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RESEARCH ARTICLE

ENVIRONMENTAL MICROBIOLOGY

Nontarget impacts of neonicotinoids on nectar-inhabiting microbes

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Abstract

Plant-systemic neonicotinoid (NN) insecticides can exert non-target impacts on organisms like beneficial insects and soil microbes. NNs can affect plant microbiomes, but we know little about their effects on microbial communities that mediate plant-insect interactions, including nectar-inhabiting microbes (NIMs). Here we employed two approaches to assess the impacts of NN exposure on several NIM taxa. First, we assayed the in vitro effects of six NN compounds on NIM growth using plate assays. Second, we inoculated a standardised NIM community into the nectar of NN-treated canola (*Brassica napus*) and assessed microbial survival and growth after 24 h. With few exceptions, in vitro NN exposure tended to decrease bacterial growth metrics. However, the magnitude of the decrease and the NN concentrations at which effects were observed varied substantially across bacteria. Yeasts showed no consistent in vitro response to NNs. In nectar, we saw no effects of NN treatment on NIM community metrics. Rather, NIM abundance and diversity responded to inherent plant qualities like nectar volume. In conclusion, we found no evidence that NIMs respond to field-relevant NN levels in nectar within 24 h, but our study suggests that context, specifically assay methods, time and plant traits, is important in assaying the effects of NNs on microbial communities.

INTRODUCTION

Neonicotinoids (NNs) are a major class of nicotinic acetylcholine receptor (nAChR) agonists and synthetic analogues of nicotine (Kovganko & Kashkan, 2004) that are the most widely used class of insecticides globally (Goulson, 2013; Hladik et al., 2018). NNs include several compounds that vary slightly in chemical structure, including imidacloprid, thiamethoxam, clothianidin and others. They are highly soluble in water and systemic in plants, translocating into multiple tissues and exudates such as nectar (Bonmatin et al., 2015). These properties render NNs effective tools in combatting a large range of insect pests in numerous crops. NNs can become pervasive in agricultural areas and their environs (Botías et al., 2016), into which residues may travel via groundwater, wind or other modes (Thompson et al., 2020). Conservation concerns exist over the short- and long-term impacts of NN exposure for nontarget organisms, that is, those species inadvertently exposed and which are not the intended foci of application (Goulson, 2013; Pisa et al., 2015; Wood & Goulson, 2017).

NNs can adversely affect many non-insect taxa despite lower binding affinity to neurotransmitter receptors of other animals than to those of insects. For example, while vertebrates are generally more likely to experience sub-lethal effects on development and reproduction than outright mortality from environmental NN exposure, evidence of NN-induced mortality in some species certainly exists (Gibbons et al., 2015). A large volume of work has explored the consequences

of nontarget NN exposure for beneficial insects in agricultural areas (Pisa et al., 2015), particularly flower-visiting pollinators (Lundin et al., 2015). In bees (Hymenoptera: Anthophila), nontarget NN exposure can result in a variety of detrimental sub-lethal effects, including declines in resistance to pests and pathogens (Alaux et al., 2010; Pettis et al., 2013), foraging and navigation (Henry et al., 2012), learning and memory (Williamson & Wright, 2013) and fecundity (Whitehorn et al., 2012).

In agroecosystems, comparatively less attention has been devoted to the nontarget effects of NNs on microbial organisms like bacteria and fungi. While microbes do not possess the receptor proteins that NNs target in insects, NNs can alter soil microbe metabolism and physiology in some cases, based on observations of their effects on soil enzyme activity levels (Cycoń & Piotrowska-Seget, 2015; Imfeld & Vuilleumier, 2012; Shahid & Khan, 2022). Earlier work suggests that natural alkaloids like nicotine (with which NNs share a mode of action) possess antimicrobial properties, though their evolutionary function (Adler, 2000; Heil, 2011) and modes of action in microbes are far less understood (Wink, 1998).

Existing studies on nontarget effects of NNs on microbes have generally focused on soil- and phyllosphere-inhabiting taxa (Pang et al., 2020), as is true of studies focusing on other pesticides in general (Imfeld & Vuilleumier, 2012). This is expected given that NNs are normally sprayed on plants or applied to the soil. Succinctly summarising this body of work is challenging due to diverging methodologies and contexts (Akter et al., 2023). Some studies have documented a range of adverse impacts from NN exposure on soil microbes (e.g., Cai et al., 2016; Streletskii et al., 2022; Yu et al., 2020). In other cases, some microbes appear to be unaffected or even benefit from exposure, especially those species that can metabolise NNs (Singh & Singh, 2005; Zhang et al., 2015). Imidacloprid, the most commonly used NN, is known to alter the structure of bacteria and fungi communities in soils and phyllospheres (Moulas et al., 2013; Parizadeh et al., 2021) by either inhibiting (Ahmed & Ahmad, 2006) or enhancing (Moulas et al., 2013; Zhang et al., 2015) growth of different taxa or altering their metabolic activity (Wang et al., 2014). A recent review (Akter et al., 2023) on soil microbes and NNs underscored the need for more studies taking place in field settings, outside the laboratory, and studies examining commercial NN formulations.

Microbes growing in flowers, including in floral nectar, may mediate the effects of NNs and other pesticides on floral traits and pollinators, yet relatively little is known about how agrochemicals affect microbes inhabiting floral nectar (Stanley & Preetha, 2016). Nectar-inhabiting microbes (NIMs) occur widely and are found among many plant species. While microbial abundance in flowers is initially low, microbes can quickly become abundant inhabitants (Lievens et al., 2014) via dispersal by flower-visiting animals (Canto et al., 2008) or from other plant tissues (Aleklett et al., 2014). NIMs have garnered attention due to their potential to influence pollination processes (Schaeffer et al., 2014) including floral reward quality (Herrera et al., 2008; Vannette & Fukami, 2018) and pollinator preference (Schaeffer et al., 2014; Yang et al., 2019). Studies on NIMs in agricultural habitats are few in number (Lievens et al., 2014) despite evidence they occur in crop flowers (Fridman et al., 2011).

NIMs may experience overlooked, nontarget effects from certain agrochemicals (Stanley & Preetha, 2016). Agricultural fungicides, for example, can reduce the richness and diversity of nectar-inhabiting yeasts (Álvarez-Pérez et al., 2016; Bartlewicz et al., 2016; Schaeffer et al., 2017; Wei et al., 2021). Other pesticides, like NNs, warrant further investigation in this regard. Due to the widespread use and systemic presence of NNs in floral nectar, it is reasonable to assume NIMs may come into prolonged contact with these compounds in flowers of treated plants (Bartlewicz et al., 2016). Nicotine is a common nectar secondary compound in various plant species (Hladik et al., 2018) and has been shown to impact the growth of certain taxa of NIMs (Vannette & Fukami, 2016). However, knowledge of how NNs may affect NIMs is lacking. Such information is important for predicting how chemical-induced impacts on floral microbes may affect pollinator behaviour, pollination, crop yield, biocontrol efforts and other critical aspects of agriculture (Burgess & Schaeffer, 2022).

As NNs are highly water soluble and plant water availability can mediate NN uptake and transport (Bonmatin et al., 2015), we suspect irrigation level may influence NN concentrations in nectar (as in Cecala & Wilson Rankin, 2021) and thus the strength of nontarget effects, if they exist. Water stress due to low soil moisture can increase the rate of uptake of NNs through xylem tissue due to higher transpiration at leaf surfaces (Stamm et al., 2015; Stein-Dönecke et al., 1992). Furthermore, increased irrigation can lead to changes in nectar attributes like sugar content (Petanidou et al., 1999; Waser & Price, 2016), which is a critical characteristic of nectar believed to filter out certain colonising microbes (Herrera, Canto, et al., 2009; Herrera, de Vega, et al., 2009; Pozo et al., 2012). Plant water availability, a function of crop irrigation regimes, may thus lead to an interesting modulation of the nontarget effects of NNs on NIMs.

In this study, we test the hypothesis that exposure to NNs, due to their chemical similarities to nicotine and systemic translocation into floral nectar, can alter the community composition of common NIMs through differential effects on the growth of specific microbe taxa. Furthermore, we hypothesise that plant water

TABLE 1 Microbe species used in the plate reader experiment in pure cultures and the greenhouse experiment in mixed culture. See Supplementary Material for growth media recipes.

Microbe taxon	Microbe species and authority	Strain designation	Lower taxonomy	Growth medium used	Plate reader experiment	Greenhouse experiment
Fungi	*Metschnikowia reukaufii* Pitt & M.W. Mill., 1968	EC52	Saccharomycetales	Yeast media	✓	✓
	Aureobasidium pullulans (de Bary & Löwenthal) G. Arnaud, 1918	EC102	Dothideales		✓	
Bacteria	*Neokomagataea thailandica* Yukphan et al. 2011	EC112	Alphaproteobacteria: Rhodospirillales	Tryptic soy	✓	✓
	Acinetobacter pollinis Alvarez-Perez et al. 2021	SCC477	Gammaproteobacteria: Pseudomonadales		✓	✓
	Rosenbergiella nectarea Halpern et al. 2013	EC124	Gammaproteobacteria: Enterobacterales		✓	
	Pantoea agglomerans (Beijerinck 1888) Gavini et al. 1989	SCC187	Gammaproteobacteria: Enterobacterales		✓	
	Apilactobacillus micheneri (McFrederick et al. 2018) Zheng et al. 2020	HV60	Bacilli: Lactobacillales	MRS	✓	✓

Note: Orange shaded cells correspond to fungi, while blue shaded cells correspond to bacteria.

availability, which governs many aspects of plant growth and nectar characteristics, interacts with the nontarget effects of NNs on NIMs by modulating nectar NN concentrations. We address these questions using two multifactorial in vitro and *in planta* experiments.

EXPERIMENTAL PROCEDURES

Overview of experiments

To determine if and how exposure to NN residues affects the growth and abundance of NIMs, we conducted two separate experiments. First, we grew seven microbes as pure (single-species) cultures in artificial broths spiked to contain one of four concentrations of each of the six NN compounds. We monitored microbial growth as optical density over 72 h and calculated the maximum growth rate (r) and carrying capacity (K). Second, we inoculated a standardised community of four microbe taxa (a subset of those from the first experiment) into floral nectar of greenhouse-grown, potted canola (Brassicaceae: *Brassica napus* L.) plants. Plants had been treated with either low or high doses of two commercial NN formulations. Plants were also irrigated at either a low or high rate to monitor for any effect of water availability on nectar characteristics, NN translocation, or microbe community metrics measured as CFU counts on agar. While each method used to quantify microbe growth has its unique limitations, previous comparisons of optical density and CFU counts (Mueller et al., 2023; Peay et al., 2011; Vannette et al., 2021) have yielded good correspondence between them.

In vitro plate reader experiment

We selected seven microbe species to assay growth in the presence of set concentrations of six major NN compounds. The selected species occur in floral nectar, are relatively well-studied, and in some cases have been found to influence behaviours of flower-visiting animals or plant reproduction (Vannette, 2020) (Table 1). All microbe strains were sourced from suspensions in autoclaved 15% v/v glycerol and 15% m/v sucrose stock at −80°C. Prior to each plate reader run, we streaked stock on agar media and incubated plates at 25°C for 72 h. Yeasts were streaked on yeast media (YM) agar and bacteria on tryptic soy (TS) agar, except for *Apilactobacillus micheneri*, which was streaked on de Man, Rogosa, and Sharpe (MRS) agar +2% m/v fructose (Vuong & McFrederick, 2019). We also prepared liquid broth analogues of each media type (omitting agarose) for use in well plates. We used nutrient-rich broths instead of an artificial nectar analogue to ensure that all seven focal microbes would exhibit sufficient growth to reliably gauge any effects of NN exposure, if present. Furthermore, attempts to create nectar analogues by approximating sugar and nitrogen concentrations of real nectar typically resulted in suboptimal growth in vitro, potentially due to the lack of unknown solutes important to microbe growth. All agar and broth media contained 0.1% v/v of a solution of either chloramphenicol (antibacterial; in yeast media) or cycloheximide (antifungal; in bacterial media) in methanol (10% m/v).

We acquired PESTANAL® analytical standards (Millipore Sigma, St. Louis, MO) for each of the six major NN compounds (Table 2). We created a separate

TABLE 2 The six neonicotinoids (NNs) used in this study. Compounds differ in molecular mass, so solutions of equal mass fraction (ppb) will differ in molarity across compounds (Wood & Goulson, 2017). We standardised concentrations across compounds by mass, instead of moles, corresponding to how NN concentrations in floral nectar are most commonly expressed in literature. In floral nectars, NN concentrations can vary considerably due to a multitude of factors. In general, nectar samples from seed-treated crops contain <10 ppb on average (Goulson, 2013; Wood & Goulson, 2017). Concentrations around 100 ppb are more unusual in nectar but represent maxima in certain scenarios (Bonmatin et al., 2015; Cecala & Wilson Rankin, 2021). Concentrations near 1000 ppb are extremely high for nectar and are unlikely to be encountered in field settings, but were included to detect any potential hormetic or stimulatory effects (Agathokleous et al., 2022) of NNs on microbial growth.

Neonicotinoid compound	Molecular mass (g/mol)	Solubility in water (g/L), 20°C	Plate reader experiment	Greenhouse experiment
Imidacloprid	255.66	0.51	✓	✓
Thiamethoxam	291.71	4.1	✓	
Clothianidin	249.67	0.327	✓	
Acetamiprid	222.68	4.2	✓	
Thiacloprid	252.72	0.185	✓	
Dinotefuran	202.21	39.83	✓	✓

stock solution for each compound in sterile distilled water by adding 2 mg of the respective compound to 100 mL of water, yielding a concentration of 2×10^4 ppb or $\mu g\ L^{-1}$. Stock solution bottles were wrapped in foil and kept in a container at 5°C to prevent photodegradation of NNs (Borsuah et al., 2020). These six stock solutions were used as spikes (as in Meikle et al., 2022) to achieve specific concentrations of each compound in the corresponding broth for each microbe assay (see below; see also Supplementary Material).

To determine if NN type and concentration influence the growth of microbes in vitro, we conducted successive runs using two spectrophotometer microplate readers (models SYNERGY HTX and 800 TS; Agilent, Santa Clara, CA) simultaneously, using a consistent plate layout for all runs (Figure S1). We chose to use a non-randomised plate layout across runs to increase the efficiency and accuracy of filling wells. To account for potential spatial effects, we included control (no NN) inoculated wells in each pair of columns (corresponding to the six types of NNs), and NN treatment wells were statistically compared only to their respective controls. We grew each microbe strain in pure culture in two 96-well plates, run at the same time in the two readers, with each run comprising one microbe. Prior to a run, we prepared separate solutions of the appropriate broth for the focal microbe spiked to contain either 1000 ppb, 100 ppb, 10 ppb, or a no-NN control of each of the six NN compounds (see Table 2 for the ecological context of these concentrations). This resulted in a total of six replicate wells for each of these 24 treatments per experimental run, alongside 24 non-inoculated control wells per plate (192 wells total per run). For the inoculum, we prepared a suspension of the focal microbe by scraping a 2-mm bolus from agar into 3.5 mL of the appropriate broth and vortexing. Per treatment, we inoculated six of the eight wells containing 180 μL of sterile broth with 20 μL of inoculum. In the remaining wells, we prepared 200 μL of non-inoculated broth to monitor for contamination across treatments. Immediately after inoculation, plates were sealed with a lid and Parafilm® (Amcor, Switzerland) (Pierce et al., 2008). While Parafilm may affect the diffusion of certain gases (Banerjee et al., 2019), we included it to minimise the risk of both uneven evaporation of wells closer to plate edges and potential contamination during incubation. Plates were then loaded into readers and incubated continuously at 25°C (30°C for *Apilactobacillus*; McFrederick et al., 2017) for 72 h. To estimate changes in cell concentration over time, readers recorded the optical density at $\lambda = 600$ nm (OD_{600}) of all wells after shaking (6-mm diameter at 6 Hz) every 15 min for 72 h. The initial OD_{600} of inoculated wells was approximately 0.1 (Table S1). After incubation, we inspected all wells for aberrant coloration or growth suggestive of contamination; none was detected.

Statistical analysis for in vitro plate reader experiment

We performed all statistical analyses in R (R Core Team, 2023). We analysed microbial growth using the function 'SummarizeGrowthByPlate' in the package *growthcurver* (Sprouffske, 2020), which fits a logistic growth equation to OD vs. time, and estimates maximum growth rate (*r*) and maximum OD (*K*) for each well over the 72-h period. OD values for inoculated wells were blank-corrected by subtracting the mean OD of all non-inoculated wells at each time point. Using the package *lme4* (Bates et al., 2015), we performed linear mixed-effect models (LMMs) for each microbe taxon, with either *r* or *K* as dependent variables, and NN type, concentration, and their interaction as independent variables, and plate reader as a random intercept effect. We obtained type III sums of squares and Kenward-Roger degrees of freedom using the 'Anova' function in the package *car* (Fox & Weisberg, 2019) and

checked all mixed models for multicollinearity (variance inflation factor, or VIF >2.0) and normality of residuals.

In planta greenhouse experiment

To assess how NN application and plant water availability impact a microbe community in floral nectar, we conducted an experiment in a glass greenhouse on the University of California, Davis campus (USA: California: Yolo County; 38.5361° N, 121.7475° W). We obtained seeds of spring canola, *Brassica napus* L. 'CP930RR' (Land O'Lakes, Inc., Arden Hills, MN, USA), not previously treated with any chemicals. We chose canola as a model plant because it is a widely grown crop that produces nectar, has flowers that are attractive to pollinators, and is often commercially treated with NNs (Sekulic & Rempel, 2016). Furthermore, canola is quick to grow from seed, produces abundant flowers, and its nectaries are easily inoculated. We sowed seeds in 60 2.5-gallon pots (25.7 × 23.2 cm) of 'UC Mix C' soil (1:1 peat and sand) in three cohorts (18 February, 4 March and 18 March 2022). After germination, we culled plants to six per pot.

We applied commercial NN formulations to pots according to label specifications once the first buds were produced in a cohort, around 14–18 days before inoculations. Pots were treated with either a high dose (25 mg active ingredient per pot, or 4.2 mg per plant), a low dose (2.5 mg AI per pot, 0.42 mg per plant), or a no-dose control of the respective formulation. For reference, a commercially treated seed typically contains from 0.2 to 1.3 mg of AI (Goulson, 2013; Wood & Goulson, 2017). We included two different NNs in the experiment, applied singly: imidacloprid, as Marathon® 1% Granular (OHP, Bluffton, SC, USA), and dinotefuran, as Safari® 20 SG (Valent U.S.A. LLC, San Ramon, CA, USA). We selected these two formulations based on multiple factors, including usage in agricultural settings, differences in solubility and leaching potential (Bonmatin et al., 2015), and approved usage in potting media and greenhouse settings.

Each pot was additionally assigned in a crossed fashion to one of two irrigation treatments. The irrigation rate was controlled by inserting one high- or low-flow irrigation spike (Primerus Products, Encinitas, CA, USA) per pot, each connected to one central irrigation line (as in Cecala & Wilson Rankin, 2021). A high-flow spike emitted 2.7 times the water (0.61 L/min) as a low-flow spike (0.23 L/min). All pots were automatically irrigated simultaneously over the soil surface at 07:00 AM daily for 60 s, or up to 120 s on hotter days to prevent wilting.

Experimental flowers were selected and inoculated with a standardised microbe community containing 10^4 cells μL^{-1} of each of a subset of four species from the plate reader experiment (Table 1) in a 20% v/v glycerol, 20% m/v sucrose stock (4×10^4 total cells μL^{-1}). Microbes were stored in pure culture aliquots at −80°C that were thawed and mixed the morning of each day of inoculations as in Francis et al. (2023). All four microbes in the inoculum were confirmed to be successfully culturable on agar media and from trial flowers prior to experiments. Using a micropipette, we delivered 0.5 μL of inoculum into each lateral nectary (total of 1 μL per flower) of newly opened canola flowers and tagged them.

After 24 h, we excised all inoculated flowers using sterilised forceps and transported them to the laboratory. Inside a laminar flow hood, we used 10-μL microcapillary tubes to remove all nectar from each inoculated flower, estimated nectar volume from the length of the fluid column and expelled it into individual strip tubes. We also measured whole flower mass after nectar removal as an additional measure of plant response to our treatments apart from nectar production. We added 50 μL of Dulbecco's phosphate-buffered saline (DPBS 1×) to each nectar sample, vortexed tubes, then plated a 15 μL aliquot of the solution onto each of YM, TS, and MRS agar in 100 × 15 mm Petri dishes using plating beads. We then incubated plates for 7 days at 25°C and stored them at 5°C. We tallied CFUs per plate and classified them into morphotypes. A representative CFU of each morphotype was sequenced by single-gene PCR and barcoded using NCBI Nucleotide BLAST ('blastn'; blast.ncbi.nlm.nih.gov). CFU morphotypes other than our four inoculated microbes were tallied, but comprised only 3.5% of all CFUs and were not included in further analyses.

To determine the concentration of NN residues in canola nectar, we collected separate samples of nectar from newly opened, uninoculated flowers. To obtain sufficient volumes for residue analysis, we pooled up to 12 flowers from plants in the same pot. Samples were kept at −20°C until analysis, at which point we diluted them in ultrapure water and analysed them via electrospray ionisation (ESI) LC–MS (Martel et al., 2013) on an Orbitrap machine. For LC–MS analysis, 5 μL of samples were injected into a Thermo C18 Accucore column (2.1 × 50 mm). A standard reverse phase gradient (solvent: Optima grade water and acetonitrile (Fisher, MS grade), plus 0.1% formic acid) was run over 12 min at a flow rate of 250 $\mu L\ min^{-1}$ and the eluent was monitored for positive ions by a Thermo Scientific Q-Exactive HF operated in profile mode. Source parameters were 4 kV spray voltage, capillary temperature of 275°C, and sheath gas setting of 20. Spectral data were acquired at a resolution setting of 60,000 FWHM with the lockmass feature, which typically results in a mass accuracy of <2 ppm. Analytical standards of imidacloprid and dinetofuran (5 ppm in Milli-Q® ultrapure water) were dissolved in methanol and diluted into a mobile phase for quantitation. Standard curves were run for every set of samples, from which we

TABLE 3 Results of linear mixed models from the plate reader experiment, testing for the effect of NN type, NN concentration (conc.), and their interaction (NN type × NN conc.) on maximum OD_{600} (*K*) and maximum growth rate (*r*) of microbe taxa over 72 h. *P*-values less than 0.05 are indicated with an asterisk.

	Maximum OD_{600}, *K*					Maximum growth rate, *r*				
	Model term	*F*	df	*P*	R^2	Model term	*F*	df	*P*	R^2
Metschnikowia	**NN type**	2.19	5119	0.060	0.92	**NN type**	1.44	5119	0.21	0.20
	NN conc.	1.02	3119	0.39		**NN conc.**	0.52	3119	0.67	
	NN type × NN conc.	0.52	15,119	0.93		**NN type × NN conc.**	0.73	15,119	0.75	
Aureobasidium	**NN type**	1.36	5119	0.24	0.88	**NN type**	0.36	5119	0.88	0.66
	NN conc.	2.83	3119	0.041*		**NN conc.**	1.23	3119	0.30	
	NN type × NN conc.	1.10	15,119	0.36		**NN type × NN conc.**	0.80	15,119	0.68	
Acinetobacter	**NN type**	0.28	5119	0.93	0.82	**NN type**	0.20	5119	0.96	0.27
	NN conc.	12.99	3119	<0.0001*		**NN conc.**	2.32	3119	0.079	
	NN type × NN conc.	1.53	15,119	0.10		**NN type × NN conc.**	0.75	15,119	0.73	
Neokomagataea	**NN type**	1.31	5119	0.27	0.77	**NN type**	0.45	5119	0.81	0.62
	NN conc.	4.85	3119	0.0032*		**NN conc.**	2.14	3119	0.099	
	NN type × NN conc.	0.75	15,119	0.73		**NN type × NN conc.**	1.35	15,119	0.18	
Rosenbergiella	**NN type**	1.19	5119	0.32	0.68	**NN type**	3.76	5119	0.0034*	0.85
	NN conc.	6.35	3119	0.00049*		**NN conc.**	7.83	3119	<0.0001*	
	NN type × NN conc.	1.21	15,119	0.27		**NN type × NN conc.**	1.74	15,119	0.053	
Pantoea	**NN type**	2.64	5119	0.026*	0.98	**NN type**	1.40	5119	0.23	0.64
	NN conc.	1.38	3119	0.25		**NN conc.**	12.63	3119	<0.0001*	
	NN type × NN conc.	0.92	15,119	0.54		**NN type × NN conc.**	0.56	15,119	0.90	
Apilactobacillus	**NN type**	1.50	5119	0.19	0.92	**NN type**	0.55	5119	0.74	0.27
	NN conc.	8.73	3119	<0.0001*		**NN conc.**	1.43	3119	0.24	
	NN type × NN conc.	1.18	15,119	0.29		**NN type × NN conc.**	1.44	15,119	0.14	

Note: Orange shaded cells correspond to fungi, while blue shaded cells correspond to bacteria.

back-calculated sample residue concentrations. Extracted Ion Chromatograms (XICs) utilising a 10 ppm mass window for each of the compounds were used for quantitation.

Statistical analysis for in planta greenhouse experiment

To determine how irrigation level and NN application impacted our model floral microbe community, we constructed linear mixed models in *lme4* with nectar volume, flower mass, CFU abundance (summed across the three media types), CFU density (per μL nectar), and CFU Shannon diversity per flower as dependent variables. As independent variables, we included irrigation rate, NN dose, their interaction and NN type (nested within dose). To test if differences in microbial community composition (as Bray–Curtis dissimilarity) were related to treatments, we also performed a permutational multivariate analysis of variance (permANOVA) using the function 'adonis' in the package *vegan* (Oksanen et al., 2020). Multivariate homogeneity of dispersions within independent variable groups was checked with the PERMDISP2 procedure using the function 'betadisper' in *vegan*. We visualised the distance between samples with non-metric multidimensional scaling using the function 'metaMDS' and confirmed ordination stress was sufficiently low in $k = 2$ dimensions using the function 'dimcheckMDS'. All figures were created using the package *ggplot2* (Wickham, 2016).

RESULTS

In vitro experiment

For both fungi assayed, growth parameters did not respond to NNs (Table 3), with the following

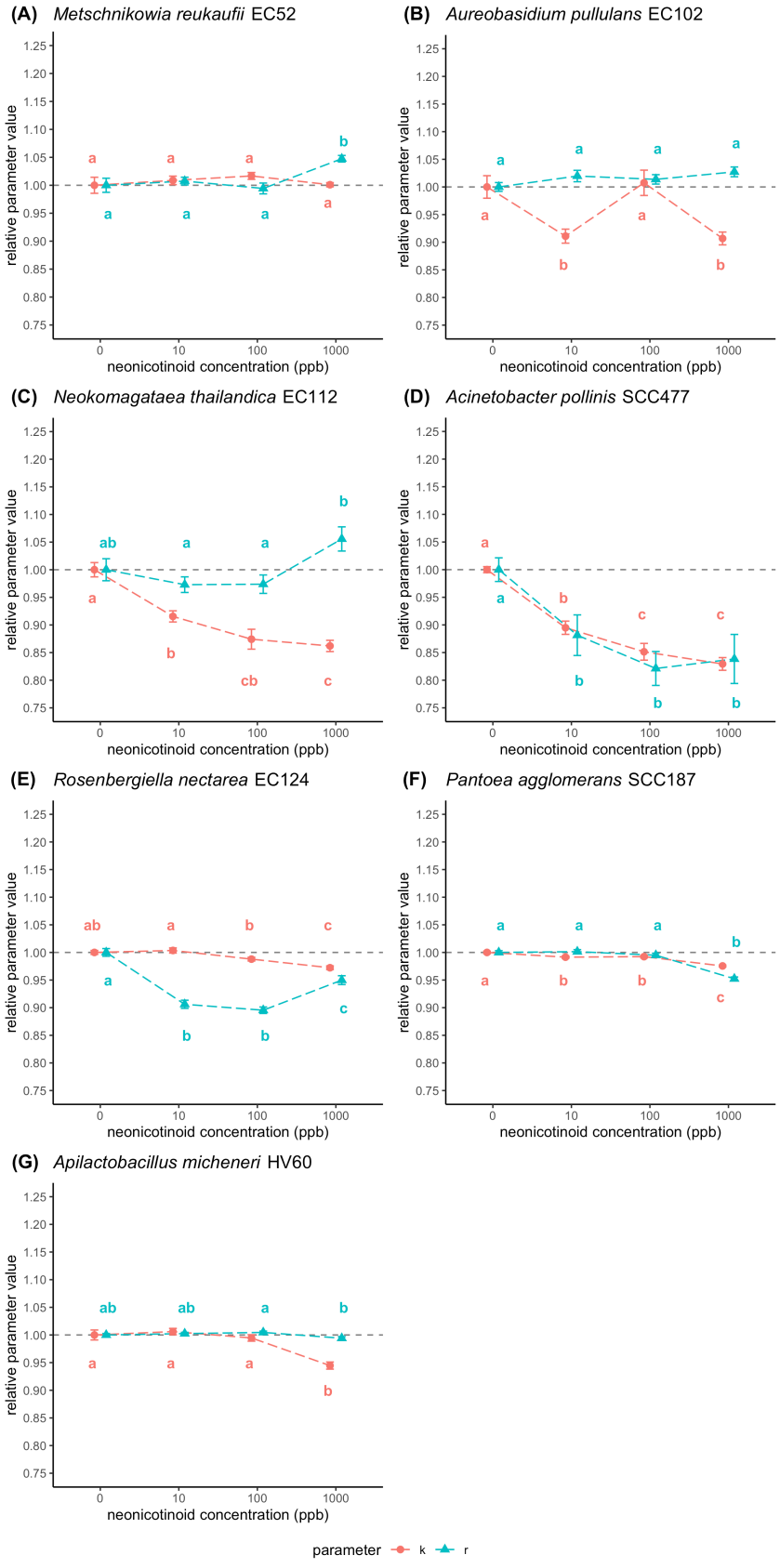

FIGURE 1 Legend on next page.

exceptions: *Metschnikowia r* was higher at 1000 ppb (Figure 1A), while *Aureobasidium K* displayed the only non-linear relationship in the experiment found between a growth parameter and increasing NN concentration. *Aureobasidium K* was lower at 10 and 1000 ppb but did not differ from the control at the intermediate 100 ppb (Figure 1B).

For bacteria, increasing concentrations of NNs generally decreased growth rate and maximum optical density relative to the controls (Figure 1C–G), with some exceptions (Figure 1; Table 3). *K* values decreased with increasing NN concentration, but the threshold concentration at which a negative response was observed differed across bacterial species. The same pattern was generally true for *r* values, however, there were two exceptions: *Neokomagataea r* was higher at 1000 ppb (Figure 1C), and *Apilactobacillus r* did not differ at any NN concentration (Figure 1G).

Generally, microbial growth parameters did not vary with respect to the type of NN compound (e.g., imidacloprid vs. dinotefuran, etc.; Table 2). There were only two exceptions, according to post-hoc Tukey's HSD tests: *Rosenbergiella r* was reduced to a greater extent by imidacloprid than acetamiprid, while *Pantoea* K was reduced more by imidacloprid than thiacloprid. In all LMMs of *r* and *K* values, the interaction term between NN type and NN concentration was not significant ($p > 0.05$) for any species.

In planta experiment

Nectar and flower properties

Irrigation level, but not NN treatment, affected canola flowers. Flowers from plants in the high irrigation treatment contained, on average, 2.1 times more nectar ($F_{1,40} = 22.71$, $p < 0.0001$; Figure 2A) and the flowers themselves weighed 1.7 times more ($F_{1,39} = 17.34$, $p = 0.00017$; Figure 2B) compared to flowers in the low irrigation treatment. In contrast, the dosage of NN formulation applied had no affect on flower mass ($F_{2,38} = 0.83$, $p = 0.45$; Figure 2B) or nectar volume ($F_{2,38} = 1.74$, $p = 0.19$; Figure 2A). Similarly, NN formulation type (Marathon vs. Safari) had no effect on flower mass ($F_{2,39} = 0.95$, $p = 0.39$) or nectar volume ($F_{2,38} = 0.23$, $p = 0.79$). Roughly 32% of inoculated flowers contained no retrievable nectar the day following inoculation. Irrigation treatment significantly affected the probability of flowers containing nectar ($\chi^2_1 = 8.07$, $p = 0.0045$), but there was no effect of NN formulation dose ($\chi^2_2 = 5.45$, $p = 0.065$) or type ($\chi^2_2 = 5.41$, $p = 0.067$).

NN treatment resulted in residues of the respective active ingredient parent compounds in canola nectar. LC–MS analyses of nectar collected from un-inoculated flowers showed detectable residues of imidacloprid and dinotefuran in flowers (Figure 3). One flower from an untreated plant screened for imidacloprid yielded 7.04 ppb, though this value was below the limit of detection (LOD) for the run. As a conservative measure, we subtracted this amount from recorded values for all flower samples. Notably, one flower from a plant treated with a 'low' dose of imidacloprid yielded no detectable residues.

Microbial growth from inoculated nectar samples

We recovered a total of 4151 CFUs (summed across the three agar media types) across our 101 plated nectar samples (Figure 4D). Most CFUs were *Apilactobacillus micheneri* (2279 CFUs, or 55%) or *Acinetobacter pollinis* (1577, or 38%). The remaining 7% comprised 141 CFUs of *Neokomagataea thailandica*, 8 of *Metschnikowia reukaufii* and 146 of various non-inoculated bacterial and fungal morphotypes, which included species of *Streptomyces*, *Erwinia*, *Arthrobacter* and others.

Total CFU abundance per nectar sample (Figure 2C) was not related to our experimental treatments (irrigation: $F_{1,35} = 0.012$, $p = 0.91$; formulation dose: $F_{2,28} = 1.52$, $p = 0.24$; irrigation × dose interaction: $F_{2,35} = 1.55$, $p = 0.23$; formulation type: $F_{2,31} = 0.57$, $p = 0.57$). Despite no significant treatment main effects, CFU abundance was positively related to nectar volume ($F_{1,89} = 17.1$, $p < 0.0001$, Figure 2D), which was higher on average in high irrigation treatment flowers (see above). Total CFU density in nectar did not vary with nectar volume ($F_{1,89} = 0.15$, $p = 0.70$) or any experimental variables (irrigation: $F_{1,35} = 0.27$, $p = 0.61$; formulation dose: $F_{2,28} = 1.80$, $p = 0.18$; irrigation × dose interaction: $F_{2,34} = 1.78$, $p = 0.18$; formulation type: $F_{2,31} = 0.72$, $p = 0.50$). Of the frequently detected inoculated microbes, *Apilactobacillus micheneri* density did not vary with nectar volume nor with any experimental variables (all $p > 0.05$), while *Acinetobacter* density was positively related to

FIGURE 1 Nectar-inhabiting microbe (NIM) growth parameters in response to neonicotinoids (NNs) in the plate reader experiment. Shown are the maximum OD_{600} (*K*, in pink) and maximum growth rate (*r*, in blue) of seven NIM taxa in artificial broths spiked to contain set concentrations (in ppb) of NN compounds. Points and whiskers represent the mean ± SEM parameter at the given concentration relative to that parameter in the respective no-NN control treatment. Within each growth parameter, points connected by the same letter are not significantly different from one another according to Tukey's HSD tests. Growth parameter values were averaged across the six tested NN compounds within each concentration, as parameters generally did not vary significantly by compound (see Results for two exceptions).

nectar volume ($F_{1,76} = 4.49$, $p = 0.037$). CFU Shannon diversity was positively related to nectar volume ($F_{1,68} = 21.38$, $p < 0.0001$; Figure 2F) but did not vary with any experimental variables (irrigation: $F_{1,34} = 1.12$, $p = 0.30$; formulation dose: $F_{2,26} = 1.13$, $p = 0.34$; formulation type: $F_{2,34} = 0.8035$; $p = 0.46$; irrigation × dose interaction: $F_{2,30} = 0.52$, $p = 0.60$; Figure 2D).

The community composition of microbes in nectar was not related to any experimental variables (PERMANOVA; all $p > 0.05$; Figure 4A,B) but was significantly influenced by nectar volume ($F_{1,82} = 2.72$, $p = 0.0211$; Figure 4C). Beta diversity of microbes did not differ with respect to any experimental treatments (irrigation rate: $F_{1,89} = 1.62$, $p = 0.21$; neonic formulation type: $F_{2,88} = 2.35$, $p = 0.10$; neonic formulation dose: $F_{2,88} = 1.39$, $p = 0.26$).

DISCUSSION

Despite the widespread use of plant-systemic (NN) compounds in agroecosystems, little is known about how these chemicals affect plant-associated microbes, especially NIMs. We found that the growth rate and/or maximum optical density of our five assayed bacteria species generally decreased with rising concentrations of NNs in vitro, with few exceptions. In contrast, the two yeast species did not display a strong response to NNs. NN effects on microbial growth mostly did not vary based on compound type (e.g., imidacloprid vs. dinotefuran, etc.). Contrary to our predictions, our model microbial community inoculated into canola nectar did not respond to NN treatment or plant irrigation level. Rather, only a subset of the inoculated NIM species survived in canola nectar, and higher nectar volumes increased microbial abundance and diversity.

In our in vitro plate reader experiment, bacteria grown in NN-spiked nutrient broths generally responded to increasing concentrations of NNs, while fungi did not (Neves et al., 2001). With a few exceptions, NNs negatively impacted bacterial growth metrics, but bacterial species differed in terms of the NN concentration at which effects on their growth were observed, if at all. This is consistent with other studies on NNs and soil-inhabiting microbes, which often find microbial taxa vary widely in their responses to NN exposure (Akter et al., 2023; Cycoń et al., 2013). Four of our five assayed bacterial species belong to various sub-groups of Pseudomonadota Garrity et al. 2021 (synonym Proteobacteria), which are common in nectar (Álvarez-Pérez et al., 2012; Fridman et al., 2011), though we did not select bacterial taxa based on taxonomy. Some other studies have also noted Pseudomonadota decreasing after exposure to imidacloprid (Garg et al., 2021; Parizadeh et al., 2021), so our a priori selection of these specific taxa could be partly responsible for the general responses to NNs we observed. However, negative responses to NNs have also been documented in non-Pseudomonadota taxa (Garg et al., 2021; Streletskii et al., 2022), and other studies find some Pseudomonadota *increase* in response to NNs in certain environments (Cai et al., 2016; Fu et al., 2022; Zhang et al., 2021).

Environmental context, in addition to genetic or physiological variation within taxa, is likely critically important when discussing bacterial responses to NNs, making it difficult to draw conclusions about general trends (Akter et al., 2023). The same considerations also apply to fungi, in that certain studies find fungal abundance is not affected by imidacloprid in soils (Cycoń et al., 2013; Zhang et al., 2015) while others find it decreases (Cai et al., 2016). The goal of our present study was to gauge the response of several diverse representatives of common nectar-inhabiting microbe taxa. One consequence of this approach is the inability to gauge variation across strains *within* each microbe species, which should serve as a key topic for future investigation.

In our greenhouse canola experiment, we found no evidence that either imidacloprid or dinotefuran application to plants affected our inoculated NIM community in floral nectar. Irrigation and NN treatments exerted quantifiable effects on the plants themselves: higher irrigation increased floral mass and nectar volume (Gallagher & Campbell, 2017; Petanidou et al., 1999), and we detected NN parent compounds in treated plant nectar. NN concentrations in nectar from non-inoculated flowers were variable—and likely similarly variable in inoculated flowers—albeit consistent with dosage level. NN application did not affect nectar volume or floral mass, however. Based on an earlier study in *Phacelia* which also examined the effects of irrigation and NN application using a similar experimental design (Cecala & Wilson Rankin, 2021), we had expected an interaction between imidacloprid application and irrigation level, wherein a high dose of imidacloprid would buffer the negative effects of decreased irrigation on nectar volume. This interaction, hypothesised to be attributable to the neonicotinoid 'stress shield' phenomenon described in other plants (Ford et al., 2010), was not observed in canola, potentially due to plant species-specific differences (Zhang et al., 2023).

One potential reason we observed microbe responses to NNs in artificial broths but not in canola nectar is the difference in the environmental conditions microbes experienced. Streletskii et al. (2022) show that which bacterial genera responded to NNs, as well as the dynamics of their reaction, depended on the addition of carbon to their environment. Changes in salinity (Zhang et al., 2015), pH, and other edaphic or abiotic factors (Zhang et al., 2021) can mediate the direction of the effect of NNs on soil bacterial diversity.

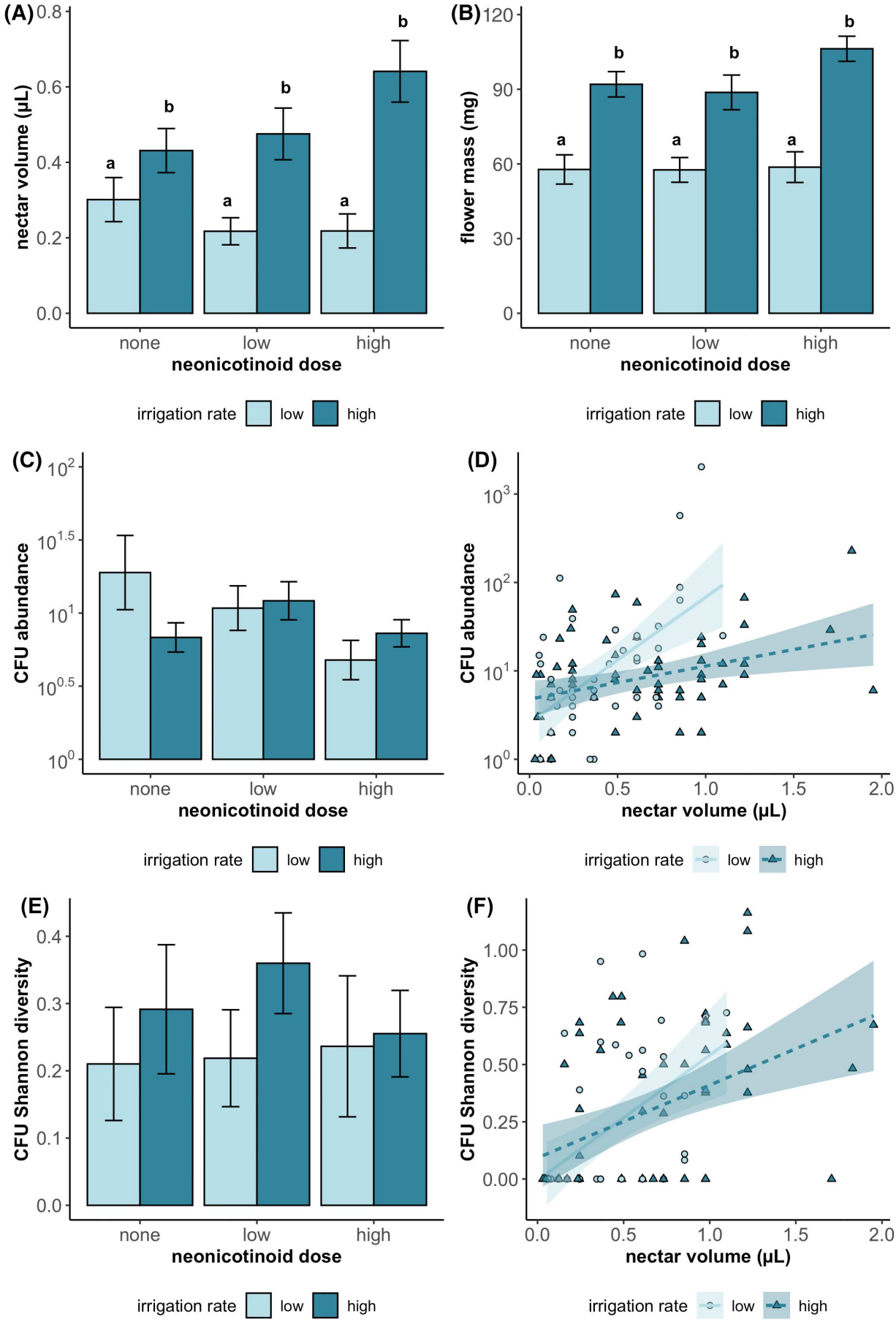

FIGURE 2 Legend on next page.

We echo calls by Akter et al. (2023) that further understanding of the physiochemical properties of microbial habitats and their effects on growth is needed—specifically how these properties are influenced by NNs, and how this mediates microbial responses.

We did not compare the chemistry of our artificial broths to actual canola nectar, but they undoubtedly differ in the types and concentrations of carbohydrates and proteins they contain. The ability of microbes to form biofilms or use other structural features in the two habitats may also differ. Artificial broths and real nectars differ further in other physiochemical properties like osmotic pressure, temperature and pH, all of which are relevant to the growth of NIMs and can in turn even be altered by microbe presence and metabolism (Jacquemyn et al., 2021; Tucker & Fukami, 2014). Another hypothesis is that NIMs may be less exposed to NN in nectar than in soils, resulting in weaker effects than those observed in soil-focused studies. Most of the active ingredients of NN formulations usually enter the soil, while comparatively little make it into the nectar (Goulson, 2013; Stewart et al., 2014).

Furthermore, the length of time microbes were allowed to grow in our two experiments differed: from 3 days in broths to only 24 h in canola nectar, constrained by floral longevity. If, at the NN exposure levels in our canola study, a microbe does not show a response until after 24 h, this could be a potential reason why we observed no effects of our experimental variables in our canola experiment. If this were the case, we could narrow down in which plant species, based on floral traits like floral longevity as well as nectary anatomy and function, we may expect to potentially see the effects of NNs on NIM communities. For example, one may not expect to observe any impact of NNs on NIMs in plants with short-lived (<24 h) flowers or nectar production.

In canola flowers, nectar volume was positively correlated with total microbial abundance, diversity, and community composition as estimated from CFU counts. We did not observe any effects of our experimental variables (irrigation rate and NN application) on the inoculated microbe community. Microbe density (CFU μL^{-1}) in nectar samples was unrelated to nectar volume and experimental treatments. While we did not explicitly test for it, we suspect that flowers with larger nectar volumes offered more resources and area, resulting in a higher carrying capacity for microbial populations. This hypothesis can be more critically evaluated in the context of species-area relationships (SARs; Lomolino, 2000) where habitat size is analogous to nectar volume. While SARs are often applied to

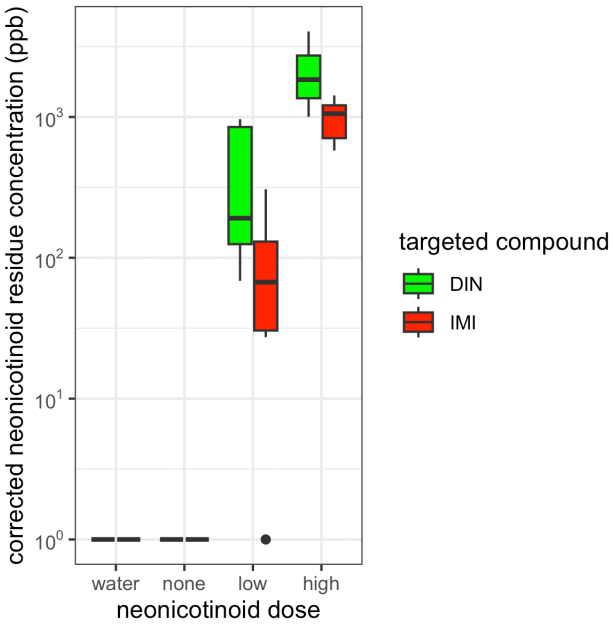

FIGURE 3 Neonicotinoid residue concentrations (ppb), calculated from electrospray ionisation (ESI) LC–MS peak areas (derived from standard curves) in samples of nectar collected from non-inoculated canola (*Brassica napus* 'CP930RR') flowers. Plants were treated either with dinotefuran or imidacloprid formulations at either a 'low' or 'high' dose (see Experimental Procedures: *In planta* greenhouse experiment), or a zero-dose control ('none'). 'Water' indicates pure MilliQ water (used in nectar dilutions) screened for either compound. Total $N = 27$ samples.

macroorganisms, studies have found that generally, microbe species number and/or diversity also tend to increase with sampled habitat size (Dickey et al., 2021; Li et al., 2020). Zemenick et al. (2018) found greater bacterial Shannon diversity, but not richness, in *Aquilegia* flowers with greater nectar volumes. However, nectar volume is a very plastic trait. While our experiment accounted for factors such as evaporation, plant secretion patterns, and temperature fluctuations, other variables should be investigated in future studies. These include removal by pollinators and the phenomenon in which some plants modulate nectar volumes within their own flowers in response to microbial colonisation and growth (Vannette & Fukami, 2018).

Aside from nectar volume, the chemical profile of canola nectar may have contributed to differential growth rates across our four inoculated microbes. Different plant species can have strong filtering effects, influencing which microbes can establish and proliferate in their nectar (Herrera, 2014; Herrera, Canto, et al., 2009; Herrera, de Vega, et al., 2009). Surprisingly, we documented only eight CFUs of

FIGURE 2 Univariate linear mixed models showing the effects of irrigation rate and neonicotinoid formulation dosage on floral nectar volume and floral mass (A, B), CFU abundance (C, D), and CFU Shannon diversity (E, F) in nectar. Bars and whiskers represent mean ± SEM values, and bars connected by the same letter (or bars not labelled) are not significantly different from one another.

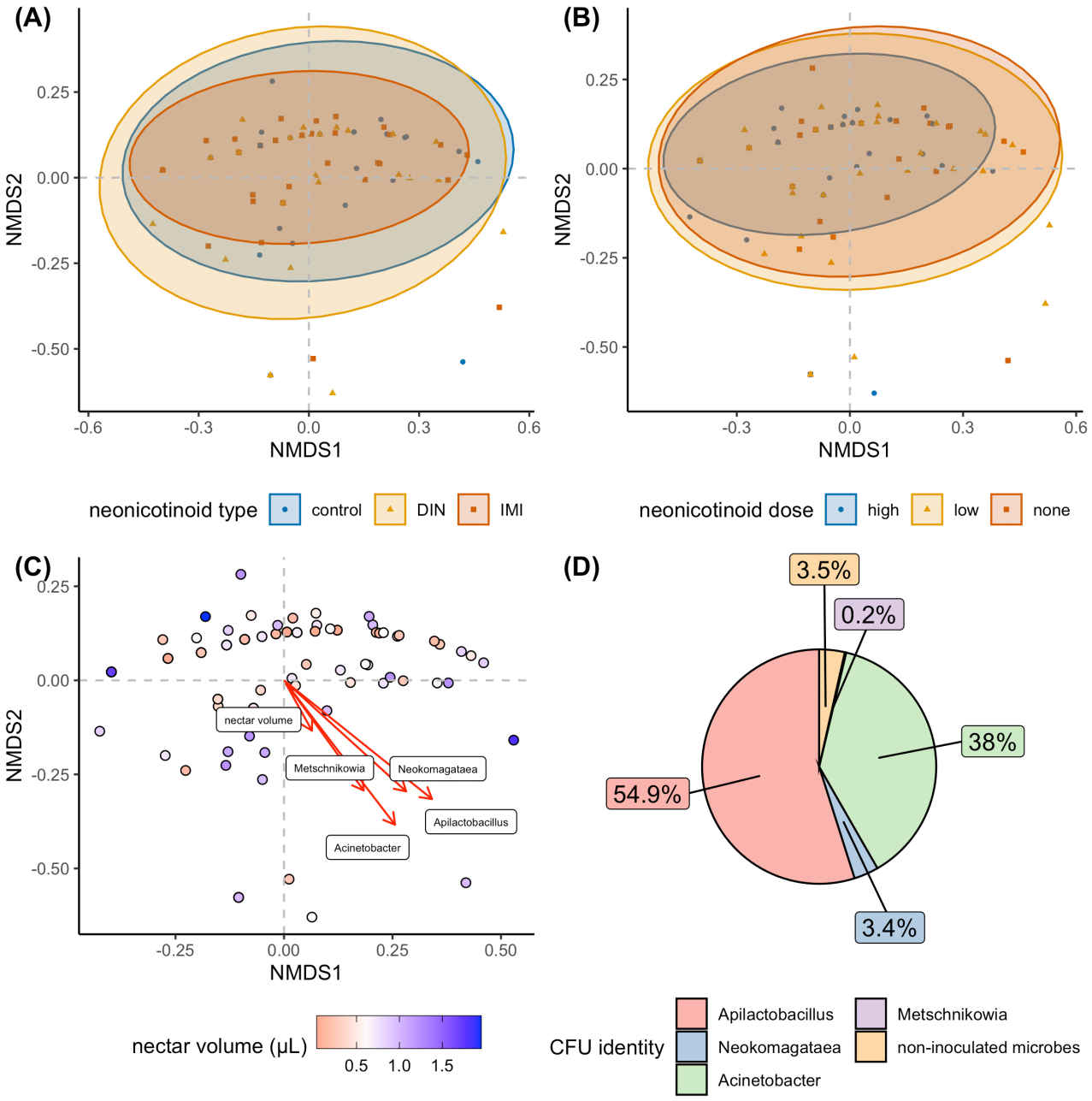

FIGURE 4 Neither the type (A) nor dose (B) of neonicotinoid formulation applied to canola plants influenced the community composition of the inoculated NIM community. In contrast, floral nectar volume was associated with shifts in the community (C). Linear vectors for nectar volume and the four inoculated species are shown as red arrows. (D) Frequency of CFUs by morphotaxon, summed across all plated nectar samples.

Metschnikowia reukaufii across all of our plated nectar samples, despite *Metschnikowia* yeasts being a common nectar-specialist taxon in many plants and ecosystems worldwide (Herrera, Canto, et al., 2009; Herrera, de Vega, et al., 2009; Lachance et al., 2001). The same *Metschnikowia* strain grew prolifically in our artificial yeast media broth in our plate reader experiment. We hypothesise low *Metschnikowia* abundance could be a result of antimicrobial compounds in *Brassica* nectar. Differences across microbe taxa in tolerance to host plant metabolites can be one factor explaining the compositions of certain plant-associated microbe communities (Thoenen et al., 2023). The lipid transfer protein BrLTP2.1 expressed in *Brassica rapa* nectar is known to exhibit antifungal properties (Schmitt et al., 2018), although we did not look for the presence of this peptide in our nectar samples. Alternatively, direct competitive interactions between microbes or inhibition through nectar habitat modification could also exclude species from these communities (Debray et al., 2022).

Interactions between NNs and NIMs can also be investigated in a broader ecological context. First, would effects on microbes become apparent if

examined in a spatiotemporal context and metacommunity framework (Miller et al., 2018) which considers intracommunity processes (e.g., exposure to pesticides) alongside dispersal patterns? Our study did not examine microbial dispersal over time or space. In nature, NIMs are picked up from flowers and dispersed by pollinators, which then periodically introduce them to other flowers via visitation. Belisle et al. (2012) considered flowers as 'islands' for microbial dispersal in a biogeographical context, finding host plant location and floral density as strong predictors of yeast presence in nectar. In an agricultural field where all plants are treated at the same time, one might expect NIM communities to be chronically exposed to these compounds throughout the entire flowering period as they are transferred between flowers by pollinators. Further work could examine whether this could lead to long-term shifts in local microbial community structure or adaptation to agrochemical tolerance.

Second, can certain NIMs actually innately degrade or bioaccumulate NNs, and thus modulate the exposure of co-occurring microbe species to NNs? Various physiological factors could result in microbes facing varied concentrations of NNs over time. We did not assay any of our study microbes for their ability to degrade NNs, but several taxa of soil-inhabiting bacteria are documented NN-degraders, including species of *Bacillus*, *Klebsiella*, *Rhizobium*, *Pseudomonas* (Pang et al., 2020) and other Pseudomonadota (Zhang et al., 2018). This topic warrants further investigation and may have practical applications in agricultural fields to mitigate NN exposure risks to pollinators at flowers. The success of such a strategy would depend on the specific metabolites produced by biodegradation (Sabourmoghaddam et al., 2015) in comparison to those normally produced via light, water, and plant metabolism, and their relative toxicity to insects (Phugare et al., 2013).

In conclusion, our study explored the effects of NNs on common and geographically widespread species of NIMs. Our work contributes to the growing interest in understanding how different factors—local and landscape, biotic and abiotic—contribute to the diversity and distribution of nectar and phyllosphere microbe communities in agricultural ecosystems (Burgess & Schaeffer, 2022; Noel et al., 2022; Schaeffer et al., 2021). A wide range of hypotheses remains to be tested regarding the nontarget effects of agrochemicals on NIMs. We suggest further investigation of how nontarget effects of agrochemicals on NIMs may vary in terms of their growing environment, for example, plant host identity and resource availability.

AUTHOR CONTRIBUTIONS

Jacob M. Cecala: Conceptualization; investigation; funding acquisition; writing – original draft; methodology; validation; visualization; writing – review and editing; software; formal analysis; project administration; data curation; supervision; resources. **Rachel L. Vannette:** Conceptualization; investigation; methodology; writing – review and editing; supervision; resources.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data generated in this study are publicly available in the Dryad data repository at https://doi.org/10.5061/dryad.gf1vhhmw2.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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