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Neonicotinoid insecticides as emerging contaminants in agricultural soil

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Abstract
Using an LC-MS-MS method for detection of 6 neonicotinoid insecticides (imidacloprid, dinotefuran, acetamiprid, clothianidin, thiamethoxam, nitenpyram) was developed a new performant extraction method based on sonication treatment of soil samples, which were previously dried, grounded, homogenized, sieved (2 mm) and subjected to the selective extraction process with acetonitrile. Then the obtained extracts were diluted with ultrapure water (ratio 1: 100) and subjected to purification by Strata C18 SPE extraction using cartridges loaded with 200 mg/6 mL of octa-dodecyl-silica adsorbent phase. The entire methodology allowed obtaining quantification limits at trace level that varied in the range 0.3-0.9 ng/g and recoveries between 71.4% and 109.6%. In the agricultural soil samples, taken from the lands cultivated with wheat, corn, sunflower, beans, located in Prahova and Giurgiu counties (Romania), only four neonicotinoids out of the total of six were quantified imidacloprid (0.38 ng/g-56.9 ng/g), acetamiprid (1.7-7.2 ng/g), thiamethoxam (1.05-6.7 ng/g), clothianidin (1.1-1.5 ng/g).

Keywords: *neonicotinoid (NN) insecticides, UE-SPE-LC-MS/MS, agricultural soil, emerging contamination*

INTRODUCTION

Neonicotinoid (NN) insecticides can be classified into three major chemical groups: N-nitro-guanidine (imidacloprid, thiamethoxan, clothianidin, dinotefuran); nitro-methylene (nitenpyram); N-cyan- amidines (acetamiprid, thiacloprid) [1]. These substances in small quantities are toxic for insects, the lethal dose for bees being 4-5 ng/insect (clothianidin and imidacloprid). Neonicotinoid compounds are the most widely used insecticides in the world. They act systemically on all plant tissues when they are applied to seeds. As neurotoxins with high toxicity to most arthropods, they provide effective control against insects and have numerous uses in agriculture and horticulture [2].
Recent research shows that these compounds are more persistent in soil than previously understood [2, 3]. Very low concentrations of neonicotinoid residues in plants, soil, and groundwater are associated with reductions in the diversity and abundance of non-target insects and insectivorous birds [4, 5]. Neonicotinoids are persistent in soils under suitable conditions. Among others, low levels of soil quality, may be due to the increase in microbial activity, temperature and precipitation dissipation time. Their reported half-lives (DT50) vary depending on the compound and environmental circumstances, but the range is 150-6900 days for Clothianidin [2]. The presence of neonicotinoid compounds in agricultural soils is insufficient studied, and detection methods for these emerging contaminants did not allow reporting of concentrations at trace levels, which generate toxicity to invertebrates.
A review of DT50 (half-life by dissipation, table 1), available in field and laboratory studies conducted between 1999 and 2013, was performed by Goulson [2]. The reported DT50s are highly

variable and usually are situated in the range from 200 - over 1000 days for imidacloprid, 7–353 days for thiamethoxam and 148-6931 days for clothianidin [6, 7]. DT50s appear to be lower for nitro-substituted neonicotinoids, at 3–1000 days for thiacloprid and 31-450 days for acetamiprid. DT50 values over 1 year would suggest the probability of accumulation of neonicotinoids in soil, assuming a continuous intake.

Table 1. Chemical properties and persistence of neonicotinoid insecticides in soil [6, 7]

Compound (abbreviation)	Lipophilicity (log Kow)	Soil affinity (log Koc)	Half-life in soil DT50 (days)
Dinotefuran (DIN)	-0.55	1.41	75-82
Imidacloprid (IMI)	0.57	2.19	100-1250
Nitenpyram (NIT)	-0.66	1.78	8
Thiamethoxam (THM)	-0.13	1.75	7-335
Clothianidin (CLO)	0.91	2.08	148-6931
Acetamiprid (ACE)	0.8	2.3	31-450
Thiacloprid (TCP)	1.26	2.78	3.4-1000

However, these reported values are highly variable. A single available field study has been conducted evaluating the accumulation of neonicotinoid in soil over several years, with continuous intake of neonicotinoid [8]. In 2005, Bonmatin examined 74 agricultural soil samples for imidacloprid [8]. The values were higher in the samples in which IMI was applied two years in a row, compared to the samples in which the compound was used only once, thus highlighting the accumulation of NN's in the soil.

Goulson [2] evaluated the studies on the presence of NN's in the soil and noticed that they accumulate over several years followed by a flattening after 5 years.

Starting with 2013, several papers were presented in international journals on the determination of NN's in soil samples from agriculture and thus DT50 was calculated in these soils but also the accumulation of these compounds in the soil. Jones et al. determined the concentrations of some NN's in soil samples taken from both the central area and the surrounding areas of 18 agricultural lands in six English counties [9]. The soil samples were taken during spring, before the cultivation of cereals. The following NN's were detected clothianidin (<0.02–13.6 ng/g), imidacloprid (<0.09–10.7 ng/g) and thiamethoxam (<0.02–1.5 ng/g). NN concentrations in the middle of the lands showed higher values than the soils taken from the marginal areas (clothianidin average of 4.89 versus 0.84 ng/g, imidacloprid average of 1.62 versus 0.76 ng/g and thiamethoxam average of 0.40 versus 0.05 ng/g).

In the spring of 2013 and 2014 Limay-Rios et al. [10] sampled soils from 25 Ontario lands prior to cultivation and observed mean values of 0.91 ng/g thiamethoxam and 3.45 ng/g of clothianidin, concentrations comparable to those obtained by Jones et al. [9].

Soil samples cultivated with rapeseed in winter and 5 wheat fields sown in winter were collected in summer 2013, 10 months after sowing crops were analyzed by Botías et al. [11]. The soils were sampled from the center of the land/field (rapeseed for oilseeds) and soil from field edges (oilseed rape). The following NN's were determined: clothianidin (0.41–28.6 ng/g), imidacloprid (≤0.07-7.90 ng/g), thiacloprid (≤0.01–0.22 ng/g) and thiamethoxam (≤0.04–9.75 ng/g). Residues in the center of rapeseed fields were higher than those at the edge of oilseed rape fields (clothianidin average of 13.28 versus 6.57 ng/g, imidacloprid average of 3.03 versus 1.92 ng/g, thiacloprid average of 0.04 compared to ≤0.01 ng/g and thiamethoxam average of 3.46 compared to 0.72 ng/g). These values were higher than those detected by *Jones* et al. [9] and Limay-Rios et al. [10].

The results of a study conducted in 2015 by Hilton et al. on 18 soils treated with thiamethoxam (uncultivated soils, soils with vegetation/grasses, soils sown with potatoes, spring barley, peas, soybean, winter barley, winter wheat) reveals that DT50 for thiamethoxam had values between 7.1 and 92.3 days [12]. This compound showed reductions in its initial applied concentration of 10% over one year.

The soil is an essential ecosystem that allows obtaining of food, nutrient regulation, raw materials, water purification, energy, carbon sequestration, pest control. Thus, soil is an important natural resource for reducing climate change, obtaining agricultural products, food security and conserving ecosystems. The investigation of the chemical quality of the soil is necessary in order to evaluate the transfer and bioaccumulation potential of toxic contaminants, in vegetables, cereals and fruits. Thus, for the detection of emerging contaminants (not regulated in environmental legislation) in the soil, it is necessary to develop continuously new high-performance analytical methods, able to identify and quantify traces of these compounds.

In 2015, neonicotinoid insecticides have been included in the watch list of substances for the European Union monitoring program in surface water and groundwater (495/2015/ EU, LOD 9 ng/L) [13]. In 2018, the European Commission approved the use of imidacloprid, thiamethoxam and clothianidin treated seeds only in permanent greenhouse. Thus, the use of these substances for agricultural purposes was forbidden [14] but the governments of the European countries, in order to avoid serious losses in agricultural production, obtain annual derogations from the legislation. The methods for detecting neonicotinoids in soil samples presented in the scientific literature have been based on liquid chromatography [15-19].

In this study was developed, optimized and validated a new extraction method (isolation, concentration, purification) with high recovery rates, followed by chromatographic separation in order to detect and quantify 6 neonicotinoids in agricultural soils by LC-MS/MS instrument (liquid chromatograph coupled with triple quadrupole mass-spectrometer). Then, in the study, the developed method was applied to assess neonicotinoid levels in agricultural soils sown with corn, wheat, sunflower, beans from two counties: Prahova and Giurgiu.

EXPERIMENTAL PART

Reagents and analytical standards

Individual standards were purchased from Sigma-Aldrich (Seelze, Germany) and were of the highest purity (97%). All solvents used in sample preparation and chromatographic analysis were of LC-MS grade. Methanol, acetonitrile, formic acid was purchased also form Sigma-Aldrich. Individual standard stock solutions were prepared in methanol and were kept at -20^0 C for 6 months. The intermediate mix standard solutions were prepared by diluting of stock solutions to an exactly acetonitrile volume. Calibration standards (1; 10; 25; 50; 75; 100 ng/mL) were prepared by successive dilutions of intermediary standard solutions with initial mobile phase (acetonitrile/formic acid 0.2%, 90/10). Ultra-pure water was produced by Millipore equipment (Millipore, Bedford, MA, USA). Strata C18 cartridges (200 mg/6 mL) were obtained from Phenomenex. All calibration standards have been protected from light and kept in the dark at 4°C for one month.

UE-SPE-LC-MS/MS method for neonicotinoids detection in soil samples

The procedure used for separation of neonicotinoid in extract samples was developed in a previous study [20]. Agilent 1260 UHPLC coupled with Agilent 6410 triple quadrupole mass spectrometer working in Multiple Reaction Monitoring (MRM) with two transitions (the first most intense from precursor to product ion was used for quantification, and the second transition from the same precursor to the second most intense product ion was applied for confirmation) was used. Agilent Mass Hunter Software was applied for the data acquisition and the data processing [20]. The compounds were injected on the Hypersil Gold column (Thermo- Electron 100 x 2.1mm, 3µm) at 20°C using 10 µL of extract or standard with a gradient of mobile phase composed by acetonitrile and 0.2% formic acid. The flow rate was 0.2 mL/min and the analytes were separated in 13 minutes. The MS neonicotinoid ionization was performed in positive mode (ESI+) at a nitrogen temperature of 300°C and a gas flow rate of 8 L/min. Nebulizer gas pressure had a value of 400 psi and a voltage of 3500 V was applied to the capillary. For the fragmentation of the analytes, a collision energy in the range of 4-20 V, a fragmentation voltage of 45-100 V, a dwell time of 100 msec and a cell acceleration voltage of 7V were applied.

The soils were dried at room temperature overnight (25°C). The dry soils were thus ground and sieved through a metal sieve with a mesh size of 2 mm. From each sample 1 g of soil was weighed and placed in glass vials over which 10 mL of acetonitrile was added and the mixture was sonicated (Bandelin, Sonnorex ultrasonic bath) for 15 min. The resulting mixture was then mechanically shacked (GFL 2004) for 10 minutes. To separate the two layers, organic and soil, the obtained mixture was centrifuged for 5 min at 3000 rpm (Hettich 100). The remaining soil was subjected to extraction a second time, consecutively, using the same procedure. The extracts obtained separately were reunited in the same 300 mL glass flask with a ground-in stopper. The resulting supernatant (10 ml of organic extract) was diluted with 100 mL ultrapure water, and for sample purification, the SPE (C18) extraction was performed. Then, the obtained extract was centrifuged (5 min) for separating the layers.

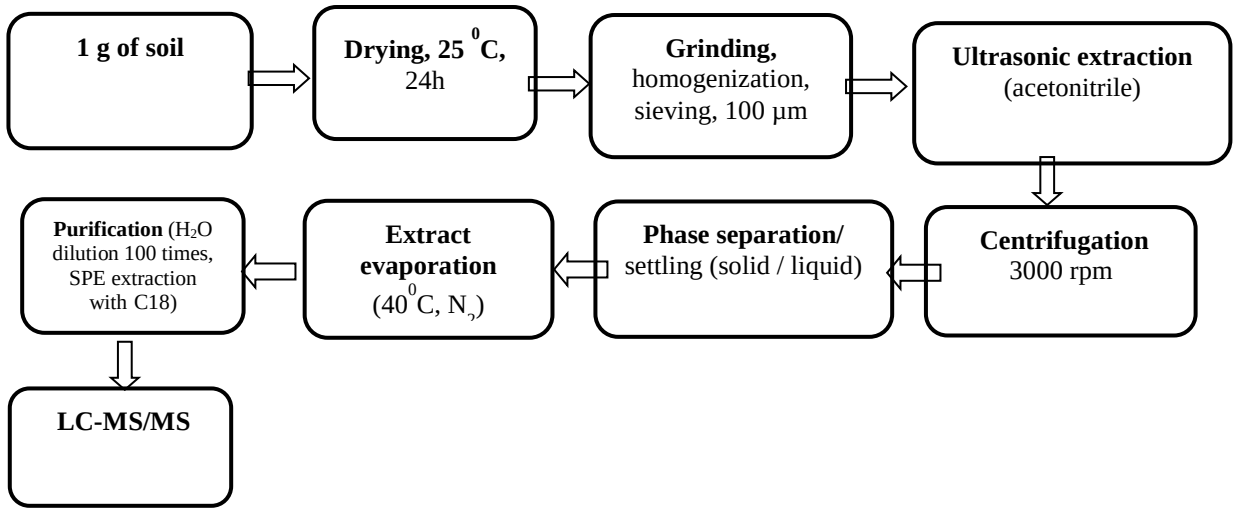


Fig. 1. Diagram containing the necessary steps in pre-treatment of solid samples for NN's extraction from soils by sonication, centrifugation and SPE purification

The final extract was evaporated at 40 °C close to dryness in a weak stream of nitrogen on a Turbo-Vap LV concentrator (Biotage) and then this was reconstituted with 1 mL of the initial mobile phase (90/10 0.2% formic acid/acetonitrile). Then, the extract was filtered through Millipore PTFE membrane (2 cm x 0.2 µm). Each 1 mL of extract was transferred into an LC vial for chromatographic analysis (figure 1).

Method validation experiments

The method was validated for all UE-SPE-LC-MS/MS procedure by calculating of performance parameters (recovery, accuracy, precision, limit of quantitation for soil samples). The recovery expressed as the ratio between the recovered and the spiked analyte concentration of the total procedure was calculated and it was used for quantification. Mixture of external standard was added to soil sample in order to evaluate the precision of the chromatographic and detection parameters. Calibration curves (1-100 ng/g) were applied for quantification using linear regression analysis. The quantification limit (LOQ) of the method was expressed as the minimum detectable amount of compound with a signal to noise ratio of 10. To evaluate the ability to extract all the analytes from soil, five recovery tests were made. For that, 1 g of soil samples was taken to which the middle calibration standard of the curve was added. On the other hand, soil samples without spike (blinds/blanks) with potential native contamination were analyzed and the obtained neonicotinoid concentrations were subtracted from spiked samples in recoveries study. Solvent blanks were used to each extraction and HPLC detection to avoid the crossover instrument contamination with NN's.

Soil sampling

Soil samples were taken from two agricultural counties (Giurgiu and Prahova) where the following crops were sown: sunflower, corn, beans, and wheat (table 2). Thus, the soils were taken in May 2019, from a depth of 10 cm, in the immediate vicinity of the plant root using the drill for the soil. Then, they were placed in 400 g brown glass jars with lids, cooled to 4 ^{0}C and transported to the processing laboratory. The method was successfully applied to soil samples taken from agricultural areas.

Table 2. Soil samples, locations and type of agricultural crops

No.	Culture type, County name
1	S1FS-GR, soil from sunflower culture, Giurgiu County
2	S2FS-PH, soil from sunflower culture, Prahova County
3	S3F-GR, soil from bean culture, Giurgiu County
4	S4F-PH, soil from bean culture, Prahova County
5	S5P-GR, soil from corn culture, Giurgiu County
6	S6P-PH, soil from corn culture, Prahova County
7	S7G-GR, soil from wheat culture, Giurgiu County
8	S8G-PH, soil from wheat culture, Prahova County

RESULTS AND DISCUSSION

Performance of analytical method

Recoveries and limits of quantification (LOQ) of neonicotinoid in soils are presented in table 3**.** Good linearity (r^2 >0.99) for selected concentration range was obtained for all neonicotinoids. A 6-point calibration line was obtained for each analyte in a concentration range with a lower linearity limit of about 1 ng/mL (1 µg/kg) for all compounds. The linearity correlation coefficient was used to evaluate the linearity of the calibration range. A very good linearity was observed for all compounds in the linear range of concentrations with determination coefficients (r^2) between 0.995% and 0.9985.

Table 3. Performance parameters of the analytical methodology (calibration range, determination coefficient, precision, recoveries, LOQ)

Compound	Calibration range (µg/kg, ng/g)	r^2	Repeatability RSD_r (%)	Reproducibility RSD_R (%)	Recovery, %	LOQ (µg/kg,ng/g)
Imidacloprid	1-100	0.9953	7.09	13.7	82.81	0.3
Dinotefuran	1-100	0.9985	8.01	12.2	79.92	0.5
Nitenpyram	1-100	0.9966	7.48	10.4	71.4	0.3
Thiamethoxam	1-100	0.9911	8.5	16.5	109.96	0.9
Clothianidin	1-100	0.9985	6.9	15.6	80.24	0.8
Acetamiprid	1-100	0.9971	5.9	13.6	82.08	0.6

Figure 2 shows the chromatogram corresponding to a mixed calibration standard of the studied compounds at 100 ppb.

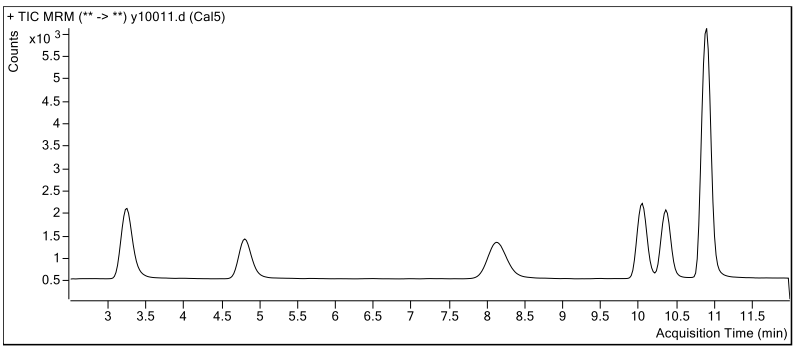


Fig. 2. MRM TIC chromatogram of a standard mixed solution containing NN's in concentration of 100 ppb

Linear regression was obtained for Imidacloprid.
The detector response was linear over the investigated area. The linear regression plotted with the regression parameters (the slope, determination coefficient), is presented in figure 3A. The detector has a corresponding linearity in the selected range, confirmed by the coefficient of determination (r^2 = 0.998). In figures 3B the MRM chromatograms and LC-MS/MS spectra of the [M-H] + ions for Imidacloprid for the measured concentration values are shown.

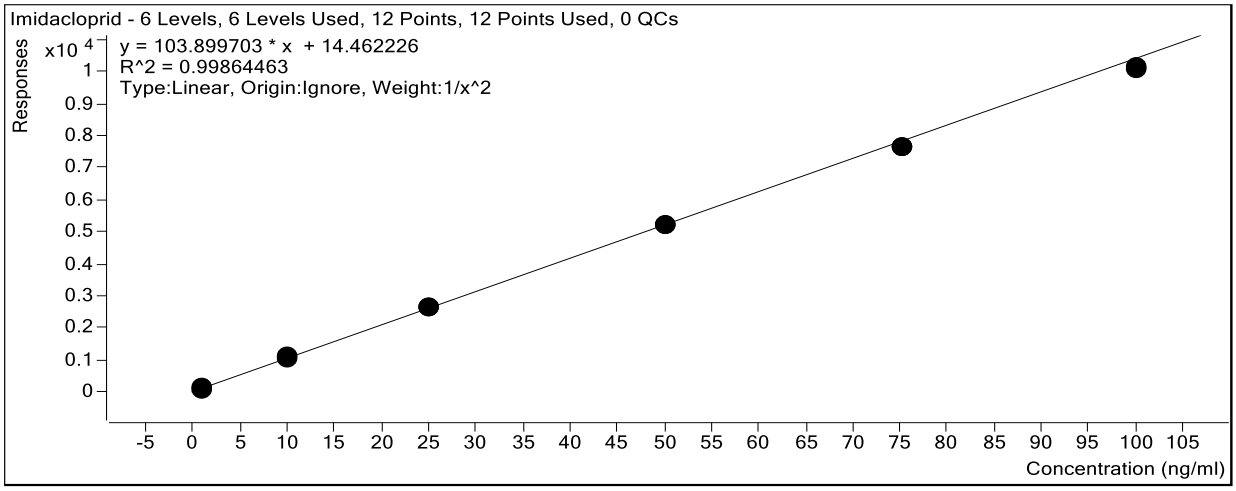


Fig. 3A. Linear regression for Imidacloprid

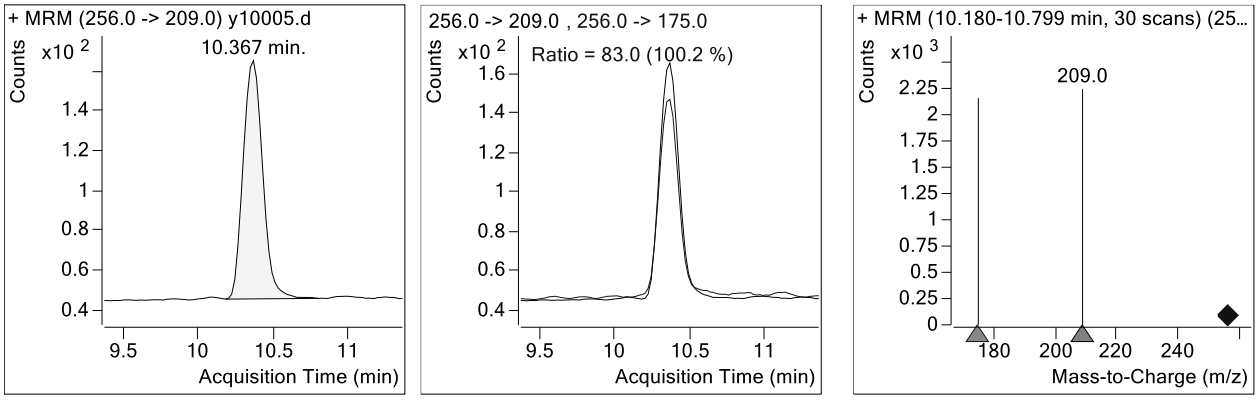


Fig. 3B. MRM chromatograms and LC-MS/MS spectra of product ions - quantifier and qualifier [M + H] + for Imidacloprid for a concentration level of 50 µg/L

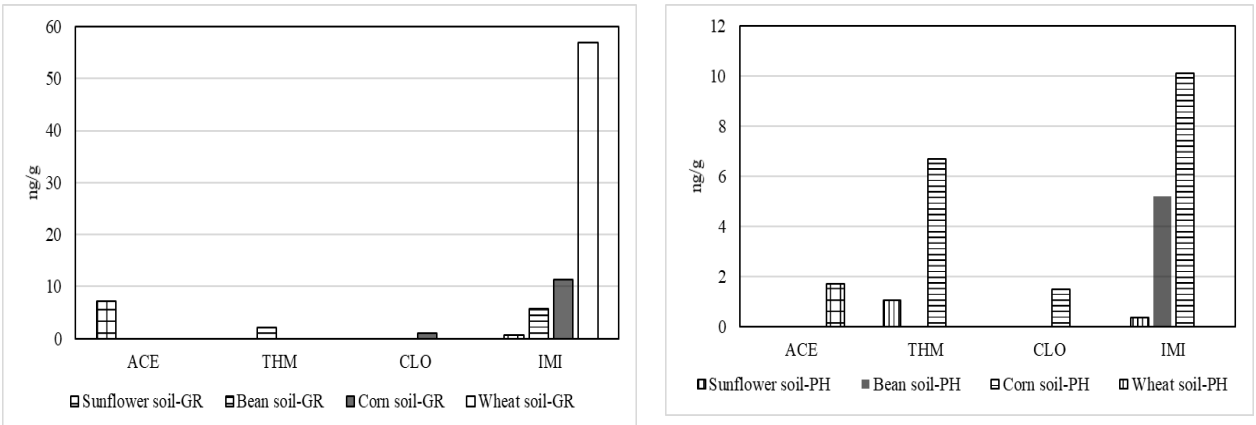
Neonicotinoid occurrence in agricultural soils
The intense use of NN's is controversial due to the lack of understanding on their persistence in the soil and due to adverse effects on microorganisms. Thus, it is necessary to evaluate the NN level and their effects on organisms in soil and aquatic environment. *This is the first report on the*

presence of NN's in the *agricultural soil from Romania*. We initially have developed and validated a method for analyzing these soil compounds. Then, the aim was to evaluate the presence of NN's in soil samples influenced by various types of crops and to highlight a pattern/use profile based on the results obtained. It is known that NN's are used by applying to a crop seed and that the plant takes up only about 5% of the applied insecticide, so that 95% of the compound is found in the environment. It is assumed that some of these pollutants are bio-transformed (87-89%) and another proportion (11-12%) persists in the environment (soil/ water) [8, 12]. NN's can be retained by soil particles depending on various factors. NN's are soluble in water and can move from soil using water molecules. Leaching from soil is reduced and adsorption is high if the soil contains organic matter. Clay soils have a high retention (retention/fixation) of NN's, in the case of humus soil, the retention was maximum, and the residual concentrations of NN's were the highest. The sandy soil allows the rapid transfer of NN's to other environmental components.
Table 4 summarizes the variability of NN concentrations in agricultural soils (detection frequency, average, minimum, maximum).

Table 4. NN concentrations determined in agricultural soil samples sown with wheat crops, corn, sunflower and beans

ng/g	Minimum	Maximum	Detection frequency	Average
Thiamethoxam	1.05	6.7	37.5	3.28 ± 0.21
Clothianidin	1.1	1.5	25	1.3 ± 0.11
Imidacloprid	0.38	56.9	87.5	12.9 ± 1.1
Acetamiprid	1.7	7.2	25	4.45 ± 0.34

In agricultural soils taken from lands (located in Prahova and Giurgiu counties), cultivated with wheat, corn, sunflower and beans, they were quantified only four NN's out of the total of 6: imidacloprid (0.38 ng/g - 56.9 ng/g), acetamiprid (1.7-7.2 ng/g), thiamethoxam (1.05-6.7 ng/g), clothianidin (1.1-1.5 ng/g, figure 4). Imidacloprid was ubiquitous and it was detected in 87.5% of the total samples, which reveals the intense use of this insecticide, followed by the frequency of thiamethoxam (37.5%). The least identified compounds were clothianidin and acetamiprid (25%).

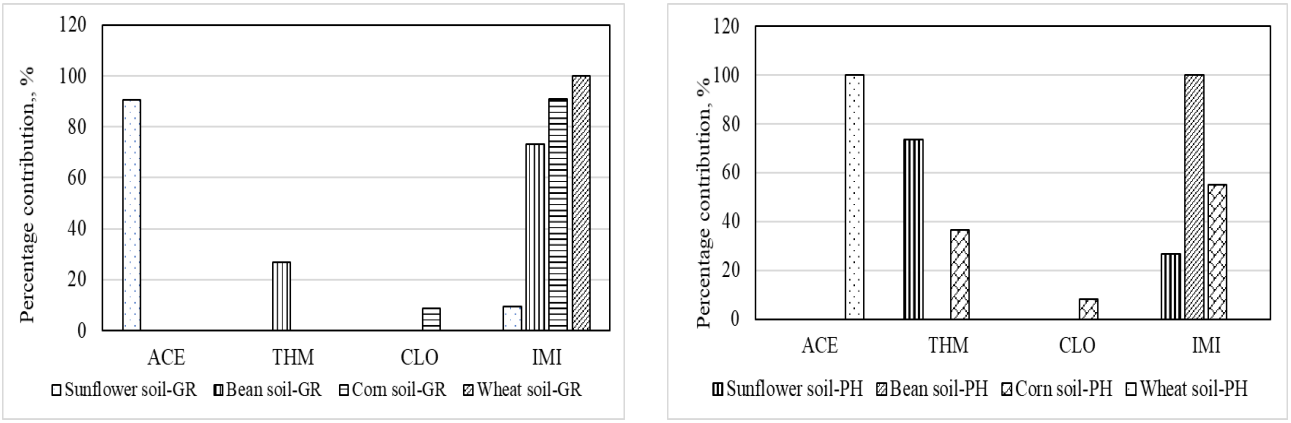


a) b)

Fig. 4. Variability of NN concentrations in the soils taken from Giurgiu (a) and Prahova (b) counties

In the case of imidacloprid, maximum concentrations were detected in Giurgiu County 56.9 ng/g in wheat-cultivated soil, and 11.3 ng/g in maize cultivated soil, and in Prahova County for wheat cultivated soil (10.1 ng/g). For clothianidin, maximum values were obtained only in Prahova County, in the case of wheat crops (10.1 ng/g), beans (5.2 ng/g). Thiamethoxam showed high values in bean crops (6.7 ng/g in Prahova land and 2.1 ng/g in Giurgiu area). Acetamiprid recorded point values in the soil of the sunflower culture of 7.2 ng/g in Giurgiu County and 1.7 ng/g Prahova County.

Thus, acetamiprid predominated in sunflower crop soil, generating a percentage contribution of 90.6% (Giurgiu area), followed by thiamethoxam with a contribution of 73.4% (Prahova area). Imidacloprid had a reduced contribution of 26.6% (Prahova area) at the total NN concentration in the soil (figure 5). In wheat culture, acetamiprid with a contribution of 100% (Prahova area) and imidacloprid with a maximum contribution of 100% in Giurgiu area predominated.
For maize sown soil, the maximum contributions were estimated for Imidacloprid: 73% in the Giurgiu area (GR) and 55.2% in the Prahova area, followed by thiamethoxam: 36.6% in the Prahova area. The low values of NN contributions (below 25%) seem to be due either to their use in previous years (knowing that NN's are persistent in the environment) or to leaks caused by precipitation on neighboring agricultural land that conservatively transported these contaminants.
For the soil sown with the legume plant (Latin) Phaseolus vulgaris, native to the USA, major contributions were found in the case of imidacloprid: 100% in the Prahova area and 73.1% in the Giurgiu area. Thiamethoxam had a reduced contribution of only 26.9% for the soil cultivated with this vegetable.



a) b)

Fig. 5. The percentage contribution of each NN at the total concentration depending on the type of cultures studied from Giurgiu (a) and Prahova (b) counties

The values of neonicotinoid concentrations reveal the pattern of use of these insecticides in agriculture in the two counties of Prahova and Giurgiu, in the south-east area of Romania. These concentrations are likely to adversely affect non-target organisms. The results of the study carried out on agricultural soils cultivated with corn, wheat and sunflower seeds, treated with NN's, generates soil pollution values that represent sources of exposure for soil life forms.

CONCLUSIONS

In the samples of agricultural soil, harvested from the fields cultivated with wheat, corn, sunflower, beans, located in Prahova and Giurgiu counties (Romania), were quantified only 4 NN's out of the total of 6: imidacloprid (0.38 ng/g-56.9 ng/g), acetamiprid (1.7-7.2 ng/g), thiamethoxam (1.05-6.7 ng/g), clothianidin (1.1-1.5 ng/g). Imidacloprid was ubiquitous and was detected in 87.5% of the total samples, which reveals the intense use of this insecticide, followed by thiamethoxam (37.5%). The compounds most frequently identified were clothianidin and acetamiprid (25%). The values of the reduced contributions of NN's (below 25%) seem to be due either to their use in previous years (being known that NN's are persistent in the environment) or to leaks caused by precipitation on neighboring agricultural lands that conservatively transported these contaminants. For the soil of the legume plant Phaseolus vulgaris, major contributions were observed in the case of imidacloprid: 100% in Prahova County and 73.1% in Giurgiu area. Thiamethoxam had a reduced contribution of only 26.9% to the total concentration of neonicotinoids detected in soil samples grown with beans.

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