are more difficult to study in the field and remain poorly known.

Studies on non-target effects of aerially sprayed insecticides in forests suffer from several methodological limitations. First, most arthropod sampling techniques are biased towards certain taxa due to the diversity in life history and habitat use among species, making it difficult to sample communities comprehensively (Ozanne, 2005). Second, taxonomic expertise is rarely available for all sampled taxa (Brown, 2005), such that non-target assessments are usually focused on a few well-known taxa rather than diverse communities. Last, insecticide effects are primarily tested on coarse taxonomic ranks (often order or family) rather than groupings reflecting hypotheses on the field sensitivity of species. Most products approved for defoliator management in temperate forests are non-systemic ingestion insecticides such as the moulting inhibitor diflubenzuron (Marx, 1977) and the Lepidoptera-specific gut poison *Bacillus thuringiensis* var. *kurstaki* (Gill et al., 1992). Thus, the communities of forest arthropods can be classified into hypothetical sensitivity groups based on their feeding habits. For example, chewing herbivores are expected to be more sensitive than leaf miners or sucking herbivores that feed on internal plant tissues. The analyses of sensitivity groups may reveal toxic effects of these substances which would be masked when ecologically diverse taxonomic groups are investigated. This approach can help improve our understanding of the insecticide effects in forests.

Recent advances in amplicon-based metabarcoding have brought new opportunities to circumvent the shortcomings of species determination based on morphological examination (Hebert & Gregory, 2005). With the implementation of next generation sequencing (NGS) within the metabarcoding framework, millions of amplified cytochrome *c* oxidase 1 (CO1) genes can now be sequenced simultaneously and searched

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against reference barcode libraries to bulk-identify species presence from raw samples (Baird & Hajibabaei, 2012). During the last decade, the rapid growth of sizeable collaborative barcode databases such as the Barcode of Life Data System (BOLD; Ratnasingham & Hebert, 2007) greatly increased the scope of metabarcoding approaches for the study of biodiversity. In BOLD, similar CO1 DNA barcodes are clustered and attributed a unique identifier (Barcode Index Number, BIN) that closely resemble species and can be used as a species proxy in groups with low taxonomic resolution (Ratnasingham & Hebert, 2013). Delineations based on genetic distance are unbiased by the asymmetric state of taxonomic expertise, enabling comparison across taxa and studies. This approach, combined with comprehensive canopy sampling techniques such as pyrethrum knockdown (Ozanne, 2005), offers a robust framework to assess diversity patterns in diverse arthropod communities. However, the use of CO1 metabarcoding for assessments of community structure is not devoid of flaws. The challenge of inferring relative abundances of species from sequence read abundances is a pivotal issue of current amplicon-based approaches. Sequence divergence and differences in the copy number of the targeted locus, as well as variation in body mass among specimen, lead to asymmetric amplification among species, hindering the detection of some taxa while making the estimation of species abundance from the number of sequence reads largely inaccurate (Elbrecht et al., 2017; Krehenwinkel et al., 2017).

In the present study, we assessed the short-term (5 weeks post-application) effects of the insecticides DFB and BTK used to control gypsy moth populations on the early summer community of canopy arthropods in Central European deciduous oak woodlands. We combined taxonomic expertise and DNA metabarcoding to maximise diversity detection and implemented a simple protocol integrating order-level sorting and subsampling of communities to obtain reliable estimates of species abundance from sequence reads numbers. We then coarsely classified species into ecological groups illustrating their expected sensitivity to direct exposure based on knowledge of toxicological and environmental dynamics of the insecticides and the species' life-history traits. Specifically, we distinguished taxa expected to be affected via primary exposure (externally feeding Lepidoptera [DFB; BTK], other externally feeding herbivores [DFB only]), potentially affected via secondary exposure (parasitoids, predators, scavengers [DFB only]) and unaffected (internally feeding herbivores and scavengers). We then assessed diversity patterns of arthropod communities in trees treated with DFB, BTK or left unsprayed, to test whether insecticide impacts can be reliably predicted on the mere basis of direct effect hypotheses.

2 | MATERIALS AND METHODS

2.1 | Experimental design

The experiment was conducted in three neighbouring oak-dominated stands, 'Bauernschlag' (52 ha; 50.05706°N, 10.08311°E), 'Vorberg' (58 ha; 50.0534°N, 10.08899°E) and 'Brunnholz' (6 ha; 50.03170°N, 10.07715°E), in the region of Schweinfurt (Lower Franconia, Bavaria, Germany) in early summer 2017. The sites were selected based on population surveys conducted during the previous winter (October 2016–February 2017) predicting patchy densities of the gypsy moth *Lymantria dispar* L. (Lepidoptera: Erebidae), ranging from endemic to potential outbreak levels. Within 10 structurally homogeneous areas across the three stands (blocks), we delimited two 3,000-m^2 (100 × 30 m) treatment plots that were randomly assigned to one insecticide treatment (DFB or BTK) and one control plot. Plots were separated by buffer zones at least 40-m wide, which is considered a safe distance to prevent off-target spraying for aerial applications in still air conditions (German Federal Office of Consumer Protection and Food Safety, 2019). A detailed description of the study sites and the experimental design is provided in Appendix S1.

2.2 | Insecticides

The commercial formulations licensed for forest use DiPel® ES (BTK strain ABTS 351 [HD-1]; 33.2 g/L; 17,600 IU/mg; Cheminova Deutschland) and Dimilin® 80 WG (800 g DFB/kg; Spiess-Urania Chemicals) were used. Both DFB and BTK are relatively selective insecticides. They are non-systemic, which means that they remain on the surfaces of plants after application, do not translocate well into plant tissues and must be ingested to be effective (Bull & Ivie, 1978; Gill et al., 1992; Grosscurt, 1978). Their main difference lies in their spectrum of action, with BTK being specific to Lepidoptera, while DFB is toxic to the juvenile stages of all arthropods upon oral contact. Table 1 summarises ecotoxicological data on the toxicity and environmental dynamics of both substances.

DiPel® ES and Dimilin® 80 WG were sprayed at their maximal legal rate of 3 L/ha and 75 g/ha respectively. The treatments were administered by a Bell 47 helicopter on 17 May 2017 between 07:00 and 09:00 in dry and still air conditions. We assessed spraying accuracy and coverage with water-sensitive spray cards (agrotop, Obertraubling, Germany) placed on the forest floor at intervals of 2–3 m along straight lines connecting the centroid of adjacent plots. A total of 131 spray cards were exposed within a transect crossing four blocks (Appendix S1) and collected and replaced after each prey flight to inspect droplet deposition patterns. Uniform spray coverage was observed on all cards exposed within the spray plot boundaries. Off-target spraying, measured as the presence of spray droplets in the buffer zones, did not occur beyond 3 m away from plot boundaries.

2.3 | Arthropod communities

We used pyrethrum knockdown (in this case canopy fogging) to sample free-living arthropods from oak crowns. One mature oak tree (i.e. DBH > 35 cm) was selected close to the centre of each spray plot. Patchy densities of gypsy moth egg masses predicted variable levels of defoliation across blocks: low (blocks 1, 2 and 10), moderate (blocks 4–9) and severe (block 11; Appendix S1). We hence selected two trees in the control area of each block to compare the impacts of high and low defoliation along with that of insecticides. To measure

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TABLE 1 Oral toxicity and persistence times of diflubenzuron (DFB) and *Bacillus thuringiensis* var. *kurstaki* (BTK)

	Insecticide		References
End point	**DFB**	**BTK**	
LC_{50} (mg/L)[a]			
Bombus terrestris	0.320 (0.310–0.330)	*non-toxic*[b]	Mommaerts et al. (2006, 2010)
Lymantria dispar	0.060 (0.001–0.170)	0.047 (0.036–0.064)	Berry et al. (1993) and van Frankenhuyzen et al. (1993)
Spodoptera exigua	15.8 (12.6–19.7)	34.0 (26.3–44.3)	MacIntosh et al. (1990) and Van Laecke and Degheele (1991)
DT_{50} (days)[c]			
Vegetation	1–21	1–64	Glare and O'Callaghan (2000) and Wimmer et al. (1993)
Soil	10–20	100–200	Beck et al. (2004) and Thompson and Kreutzweiser (2006)

[a]Toxicity expressed as the median lethal concentration (LC_{50}), that is the concentration of active substance (CryIA protein in the case of BTK) per volume of diet. Toxicity values are given for the target pest of the present study (gypsy moth, *Lymantria dispar*, Lepidoptera: Erebidae), a standard agricultural pest (beet armyworm, *Spodoptera exigua*, Lepidoptera: Noctuidae) and a standard non-target species (buff-tailed bumblebee, *Bombus terrestris*, Hymenoptera: Apidae).

[b]No mortality was observed at the maximum recommended field concentration.

[c]Persistence expressed as the biological half-life (DT_{50}), that is the time required to half the applied concentration, on vegetation and in the soil. Persistence times are influenced by precipitations, UV radiation and the target tree species and are hence expressed as ranges.

the efficacy of the treatments, we placed one 10-cm-wide band of barricade tape around the trunk of each focal tree, which allowed us to sample late-instar gypsy moth taking shelter during the daytime. The caterpillars were collected and counted on 12 and 13 June 2017. We sampled canopy arthropods on a single occasion between 19 and 22 June 2017, 5 weeks after treatment application, which corresponds to the peak feeding of gypsy moth caterpillars (and hence defoliation).

We used Swingfog® SN-50 fogging machines (Swingtec GmbH) and a 1% pyrethrum suspension in petroleum white oil to generate a thermal cloud that carries pyrethrum droplets into the canopy. Fogging was conducted from dusk to dawn in still air conditions to maximise efficiency (Ozanne, 2005) and simultanesouly in adjacent plots (i.e. within block) to nullify the impact of a potential drift of the fog cloud into neighbouring plots. The fogging machine was operated until most of the focal tree crown was coated by the fog, which was determined visually using electric torch lights. The resulting fogging time ranged between 5 and 10 min per tree. We collected fallen arthropods on four 15-m^2 tarpaulin sheets exposed beneath tree crowns for 30 min and stored them in 99% ethanol until further analysis (one sample per sheet and four samples per tree).

Permits to sample arthropods by pyrethrum knockdown in the three study stands were granted by the Regional Council of Lower Franconia (Regierungspräsidium Unterfranken) per §45 para 7 cl 1 no 3 of the Federal Nature Conservation Act. No ethical approval was required for this study.

2.4 | Arthropod processing

2.4.1 | Arthropod sorting

Adults and heterometabolous (i.e. taxa undergoing incomplete metamorphosis such that adults and juveniles are morphologically similar) juveniles were sorted into 28 taxonomic groups (mostly order and suborder, further referred to as 'sorted taxa'; see Figure S1 for the complete list). Holometabolous (i.e. taxa undergoing complete metamorphosis such that adults and juveniles radically differ in appearance) were sorted into two groups: Lepidoptera/Symphyta (i.e. prolegs present) and Coleoptera/Neuroptera/Raphidioptera (i.e. prolegs absent). Because gypsy moth caterpillars were already collected from the tree stems a few days before sampling the canopy, individuals collected during fogging were excluded from the samples for further analyses.

2.4.2 | Species determination

Based on specific information relevant to the systematics of the sorted taxa, we assigned each taxon to its 'optimal' determination method: (a) morphological examination by expert taxonomist (MPH) and (b) DNA metabarcoding through NGS. The MPH group included Araneae, Opiliones, Pseudoscorpiones, adult Coleoptera, Orthoptera, Myriapoda and Isopoda. The vast majority of Central European oak-associated species in these taxa are well described and easily identifiable by experts in our network. By contrast, a high species determination efficiency was deemed challenging for taxa assigned to the NGS group, owing to various obstacles, such as the presence of many cryptic juvenile stages in the samples (e.g. Hemiptera), the dependence on non-ubiquitous characters such as male genitalia (e.g. several families within Diptera and Neuroptera), a largely incomplete current taxonomic knowledge (e.g. Diptera and Hymenoptera) or the lack of available taxonomic expertise (e.g. Psocodea).

2.4.3 | Community subsampling

To derive species abundances in the assemblage to be barcoded, we first conducted a subsampling of the taxa differentiated by sorting (Figure S1). For each tree, we divided each of the four samples

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into three equal-size fractions, for a total of 12 subsamples per tree. For each sorted taxon, individual arthropods were distributed across three numbered vials, starting from a random number and henceforth proceeding sequentially, individual by individual, in ascending order (Figure 1). This was necessary because the number of individuals of a sorted taxon was not always a multiple of 12 and the random starting vial ensured that there was no systematic bias in what vial would receive a higher number of individuals. The subsampling approach aimed to increase our detection capacity, by reducing the amount of DNA per sequenced sample. It also allowed an estimation of species relative abundance by aggregating incidence data at the tree level (i.e. the sum of presence/absence across subsamples), such that non-null abundances of the sorted taxa per tree ranged from 1 (only present in one subsample) to 12 (present in all subsamples). Each of the resulting 480 subsamples was filled with 99% ethanol to preserve arthropod DNA.

2.4.4 | DNA metabarcoding

We used standard methods for DNA extraction, DNA amplification, NGS, sequence analysis and taxonomic assignment using the BIN framework (i.e. putative species; Ratnasingham & Hebert, 2013). We used BINs as a proxy for species to include undescribed diversity in subsequent analyses. The full metabarcoding pipeline (DNA extraction, amplification, sequencing and bioinformatic processing) is described in detail in Appendix S2.

2.4.5 | Filtering of artefactual and contaminant reads

High-quality low abundance reads originating from sporadic contamination may artificially inflate diversity estimates (Alberdi et al., 2018). In our approach, the sorting and subsampling steps imply manipulation of the arthropods before sequencing, which may produce substantial cross-contamination between the subsamples of one sample (e.g. when alcohol attached to an individual transferred to a new vial contains DNA from other organisms, free-floating or within cells). To eliminate the resulting contaminant reads without excessively suppressing true diversity, we performed a three-step filtering protocol. First, OTUs with a read abundance below 0.005% of the total per subsample were discarded. The use of relative threshold is a standard practice to deal with artefactual sequences in metabarcoding studies (Alberdi et al., 2018). Second, we devised a protocol to filter out higher abundance contaminants. We first calculated a 'bias coefficient' for each combination of sorted taxon and subsample to correct for taxon-specific biases in read number characteristics of arthropod DNA (Krehenwinkel et al., 2017), using the following equation:

$$A_{j,k} = \frac{\sum_{i=1}^{n_k} R_{i,k} / \sum_{j=1}^{n_k} N_{j,k}}{\sum_{i=1}^{n_{j,k}} R_{i,j,k} / N_{j,k}}, \quad (1)$$

where A is the bias coefficient, R is the number of reads and N is the observed number of individuals, for a BIN i belonging to the jth sorted taxon in the kth subsample.

We then applied the correction by multiplying each read value by its corresponding bias coefficient:

$$Corr\ R_{i,j,k} = A_{j,k} \times R_{i,j,k}, \quad (2)$$

where *Corr R* is the number of reads corrected for taxon-specific biases.

Last, we filtered out bias-corrected reads below 1% of the maximum read abundance per BIN across all subsamples, as these were considered likely to be contaminants. To evaluate the performance

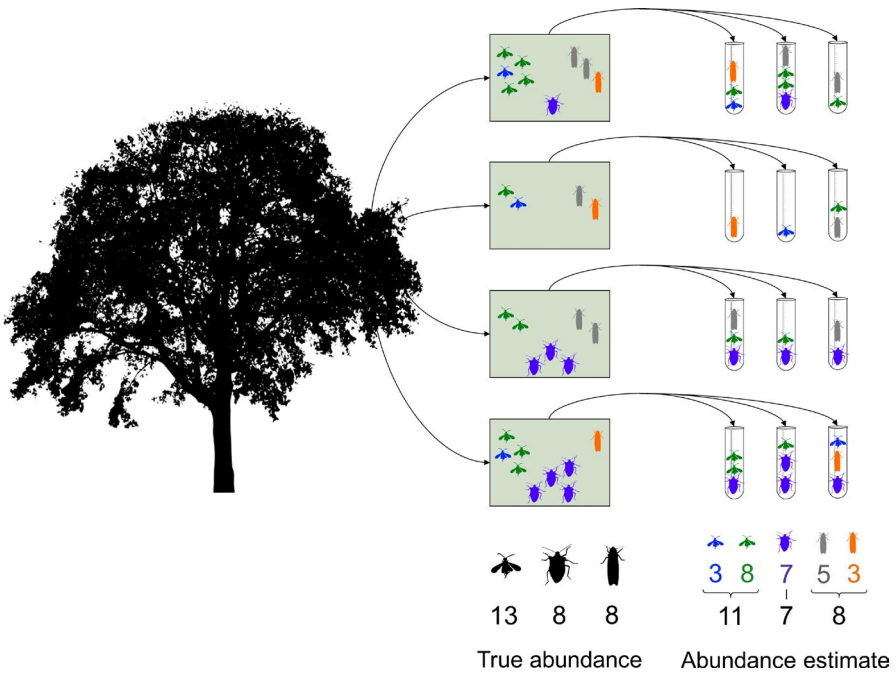

FIGURE 1 Overview of the subsampling procedure. Arthropods were collected separately from four sampling sheets per tree. Each sample was sorted into high rank taxa (represented by different silhouettes), and individuals in each taxon were equally redistributed into three subsamples (vials 1, 2 and 3), resulting in 12 subsamples per tree. Different colours represent effective species

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of our approach, we calculated the proportion of observable mismatches (false presence and false absence at the level of the sorted taxa) for each sorted taxon by cross-checking the species matrix against the sorting data (i.e. data obtained by counting individuals within high taxonomic ranks before metabarcoding; see Section 2.4.1) after pooling all BINs within sorted taxa. We then compared the proportion of mismatches before and after filtering.

2.4.6 | Abundance estimates

To estimate the abundance of each BIN per tree, we converted the number of reads to presence–absence and aggregated the data. The resulting value ranging from 0 to 12 was used as a proxy for abundance in subsequent analyses. To evaluate the accuracy of the method, we fitted linear regressions to test the strength of the relationship between the observed abundance (i.e. number of individuals counted during arthropod sorting per tree) and the abundance proxies obtained at each step of the protocol, namely (a) raw reads (i.e. number of reads per tree before contaminant filtering), (b) bias-corrected reads (i.e. number of reads per tree after bias correction and filtering) and (c) abundance estimates (i.e. aggregated presence–absence per tree). For the latter, we fitted two models to assess the influence of the number of subsamples on the accuracy of the estimation: one at the sample level (4 samples per tree) and one at the subsample level (12 subsamples per tree).

2.5 | Insecticide sensitivity groups

We assigned arthropod species into hypothetical sensitivity groups by scoring their physiological and ecological sensitivity. In the absence of single-species toxicity data for our study system, physiological sensitivity was coarsely defined based on the mode of action of the insecticides: all arthropod species were deemed physiologically sensitive to DFB, whereas only Lepidoptera were regarded as physiologically sensitive to BTK. Ecological sensitivity was defined as the possibility of exposure to insecticide based on feeding ecology. As both insecticides are only truly effective via oral exposure, all species feeding externally on the surface of plant tissue (i.e. chewing herbivores and epiphyte grazers) were deemed directly exposed to both insecticides. By contrast, species feeding on internal tissues and within concealed shelters (i.e. sucking herbivores, leaf miners, shoot and wood borers) were considered to avoid exposure. Predators, parasitoids and scavengers were regarded as potentially exposed, as they may come into direct contact with insecticide residues through contaminated prey or host. Importantly, non-predatory species with no known association with any of the overstorey and understorey tree species recorded in our study were considered to be 'tourists' (in the sense of Moran & Southwood, 1982) and excluded from the analyses, as their presence in the sampled habitat is likely very sporadic. Fully concealed herbivores such as leaf miners and wood borers were only sampled in their free-living adult stage but may have been active as juveniles during the acute phase of toxicity (i.e. in the days following application). Hence, we decided to include them in the analysis, regardless. Saproxylic (i.e. species feeding on dead wood and associated fungi) and wood-boring beetles (i.e. species feeding on the wood of live trees) were grouped within the internal herbivore guild due to the plasticity between both feeding guild and identical predictions regarding their sensitivity. Based on these scores of physiological and ecological sensitivities, we drafted sensitivity hypotheses for six so-called sensitivity groups (tourists not included; Table 2).

Feeding trait data were retrieved by mining specialised literature (see Appendix S3 for a comprehensive bibliography). In families in which the relevant traits are highly conserved, these were assigned

TABLE 2 Sensitivity groups used in the analyses based on their expected physiological and ecological sensitivity to diflubenzuron (DFB) and *Bacillus thuringiensis* var. *kurstaki* (BTK). 0 = no sensitivity; 1 = sensitivity present; a = primary exposure (direct ingestion); b = secondary exposure (trophic transfer)

		Physiological sensitivity		Ecological sensitivity	
Sensitivity group	**Feeding guilds**	**DFB**	**BTK**	**DFB**	**BTK**
Lepidopteran external feeders	Leaf chewers, epiphyte grazers	1	1	1a	1a
Non-lepidopteran external feeders	Leaf chewers, epiphyte grazers	1	0	1a	1a
Internal herbivores[1]	Sapsuckers, leaf miners, gall-feeders…	1	0/1[2]	0	0
Scavengers	Detritivores, xylophages, necrophages…	1	0	0/1b	0/1b
Predators[1]	Predators	1	0	1b	1b
Parasitoids	Parasitoids	1	0	1b	1b

[1]Species presenting multiple feeding habits (e.g. zoophytophagous species) were included in all relevant groups.

[2]Only leaf-mining Lepidoptera are physiologically sensitive.

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to the family level to avoid systematically dropping BINs not matched with a described species (e.g. it seems safe to assume that all braconids in our study system are likely to be parasitoids, regardless of the species names). In biologically diverse or poorly known families, and for all phytophagous species, traits were assigned at the species level and BINs unassigned to taxonomic species were dropped. The relative contribution of different taxonomic orders to the diversity of the sensitivity groups is shown in Figure S2.

2.6 | Statistical analyses

The statistical procedures were conducted separately for groups identified morphologically (further referred to as the MPH assemblage) using observed abundances and taxonomic species as a reference type, and for groups identified by metabarcoding (further referred to as the NGS assemblage) using aggregated incidence and BINs. For clarity, we will further refer to abundance and species regardless of the reference metric and type. We compared the average abundance per tree, the abundance of the most common species, the total species diversity (γ-diversity), the mean species diversity per tree (α-diversity) and the compositional heterogeneity among tree communities (β-diversity) between treatment for the whole community and five sensitivity groups (i.e. lepidopteran external feeders, non-lepidopteran external feeders, internal herbivores, parasitoids and predators). Only internal herbivores (represented exclusively by xylophagous beetles) and predators were sufficiently represented to be analysed in the MPH community. Because of a pyrethrum dosing mistake during the first night of pyrethrum knockdown, four blocks (16 trees) were too under-sampled to be included in the statistical analyses. Therefore, diversity patterns were only analysed in the six remaining blocks (24 trees).

We tested the influence of DFB and BTK on the abundance of gypsy moth caterpillars using a generalised linear mixed-effect model with DFB and BTK (both dummy-coded) as fixed effects and a random intercept for block nested into site. We used the same approach to investigate the quantitative response of sensitivity groups to insecticide treatments.

One major shortcoming of pyrethrum knockdown as a sampling method is its sensitivity to changes in atmospheric conditions that can substantially affect crown coverage by the fog cloud, leading to considerable variation in the sampled abundance among trees. We took note of the large extent of sampling heterogeneity in our data by computing individual rarefaction curves for each tree (Figure S3). Abundance-based rarefaction allows for correcting for uneven sampling effort by standardising communities to the smallest sample size (Gotelli & Colwell, 2001). However, effects driven by true abundance are muted in the process. Because insecticide impacts on diversity are expected to be abundance driven, we instead standardised samples by sampling coverage, which estimates the proportion of the total number of individuals of the asymptotic community present in a sample (Chao & Jost, 2012). We extrapolated species richness up to twice the observed sample size for each tree and sensitivity group using abundance-based rarefaction and extrapolation curves. We selected the associated lowest coverage value as our 'base coverage' following the method described by Chao and Jost (2012). Species richness was then interpolated or extrapolated to the abundance value corresponding to the base coverage for each tree (i.e. 'base sample size'). Following this approach, we estimated the diversity per treatment (γ-diversity) using the sum of base sample sizes for each treatment. For the control treatment, this number was halved to correct for the doubled number of replicates ($n = 12$ in control and $n = 6$ in DFB and BTK).

We decomposed species diversity in each treatment using the multiplicative diversity partitioning framework (Jost, 2007) where:

$$\gamma_i = \alpha_i \times \beta_i, \tag{3}$$

where γ is the total species diversity in treatment i, α is the average diversity per tree within treatment i and β is the effective number of compositionally distinct tree-level communities within treatment i. In the present study, we compare species assemblages within a small-scale block design, meaning that they are expected to be relatively homogeneous in the absence of treatment effect. In this context, α- and γ-diversity quantify the effect of insecticides on single tree communities and across different communities, respectively, while β-diversity measures the heterogeneity of these effects among communities.

To identify significant responses from individual species, we looked for significant abundance-based species–treatment associations by performing an indicator species analysis (De Cáceres et al., 2010) involving the 67 most common species (i.e. mean abundance ≥2 individuals per tree in at least one treatment). The strength of the associations was calculated as the point-biserial correlation coefficient (r_{pb}) corrected for unequal sample sizes. The statistical significance of the associations (P) was determined by means of two-sided permutations tests (1,000 permutations), and p-values were subsequently adjusted for multiple testing with the Šidák correction.

All analyses were performed in R 4.0.3 (R Core Team, 2020). Mixed models were fitted by maximum likelihood estimations using 'glmmTMB' (Brooks et al., 2017) and diagnosed with the package 'DHARMa' (Hartig, 2020). We tested for significant differences ($p < 0.05$) between treated and control trees using type II Wald tests with the function *Anova* in the 'car' package (Fox & Weisberg, 2019). Interpolation and extrapolation of γ- and α-diversity and estimation of β-diversity by multiplicative partitioning were performed with the 'iNEXT' package (Hsieh et al., 2016). We estimated the uncertainty of diversity estimates by constructing 95% bootstrap confidence intervals (500 replicates) using the bootstrap approach implemented within 'iNEXT'. Confidence intervals were corrected for multiple testing with the Šidák method (family-wise error rate for 18 hypotheses FWER = 0.28%). We determined statistically significant differences at a level of 5% as indicated by non-overlapping bootstrap confidence intervals between treatment levels (Chao & Jost, 2012). The indicator species analysis was performed with the 'indicspecies' package (De Cáceres & Legendre, 2009).

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3 | RESULTS

3.1 | Species identification procedures

3.1.1 | Sequence reads filtering

The full dataset included a total of 13,634,135 sequence reads clustered into 9,042 OTUs that were matched to 2,501 BINs and 85 matches not assigned to a BIN (i.e. reference COI sequences <500 bp or with more than 1% ambiguous bases, or BIN assignment pending at the time of analyses). The number of BINs was reduced to 2,334 after the correction of BIN-species discordances (Appendix S2). In total, 3,667 OTUs (11,563,819 reads) matched to 1,214 BINs were retained after removal of non-sorted matches (e.g. human contamination), matches with pairwise identity below 97% and low confidence reads (<0.005% reads per sample). After standardising read numbers to abundance and filtering out low abundance reads (<1% of maximum copy number per BIN), the final sequence read data consisted of 10,846,068 total sequence reads.

Before filtering raw reads, observable mismatches between the sorted arthropod data and the metabarcoding output consisted of 5.6% of false positives (i.e. contaminants or artefacts) and 4.7% of false negatives (i.e. undetected diversity; Figure 2, a1) across 17 sorted taxa. Small-sized Acari (36.8% false negatives), Thysanoptera (12.2%) and Sternorrhyncha (11.8%) had the most detection failures and the lowest incidence of false detections compared with larger sized taxa. The filtering procedures brought the fraction of false positives down to 0.8% and raised false negatives to 8.3% (Figure 2, a2). Most false positives were filtered out across all sorted taxa. False negatives slightly increased in all taxa, the most affected being Acari (+12.5%), Collembola (+8.3%) and the grouping of Coleoptera larvae, Neuroptera and Raphidioptera (+6.6%; Figure 2, a2).

3.1.2 | Taxonomic resolution

Metabarcoding data. In total, 25,949 sorted individuals were processed for species delineation via metabarcoding. All the 1,213 detected BINs were assigned taxonomy at the order level, 1,145 (94%) at the family level, 960 (79%) at the genus level and 757 (62%) at the species level. Among taxa, the percentage of BINs associated with a described species varied from 96% for Lepidoptera (113 BINs) to only 22% for Acari (23 BINs). For highly diverse taxa, the percentage of species-level assignment ranged from 48 for Hymenoptera to 63 and 74% for Diptera and Hemiptera respectively (Figure 2, b1).

Morphology data. A total of 4,171 of the 4,771 individuals determined by morphological examination were successfully identified to species level. These included most adult beetles (Coleoptera; 2,715 out of 2,717), all orthopterans (275), harvestmen (Opiliones; 14), pseudoscorpions (14), myriapods (36) and isopods (3), as well as 65% of all spiders (Araneae; 1,114 out of 1,712). The remaining fraction comprised almost exclusively juvenile spiders that could only be identified to genus (27; 2%) or family (571; 34%; Figure 2, b2).

3.1.3 | Abundance estimates

The relationship between raw reads and true abundance was weak and not statistically significant ($t[22] = 0.93$, $p = 0.364$; $R^2 = 0.038$; Figure 2, c1). Scaling reads to the relative abundance of each sorted taxon only marginally increased the strength of the relationship ($t[22] = 1.46$, $p = 0.160$; $R^2 = 0.088$; Figure 2, c2). By contrast, the aggregated incidence at the sample ($t[22] = 11.08$, $p < 0.001$; $R^2 = 0.810$; Figure 2, c3) and the subsample levels ($t[22] = 13.56$, $p < 0.001$; $R^2 = 0.860$; Figure 2, c4) was strongly and linearly related to true abundance. Increasing the number of subsamples per tree decreased the shrinkage of abundance estimates, from a slope of 6.74 at the sample level (4 units) to 4.79 at the subsample level (12 units).

3.2 | Effects of insecticides on the abundance of gypsy moth caterpillars

The number of gypsy moth caterpillars under tree bands was strongly reduced by BTK and DFB (5- and 43-fold compared to the mean abundance in control trees respectively; $\chi^2(2) = 60.01$, $p < 0.001$; Figure 2). Gypsy moth abundance in trees treated with DFB was only 13% of that of trees treated with BTK. Thus, although both insecticides strongly reduced the abundance of caterpillars of the pest species, DFB was substantially more effective.

3.3 | Effect of insecticides on the structure of the non-target community

3.3.1 | Diversity patterns in the different sensitivity groups

The observed number of individual arthropods did not differ between treatments across all sensitivity groups (Table S1).

Insecticide treatments had no significant effect on the overall species richness (γ-diversity) of canopy arthropods (Figure 4). However, the species diversity across treated communities was lower than that across controls for most sensitivity groups. The difference was particularly pronounced and statistically significant for xylophagous beetles (55% fewer species) and parasitoids (25% fewer species) in DFB. Although the trend was similar in the BTK treatment, it was not significant for any sensitivity group. The overall pattern of species loss in treated trees was approximately identical at the tree level (α-diversity; Figure 4), suggesting a homogeneous effect of insecticides across the different trees. The mean species diversity was 52% and 27% in DFB-treated trees relative to controls for xylophagous beetles and parasitoids respectively.

Sensitive groups did not appear significantly affected by either insecticide treatment, with the lower diversity trend in treated trees being at best marginally significant at the tree level for non-lepidopterous external feeders (Figure 4). Diversity patterns in

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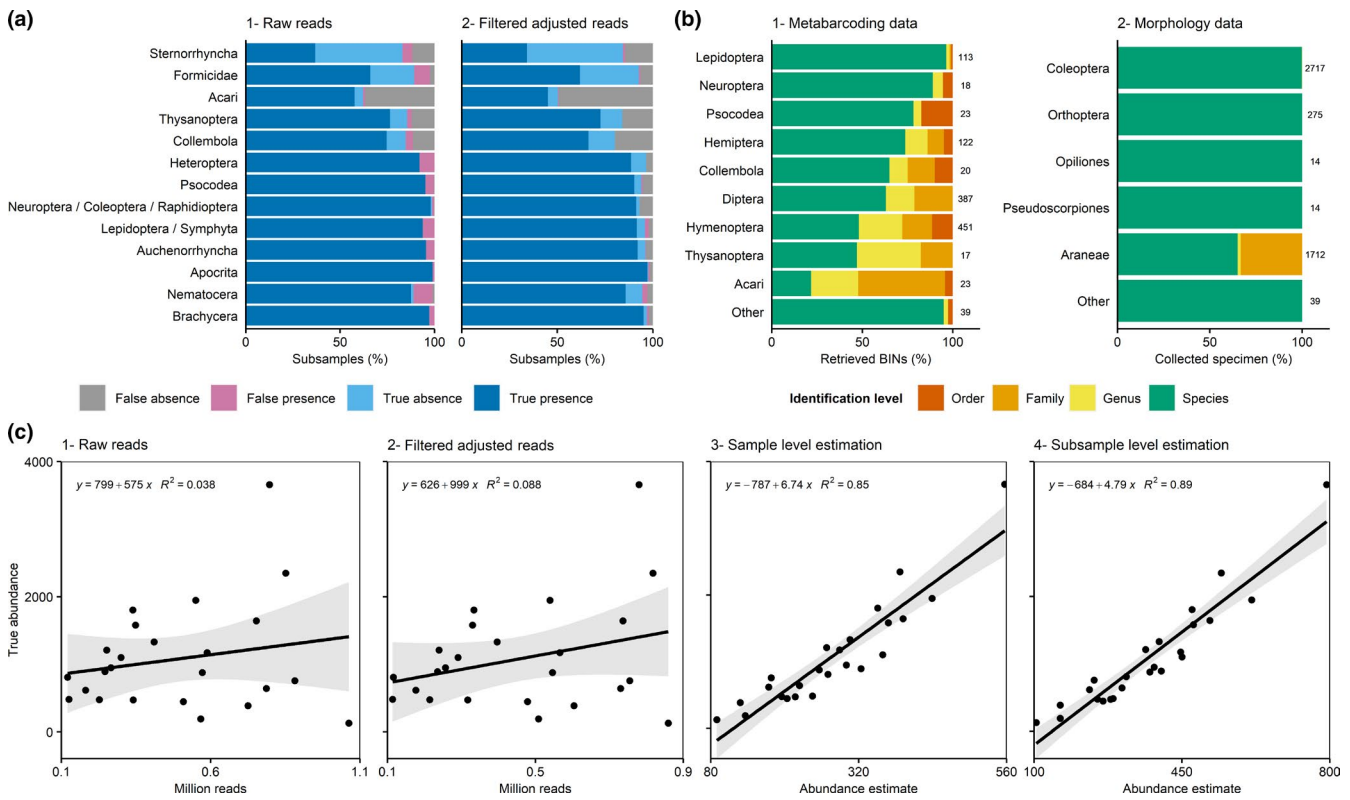

FIGURE 2 Analysis of the data processing procedures. (a) Evaluation of contaminant filtering. Proportion of true presences (dark blue), true absences (light blue), false presences (pink) and false absences (grey) observed per sorted taxon upon comparing the sorting data with the metabarcoding data before (a1) and after filtering contaminant reads (a2). (b) Taxonomic resolution. Taxonomic support for BINs matched to sequence clusters in BLAST searches (>97% pairwise identity; b1) and individuals examined by taxonomists (b2), expressed as the percentage of the BINs (b1) and individuals (b2) per taxon identified to family (orange), genus (yellow) and species (green). Absolute numbers of BINs (b1) and individuals (b2) in each taxon are indicated on the right of the bars. Taxa with less than 10 BINs or individuals are pooled into the group 'Other'. (c) Evaluation of the abundance estimates. Relationship between true abundance (i.e. the number of individuals counted during sorting) and the different estimates of species abundance at the tree level: raw reads (c1), filtered and abundance-scaled reads (c2), sample-level aggregated incidence (4 units per tree; c3) and subsample level aggregated incidence (12 units per tree; c4). Each dot represents a tree. The line shows the best linear fit to the data, described by the regression equation and the coefficient of determination (R^2) of the fit displayed on the top left of each facet. Grey shading depicts the standard error

internal feeding herbivores (excluding xylophagous beetles) and predators appeared completely unaffected by either treatment.

Weighting species by their relative abundance reduced the strength of insecticide effects on diversity. However, the strongest effects on xylophagous beetles and parasitoids were consistent and remained significant, except for the impact of DFB on the γ-diversity of parasitoids (Figure S4).

3.3.2 | Species–treatment associations

Among the most common species, only three had a significantly depressed abundance in treated trees relative to controls (Figure 5). These were the moth *Eilema sororcula* Hufnagel (Erebidae; −84.6 and −69.2% in DFB and BTK respectively), the barklouse *Valenzuela flavidus* Stephens (Psocodea: Caeciliusidae; −56.6% in DFB) and the katydid *Meconema thalassinum* De Geer (Orthoptera: Tettigoniidae; −93.1% in DFB). The parasitoids *Syntretus falcifer* Tobias (Hymenoptera: Braconidae) and *Chalarus holosericeus* Meigen (Diptera: Pipunculidae) were respectively positively and negatively associated with BTK, though only the former association remained significant after correction for multiple testing. Similarly, one undetermined species of Lauxaniid fly (genus *Calliopum*) was positively associated with DFB, but the association was not robust to *p*-value adjustment. All of the other common species did not show any clear pattern of association with any of the insecticide treatments or the control (see Table S2 for the complete analysis output).

4 | DISCUSSION

In this paper, we introduce several methodological procedures that contribute to increasing the comprehensiveness of arthropod community analysis. We sampled a very diverse community of crown-dwelling arthropods using pyrethrum knockdown and delineated a large proportion of the individuals to species by combining metabarcoding and morphological examination. We implemented these methods to address the impact of forestry insecticides on arboreal

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arthropods communities, unveiling non-target effects of the insecticide DFB across multiple taxa. While marked negative responses of a handful of species were evidence of direct toxicity, diversity patterns appear to be instead driven by indirect processes. Diversity patterns in communities treated with DFB and BTK were consistent, but almost invariably more pronounced and only significant in the former. We hereby first discuss the value and implications of our methodology for the analysis of species-rich communities. We then explore the ecological processes underlying the non-target effects reported here, and the implications of our findings for pest management strategies in forests.

4.1 | Species determination and abundance estimation

The main limitation of our method to estimate species abundances lies in the increased occurrence of cross-contamination between samples during sample pre-processing. In the present study, our taxon-specific filtering approach yielded satisfying results by removing 87% of the observed false presences while keeping the inflation of false absences to modest levels (Figure 2a). We opted for relatively conservative filtering thresholds to maximise the reduction of false positives, as we considered false negatives less problematic in community analyses. Undetected species are a common challenge in diversity analyses of insufficiently or unevenly sampled communities and can be reliably dealt with using rarefaction and extrapolation sampling curves (Colwell et al., 2012). Although we believe that upstream mitigation of contamination is the best course of action (e.g. Greenstone et al., 2012), our approach offers a viable alternative in situations when strict anti-contamination procedures cannot be implemented.

Using the BIN framework for species delimitation allowed us to retain in our analyses all individuals that were not identified as taxonomic species, which amounted to about a third of the total number of BINs (Figure 2, b1). Because cloud-based databases such as BOLD centralise taxonomic knowledge, we assume that the fraction of unidentified specimen would have likely been higher had determination been exclusively carried out by taxonomists. Taxonomic bias due to incomplete species determination is a common and serious problem in arthropod studies. As an example, an inaccurate assessment of the proportion of juvenile spiders in our sample led to a third of the individuals not being included in diversity analyses (Figure 2, b2). Our results show the considerable potential of DNA metabarcoding in improving the quality of arthropod diversity surveys (Morinière et al., 2019).

Our subsampling approach provided abundance estimates that effectively approximated true abundances (Figure 2c). Increasing the number of units by subsampling only slightly increased goodness-of-fit but substantially reduced the slope of the relationship and can thus provide a finer approximation of community evenness. Several other methods have been put forward to improve the predictability of the relationship between read numbers and abundance (e.g. Elbrecht et al., 2017; Krehenwinkel et al., 2017). However, all these methods, including the one described here, have in common to significantly inflate costs and workload. Researchers should thereby carefully consider trade-offs between information output and resource investment before attempting quantitative diversity surveys using metabarcoding.

4.2 | Treatment efficacy and outbreak effects

Both DFB and BTK were very efficacious in suppressing gypsy moth population in the treated trees (Figure 3). While caterpillar numbers were comparatively high in control trees, the predictions made based on egg mass surveys largely overestimated the risk of defoliation (Leroy, Gossner, et al., 2021). It is likely that late spring frost strongly damaged freshly hatched larval populations and stopped the outbreak altogether. Therefore, we considered the abundance of gypsy moths as an implausible driver of community structure and did not account for it in our analyses.

In practice, putting in perspective the impacts of outbreaks with those of their treatment with insecticides should always be a primary objective of impact assessment studies, yet it is challenging and rarely achieved (but see Sample et al., 1996). Beyond economic losses resulting from depressed growth rates and increased tree mortality (Davidson et al., 1999; MacLean, 2016), defoliator outbreaks can alter habitats, ecosystem processes and animal communities (e.g. Lovett et al., 2006; Scriber, 2004; Twery, 1991). Estimating the relative impacts of these different types of disturbances is crucial to support information-driven policies on the management of

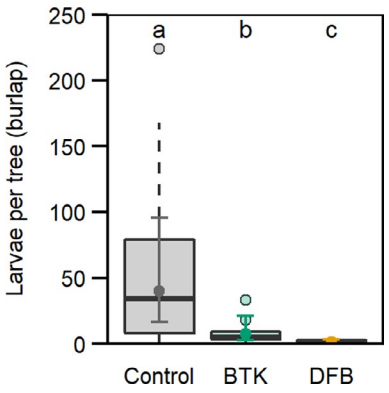

FIGURE 3 Abundance of gypsy moth larvae (*Lymantria dispar*) on trunks of control and insecticide-treated trees. Larvae were collected under tree bands ('burlaps') on trees aerially sprayed with diflubenzuron (DFB; Dimilin® 80 WG; 60 g a.i./ha; $n = 10$, orange colour) or *Bacillus thuringiensis* var. *kurstaki* (BTK; DiPel® ES; 1.75296 BIU/ha; $n = 10$; green colour) or left unsprayed ($n = 20$; grey colour). Boxplots depict the raw data, dots and error bars indicate the estimated marginal means and 95% confidence intervals derived from the model fit. Different letters indicate significant differences between treatments (Šidák-adjusted comparisons of estimated marginal means, $\alpha = 0.05$, FWER = 0.0170)

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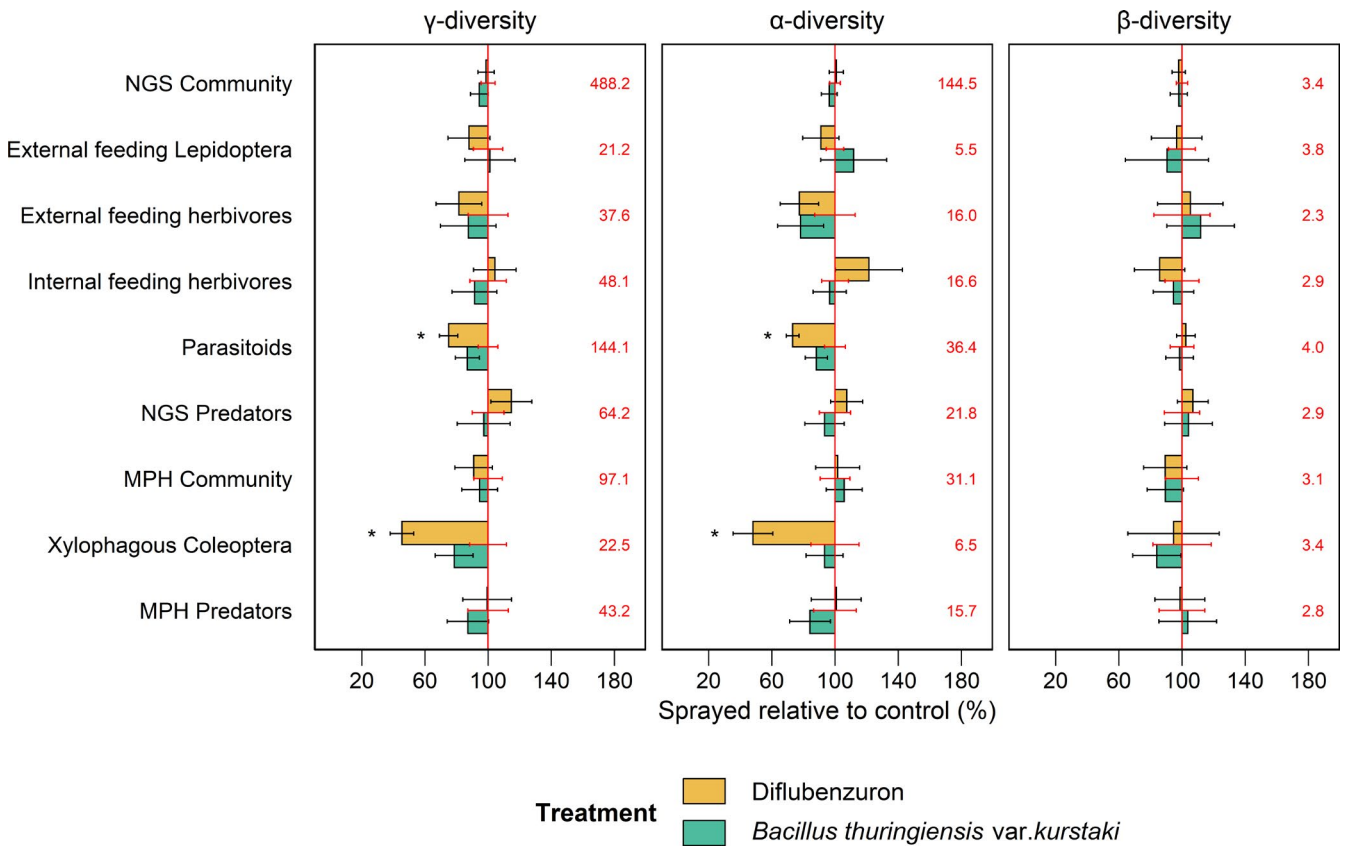

FIGURE 4 Diversity patterns in insecticide-treated trees relative to control trees. Trees were sprayed by helicopter with diflubenzuron (Dimilin® 80 WG; 60 g a.i./ha; $n = 6$; orange colour) or *Bacillus thuringiensis* var. *kurstaki* (DiPel® ES; 1.75296 BIU/ha; $n = 6$; green colour). Analyses were conducted separately for arthropods determined by DNA metabarcoding (NGS) and by morphological examination (MPH), on whole sampled communities and different sensitivity groups. Species diversity is expressed as species richness interpolated or extrapolated at even sample coverage. Diversity is partitioned into γ (total diversity per treatment), α (mean diversity per tree) and β components (species turnover, i.e. number of fully distinct tree communities: $\beta = \gamma/\alpha$). Treatment-level diversity patterns are displayed as percentage equivalents of controls, represented by the vertical red line. The baseline absolute values are indicated on the right of the bars. Error bars indicate 95% bootstrap confidence intervals (500 replicates) corrected for multiple testing with the Šidák method ($\alpha = 0.05$, two hypotheses for each of the nine assemblages: FWER = 0.0028). Non-overlapping confidence intervals indicative of a statistically significant difference to the control are marked with an asterisk (*)

forest resources and the conservation of forest ecosystems (Leroy, Lemme, et al., 2021).

4.3 | Impacts of insecticides on arboreal arthropod communities

We found that the species diversity of parasitoids and xylophagous beetles was significantly depressed in trees treated with DFB 5 weeks after treatment (Figure 4). These differences were slightly attenuated when weighing species by their relative abundance but remained pronounced and significant nonetheless (Figure S4). This indicates that even though rare species significantly contribute to the observed pattern, more common, 'typical' species are also affected, implying more severe impacts on community stability. In contrast with DFB, and despite similar patterns, BTK has no significant effect on any group. The lack of clear difference in β-diversity between treatments and the control indicates that diversity patterns were homogeneous among different arthropod communities treated with the same insecticides. While this tendency may not hold over larger spatial scales, it nonetheless suggests a certain consistency in the ecological processes underlying these effects locally.

The impacts of forestry insecticides on parasitoids have received particular attention, as this guild is considered to fulfil an important function in regulating defoliator populations (Berryman, 1996). Past studies involving parasitoids display a wide range of outcomes, from an absence of effect to 100% mortality (Flexner et al., 1986; Madrid & Stewart, 1981; Weseloh et al., 1983). These discrepancies were suggested to be driven by relative timings of host contamination and parasitisation and impacts of parasitism and insecticides on the host development rates (Flexner et al., 1986). However, we expect parasitoids to be primarily affected by insecticide-induced host scarcity (Sample et al., 1996). In particular, several parasitoid species of spring caterpillars display low reproductive rates, narrow host ranges and long generation times (K-strategists; Barbosa, 1977; Kenis et al., 2005). These species are poorly resilient to disturbance

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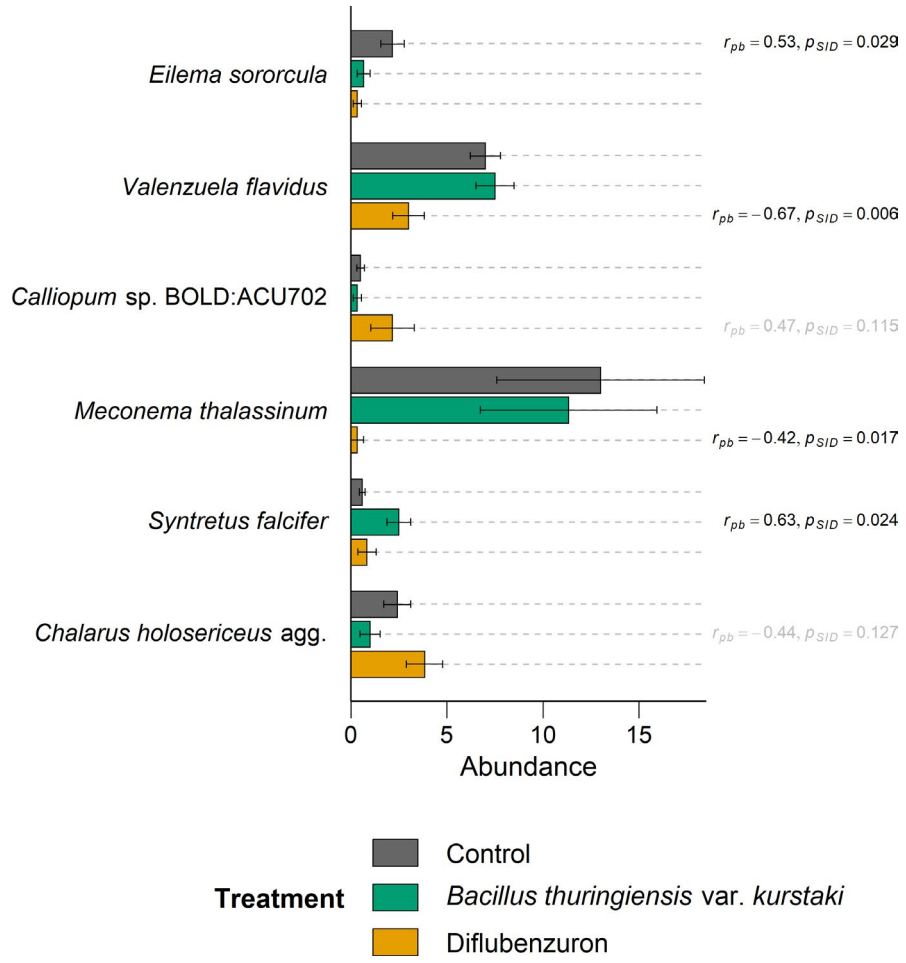

FIGURE 5 Strongest abundance-based species–treatment associations. Species abundances per treatment are expressed as mean aggregated incidences over 12 sequenced subsamples for all seven species but *Meconema thalassinum* (morphologically identified), for which true abundances are shown. Error bars indicates ±1 SE. The strength of species–treatment associations was calculated as the point-biserial correlation coefficient (r_{pb}) corrected for unequal sample sizes. The significance was calculated by means of a two-sided permutation test (1,000 permutations). The species displayed are those among the most commonly sampled species that were found to be significantly associated ($p < 0.05$) with one of the three experimental treatments: diflubenzuron (Dimilin® 80 WG'; 60 g a.i./ha; $n = 6$; orange fill), *Bacillus thuringiensis* var. *kurstaki* (DiPel® ES; 1.75296 BIU/ha; $n = 6$; green) and unsprayed control ($n = 12$; grey). *p*-values were further corrected for multiple testing using the Šidák method (p_{SID}). Full results for all 67 species tested are shown in Table S2

and should be particularly affected by the suppression of host populations.

The fact that we found more than 50% fewer xylophagous beetle species in DFB-treated trees than in controls is an obvious cause for concern. These species feed on plant and fungal tissues within live or dead wood through their larval stage, which prevents them to come into contact with insecticide residues. Heavy species loss is most likely the result of indirect processes triggered by the initial effects of DFB. Because leaf chewers are so dominant in the spring, DFB essentially suppresses most of the insect biomass despite its apparent selectivity. This asymmetric but large quantitative response is likely to cause further disruption of the community structure by altering trophic interactions, with cascading consequences affecting other arthropod groups, including non-sensitive ones (Fleeger, 2020). For example, many species of breeding birds rely on the superabundance of caterpillars in spring to feed their nestlings (Cooper, 1988; Perrins, 1991) and were shown to shift to other insect prey when this resource was suppressed by insecticides (Rodenhouse & Holmes, 1992; Sample et al., 1993). Such dietary shifts may trigger negative feedback effects on arthropod populations (Berryman et al., 1987). Beetles may be particularly vulnerable, as predators foraging primarily by foliage-gleaning caterpillars were shown to shift primarily towards poor flyers in response to declining caterpillar abundance (Cooper, 1988). However, while indirect effects of forest spraying on the fitness of insectivorous birds have received substantial attention (Awkerman et al., 2011; Cooper et al., 1990; Sample et al., 1993), the existence of feedback loops on arthropod communities has yet to be explicitly identified.

In contrast with diversity patterns, the species–treatment associations revealed by the indicator species analysis are more readily relatable to direct mechanisms (Figure 5). For example, the barklice *V. flavidus* is an obligate folicolous (i.e. leaf-frequenting) grazer that

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colonises trees in spring and only mature in June (Schneider, 2007). This delayed colonisation is driven by its specific habitat requirements, as leaf microbiota on which it feeds builds up from zero at budburst and becomes increasingly available over time (New, 1987). By contrast, the early spring species *E. moebiusi* and *G. cruciatus* have already reached the adult stage at the time of application (Lienhard, 1998) and are hence no longer susceptible to DFB (Table S2). The near-complete eradication of the oak bush cricket *M. thalassinum* in DFB-treated trees was more surprising (Figure 5). Juvenile stages can be found in large numbers in oak woodland canopies through the spring. They are primarily carnivorous, and often target caterpillars (Ingrisch & Köhler, 1998), suggesting that their collapse in DFB-treated trees resulted from secondary exposure through intoxicated prey. Trophic transfers of DFB have been reported for various predatory species under controlled conditions (Castro et al., 2012; Medina et al., 2002; Smith & Lockwood, 2003), but has so far not been considered as a major impact pathway *in situ*.

5 | CONCLUSIONS

With this study, we uncovered the existence of indirect and secondary direct impacts of one forestry insecticide on non-target species, providing evidence that the side effects of aerial spray treatments are not restricted to their predictable toxic effects. Our results underpin the view that our knowledge of the full extent of insecticide side effects on forest ecosystems remains largely incomplete. Ecological impact assessment studies frequently overlook taxa, lack clearly framed hypotheses and rarely account for the role of life history and species interactions in mediating insecticide effects. The resulting knowledge gaps may lead to a systematic underestimation of the environmental impacts on aerial treatments, which calls for an urgent methodological overhaul of impact assessment protocols to support sustainable pest management in forests. While the narrow spatial and temporal scope of our experiment does not allow us to appraise the ecological significance of our findings, their mere existence is a cause for concern and justifies closer scrutiny from forest managers, scientists and policymakers alike. With the advent of DNA metabarcoding, there are fewer and fewer practical obstacles to the realisation of impact assessment in species-rich communities. In view of our findings, we would hence recommend the adoption of community-level end points as regulatory requirements in the legal framework for authorisation of forest use of plant protection products.

All things considered, the absence of any significant effect of BTK across all tested taxa come as a silver lining in our findings. Unknown ecological impacts of forest spraying may have already been greatly mitigated by past efforts to reduce the potency and increase the specificity of forest-use substances (Holmes & MacQuarrie, 2016; Liebhold & McManus, 1999). However, even biorational insecticides should be thoroughly controlled for environmental risk. The recent rise in popularity of tebufenozide, a Lepidoptera-specific insecticide praised for its high efficacy and reliability compared to BTK (Lemme et al., 2019), should be accompanied by strict monitoring of its side effects through robust impact assessment methods. To this end and against the backdrop of the large-scale gypsy moth outbreak which recently developed in Northern Bavaria, a region-wide multidisciplinary impact assessment experiment has been set up in 2019 to gather comprehensive data on the implication of defoliator outbreaks and their management with tebufenozide (Leroy, Lemme, et al., 2021). With this project, we aim to bring the approach presented in this study into larger spatial and time-scales, to produce data that can be readily exploited by practitioners and policymakers to promote sustainable pest management strategies in forests.

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CONFLICT OF INTEREST

J.M. is the founder & CEO of AIM Advanced Identification Methods GmbH. V.B. is a permanent employee at AIM Advanced Identification Methods GmbH. All authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

W.W.W., R.P., S.S., B.M.L.L. and S.Z. conceived the original ideas and methodologies; B.M.L.L. designed the experiment; J.J., S.S., N.R., S.V., P.E. and B.M.L.L. collected the data; J.M. and V.B. conceived and implemented the metabarcoding pipeline; B.M.L.L. processed and analysed the data; B.M.L.L. wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Statement on inclusion. Our study brings together authors from three different countries (France, Germany and the United Kingdom), all of whom were based in the region where the study was carried out (Bavaria, Germany) during the conceptional and practical phase of the study. This study is a result of an active collaboration between ecologists from different Universities and applied scientists from the forest protection department of the Bavarian State Institute for

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Forestry. Knowledge originating from both international academic works and local applied research was used in the conception of the study and appropriately cited.

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository https://doi.org/10.5061/dryad.s1rn8pk95 (Leroy, Seibold, et al., 2021).

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