# Enzymatic and Molecular Biomarkers Reveal Soil Sensitivity to Neonicotinoid Pesticides in Two Typical Chilean Soils

Gabriela Briceño[1,2]*, Graciela Palma[1,2], Lukas Y. Wick[3], Milko Jorquera[1], Claudia S. Benimeli[4], Heidi Schalchli[1,2], M. Cristina Diez[2,5]

[1] Departamento de Ciencias Químicas y Recursos Naturales, Facultad de Ingeniería y Ciencias, Universidad de La Frontera, Av. Francisco Salazar 01145, Temuco 4780000, Chile

[2] Centro de Excelencia en Investigación Biotecnológica Aplicada al Medio Ambiente (CIBAMA-BIOREN), Universidad de La Frontera, Av. Francisco Salazar 01145, Temuco 4780000, Chile.

[3] Helmholtz Centre for Environmental Research UFZ Department Applied Microbial Ecology, Leipzig, Germany

[4] Planta Piloto de Procesos Industriales Microbiológicos (PROIMI-CONICET), Avenida Belgrano y Pasaje Caseros, Tucumán 4000, Argentina

[5] Departamento de Ingeniería Química, Facultad de Ingeniería y Ciencias, Universidad de La Frontera, Av. Francisco Salazar 01145, Temuco 4780000, Chile.

Corresponding Author: Gabriela Briceño Muñoz, gabriela.briceno@ufrontera.cl, ORCID: 0000-0001-7058-3377

**Abstract**

**Purpose:** This study aimed to elucidate how soil type governs the dissipation and biological effects of three widely used neonicotinoid insecticides (imidacloprid, thiamethoxam, and clothianidin) on key microbial functions linked to soil health. We evaluated whether contrasting soils differ in their sensitivity and resilience to pesticide exposure.

**Methods:** Microcosm experiments were conducted using two representative Araucanian agricultural soils of Chile: an organic matter–rich Andisol (Freire series) and a clay-rich Inceptisol (Chufquen series). Soils were treated with recommended (1×) and elevated (10×) doses and incubated for 30 days. Enzymatic activities, bacterial abundance (16S rRNA), nitrogen fixation potential (*nifH*), nitrification potential, inorganic nitrogen dynamics, and pesticide dissipation were monitored.

**Results:** Neonicotinoid effects were strongly soil-dependent. The Andisol showed higher functional sensitivity, with marked inhibition of urease, dehydrogenase, and nitrification shortly after application. In contrast, the Inceptisol exhibited greater microbial stability and resilience, likely related to higher clay-mediated adsorption. Across both soils, effects were mostly transient, with recovery of microbial functions despite persistent residues. Dissipation patterns also varied between soils and compounds.

**Conclusions:** This study provides novel evidence that neonicotinoid effects on enzymatic and molecular indicators have not been previously evaluated in an integrated manner in Araucanian soils of Chile. Soil type is a key determinant of pesticide fate and impact. Although short-term disturbances occur, microbial communities show resilience; however, the persistence of certain compounds highlights the need to consider potential accumulation and effects on non-target organisms.

**Keywords**: insecticides, soil enzymes, nitrogen cycling, soil health indicators, agricultural soils

## 1 Introduction

Accounting for approximately 24 % of the global insecticide market, neonicotinoids (NNIs) are extensively used in over 120 countries (Hladik et al. 2018; Sparks et al. 2020). NNIs are neuro-active systemic insecticides that are highly effective against a broad range of pests. Their use, however, has generated controversy due to high adverse effects on non-target organisms, including bees and other pollinators (Paoli & Giurfa, 2024). Although many newer compounds exist to reduce the NNI's environmental toxicity and ecological impact, imidacloprid (IMI), clothianidin (CLO), and thiamethoxam (THM) are still commonly used in agriculture (Elumalai et al. 2022). NNIs are used as seed coatings, foliar sprays, or in direct application to soil for a variety of crops, including maize, soybeans, oilseed rape, sunflower, cereals, beets, potatoes, and pastures, among others. Typically, 2 - 20% of the applied doses are taken up by the roots and translocated to all plant parts (Radolinski et al. 2019), enabling protection against insect pests during plant growth (Sanchez-Bayo et al. 2013; Mörtl et al. 2020). Simultaneously, substantial fractions of the applied NNIs do not get absorbed by the plants and are prone to be released to soil and may affect soil microbial processes (Bonmatin et al. 2015; Karpouzas et al. 2016).

NNIs typically exhibit aqueous solubilities of 180-570,000 mg $L^{-1}$ (PPDB, 2024), exhibit low adsorption onto soil particles compared with other pesticide groups (Zhang et al. 2018; Pietrzak et al. 2020; Briceño et al. 2025), and have a half-life ($DT_{50}$) in soil between 3 to 174 days (Mörtl et al. 2020; PPDB, 2024). Their environmental fate is influenced by many factors including their physico-chemical properties, soil properties, or the composition and activity of the soil microbial community (Gangola et al. 2023). If present in soils, NNIs may also impact soil microbiomes and their ecosystem functions such as nutrient cycling or the biotransformation of pesticides (Cycoń et al. 2013; Zhang et al. 2018). For instance, Briceño et al. (2024) reported that NNIs may affect both the diversity and activity of soil microbial communities or specific groups thereof that play a crucial role in soil structure, function and fertility (Meena et al. 2020).

The activity of soil microbial enzymes is a key indicator of soil quality and health (Wołejko et al. 2020). The effects of IMI and other NNIs on soil activity have been studied for a variety of enzymes, including dehydrogenase, urease, β-glycosidase, fluorescein diacetate hydrolase, acid phosphatase, phosphomonoesterase, protease, catalase, phosphatase, amylase, and arginine deaminase activities according to the review carried out by Akter et al. (2023). Knowledge of NNI effects on nitrogen cycling (N-cycling) is of predominant importance for plant growth and health (Singh et al. 2015). Soil N-cycling includes microbial N fixation by diazotrophic prokaryotes carrying nitrogenase activity and *nifH* gene. Nitrification is the process by which bacteria carrying amoA gene convert $NH_4^+$-N via $NO_2^-$-N to $NO_3^-$-N. Finally, denitrification is the conversion of $NO_3^-$-N to N gases that are released from the soil to the atmosphere. Given that microbial diversity and activity

(Gangola et al. 2023) as well as the fate and bioavailability of NNI may be site and soil-specific, it is necessary to understand the response in soil across different soil types (Wołejko et al. 2020).

While the European Union has banned outdoor agricultural uses of IMI, THM, and CLO since 2018, these compounds are still widely used in many South American countries and remain approved with restrictions in the United States. IMI has emerged as a global contaminant (Pang et al. 2024) due to its persistence ($DT_{50}$) of 40-124 days and its mobility in soil, with a $K_{oc}$ of 249-336 mL $g^{-1}$ and a solubility of 610 mg $L^{-1}$. As for IMI, CLO is considered to be persistent, with an approximate $DT_{50}$ of 214 days. With aqueous solubility of 327 mg $L^{-1}$ and a $K_{oc}$ of 160 mL $g^{-1}$ it is mobile and has the potential to leach into groundwater and runoff into surface waters. Unlike IMI, both CLO and THM incorporate a sulfur atom into its molecular structure. THM has very high-water solubility (4,100 mg $L^{-1}$) and a low to moderate $K_{oc}$ of 23–265 mL $g^{-1}$ and has a lower environmental half-life ($DT_{50}$ = 16-75 days (Hilton et al. 2019)).

In the context of our research into site-specific analysis of NNI-induced environmental risks and optimised agricultural pesticide management, we conducted a thorough assessment of the dissipation and effects of three widely used NNI insecticides (IMI, THM, CLO) on enzymatic activities, microbial abundance and N-related functions in two distinct agricultural soils (i.e. an Andisol and an Inceptisol) commonly encountered in southern Chile, varying in terms of organic matter content and texture.

## 2 Materials and Methods

### 2.1 Soils and pesticides

Two soils were collected from two different agricultural sites in the Araucanía region of Chile. The first soil was an Andisol of the Freire series (medial, mesic, typical placuands; 38°50′S, 72°35′W), and the second was an Inceptisol of the Chufquen series (mixed, mesic, fluventic humic dystrudepts; 38°22′S, 72°37′W) (CIREN, 2002). The Andisol was obtained from the Maquehue Experimental Field, owned by the Universidad de La Frontera, while the Inceptisol was collected from a privately owned field. In both cases, the selected plots were not under active crop management and had no recent history of pesticide application. At each site, at least five subsamples were collected from the topsoil (0–20 cm depth) across the sampling area and subsequently homogenized to obtain a representative composite sample for each soil type. After sampling, the soils were air-dried at room temperature (20 ± 2 °C) for 5 days, sieved through a 2 mm mesh, and stored at 4°C until use. The soil physico-chemical properties are shown in Table 1.

The insecticides IMI ($C_9H_{10}ClN_5O_2$), THM ($C_8H_{10}ClN_5O_3S$) and CLO ($C_6H_8ClN_5O_2S$) were obtained from Sigma-Aldrich GmbH, Switzerland and were of analytical or high-performance liquid chromatography (HPLC) grade.

**2.2 Microcosm set up and sampling**

Stored soils were incubated at 20 °C and at 40-50 % of its water-holding capacity for two weeks in the dark prior to use. For each of the sampling time points six replicates microcosms were prepared for each of the three NNIs applied: three were sacrificed for pesticide quantification and three for genetic and soil biochemical analyses. The setup consisted of 50 mL glass Falcon tubes to which 20 g of soil was placed. Each NNI was applied separately to independent soil microcosms, and no mixtures of compounds were used. The average dose recommended by AFIPA Chile for various crops for pest control or an elevated dose ten times (10x) higher than the recommended dose was used. For the calculation of the nominal application rates of each pesticide in the studied soils, a distribution in the first 5 cm of depth and a bulk density of 0.75 g $cm^{-3}$ was considered. In this case, the doses of the active ingredient (a.i.) were 0.20 mg $kg^{-1}$ (IMI), 0.16 mg $kg^{-1}$ (THM), and 0.038 mg $kg^{-1}$ (CLO). An aliquot of a standard solution of each pesticide resuspended in acetonitrile was added, and soil samples were homogenised with a sterilised spatula and manual stirring, the control sample was also spiked with acetonitrile. Then, the moisture content was adjusted to 40-50 % of the water-holding capacity. Incubation was performed in the dark at 22 ± 1 °C, and microcosms were destructively sampled on days 1, 15, and 30.

**2.3. Enzymatic activities**

Urease activity was quantified according to the method described by Kandeler and Gerber (1988). Briefly, 1 g of soil was incubated with 1.5 mL of 0.08 mol $L^{-1}$ urea solution at 37 °C for 2 h, then 13.5 mL of 2 mol $L^{-1}$ KCl solution was added and shaken for 30 min. The resulting suspension was filtered, and the filtrate was analysed for ammonia by the previously described colorimetric method. Determination of the acid phosphatase activity followed the method described by Tabatabai and Bremmer (1969). Briefly, 1 g of soil was incubated with modified universal buffer (MUB) adjusted to pH 6.5, and 1-mL p-nitrophenyl phosphate (pNPP) at 30°C for 1 h, then 1 mL of $CaCl_2$ (0.5 M) and 4 mL of NaOH (0.5M) were added to stop the reaction. After centrifugation, sample was read in microplate reader at 400 nm and expressed as µg p-nitrophenol $g^{-1}$ soil. The β-glucosidase activity was determined as described by Tabatabai (1982). Briefly, 1 g of soil was incubated with buffer MUB-HCl, pH 6.0, and 1-mL p-nitrophenyl-β-D-glucopyranoside (25 mM) at 37°C for 1 h, then was added 1 mL of $CaCl_2$ (0.5 M) and 4 mL of a mixture of Tris (hydroxymethyl) aminomethane (THAM)/NaOH, pH 12, were added to stop the reaction. After centrifugation, the sample was read in a microplate reader at 400 nm and expressed as µmoles p-nitrophenol

128 $g^{-1}$ soil $h^{-1}$. Dehydrogenase activity was quantified by the reduction of 2,3,5-triphenyltetrazoliumchloride (TTC) to triphenyl
129 formazan (TPF), according to Serra-Witting et al. (1995). Briefly, 3 g of soil samples were mixed with TTC solution (3 %)
130 and deionised water (DI) (1:1) and incubated at 37 °C for 24 h in the dark. After incubation, the TPF produced was extracted
131 with methanol, filtered, and determined by a microplate reader at 485 nm.
132
133 **2.4. DNA extraction and real-time PCR**
134 On days 1 and 30, DNA from the soil was extracted using the commercial NucleoSpin® Soil kit (Macherey-Nagel) following
135 the manufacturer's instructions. For DNA quantification, 2 µL of each sample were quantified using the QuantiFluor ONE
136 dsDNA reagent in a Quantus Fluorometer (Promega Inc., USA). Total bacterial abundance was quantified by qPCR targeting
137 the 16S rRNA gene, while N-cycle-related *nifH* genes were also quantified. All measurements were carried out in
138 QuantStudio3 (ThermoFisher Inc, USA) using the standard curve protocol with EvaGreen as dye with four replicates per
139 treatment. The reactions were set up to a 10 µL final volume using HotFire Firepol Hotstart Supermix 2x (Solis Biodyne,
140 Estonia) as mastermix. For each gene, 500 µM of each primer was added to the reactions, and 1 µL of each DNA sample was
141 used as template. An initial incubation of 12 min at 95 °C was used to activate the polymerase, followed by 40 cycles of 95
142 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s. A melt curve (60 – 95 °C) was performed after the amplification cycles to
143 assess the reaction specificity. All standard curves used in this experiment were synthesised as dsDNA gBlocks by IDT DNA
144 (IDT, USA). The standard curves data was linearised, and sample Ct values were interpolated to estimate copy numbers.
145
146 **2.5. Nitrification potential**
147 The potential for nitrification in soil was determined as described by Berg & Rosswall (1985): 5 g of fresh soil was placed in
148 50 mL centrifuge tubes, to which 20 mL of a solution containing 1 mM $(NH_4)_2SO_4$ and 1.5 M $NaClO_3$ was added. Tubes were
149 incubated on a mechanical shaker at 400 rpm at approximately 20 °C for 5 h, and the control was stored at -20 °C for the same
150 period. At the end of the incubation period, the control samples were thawed and, together with the treated samples, 1 mL of
151 2 M KCl was added. After shaking, the suspensions were centrifuged at 3,820 g for 10 min and the supernatant was collected.
152 The nitrite concentration in the supernatant was determined colourimetrically using a buffer solution of $NH_4Cl$ and the colour
153 reagent *N*-(1-naphthyl)ethylenediamine. After 5 min, the absorbance was measured at 520 nm in a microplate reader. The $NO_2$
154 concentration in the soil samples was calculated from calibration curves of sodium nitrite ($NaNO_2$) solution at concentrations
155 ranging from 0 - 1 µg $NO_2$-N $mL^{-1}$.

### 2.6. Determination of ammonium ($N\text{-}NH_4^+$) and nitrate ($N\text{-}NO_3^-$)

Extractable $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ from microcosms at all sampling dates was quantified as described earlier. For extraction, 2.5 g of soil was shaken with 12.5 mL of 1 mol $L^{-1}$ KCl for 30 min, centrifuged at 950 g for 15 min, and filtered. Concentration of $N\text{-}NH_4^+$ was determined colourimetrically as described by Kandeler and Gerber (1988). For this, 1 mL of filtrate was diluted to 10 mL with DI water, then 2 mL of 0.1 % sodium dichloroisocyanurate and 5 mL of sodium salicylate solution (prepared from 0.12 % sodium nitroprusside, 17 % sodium salicylate, and DI water) were added. After 30 min incubation at room temperature, absorbance was measured at 620 nm. Nitrate was quantified by a vanadium (III) and Griess reagent method (Doane and Horwath 2003; Keeney and Nelson 1982) with spectrophotometric reading at 540 nm. A reagent solution containing 200 mg sulfanilamide and 10 mg *N*-(1-naphthyl)ethylenediamine dihydrochloride in 400 mL DI water was mixed with 400 mg vanadium (III) chloride in 50 mL of 1 mol $L^{-1}$ HCl. Then, 0.4 mL of filtrate was combined with 2 mL of reagent, incubated at 60 °C for 2 h, and left at room temperature for 20 min before measurement.

### 2.7. Determination of pesticide concentration in soil

The extraction of IMI, THM, and CLO from soil microcosm samples was performed using acetonitrile. Briefly, 20 g of soil and 40 mL of solvent were placed in a 100 mL Erlenmeyer flask, shaken at 300 rpm for 30 min, sonicated for 20 min, and then centrifuged at 8,600 g for 10 min at 4 °C. The method showed high extraction efficiency, with recovery rates exceeding 85% for all pesticides. Subsequently, 10 mL of the supernatant were concentrated to dryness using an SPD121P SpeedVac® concentrator (Thermo Scientific Savant®), reconstituted in 2 mL of acetonitrile, filtered through a 0.22 µm PTFE membrane filter, and analysed using a Shimadzu Prominence HPLC chromatograph LC-20AT with a diode-array detector (SPD-M20A) and a Purospher® STAR RP-18 column (150 x 4.6 mm). The mobile phase was acetonitrile and 0.1 % phosphoric acid (40:60 v $v^{-1}$ for IMI and THM and 50:50 v $v^{-1}$ for CLO) injected at a flow rate of 1 mL $min^{-1}$. The column temperature was maintained at 40 ± 1 °C, and the detector was set to acquire data at 270 nm, 252 nm, and 267 nm for IMI, THM, and CLO, respectively. Instrument calibration and quantification were performed using pure reference standards (0.05–50 mg $L^{-1}$) for each pesticide. The limits of detection (LOD) and quantification (LOQ) were determined according to the IUPAC Harmonized Guidelines for single laboratory validation of analytical methods (Thompson et al. 2022). The LOD was calculated using the formula LOD = (Sy/S), where Sy is the standard deviation of the response and S is the slope of the calibration curve. The resulting LODs for IMI, THM, and CLO were 0.012, 0.097, and 0.025 mg $L^{-1}$, respectively. LOQs were calculated using the formula

LOQ = 10(Sy/S), yielding values of 0.036, 0.294, and 0.075 mg $L^{-1}$ for IMI, THM, and CLO, respectively (Briceño et al. 2025).

### 2.8. Statistical Analysis

All experiments were performed in triplicate. The results were expressed as mean values ± standard deviation. The data were subjected to statistical analyses of variance (ANOVA), and all mean separations were determined using the Tukey test ($p < 0.05$). Homogeneity of variances was verified using Levene's test. The analyses were conducted using SPSS Statistics for Windows, Version 26.0 (IBM Corp., Armonk, NY, USA).

## 3 Results

### 3.1 Effects on soil enzymatic activities

Key enzymes involved in the cycling of carbon, N, and phosphorus (i.e. urease, *β*-glucosidase, acid phosphatase and dehydrogenase) exhibited significant, albeit mostly transient, changes in soil enzymatic activity in response to IMI, THM and CLO, with the highest sensitivity being shown by urease and dehydrogenase. IMI and CLO had a greater effect than THM, particularly at higher doses (Figure 1). Overall, the organic matter–rich Andisol exhibited greater functional sensitivity, whereas the clay-rich Inceptisol showed higher enzymatic stability, as described in more detail below.

*Urease:* Urease activity (Fig. 1A, B) was lower in the Andisol (≤ 8 µmol $NH_4^+$ $g^{-1}$ $h^{-1}$) than in the Inceptisol soil (≤ 17 µmol $NH_4^+$ $g^{-1}$ $h^{-1}$). IMI significantly influenced the urease activity on days 15 and 30 in both soils, yet not on day 1. Except for the CLO (10x) on day 1, which revealed a significant reduction in activity, a similar trend was observed for CLO in the Andisol. In the Inceptisol, however, no significant differences were observed compared with the uncontaminated control after 30 days. THM generally showed minor effects in both soils; however, it induced a significant drop in activity (~ 50 %) on day 30 in the Inceptisol.

*Acid phosphatase*: Acid phosphatase activity (Fig. 1A,B) was consistently lower in the Andisol than in the Inceptisol but increased over time in both soils. In the Andisol, enzyme activity ranged from 138–183 µg pNP $g^{-1}$ $h^{-1}$ on day 1 and reached up to 310 µg pNP $g^{-1}$ $h^{-1}$ by day 30, with the highest values observed under THM. In the Inceptisol, activity ranged from 326–391 µg pNP $g^{-1}$ $h^{-1}$ on day 1, with no significant differences among treatments. A reduction was observed on day 15 under IMI (10×) and CLO treatments, whereas THM (10×) increased activity by approximately 20% relative to the control at day 30.

*β-glucosidase:* β-glucosidase activity (Fig. 1A,B) showed minor changes in the Andisol on days 1 and 30. However, on day 15, activity decreased across all treatments by 32–67% relative to day 1, with the smallest reductions observed in the control and THM-treated soils. Activity recovered by day 30, reaching 58–69 µmol pNP $g^{-1}$ $h^{-1}$. In the Inceptisol, β-glucosidase activity remained largely unaffected on days 1 and 15, with a slight increase observed under THM treatments at day 30.

*Dehydrogenase*: Dehydrogenase activity (Fig. 1A,B) clearly fluctuated between days 1, 15, and 30 in the Andisol. On day 1, activity was low (< 0.5 µg $g^{-1}$ $h^{-1}$) and revealed significant impacts of IMI, IMI (10×), and CLO. By day 15, a ~230–1060 % increase in dehydrogenase activity to > 1.0 µg $g^{-1}$ $h^{-1}$ was evident for all treatments except the control and THM (10×), which increased only ~ 0.7 µg $g^{-1}$ $h^{-1}$. The enzyme activity of the contaminated soils dropped sharply again to ~0.15–0.70 µg $g^{-1}$ $h^{-1}$ on day 30. However, the control and THM (10x) remained at levels observed on day 15. Contaminating the Inceptisol resulted in significantly reduced dehydrogenase activity except for CLO (10x). On day 15, the enzyme activity of soils treated with THM and IMI was clearly enhanced compared to the control and the other treated soils. At day 30, soil activity remained relatively stable, with the control, CLO and THM treatments maintaining values above 1.0 µg $g^{-1}$ $h^{-1}$.

### 3.2 Effects on 16S rRNA and *nifH* gene copy numbers

The effect of IMI, THM and CLO on bacterial abundance and nitrogenase activity was quantified by 16S rRNA and *nifH* gene copy numbers on days 1 and 30 (Figure 2A,B). In the Andisol (Fig. 2A), a significant 1.3–2.3-fold increase (~35–130%) in 16S rRNA copy numbers was observed on day 1 in the presence of pesticides, reaching values close to 5 × $10^6$ copies $g^{-1}$. By day 30, 16S rRNA abundance decreased across all treatments to around 2 × $10^6$ copies $g^{-1}$, with no significant differences among treatments. In the Inceptisol (Fig. 2B), a slight increase was observed under THM (10×) on day 1. By day 30, a slight decreasing trend was observed; however, no significant differences relative to the control were detected. In the Andisol (Fig. 2A), *nifH* gene abundance on day 1 ranged between 2–5 × $10^6$ copies $g^{-1}$, with the highest values observed under CLO treatments. By day 30, copy numbers decreased across all treatments to approximately 2–3 × $10^6$ copies $g^{-1}$, with no significant differences among treatments. In the Inceptisol (Fig. 2B), *nifH* gene abundance increased in all contaminated treatments on day 1 by >50% relative to the control, with IMI (10×), THM, and CLO showing the highest values, reaching up to ~6 × $10^6$ copies $g^{-1}$. By day 30, *nifH* abundance in IMI (10×)-treated soil reached approximately 8 × $10^6$ copies $g^{-1}$ and was significantly higher than in all other treatments.

### 3.3 Effects on the soil nitrification potential

Figure 3 shows the nitrification potential (μg $NO_2^-$–N $g^{-1}$ $5h^{-1}$) in the Andisol and Inceptisol exposed to IMI, THM and CLO. At day 1 in the Andisol (Figure 3A), a significant inhibition of the nitrification potential was observed in IMI (10x), THM (10x) and CLO (10x) treated soils (≤ 0.2 μg $NO_2^-$–N $g^{-1}$ $5h^{-1}$) as compared to the control or IMI, THM and CLO treatments. At days 15 and 30, the inhibitory effect disappeared, and nitrification rates in all treatments converged to values around 0.5 μg $NO_2^-$–N $g^{-1}$ $5h^{-1}$ with no significant differences among the treatments detected. The nitrification potential of the Inceptisol (Figure 3B) on day 1 was approximately 3-fold higher than in the Andisol, except for IMI (10×), THM (1×) and THM (10×), which caused a 40–95% inhibition of soil nitrification. The inhibition however was reversible, as nitrification activity of all Inceptisol treatments stabilised at around 1.5 μg $NO_2^-$–N $g^{-1}$ $5h^{-1}$, with no significant differences among treatments.

**3.4 Effects on soil ammonium and nitrate concentrations**

*Ammonium:* The ammonium (N-$NH_4^+$) concentrations varied between treatments and soils over time (Tables 2) with the uncontaminated Inceptisol soil having a ~4-fold higher N-$NH_4^+$ concentration (421.2 mg $kg^{-1}$) than the Andisol at day 1. In the Andisol, initial N-$NH_4^+$ concentrations were 92.0 – 100.0 mg $kg^-$, with no significant differences among treatments. By day 30, a marked decrease in the N-$NH_4^+$content was observed in all treatments, particularly in the case of IMI (10x), with a value of 27.0 mg $kg^{-1}$. In the Inceptisol, N-$NH_4^+$ concentrations ranging from 272.7 to 421.2 mg $kg^{-1}$ at day 1 and were 3-4-fold higher than at day 30 yet didn't exhibit any significant differences among the soil treatments. In most cases, the effect of pesticides did not exceed a deviation of >25% compared to soil control. The greatest deviation representing inhibition was observed in Andisol soil with IMI and IMI 10X, and in Inceptisol soil on day 1 of application.

*Nitrate*: IMI, THM and CLO treatments of the Andisol soil resulted in lower N-$NO_3^-$ concentrations at day 1 (Table 3), with reductions relative to the control being significant for IMI (10x) (80.2 mg $kg^{-1}$), THM (87.4 mg $kg^{-1}$), THM (10x) (68.9 mg $kg^{-1}$), and CLO (68.3 mg $kg^{-1}$). By day 30, N-$NO_3^-$ concentration increased in all treatments indicating recovery of nitrification activity independent of the treatment history. In the Inceptisol soil, N-$NO_3^-$ contents ranged from 171.6 to 255.3 mg $kg^{-1}$ at day 1 and increased slightly by day 30. At this time point, THM showed a response comparable to the other pesticides, despite the slight positive deviation from the control. The strongest inhibition of nitrification was observed in the Andisol on day 1 after application of IMI (10×), THM, and THM (10x). This inhibitory effect persisted for THM (10x) after 30 days.

**3.5 Neonicotinoid dissipation**

On day 1, >98% of the initially applied IMI, THM and CLO could be recovered in both soils; however, compound-dependent dissipation patterns were observed at days 15 and 30 (Figure 4). In the Andisol, continuous dissipation of THM (52–72%) over 30 days was observed, whereas removal of IMI (25–30%) and CLO (8–16%) slowed after 15 days and remained largely unchanged until day 30. In the Inceptisol, CLO showed the greatest and continuous dissipation, reaching 55–75% removal after 30 days, while IMI (20–50%) and THM (18–40%) showed limited dissipation that occurred mainly within the first 15 days.

## 4 Discussion

In our study, we quantified the dissipation and impact of three commonly used NNI insecticides on enzymatic activities involved in carbon, nitrogen, and phosphorus cycling (urease, β-glucosidase, acid phosphatase, and dehydrogenase) chosen as indicators of soil health, as enzymes quickly respond to environmental or human-induced soil changes (Wołejko et al. 2020). Furthermore, microbial abundance, and N-related functions in two representative agricultural soils from southern Chile; i.e. an Andisol (Freire series) and an Inceptisol (Chufquen series) were evaluated.

### 4.1 Soil enzyme activity and bacterial biomass

The enzymatic activities evaluated in this study revealed that NNI application significantly modified the functional responses of soil microbial communities, with variations depending on soil type, pesticide type and concentration, and exposure time. The soil type also played a decisive role in modulating microbial responses. The Andisol was generally more sensitive to pesticide application than the Inceptisol with high organic matter and high clay content. Andisol also showed reduced enzyme activity even in the absence of pesticides than the Inceptisol. The composition of microbial communities normally varies across different soil types (Yuan et al. 2024), with typically increased microbial biomass and diversity in soils with higher clay content (Gregory et al. 2007). Moreover, the relative abundances of certain fungi and filamentous bacteria are often higher in clay-rich soils (Xia et al. 2020), which could be influencing the increased activity observed in the Inceptisol soil. Fine soil particles are important for soil recovery and resilience, suggesting that clayey soils have a strong capacity to self-organise after disturbance (Bonetti et al. 2017).

The Inceptisol maintained microbial activity and overall functionality under chemical stress, likely due to its higher clay-driven adsorption capacity for NNIs, which seems to play a more decisive role than the organic matter content of the Andisol (Briceño et al. 2025). Moreover, the high nutrient abundance in the Inceptisol soil, particularly phosphorus,

potassium, and total bases (Table 1), may have contributed to Andisol being more easily disturbed by NNI application, as reported by Zhang et al. (2018). In addition to adsorption processes, the nature of the organic matter associated with Al and Fe compounds in Andisols may also contribute to the greater functional sensitivity observed in this soil. Volcanic soils are characterized by the stabilization of organic matter through interactions with short-range-order minerals and Al/Fe complexes. In Andisols, humic substances are strongly associated with these mineral fractions, resulting in organic matter pools with long turnover times and reduced availability for microbial mineralization (Sollins et al. 1996; Takahashi & Dahlgren, 2016). Furthermore, these interactions may promote enzyme immobilization and decrease extracellular enzymatic activity (Diez et al. 2006). NNIs' toxicity alters enzyme activities, leading to a time-dependent inhibitory effect in some cases. In general, IMI and CLO would be the most toxic pesticides, affecting specific soil functions, such as N and carbon mineralisation processes by suppressing microbial urease producers, or affecting microbial vitality for carbon cycling (Nannipieri et al. 2012), as indicated by our results for urease and β-glucosidase activity, respectively. Inhibitory patterns of urease activity under IMI exposure have been reported, attributed to a decrease in microbial biomass or shifts in community structure (Cycoń et al. 2013; Zhang et al. 2021). However, the adverse effect of IMI on urease activity was observed immediately after application. In contrast, a stimulatory response was detected at the higher IMI (10 mg kg-1) dose at subsequent sampling times (Cycoń et al. 2015). There are no reported studies on CLO for comparative purposes.

Pesticide application can decrease, increase or have no effect on soil activity (Palma et al. 2023). In our study, the central tendency showed a slight increase in acid phosphatase and β-glucosidase activity in Andisol, possibly associated with a transient stimulation of microbial metabolism. However, an inhibitory effect of NNIs on day 15 of urease activity in the Andisol and Inceptisol, acid phosphatase in Inceptisol, and β-glucosidase in Andisol soil indicates an eventual toxic impact, possibly due to accumulation of the pesticides or the eventual apparition of intermediate degradation products of IMI, some of them widely reported, e.g., 6-chloronicotinic acid (Castillo-Diaz et al. 2017; Zhang et al. 2023). No similar or related studies were found for CLO; however, their toxicity has been reported on non-target soil invertebrates (Bandeira et al. 2021).

Dehydrogenase, which reflects the overall oxidative activity of soil microorganisms, showed the most significant differences among soils. Its initial inhibition in both soils confirms that NNI exposure rapidly suppresses microbial respiration, as observed in other studies with IMI and acetamiprid, suggesting that dehydrogenase activity is sensitive to NNIs (Wang et al. 2014). The subsequent increase by day 15 suggests that surviving microbial populations adjusted their metabolism or that specific resistant groups proliferated. However, the substantial decline in Andisol by day 30 indicates that prolonged exposure to lethal compounds limited microbial adaptation (Jyot et al. 2015), potentially due to sustained pesticide accumulation or

energy metabolism impairment. In the Inceptisol, the more stable dehydrogenase levels after 30 days reveal greater resilience and metabolic recovery. Pesticides, as the NNIs studied here, can temporarily inhibit soil activity, and this inhibition can be recovered or even enhance the enzyme's activity once the insecticide has been degraded (Wang et al. 2014). The THM was the pesticide that showed a milder effect, especially at early stages, similar to that reported by Jyot et al. (2015), which may be related to its lower persistence, limiting microbial contact. However, this apparent lower impact should be interpreted carefully, since THM can be transformed into CLO, a more persistent NNI metabolite with greater environmental persistence and mobility (Hilton et al. 2019). Interestingly, in the Inceptisol at day 1, lower pesticide doses appeared to exert a greater inhibitory effect on dehydrogenase activity than the corresponding higher doses. This non-linear response may be associated with differences in pesticide bioavailability. At higher concentrations, stronger adsorption onto clay minerals may reduce the immediate availability of NNIs to microorganisms, partially attenuating their inhibitory effects (Zhang et al. 2018; Wołejko et al. 2020). In addition, rapid microbial adaptation or shifts in microbial community composition may also contribute to this response pattern (Wang et al. 2014).

Modification in potential enzyme activities and functional gene abundances offer insights into alterations in the microbial communities to perform ecosystem function (Stache et al. 2025). In our study, the effect of the NNIs on 16S gene abundance was negligible, with only differences observed at day 1 in Freire soil, where an upward trend was observed, but the THM (10x) treatment was significantly different ($P<0.05$) from the soil without pesticide application. With respect to N cycling, *nifH* gene abundance was most influenced by pesticide application in the Inceptisol, with increases observed on days 1 and 30 after application, highlighting all treatments and IMI (10x), respectively. Studies have reported that NNIs such as IMI may differentially affect part of the microbial community, observing that Gram-negative bacteria are more tolerant to this exposure than Gram-positive bacteria (Cycon et al. 2013). This could indicate a small proliferation of certain groups of microorganisms associated with N-fixation. Chen et al. (2017) reported that the phylum Bacteroidota was the most abundant in soils exposed to paichongding, a novel insecticide with higher insecticidal activity than IMI. In the same way, Zhang et al. (2018) reported that Proteobacteria increased after IMI, THM, and thiacloprid in different types of soil. Bacteroidetes and Proteobacteria play key roles at different steps of the N cycle (Ren et al. 2018; Zhang et al. 2025).

**4.2 Short-term inhibition of nitrification potential in soils exposed to neonicotinoid insecticides**

In this study, we evaluated whether NNI pesticides cause unacceptable long-term effects, defined as >25% deviation from the control, on N transformation by soil microorganisms, as described by Starche et al. (2025). A decrease in nitrification potential

in pesticide-treated soil, as observed in our study, means that the microorganisms responsible for oxidising $NH_4^+$ to $NO_2^-$ and then to nitrate $NO_3^-$ have reduced their metabolic activity or abundance. However, this response was observed immediately after application of the highest NNIs doses, and both soils recovered quickly. Generally, nitrification is more sensitive to pesticides than ammonification (Cycon et al. 2015); therefore, the nitrification potential has been identified as one of the most sensitive microbial indicators for assessing the impact of pesticides on soil (Sim et al. 2022). Akter et al. (2022) reported that long-term treatment of paddy soil with pesticides, such as THM, affected both the nitrification rate and the potential nitrification rate, likely due to inhibition of the nitrifying activity of specific microbial groups, such as ammonia-oxidising bacteria. Similar results were observed for nitrification potential, which was significantly inhibited in a Loam soil by the insecticide fipronil, herbicides trifluralin, chlorsulfuron, isoxaflutole, and paraquat, and by the fungicides azoxystrobin, flutriafol, and metalaxyl-M (Sim et al. 2022).

The deviation criterion for the $NH_4^+$ concentration exceeded 25% in the Andisol at day 30, particularly with the IMI treatment, and at day 1 for almost all treatments, indicating a reduction in $NH_4^+$ concentration (Table 2). In soils treated with pesticides, a decrease in $NH_4^+$ may be due to (i) increased nitrification, which is usually accompanied by an increase in $NO_3^-$, (ii) inhibition of ammonification, reflecting lower microbial production of $NH_4^+$. According to our results, the first explanation could be a temporary nitrification activation in the Inceptisol soil on day 1, as indicated by an increase in $NO_3^-$ concentration. However, this effect was not long-lasting, since the deviation criterion did not exceed 25 %. Nitrification is an essential process in the soil N cycle that contributes to plant N availability. However, excessive $NO_3^-$ production may increase the risk of $NO_3^-$ leaching due to its high solubility and mobility in soil (Fenice, 2021). On the contrary, a decrease in $NO_3^-$ concentration exceeding the 25 % was observed mainly in treated soil with IMI and THM (Table 3). These findings are consistent with Cycoń et al. (2015) and Sim et al. (2022) because insecticides such as IMI or fipronil significantly inhibit potential nitrification, reducing the formation of $NO_3^-$ compared to controls. The pesticide treatment reduced the abundance of nitrifying bacteria in Andisol soil, as previously observed with 2,4-D (Palma et al. 2023). Therefore, it is necessary to pay special attention to microorganisms such as ammonium-oxidising Archaea (AOA) and Bacteria (AOB) in future observations.

**4.3 Neonicotinoid dissipation depends on the compound and soil type**

Dissipation of IMI, THM, and CLO applied in 1- and 10-fold the recommended application dose in two Araucanian soils (Andisol and Inceptisol) was tested (Fig. 4). Because the soils were not sterilised, the observed pesticide dissipation likely

reflects the combined contribution of abiotic processes and microbial activity, which could not be distinguished in the present experiment.

We found that dissipation rates of NNIs after 30 days in non-sterile soils did not depend on the initial concentration but differed between soils. In the Andisol the dissipation rates followed the order THM > IMI > CLO, while in the Inceptisol it was CLO > THM ≥ IMI. The slow dissipation of IMI may be explained by its polar nature and its stability under neutral to acidic conditions, whereas THM degradation is favored under acidic conditions such as those present in the studied soils. For instance, although CLO was poorly removed in the Andisol, its dissipation was considerably higher in Inceptisol. Such differences may be explained among others by differing adsorption capacity and subsequent varying surface-catalysed hydrolysis rates. According to Wu et al. (2023) degradation efficiencies of the CLO and TMX decreased with high SOM contents in the soil. However, the higher clay content of the Inceptisol may enhance the adsorption of IMI, THM and CLO (Briceño et al. 2025), thereby facilitating their dissipation and contributing to shorter half-life (DT50) values, as also reported by Li et al. (2023). This suggests that soil mineral composition and texture play an important role in regulating the environmental fate of NNIs and cannot be ignored when they are used (Wu et al. 2023).

The relative dissipation of the compounds in the Freire soil also coincided with reported $DT_{50}$ values from the literature, with 545 days for CLO, ≥187 days for IMI, and >121 days for THM (PPDB, 2024; Morrissey et al. 2015; Lewis et al. 2016), determined under previously reported environmental conditions. However, THM dissipation was within the $DT_{50}$ ranges previously reported by Hilton et al. (2019) (24.9–43.5 days). This behavior may be associated with the higher soil organic carbon content of the Andisol, which could support greater microbial abundance and enhanced NNI biodegradation (Zhang et al. 2018; Wołejko et al. 2020). Another possible explanation is that THM dissipation in the Andisol may be related to its higher water solubility and lower Koc compared with IMI and CLO, which may reduce its adsorption capacity (Briceño et al. 2025) and increase its bioavailability for microbial degradation. The results obtained in this study are an important contribution considering that Andisols are soils widely distributed throughout the world, and supporting high agricultural production (Palma et al. 2023). Some characteristic properties of Andisols include high organic matter content, acidic pH, and a mineral fraction dominated by short-range-order minerals such as allophane and imogolite (Shoji et al. 1993; McDaniel & Lowe, 2012). According to our results, THM appeared to exert lower short-term effects on the evaluated soil parameters compared with IMI and CLO, whose chemical properties likely influenced the response in these soils, as observed in other studies (Modrić et al. 2024). However, this lower apparent risk should be interpreted cautiously, since CLO is one of the major metabolites of THM and is recognized for its persistence in soils. Although soil enzyme activities are useful indicators of soil

metabolic functioning, they do not fully represent overall soil health. Furthermore, the environmental risk associated with NNIs is related not only to their persistence in soils but also to their mobility and potential leaching into water bodies, which may contribute to broader ecological impacts, including effects on pollinators and aquatic ecosystems (Mandal et al. 2026). These impacts remain poorly studied in Chile.

## 5 Conclusion

Overall, this study provides the first integrated assessment of how commonly used NNI insecticides influence multiple components of the soil N cycle under contrasting soil conditions. By combining measurements of enzymatic activity, inorganic N dynamics, nitrification potential, and functional gene abundance, our results show that NNIs such as IMI, THM, and CLO can induce short-term and process-specific disturbances, particularly affecting nitrification, while other N-cycling processes appear more resilient. Importantly, the recovery of most microbial functions despite the persistence of detectable pesticide residues suggests that chemical persistence does not necessarily translate into sustained biological impact, likely due to reduced bioavailability and microbial functional adaptation. These findings are especially relevant for volcanic soils such as Andisols, which dominate large agricultural areas of southern Chile. Understanding how neonicotinoids interact with these soils and their microbial communities is therefore essential for predicting their environmental behaviour and potential impacts on soil ecosystem functioning.

Soil type played a decisive role in modulating microbial responses. The Andisol (Freire series) exhibited higher functional sensitivity, reflected in stronger inhibition of urease and dehydrogenase activities and slower recovery of N-related processes. In contrast, the clay-rich Inceptisol (Chufquen series) showed greater microbial and enzymatic resilience. Molecular analyses revealed that total bacterial abundance remained relatively stable, whereas the *nifH* gene responded selectively to NNI exposure, suggesting adaptive shifts within N-fixing communities rather than broad microbial suppression. The temporary reduction in nitrification potential at elevated pesticide doses, followed by recovery, indicates that N-cycling processes are resilient but vulnerable to short-term disturbances immediately after pesticide application.

Pesticide dissipation was strongly influenced by both soil properties and insecticide identity, with CLO exhibiting the greatest persistence, followed by IMI and THM. These findings underscore the importance of accounting for soil-specific properties in pesticide risk assessment and support the use of soil enzymes and microbial functional genes as practical indicators for evaluating soil health and guiding sustainable agricultural management.

## References


Akter R, Binte Mukhles M, Rahman MM, Rana MdR, Huda N, Ferdous J, Rahman F, Rafi MH, Biswas SK (2022) Effect of pesticides on nitrification activity and its interaction with chemical fertiliser and manure in long-term paddy soils. Chemosphere 304: 135379. https://doi.org/10.1016/j.chemosphere.2022.135379

Akter S, Hulugalle NR, Jasonsmith J, Strong CL (2023) Changes in soil microbial communities after exposure to neonicotinoids: A systematic review. Environ Microbiol Rep 15:431– 444. https://doi.org/10.1111/1758-2229.13193

Bandeira FO, Lopes PR, Hennig TB, Brancalione J, Nogueira DJ, Matias WG (2012) Chronic effects of clothianidin to non-target soil invertebrates: Ecological risk assessment using the species sensitivity distribution (SSD) approach. J Hazard Mater 419: 126491. https://doi.org/10.1016/j.jhazmat.2021.126491

Berg P, Rosswall T (1985) Ammonium oxidizer numbers, potential and actual oxidation rates in two Swedish arable soils. Biol Fertil Soils 1: 131–140. https://doi.org/10.1007/BF00301780

Bonetti JA, Anghinoni I, Tuzzin de Moraes M, Fink JR (2017) Resilience of soils with different texture, mineralogy and organic matter under long-term conservation systems. Soil Tillage Res 174: 104–112. https://doi.org/10.1016/j.still.2017.06.008

Bonmatin J, Giorio C, Girolami V, Goulson, D, Kreutzweiser DP (2015) Environmental fate and exposure; neonicotinoids and fipronil. Environ Sci Pollut Res 22: 35–67. https://doi.org/10.1007/s11356-014-3332-7

Briceño G, Diez MC, Palma G, Jorquera M, Schalchli H, Saez JM, Benimeli CS (2024) Neonicotinoid effects on soil microorganisms: Responses and mitigation strategies. Sustainability 16: 3769. https://doi.org/10.3390/su16093769

Briceño G, Palma G, Schalchli H, Durán P, Llafquén C, Huenchupán A, Rodríguez-Rodríguez C, Diez MC (2025) Adsorption–desorption behaviour of imidacloprid, thiamethoxam, and clothianidin in different agricultural soils. Agriculture 15:1380. https://doi.org/10.3390/agriculture15131380

Castillo-Díaz JM, Martin-Laurent F, Beguet J, Nogales R, Romero E (2017) Fate and effect of imidacloprid on vermicompost-amended soils under dissimilar conditions: Risk for soil functions, structure, and bacterial abundance. Sci Total Environ 579: 1111–1119. https://doi.org/10.1016/j.scitotenv.2016.11.113

CIREN. Estudio Agrológico, IX Región: Descripciones de Suelos, Materiales y Símbolos (Pub. CIREN N◦122); Centro de Información de Recursos Naturales: Santiago, Chile, 2002. Available online: https://bibliotecadigital.ciren.cl/items/0c56d801-ba1e-476a-a634-b40b819b66e5 (accessed on 15 July 2024).

Cycoń M, Markowicz A, Borymski S, Wójcik M, Piotrowska-Seget Z (2013) Imidacloprid induces changes in the structure, genetic diversity and catabolic activity of soil microbial communities. J Environ Manage 131:55–65. https://doi.org/10.1016/j.jenvman.2013.09.041

Cycoń M, Piotrowska-Seget Z (2015) Community structure of ammonia-oxidising archaea and ammonia oxidising bacteria in soil treated with the insecticide imidacloprid. Biomed Res Int 2015:582938. https://doi.org/10.1155/2015/582938

Chen J, Zhou S, Rong Y, Zhu X, Zhao X, Cai Z (2017) Pyrosequencing reveals bacterial communities and enzyme activities differences after application of novel chiral insecticide Paichongding in aerobic soils. Appl Soil Ecol 112: 18–27. https://doi.org/10.1016/j.apsoil.2016.12.007

Diez MC, Gallardo F, Saavedra G, Cea M, Gianfreda L, Alvear M (2006) Effect of pentachlorophenol on selected soil enzyme activities in a chilean Andisol. J Soil Sci Plant Nutr 6: 40–51. https://doi.org/10.4067/S0718-27912006000300004

Doane TA, Horwáth WR (2003) Spectrophotometric determination of nitrate with a single reagent. Anal Lett 36:2713–2722. https://doi.org/10.1081/AL-12002 4647

Elumalai P, Yi X, Chen Z, Rajasekar A, Brazil de Paiva T, Hassaan M, Ying G, Huang M (2022) Detection of neonicotinoids in agriculture soil and degradation of thiacloprid through photo degradation, biodegradation and photo-biodegradation. Environ Poll 306: 119452. https://doi.org/10.1016/j.envpol.2022.119452

Fenice M (2021) The Nitrogen Cycle: An Overview. In: Gonzalez-Lopez J, Gonzalez-Martinez A. Nitrogen cycle ecology, biotechnological applications and environmental impacts, 23 pp. CRC Press is an imprint of Taylor & Francis Group, LLC, Boca Raton, FL.

Gangola S, Joshi S, Bhandari G, Pant G, Sharma A, Perveen K, Bukhari N.A, Rani R (2023) Exploring microbial diversity responses in agricultural fields: a comparative analysis under pesticide stress and nonstress conditions. Front Microbiol 14: 1271129. https://doi.org/10.3389/fmicb.2023.1271129

480 Gregory AS, Watts CW, Whalley WR, Kuan HL, Griffiths BS, Hallett PD, whitmore AP (2007) Physical resilience of soil to
481 field compaction and the interactions with plant growth and microbial community structure. Eur J Soil Sci 58: 1221–
482 1232. https://doi.org/10.1111/j.1365-2389.2007.00956.x

483 Hilton M, Emburey SN, Edwards PA, Dougan C, Ricketts DC (2019) The route and rate of thiamethoxam soil degradation in
484 laboratory and outdoor incubated tests, and field studies following seed treatments or spray application. Pest Manag
485 Sci 75:63–78. https://doi.org/10.1002/ps.5168

486 Hladik ML, Main AR, Goulson D (2018) Environmental risks and challenges associated with neonicotinoid insecticides.
487 Environ Sci Technol 52: 3329–3335. https://doi.org/10.1021/acs.est.7b06388

488 Jyot G, Mandal K, Singh B (2015) Effect of dehydrogenase, phosphatase and urease activity in cotton soil after applying
489 thiamethoxam as seed treatment. Environ Monit Assess 187: 298. https://doi.org/10.1007/s10661-015-4432-7

490 Kandeler E, Gerber H (1988) Short-term assay of soil urease activity using colorimetric determination of ammonium. Biol
491 Fertil Soils 6:68–77. https:// doi.org/10.1007/ BF002 57924

492 Karpouzas DG, Tsiamis G, Trevisan M, Ferrari F, Malandian C, Sibourg O, Martin-Laurent F (2016) "LOVE TO HATE"
493 pesticides: felicity or curse for the soil microbial community? An FP7 IAPP Marie Curie project aiming to establish
494 tools for the assessment of the mechanisms controlling the interactions of pesticides with soil microorganisms. Environ
495 Sci Pollut Res Int 23:18947–51. https://doi.org/10.1007/s11356-016-7319-4

496 Keeney DR, Nelson DW (1982) Nitrogen-inorganic forms. In: Page AL, Miller RH, Keeney DR (eds) Methods of soil analysis
497 part 2: Chemical and microbiological properties, 2nd edn. American Society of Agronomy Madison, WI, pp 643–698

498 Li Y, Li Y, Bi G, Ward TJ, Li L (2023) Adsorption and degradation of neonicotinoid insecticides in agricultural soils. Environ
499 Sci and Pollut Res 30:47516–47526. https://doi.org/10.1007/s11356-023-25671-9

500 Lewis KA, Tzilivakis J, Warner DJ, Green A (2016) An international database for pesticide risk assessments and management.
501 Hum Ecol Risk Assess 22: 1050–1064. https://doi.org/10.1080/10807039.2015.1133242

502 McDaniel PA, Lowe DJ, Arnald O, Ping C-L (2012) Andisols. In: Huang PM et al. (ed) Handbook of soil sciences, 2nd edition.
503 Vol 1: Properties and Processes. CRC Press (Taylor and Francis), Boca raton, FL, pp 29-33.

Meena RD, Kumar S, Datta R, Lal R, Vijayakumar V, Brtnicky M, Sharma MP, Yadav GS, Jhariya MK, Jangir CK, Pathan SI, Dokulilova T, Pecina V, Marfo TD (2020) Impact of agrochemicals on soil microbiota and management: A Review. Land 9: 34. https://doi.org/10.3390/land9020034

Modrić S, Didović M, Dubrović I, Žurga P, Broznić D (2023) Those that remain: sorption/desorption behaviour and kinetics of the neonicotinoids still in use. Int J Mol Sci 24: 6548. https://doi.org/10.3390/ijms24076548

Mörtl M, Vehovszky Á, Klátyik S, Takács E, Győri J, Székács A (2020) Neonicotinoids: Spreading, translocation and aquatic toxicity. Int J Environ Res Public Health 17:2006. https://doi.org/10.3390/ijerph17062006

Morrissey CA, Mineau P, Devries JH, Sanchez-Bayo F, Liess M, Cavallaro MC, Liber K (2015) The route and rate of thiamethoxam soil degradation in laboratory and outdoor incubated tests, and field studies following seed treatments or spray application. Pest Manag Sci 75: 63–78. https://doi.org/10.1002/ps.5168

Nannipieri P, Giagnoni L, Renella G, Puglisi E, Ceccanti B, Masciandaro G, Fornasier F, Moscatelli M.C, Marinari S (2012) Soil enzymology: classical and molecular approaches. Biol Fertil Soils 48: 743–762. https://doi.org/10.1007/s00374-012-0723-0

Palma G, Spuler MJ, Jorquera M, Briceño G (2023) Effects of the combined application of nitrogen fertiliser and 2,4-D on nitrification ammonia oxidisers and herbicide bioavailability in a volcanic soil. A microcosm study. J Soil Sci Plant Nutr 23:4309–4317. https://doi.org/10.1007/s42729-023-01350-z

Pang S, Lin Z, Zhang Y, Zhang W, Alansary N (2020) Insights into the toxicity and degradation mechanisms of imidacloprid via physicochemical and microbial approaches. Toxics 8: 0065. https://doi.org/10.3390/toxics8030065

Paoli M, Giurfa M (2024) Pesticides and pollinator brain: How do neonicotinoids affect the central nervous system of bees? Eur J Neurosci, 60:5927–5948. https://doi.org/10.1111/ejn.16536

Pesticide Properties DataBase PPDB:. Available at: https://sitem.herts.ac.uk/aeru/ ppdb/en/index.htm, accessed on 15 December 2025.

Pietrzak D, Kania J, Kmiecik E, Malina G, Wątor K (2020) Fate of selected neonicotinoid insecticides in soil–water systems: Current state of the art and knowledge gaps. Chemosphere, 255: 126981. https://doi.org/10.1016/j.chemosphere.2020.126981

529 Radolinski J, Wu J, Xia K, Hession WC, Stewart RD (2019) Plants mediate precipitation-driven transport of a neonicotinoid
530 pesticide. Chemosphere 222: 445-452. https://doi.org/10.1016/j.chemosphere.2019.01.150

531 Ren M, Zhang Z, Wang X, Zhou Z, Chen D, Zeng H, Zhao S, Chen L, Hu Y, Zhang C, Liang Y, She Q, Zhang Y, Peng N
532 (2018) Diversity and contributions to nitrogen cycling and carbon fixation of soil salinity shaped microbial
533 communities in Tarim basin. Front Microbiol 9:431. https://doi.org/10.3389/fmicb.2018.00431

534 Sanchez-Bayo F, Tennekes H, Goka K (2013) Impact of systemic insecticides on organisms and ecosystems. Insectic -
535 development of safer and more effective technologies, Stanislav Trdan, IntechOpen, https://doi.org/ 10.5772/52831

536 Serra-Wittling C, Houots S, Barriuso E (1995) Soil enzymatic response to addition of municipal solid-waste compost. Biol
537 Fertil Soils 20:226–236. https://doi.org/10.1007/BF003 36082

538 Shoji S, Nanzyo M, Dahlgren R (1993). Terminology, concepts and geographic distribution of volcanic ash soils. In: Shoji et
539 al. (ed). Volcanic Ash Soils: Genesis, Properties and Utilization. Elsevier.

540 Sim JXF, Doolette CL, Vasileiadis S, Drigo B, Wyrsch ER, Djordjevic SP, Donner E, Karpouzas DG, Lombi E (2022) Pesticide
541 effects on nitrogen cycle related microbial functions and community composition. Sci Total Environ 807: 150734.
542 https://doi.org/10.1016/j.scitotenv.2021.150734

543 Singh S, Gupta R, Kumari M, Sharma S (2015) Nontarget effects of chemical pesticides and biological pesticide on
544 rhizospheric microbial community structure and function in *Vigna radiata*. Environ Sci Pollut Res 22: 11290–11300.
545 https://doi.org/10.1007/s11356-015-4341-x

546 Sollins P, Homann P, Caldwell BA (1996) Stabilization and destabilization of soil organic matter: mechanisms and controls.
547 Geoderma 74: 65–105. https://doi.org/10.1016/S0016-7061(96)00036-5

548 Sparks TC, Crossthwaite AJ, Nauen R, Banba S, Cordova D, Earley F, Ebbinghaus-Kintscher U, Fujioka S, Hirao A, Karmon
549 D, KennedyR, Nakao T, Pophamj HJR, Salgado V, Watson GB, Wedel BJ, Wessels FJ (2020) Insecticides, biologics
550 and nematicides: Updates to IRAC's mode of action classification- a tool for resistance management. Pestic Biochem
551 Physiol 167: 104587. https://doi.org/10.1016/j.pestbp.2020.104587

552 Stache F, Ditterich F, Hochmanová Z, Hofman J, Poll C, Kandeler E (2025) Quantification of soil microbial functional genes
553 as potential new method in environmental risk assessment of pesticides. Ecotoxicology 34:1312–1324
554 https://doi.org/10.1007/s10646-025-02920-w

555 Tabatabai MA, Bremmer JM (1969) Use of p-nitrophenylphosphate to assay of soil phosphatase activity. Soil Biol Biochem
556 1:301–307. https://doi.org/10.1016/0038-0717(69)90012-1

557 Tabatabai MA (1982) Soil Enzymes. In: Page, A.L., Miller, R.H. and Keeney, D.R., Eds., Methods of Soil Analysis, ASA,
558 SSSA, Publisher, Madison, WI, 903-947.

559 Takahashi T, Dahlgren RA (2016) Nature, properties and function of aluminum–humus complexes in volcanic soils. Geoderma
560 263: 110–121. http://dx.doi.org/10.1016/j.geoderma.2015.08.032

561 Thompson M, Ellison SLR, Wood R (2002) Harmonized guidelines for single-laboratory validation of methods of analysis
562 (IUPAC Technical Report). Pure Appl Chem 74: 835–855. http://dx.doi.org/10.1351/pac200274050835

563 Wang F, Yao J, Chen H, Yi Z, Choi M.M F (2014) Influence of short-time imidacloprid and acetamiprid application on soil
564 microbial metabolic activity and enzymatic activity. Environ Sci Pollut Res 21:10129–10138.
565 https://doi.org/10.1007/s11356-014-2991-8

566 Wołejko A, Jabłońska-Trypuć A, Wydro U, Butarewicz A, Łozowicka B (2020) Soil biological activity as an indicator of soil
567 pollution with pesticides – A review. App Soil Ecol 147: 103356. https://doi.org/10.1016/j.apsoil.2019.09.006

568 Wu J, Zhuang M, Zou Z, Jianing X, Wang F, Jia C, Zhang H (2023) Efficient degradation of clothianidin and thiamethoxam
569 in contaminated soil by peroxymonosulfate process. Environ Sci Pollut Res 30:48211–48219.
570 https://doi.org/10.1007/s11356-023-25738-7

571 Xia Q, Rufty T, Shi W (2020) Soil microbial diversity and composition: Links to soil texture and associated properties. Soil
572 Biol Biochem 149: 107953. https://doi.org/10.1016/j.soilbio.2020.107953

573 Yuan Y, Li X, Liu F, Tian X, Shao Y, Yuan Z, Chen Y (2024) Differences in soil microbial communities across soil types in
574 China's temperate forests 15: 1110. https://doi.org/10.3390/f15071110

575 Zhang P, Ren C, Sun H, Min L (2018) Sorption, desorption and degradation of neonicotinoids in four agricultural soils and
576 their effects on soil microorganisms. Sci Total Environ 615:59–69. https://doi.org/10.1016/j.scitotenv.2017.09.097

Zhang H, Zhang Z, Song J, Mei J, Fang H, Gui W (2021) Reduced bacterial network complexity in agricultural soils after application of the neonicotinoid insecticide thiamethoxam. Environ Pollut 274: 116540. https://doi.org/10.1016/j.envpol.2021.116540

Zhang X, Huang Y, Chen WJ, Wu S, Lei Q, Zhou Z, Zhang W, Mishra S, Bhatt P, Chen S (2023) Environmental occurrence, toxicity concerns, and biodegradation of neonicotinoid insecticides. Environ Res 218: 114953. https://doi.org/10.1016/j.envres.2022.114953

Zhang S, Hou M, Li B, Guan P, Chi Q, Sun H, Xu H, Cui D, Zhu Y (2025) Roles of core *nosZ* denitrifiers in enhancing denitrification activity under long-term rice straw retention. Front Plant Sci 16:1541202. https://doi.org/10.3389/fpls.2025.1541202

## Declarations

**Funding:** This research was funded by the ANID FONDECYT project N° 1230965, DIUFRO project N° DI22-0029, and ANID/FONDAP/1523A0001.

**Conflict of interest:** The authors declare no competing interests.

**Availability of data and material:** The authors confirm that the data supporting the findings of this study are accessible within the article.

**Author's Contribution:**

Conceptualization: Gabriela Briceño & Graciela Palma; methodology: Gabriela Briceño & Graciela Palma; formal analysis: Gabriela Briceño & Milko Jorquera; investigation: Gabriela Briceño, Heidi Schalchli, María Cristina Diez; resources: Gabriela Briceño; writing—original draft preparation: Gabriela Briceño; writing—review and editing: Lukas Y Wick; project administration and funding acquisition: Gabriela Briceño. All authors have read and agreed to the published version of the manuscript.

**Legend of the tables**

**Table 1.** Physicochemical properties and textural characteristics of the Andisol (Freire Serie) and Inceptisol (Chufquen Serie) soils used in the microcosm experiments.

**Table 2.** Ammonium ($NH_4^+$) concentrations in Andisol (Freire Serie) and Inceptisol (Chufquen Serie) at days 1 and 30 after application of imidacloprid (IMI), thiamethoxam (THM), and clothianidin (CLO) at recommended (1×) and elevated (10×) doses under microcosm conditions.

**Table 3.** Nitrate ($NO_3^-$) concentrations in Andisol (Freire Serie) and Inceptisol (Chufquen Serie) at days 1 and 30 following application of imidacloprid (IMI), thiamethoxam (THM), and clothianidin (CLO) at recommended (1×) and elevated (10×) doses.

**Legend of the figures**

**Figure 1.** Soil enzymatic activities in two soils: Andisol (A) and Inceptisol (B) exposed to neonicotinoid insecticides. Urease, β-glucosidase, acid phosphatase, and dehydrogenase activities were measured at 1, 15, and 30 days after application of imidacloprid (IMI), thiamethoxam (THM), and clothianidin (CLO) at two doses (1× and 10×). Bars represent mean values ± standard deviation (n = 3). Different letters indicate significant differences among treatments within the same soil and sampling time (Tukey test, $p < 0.05$).

**Figure 2.** Abundance of total bacteria (16S rRNA gene) and nitrogen fixation potential (nifH gene) in two soils: Andisol (A) and Inceptisol (B) exposed to neonicotinoid insecticides. Gene abundances were evaluated at 1 and 30 days after application of imidacloprid (IMI), thiamethoxam (THM), and clothianidin (CLO) at two doses (1× and 10×). Bars represent mean values ± standard deviation (n = 4). Different letters indicate significant differences among treatments within the same soil and sampling time (Tukey test, $p < 0.05$). Gene abundances are expressed as copies $g^{-1}$ dry soil.

**Figure 3.** Nitrification potential in two soils: Andisol (A) and Inceptisol (B) exposed to neonicotinoid insecticides. Nitrification potential was evaluated at 1, 15, and 30 days after application of imidacloprid (IMI), thiamethoxam (THM), and clothianidin (CLO) at two doses (1× and 10×). Bars represent mean values ± standard deviation (n = 3). Different letters indicate significant differences among treatments within the same soil and sampling time (Tukey test, $p < 0.05$).

**Figure 4.** Residual percentages of imidacloprid (IMI), thiamethoxam (THM), and clothianidin (CLO) in (A) Andisol (Freire series) and (B) Inceptisol (Chufquen series) at days 1, 15, and 30 following application at recommended (1×) and elevated (10×) doses. Bars represent mean values ± standard deviation (n = 3). Different letters indicate significant differences among treatments within the same soil and sampling time (Tukey test, $p < 0.05$).