OTKA zárójelentés 2014-19

Identification of metabolites of biologically active ingredients and characterization of their chemical/ecotoxicological effects

Environmental levels, mobility in soils, decomposition

In the first period of the project we have developed and optimized the analytical procedures for determination of neonicotinoid insecticide active ingredients clothianidin (CLO) and thiamethoxam (TMX) in surface water and in soil using liquid chromatography. We have also determined the limits of detections (LODs) and recoveries of the methods and checked the ingredient contents of coated seeds applied in the experiments (Mörtl et al., 2016).

Based on the results of preliminary sampling we have established that contamination levels in environmental water samples are low (4-30 ng/L and 17-40 ng/L for TMX and CLO, respectively) and the vast majority of samples (92%) contained neonicotinoid insecticide below LOD. Some residues (10–41 ng/mL) were detected in temporary shallow water bodies near to treated crops and among them CLO occurred more frequently than TMX (Székács et al, 2015).

Monitoring of contaminated sampling sites was discontinued as neonicotinoids (CLO and TMX) in our earlier results occurred rarely in environmental samples collected. This is probably due to the decision of the European Commission in 2013 that imposed restrictions for use of pesticides containing CLO and TMX and banned seed treatment for different crops. Monitoring of neonicotinoids in surface water has been later continued (2017) in the frame of an international cooperation. Samples taken from Lake Balaton in May contained only CLO in a single case at level of 15.4 ng/L. In Danube water weekly sampled at Budapest only CLO was detected initially (May), but samples contained both TMX and CLO in June. Levels ranged between 4.7 and 16.8ng/L for TMX, and 3.5 and 11.4 ng/L for CLO. Similar levels were observed in Danube at Novi Sad and Beograd for CLO and TMX residues.

Movement of ingredients was studied by determination of elution profiles in different soil columns under laboratory conditions. Adsorption characteristics of soil columns and retention of these water soluble ingredients are strongly influenced by clay and/or organic matter content of soils. Rapid leaching was observed for sand characterized by low binding capacity, while loam with high organic content retained the insecticides (Mörtl et al 2016).

Decomposition rates of TMX were determined under laboratory and field conditions as well. The ratios of CLO metabolite compared to both compounds seem to be independent from the circumstances and become constant after two or three weeks after plantation.

Uptake of ingredients

Uptake of ingredients in plants was investigated by determination of insecticide levels in guttation liquids and because of its high guttation frequency maize was chosen as a model species in the present work. Regarding the amounts of drops and the levels in guttation liquids the age of plantation is substantial. Among the micrometeorological parameters the effect of relative moisture is prevalent. However, mild air movement facilitated evaporation of drops and concentrated the ingredients resulting extreme values. On the other hand uptake of ingredients and their appearance in guttation liquid is influenced by soil type as well. We have observed that ingredients could be detected for two month in guttation liquids of maize plants emerged from coated seeds. Drops collected at leaf edges contained significantly higher amounts of insecticides as those of in crown cups. Short distances between the potted seeds resulted in slightly higher concentrations in guttation liquids compared to usual planting mode. CLO could be detected even in drops collected at leafs of plants emerged from TMX coated seeds as it is a well-known metabolite of TMX. Due to metabolic transformation the ratio of CLO increases to approximately 20-30 percent of TMX.

Application mode and soil type also influence the levels in the guttation drops (Mörtl et al 2017). Non-coated seeds were potted and surface of soils were sprayed with a solution containing both CLO and TMX. Ingredients appeared in guttation liquids of maize plants after 7, 15 and 24 days for sand, clay and loam, respectively. Highest values measured for CLO and TMX were 1.63 µg/mL and 1.82 µg/mL, respectively. The concentrations determined were significantly lower than those obtained for plants emerged from coated seeds, when the peak values exceeded the level of 100 µg/mL. We have also assessed the doses in different applications (seed coating, spray and granule), and concluded that there is no significant difference in recommended application rates and similar amounts are applied in all application modes. However, seed coating results in higher levels of neonicotinoids in guttation liquid compared to spray application.

The apparent levels in guttation liquid were found to be are affected, among others, by the amount of the insectide in the coating material. When coating contained only about 5% of the recommended rate of thiacloprid (TCL), levels in guttation drops were about one order of magnitude lower (peak concentration 14.7 µg/ml) than if the recommended dose was applied. The effect of soil type, studied earlier, showed that higher organic material or clay content results in lower ingredient uptake.

Spread of insecticides and cross-contamination between closely potted coated and/or non-coated seeds were studied (Mörtl et al., 2017). As expected, neonicotinoids were also uptaken by plants emerged from non-coated seeds via soil and excreted to guttation drops. Insecticide (CLO or TMX) concentrations taken up by plant from non-coated seeds and detected in the guttation liquids during the sampling period were over 40% compared to plants from coated seeds. Cross-contamination between differently coated seeds (CLO or TMX) was also studied in different soil types (sandy, clay and loam) and in pumice (soilless growing medium). Based on the results cross-contamination rates and appearance of insectides in guttation liquid have been shown to be influenced by organic matter and clay content. These components retain the ingredients, and their uptake was limited.

Cross-contamination via soil and appearance of CLO and TMX in the guttation liquid of weeds emerging in the proximity of neonicotinoid coated maize seeds was also studied (Mörtl et al., 2019a). Two common weeds were investigated: a well-known, aggressive perennial maize weed, the creeping thistle (Cirsium arvense) and a common annual weed, red poppy or Flanders poppy (Papaver rhoeas) as model species for laboratory and field studies. The results confirmed that cross-contamination may occur by uptake through soil from neighbouring maize plants that emerged from coated seeds. TMX and CLO residues were detectable in the guttation liquid of the weeds for up to 20 to 35 days after planting of maize seeds, although levels were substantially lower than those in maize emerged from coated seeds. Peak concentrations of TMX and CLO in the guttation liquid of maize were 160 and 70 µg/mL, while both appeared in creeping thistle and red poppy at 21 and 0.7 µg/mL, respectively. The significantly lower levels detected in red poppy are explained by its higher guttation intensity.

We have investigated the effect of coformulants to decomposition of MOSPILAN 20 SG in different water matrices, in distilled water and in surface water samples originated from River Danube under laboratory conditions (Mörtl et al., 2019b). Analytical determination of surfactant (linear alkylbenzene sulfonates, LAS) were performed both by HPLC-UV and commercial ELISA methods. These methods were optimized and validated as well. Losses of LASs in a neat surfactant mixture, in an insecticide formulation MOSPILAN 20 SG, and in solutions with different neonicotinoid active ingredients (AIs) were monitored. Dissipation rates of LASs were found different in these aqueous matrices, with decomposition rates higher for the neat surfactant mixture than for MOSPILAN 20 SG (nearly 2- and 9-fold in distilled water and in surface water from River Danube, respectively). Half-lives determined in surface water from River Danube were shown to be affected by the presence of neonicotinoid AIs TCL > imidacloprid (IMI) > acetamiprid (ACE), while CLO and TMX did not occur to affect LAS decomposition.

This summer (2019) we have performed experiments to study the effect of corn species to the guttation liquid levels. Seeds were set in a capsule containing equal amounts of TCL and plants were grown in a greenhouse. Four types of corn species have been studied (3 landrace, 3 exotic, 2 cultivated and 2 GM) maize seeds were used. The amounts of excreted guttation liquids were similar, but there were significant differences, observed between levels and the amounts of excreted TCL by a plant.

Toxic effects

The *Daphnia magna* immobilization tests are performed on the basis of the standard OECD 202 Test Guideline. The toxicity of neonicotinoids tested is considered negligible because toxic effects in this aquatic system are only caused by high concentrations, and probably not found often in the environment. Toxic effects of pesticide AIs and formulated pesticides were compared on *D. magna* test organism for three ingredients (TMX, CLO and TCL). Apache containing CLO was more toxic than the corresponding ingredient. The surfactant additive (a mixture of sulfonates) was characterized and measured by LC-MS (Takács et al., 2017).

Aquatic toxicity of MOSPILAN 20 SG, along with that of its AI ACE and co-formulant LAS, as well as the mixture of ACE and LAS was also investigated in the 48-hr acute immobilization assay on the water flea (*D. magna*) aquatic indicator organism. LAS appeared to be significantly (8-fold) more toxic in the *D. magna* test than ACE, and the toxicity of the formulated insecticide was found to be 1.3 and 19.6 times higher than explained by its AI and LAS content, respectively, indicating synergistic toxicity. The strongest synergy between ACE and LASs was observed, when the neat forms of the two substances were applied in combination at concentrations equivalent to those in MOSPILAN 20 SG. The results contribute to a complex assessment of the time dependent dissipation pattern of LASs in surface water (Mörtl 2019b).

APACS WG formulated pesticide and its pesticide AI CLO were subjected to a standardized ecotoxicity (Fish Embryo Acute Toxicity, FET) test on *Danio rerio* embryos according to OECD Test Guideline 236. Preliminary results indicated that there is significant difference in the toxicity of formulated pesticide and its ingredient CLO. In contrast to APACS WG when application level of 25 mg/L caused 100% mortality, indications of lethality were seldom observed for CLO even at the highest concentration (100 mg/L) applied in acute toxicity test. Some mortality could be recorded for CLO at higher concentrations (120 mg/L and 230 mg/L, near to water solubility), but further experiments are required determine the exact value of LC_{50}. Results of experiments with APACS WG in the range of 0.5 mg/L and 5 mg/L indicated that LD_{50} value is expected between 1 mg/L and 3 mg/L.

Toxicity of MOSPILAN 20 SG and its components were also determined in FET test. For the active ingredient ACE only preliminary assay was performed at 200 mg/l concentration, since lethal and sublethal effects at this concentration were not detected. Higher concentration is not relevant in the environment because of the water solubility of ACE. The highest toxicity was determined for LAS additive ingredient, 50% mortality was detected at 5.13±0.21 mg/L. Results showed that the test organism is more sensitive to LAS than the active ingredient. Combined toxic effect of ACE and LAS was also investigated where 46.08±5.21 mg/L and 5.21±0.15 mg/L LC_{50} values were determined, respectively. Results show that toxicity of ACE increased in the presence of the surfactant. LC_{50} value of the insecticide formulation was 133.14±2.5 mg/L, which value corrected for active ingredient and LAS content (20.2% and 2.4%, respectively) of the formulation is 26.89±0.51 mg/L for ACE and 3.2±0.06 mg/L for LAS. This correction shows that both known compounds are more toxic in formulation than in their pure form. This effect is also influenced by other not known ingredients in the formulation. Presence of the chorion as a barrier between the embryo and the environment is crucial in toxic effects of the compounds. Embryos in eggs were protected from harmful components in the medium, however after hatching all juveniles were coagulated at higher concentrations. For MOSPILAN 20 SG and LAS sublethal effects, like oedema, decarease in heart beat and amorphous vertebrate shape were observed in some cases.

As testing the functional consequence of the cholinergic modulation by neonicotinoid insecticides the identified cholinergic synapses between the VD4 and RPeD1 neurons of the pond snail *Lymnaea stagnalis* were recorded in the presence of neonicotinoids In the concentration range used (0.01-1 mg/mL) all the chemicals acted displayed antagonist activity, inhibiting the cholinergic excitatory components of the VD4-RPeD1 connection. TCL (0.01 mg/mL) blocked almost 90 percent of excitatory postsynaptic potentials (EPSPs), while the less effective TMX (0.1 mg/mL) reduced the synaptic responses by about 15 percent. The acetylcholine (ACh)-evoked membrane responses of RPeD1 neuron were similarly inhibited by the neonicotinoids, confirming the involvement of the same target, the ACh receptors (AChRs). Moreover, the inhibitory effects on the EPSPs were often stronger, longer lasting and less reversible, suggesting that synaptic and extrasynaptic AChRs may also be different in their availability and sensitivity to bath applied chemicals. We concluded that neonicotinoids act on nicotinergic acetylcholine receptors (nAChRs) in the snail CNS. This has been established previously in the insect CNS; however, our data indicate differences in the background mechanism or the nAChR binding site in the snail. We provided the first results concerning neonicotinoid-related toxic effects on the neuronal connections in the molluscan nervous system. Aquatic animals, including molluscs, are at direct risk while facing contaminated surface waters, and snails may provide a suitable model for further studies of the behavioral/neuronal consequences of intoxication by neonicotinoids (Vehovszky et al 2015).

To assess the exposure of bees to neonicotinoid containing guttation liquids, an evaluation of AChE activity was suggested as an *in vivo* marker for monitoring the toxic effects associated with insecticides. To test the possible direct involvement of neonicotinoids on AChE activity we applied *in vitro* experiments using purified eel AChE to determine whether or not ACTM, and TMX are effective blockers of the AChE enzyme. The tested neonicotinoids were not a uniform group regarding their blocking ability. We found that ACTM exhibited much higher potency of AChE inhibition than TMX, probably due to its cyano-substituted structure. Our results suggest a previously not established, direct AChE blocking mechanism of neonicotinoids tested, thus the neuronal AChE enzyme is likely among the direct targets of the neonicotinoid insecticides. We conclude, that these AChE inhibitory effects may also contribute to toxic effects on the whole exposed animal (Győri et al. 2017).

We examined the interspecies variability of various biomarkers of defence (metallothioneins (MT), (EROD), biomarkers of damage (lipid peroxidation (LPO) and DNA strand breaks (DNA_sb) and reproduction (vitellogenin-like proteins (Vtg) in *Dreissena bugensis* inhabiting either pristine- or moderately impacted sites of Lake Balaton. Each biomarker considered was elevated by fall, with significantly higher values in the mussels inhabiting harbours, underlining the reliability of these stress markers to highlight even low- to moderate anthropogenic pressure. The implemented biochemical assessment techniques and the gained knowledge on the natural variability of selected biomarkers will serve as basis for future monitoring programmes aimed at detect pollution events by agrochemicals in in situ circumstances. Results were published in Science of the Total Environment (Farkas et al, 2017). We also tested the isolated gill tissue model of the mussels *Dreissena bugensis* to study the activity of the MXR system in the presence of neonicotinoid insecticides. We used the formulated products commercially available in Hungary namely IMI (Kohinor, Makteshim Agan), acetamiprid (Mospilan, Sumi Agro), TMX (Actara, Syngenta), CLO (Apacs, Arysta Life Science) and TCL (Calypso, Bayer). The accurate concentrations of the active ingredients in each neonicotinoid product were confirmed by GC/MS chromatography. Our results showed that sublethal (50-100 mg/L) concentrations of Actara, Calypso and Kohinoor enhanced the accumulation of (0.5 μM) concentration of rhodamine B (used as model subtrate of the MXR) in the gill tissue. These results demonstrated direct inhibitory effects of the MXR system similarly to (20 μM) verapamil used as model inhibitor for reference. Testing Mospilan (Sumi Agro, containing TMX) by the same tests, however, did not change rhodamine B accumulation. Neonicotinoid type insecticides are generally regarded as selective neurotoxins for insects, our presented results suggest, however, that neonicotinoid containing insecticides may also act on non-target animals, potentially inhibiting the MXR system as chemosensitizers (Vehovszky et al, 2018). The cellular effect of neonicotinoids were started to investigate in the model nervous system. The involvement of a class of ser/tre protein kinases, the arginine-directed kinases (AGC-kinases), and p42/44 MAPK which play central role in the regulation of different cell physiological processes were tested preliminary. Exposition of the animals to 10 μg/mL Mospilan (ACTM) for three hours elevated the phosphorylation level of a 50 kDa protein by AGC-family kinases in the brain of 5 out of 6 animals compared to control. However, the difference of the density of this phosphorylated protein was too high between the individuals of the treated group which yielded a relatively high error of the result. 24 h treatment did not show any differences in the phosphorylation pattern of protein targets of AGC-kinases. A slight but still uncertain elevation of activated (phosphorylated) MAPK was detected after 24 of exposition.

Project management, publication activities

The OTKA project was extended by 1 year.

The OTKA Project Office was informed about slight modifications of the staff at NARIC. Two PhD Students leaved the research team, but also one new PhD Student (Szandra Klátyik) was involved.

Costs were used according to the preliminary plan, with the following modifications. Team in the NARIC did not use the whole conference cost planned. The budget remained was transferred for purchasing consumables (chemicals, plates, cartridges, etc.). Team in the BLI did not use all the daily allowance, the budget remained was transferred for extended salary of our qualified technician.

The OTKA Project Office was informed about to buy a TissueLyser instead of upgrading of the Wallac 1420 (Perkin-Elmer) microplate reader (BLI)

Results were reported in leading journals (5 publications in D1 journal), two and three articles were published *Aquatic Toxicology* and *Science of the Total Environment*, respectively. Additional two manuscripts were published in *International Journal of Environmental Analytical Chemistry* (Q2) after the conference in Santiago de Compostela, two papers in *Journal of Chemistry* (Q3, open access) and one in *Acta Biologica Hungarica* (Q3). The cumulative impact factor (IF) of published papers is 29.20 (according to ISI Journal Citation Reports & Ranking). Results were also presented in international and national conferences as well.

After detailed statistical evaluation of the outcome of the field study on neonicotinoid occurrence in corn guttation liquid obtained this summer (see above), the results will be summarized in a new manuscript, also in a prestigious scientific periodical. In addition, a review, completed with so far unpublished results of the research project have been presented in a paper submitted to *International Journal of Environmental Research and Public Health* (Open Access, IF 2.468). The **s**tudy involves new results, including levels measured in River Danube and Lake Balaton, discussion of the measured seed coating rates, unpublished results of some field experiments as well as neurotoxic experiments. Physiological results suggested behavioural effects, modulated locomotion and feeding activity of the pond snail *Lymnaea stagnalis* in the presence of formulated neonicotinoids. Moreover, electrophysiological results on the isolated CNS of *Helix pomatia* also suggests an additional, previously not described effect on the neuronal level. Dose-effect studies revealed a biphasic modulation of the ACh responses suggesting the possible contribution of AChE ezyme inhibition in the lower concentration neonicotinoids, and an AChR inhibition in higher concentrations. The IF of these latter two publications will further raise the cumulative IF value achieved by the project.

Published Papers (2015-2019)

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Vehovszky, A., Farkas, A., Acs, A., Stoliar, O., Szekacs, A., Mortl, M., Gyori, J., 2015. Neonicotinoid insecticides inhibit cholinergic neurotransmission in a molluscan (Lymnaea stagnalis) nervous system. Aquatic Toxicology 167, 172-179.

Mortl, M., Kereki, O., Darvas, B., Klatyik, S., Vehovszky, A., Gyori, J., Szekacs, A., 2016. Study on Soil Mobility of Two Neonicotinoid Insecticides. Journal of Chemistry -NY. Article ID 4546584, 9 pages

Farkas, A., Acs, A., Vehovszky, A., Falfusynska, H., Stoliar, O., Specziar, A., Gyori, J., 2017. Interspecies comparison of selected pollution biomarkers in dreissenid spp. inhabiting pristine and moderately polluted sites. Science of the Total Environment 599-600, 760-770.

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Mortl, M., Darvas, B., Vehovszky, A., Gyori, J., Szekacs, A., 2017. Occurrence of neonicotinoids in guttation liquid of maize - soil mobility and cross-contamination. International Journal of Environment and Chemistry 97, 868-884.

Takács, E., Klatyik, S., Mortl, M., Racz, G., Kovács, K., Darvas, B., Szekacs, A., 2017. Effects of neonicotinoid insecticide formulations and their components on Daphnia magna - the role of active ingredients and co-formulants. International Journal of Environment and Chemistry 97, 885-900.

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Mortl, M., Takacs, E., Klatyik, S., Szekacs, A., 2019b. Aquatic toxicity and loss of linear alkylbenzenesulfonates alone and in a neonicotinoid insecticide formulation in surface water. Science of the Total Environment 652, 780-787.

Mörtl M, Vehovszky Á, Klatyik Sz., Takács E, Győri J, Szekacs A, Neonicotinoids: spreading, translocation and aquatic toxicity (submitted) of *International Journal of Environmental Research and Public Health* (Open Access**, IF 2.468**).