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# MONITORING AND BIOLOGICAL EVALUATION OF SURFACE WATER AND SOIL MICROPOLLUTANTS IN HUNGARY

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**Abstract** In the development of a complex soil contamination monitoring system including the detection of agriculture-related micropollutants, heavy metal contamination and ecotoxicity, a survey has been carried out in Békés county and at certain water catchment areas in Hungary, using different techniques for the characterisation of soil and surface water status. Besides the representativity optimisation of the sampling technique, instrumental analysis, biological tests (soil biology and aquatic toxicity) were also applied, and results obtained were presented in a spatial informatics system. The target analyte group, indicators and methodology is in compliance with recommendations of the European Environment Agency monitoring working group. Contaminant concentrations of soil profiles have been characterised down to the ground water table. Pesticide residues were monitored by using gas chromatography coupled with mass spectrometry and enzyme-linked immunosorbent assay. Target analytes included triazine, phenoxyacetic acid, acetanilide, dinitroaniline and phosphonomethylglycine type herbicides, chlorinated hydrocarbons (CHCs), organophosphate and carbamate insecticides, an insect hormonal agonist and a triazole fungicide. Besides banned persistent CHC insecticides (DDT, HCH, etc.), atrazine and acetochlor herbicides are common contaminants in Hungary, reaching 200 ng/g and 300 ng/mL concentration in the soil and surface water samples studied, and trifluralin, glyphosate and metolachlor were also detected in some cases. Heavy metal and other microelement contamination was detected by inductively coupled plasma atomic emission spectroscopy, and within-plot heterogeneities were studied throughout soil profiles. Nickel has been found as a relatively common contaminant in arable lands in the area, however, relation of the contamination pattern to fertiliser usage in the region could not be confirmed. Total microbiological activity was analysed by using fluoresce in diacetate (FDA) hydrolysis. The results of this measurement did not show correlation with heavy metal content or with land use types. Toxic effects of water and soil samples were determined on *Daphnia magna* Straus (Cladocera, Crustacea) according to the ISO 6341:1996 standard. The vast majority of the samples exerted no observable toxicity on this bioindicator organism. Overall toxicity often occurred not asthe sum of the reported toxicity of the individual contaminants found: cases of antagonistic and synergistic effects in toxicity were both observed.

**Keywords:** Monitoring; Pesticides; Microelements; Soil; Surface water; Ground water; Soil microbial life; Aquatic toxicity

## 1. BACKGROUND, AIM AND SCOPE

The European Environment Agency (EEA) estimated that potentially polluting activities have occurred at over three million sites in EEA member countries, and national estimates show that more than 8% (or nearly 250000 sites) are contaminated and need to be remediated (European Environment Agency, 2007). According to projections based on the analysis of current changes, the total number of contaminated sites needing remediation may increase by more than 50% by 2025. Heavy metals, mineral oil, simple and polycyclic aromatic hydrocarbons, phenols, as well as chlorinated

hydrocarbon type and other pesticides have been identified as main soil contaminants in Europe (Rodrigues et al., 2009). Inorganic anions, various hydrocarbons, heavy metals and pesticides are reported as the most relevant contaminants for groundwater. Detrimental effects of agricultural practices, including agrochemicals, on soil quality also include persistent pollution, salinisation, subsequent fertility loss or desertification (Lal & Stewart 1990).

Sustainability of agricultural technologies, including intensive agriculture, integrated pest management and ecological farming, is focused on soil quality, the relations between its use and management, and the environment (Larson & Pierce 1994, Anton et al., 2014). Corresponding impacts on water resources include drift-off and leaching into natural aquifers, causing permanently reoccurring surface water pollution. The most important motives for the application of risk reduction measures have been to protect groundwater resources and to avoid human exposure to contaminants *via* drinking water from ground sources. The more severe consideration of water pollution than soil contamination is may be, in part, due to broader dispersion of contaminants in groundwater compared to soil. Due to their impact on biodiversity, it has been emphasized for over a decade now that pesticide inputs should be reduced to a minimum (McLaughlin & Mineau 1995).

Impact assessment on the soil and groundwater contamination requires a detailed knowledge of the local situation in each site (e.g. type and amount of contaminants, hydro-geological conditions, land use, receptors, etc.) and therefore cannot be generalised. It has to be considered at local level: the nature, level and complexity (composition) of the contamination has to be measured, and the extent (both by area and by depth) has to be determined.

To provide sustainable prevention of such contamination or solutions to such problems a detailed and accurate soil quality monitoring system is required, with special emphasis on soil characteristics, functional evaluation and agricultural or industrial activities exerted. The Hungarian Soil Information Monitoring System (TIM) defined within the national Environmental Information Monitoring System created in 1991 (Hungarian Central Agricultural Office 2007) covers the entire country providing an opportunity to create a detailed and objective national information system for soil natural resources, and serves as the basis of up-to-date environmental and agricultural management. The territorial measuring grid of the system consists of 1236 measuring points designated by environmental and biogeographical considerations. These points are representatives of all varieties of soil types of throughout the country, and soil quality in agricultural utilisation is systematically followed and reported, mainly on the basis of physicochemical analyses. Problems identified in the system are further specified based on regional and local subsystems. In addition, merger of this system with databases on other environmental resources (the atmosphere, water supplies, vegetation, biological resources, etc.), existing or in development, has been projected.

The objective of the research that initiated our survey was to develop a soil contamination monitoring subsystem capable of complex survey of typical soil contaminants in a south-eastern county (Békés county) of Hungary, using up-to-date sampling and measurement techniques, and therefore, eliminating environmental protection and environmental analytical limitations due to the agricultural focus of the presently functioning Soil Information Monitoring System. Additional important objectives of the projects include: (i) optimisation of the sampling technique to reveal the contamination source, (ii) the development of *in situ* sensoric methods to identify volatile contaminants; (iii) the expansion of the range of investigation beyond analytical determination to biological tests (soil biology, ecotoxicity and mutagenicity), with particular emphasis on possible interactions among pesticide active ingredients and other substances (e.g. surfactants, accompanying contaminants) in order to provide risk based soil and ground water contaminant identification; and (iv) to interpret results obtained in a selected sampling region in a spatial informatics system in order to obtain a measurement protocol flexibly adaptable to ecological indications.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals

Chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) and Sigma Chemical Co. (St. Louis, MO), unless otherwise stated. Analytical standards of the target analyte pesticides were provided by the Hungarian Central Agricultural Office, Plant Protection, Soil Conservation and Agri-environment Directorate, from official standard reference materials received from the manufacturers/distributors of acetochlor, atrazine (Nitrokémia Rt., Fűzfőgyártelep, Hungary), carbofuran (Agro-Chemie Kft., Budapest, Hungary), diazinon, fenoxycarb, prometryn (Syngenta Kft.,

Budapest, Hungary), metribuzin (Bayer HungáriaKft., Budapest, Hungary), phorate (BASF HungáriaKft., Budapest, Hungary), terbutryn (Agrosol Bt., Gödöllő, Hungary) and trifluralin (BudapestiVegyiművek Rt., Budapest, Hungary). Solvents purchased from Merck KGaA (Darmstadt, Germany) were of analytical grade. CarboPrep-90 (500 mg, 6 mL) and Carbograph (200 mg, 6 mL) columns were purchased from Restek (Bellefonte, PA, USA) and Alltech Associates, Inc. (Deerfield, IL, USA), respectively. HPLC grade distilled water was prepared on a MilliQ RG ion-exchanger from Millipore (Bedford, MA, USA). MN (Macherey-Nagel) 640W filter paper was obtained from Reanal Rt. (Budapest, Hungary).

### 2.2 Sampling and sample extraction

#### *2.2.1 Sample collection*

In the scope of a national monitoring program, 423 soil samples and 202 surface and ground water samples were collected between 2008 and 2013, in uneven annual distribution,from agricultural fields and industrial sites. The geographic location of the sampling sites is depicted on figure 1.

Contamination in arable lands and industrial areas has been investigated on 13 plots in 5 replicates. Among agricultural areas, three types of land usage have been involved: arable lands under intensive cultivation, organic farming and pasture. The study area in the case of contamination of agricultural origin covered 4 settlements in Békéscounty (Köröstarcsa, Medgyesegyháza, Csorvás, Battonya). Both intensive and organic parcels were chosen in all 4 settlements (4 organic and 4 intensive), the pasture was designated in Csorvás. Contamination of industrial origin was examined in 3 settlements (Orosháza, Gyomaendrőd, Békéscsaba), at 5 sites (Orosháza – Linamar, Public Road Manager Corp., Glass Factory; Gyomaendrőd – Nagylapos; Békéscsaba – Sludge Desposition Site). Spatial setting of sampling accuracy was supported by a global positioning system.

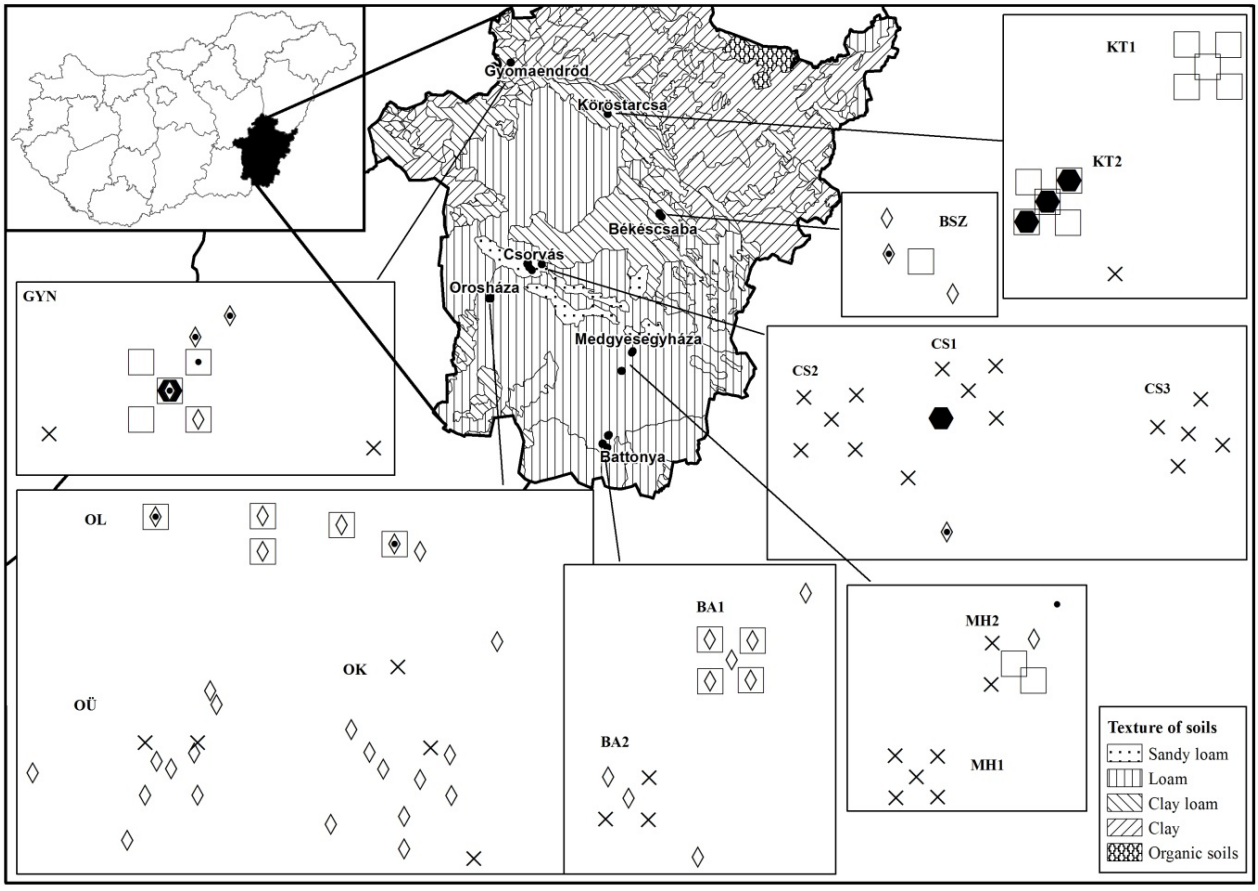


Figure 1. Spatial distribution of sampling and contaminated sites in Békés county, Hungary with the 14 sampling sites. Identified site contamination may indicate pollution by several organic micropollutants (pesticides, total petroleum hydrocarbons, etc) and toxic microelements. Contamination is indicated if contaminant concentration exceeds the threshold (0.1 ng/mL for given pesticides or 0.5 ng/mL for total pesticides (European Parliament and Council 2006), "B" limit value for microelements (Hungarian Ministries 2009) defined in the corresponding effective legal regulations).

Soil sampling was carried out according national standard MSZ 21470-1:1998 (Hungarian Standards Institution 1998) during the April-May period by using a motorised Eijkelkamp soil drilling equipment. Contaminant concentrations of soil profiles from topsoil to subsoil were characterised down to ground water table, creating one sample in every 30 or 50 cm. Parcels of diffuse agricultural load were further narrowed to define a 5 ha Representative Parcel Part (RPP), preferably as a quadrat. RPP was designed on the representative, homogeneous part of the parcel. This was carried out to characterise the nutrient content of the surface soil layer. In our study, a sampling allocation in regular design was used for the mechanical drillings, thus a parcel of 50x50 m territory was designated in one corner of the RPP. The soil samples were taken from drillings in the corners and in the centre point of this part of the RPP, in five replications each.

Water sampling was carried out according to national standard MSZ ISO 5667 (Hungarian Standards Institution 1995), twice a year, before and after agricultural pesticide applications, during the months of April-May and June-September. Surface water samples (from depths not exceeding 50 cm) were collected by immersion of a sampling vessel, while groundwater samples were taken from the soil drillings or from already existing groundwater monitoring wells. Both kinds of water samples were transferred into clean, 2.5 litre volume dark glass bottles sealed with a watertight screw-cap insulated with teflon lining, and were transported in cool boxes to the laboratory.

### *2.2.2 Sample preparation*

To provide appropriate sample preparation for gas chromatography – mass spectrometry (GC-MS) determinations, solvent extraction and solid phase extraction (SPE) methods were applied. Soil samples were air-dried, ground on a Retsch GM 200 cutting mill (Retsch GmbH, Haan, Germany), and subjected to solvent extraction. Thus, 10 g dried soil was extracted with 15 mL of hexane/acetone (1:1) and centrifuged at 4000 rpm. Finally, 10 mL of the supernatant was evaporated and resuspended in 1 mL of ethyl acetate (Cairns & Sherma 1992, Oldal et al., 2006). Water samples were filtered in a suction filtration apparatus using MN 640W filter paper to remove floating particles, stirred for 1 min, left to settle for 10 min, and then subjected to SPE (Majzik-Solymos et al., 2001, Maloschik et al., 2007) using graphitized carbon based SPE cartridges. SPE columns (CarboPrep-90, 500 mg, 6 mL) were conditioned, applying low eluent flow velocity, with 5 mL of dichloromethane/methanol (8:2), 2 mL of methanol, and 10 mL of distilled water containing 10 mg/mL ascorbic acid. After the conditioning step, 1000 mL of the water sample was passed through the column at a flow rate of 10-15 mL/min. The column was rinsed with 7 mL of distilled water, air-dried for 10 min with suction by vacuum, washed with 1 mL of methanol/distilled water (1:1), and air-dried again. Neutral and alkaline components absorbed into the column were eluted, at a low eluent flow velocity, with 1 mL of methanol and 1 mL of dichloromethane/methanol (8:2). Combined eluates were concentrated to 0.1 mL under nitrogen gas flow. Then 2 mL of isooctane was added to the extract, and the solution was evaporated to a final volume of 1 mL. Extract samples were applied for measurement with GC-MS.

In order to evaluate the SPE/GC-MS process, water samples were spiked with standards of the target compounds at concentrations between 0.001 and 25 ng/mL, and subjected to the above SPE protocol and to instrumental analysis. Analytical standards of the active ingredients were added to HPLC grade distilled water (MilliQ) in methanol stock solutions, except for phorate, where stock solution was prepared in acetone. Spike levels included 2- and 5-fold values of the limit of detection (LOD), except for fenoxycarb. Five parallel detections were carried out at these levels for each active ingredient.

For sample preparation for analysis by inductively coupled plasma atomic emission spectroscopy (ICP-AES) dried soil samples were ground and homogenised to achieve appropriate texture for the spectroscopic measurement. For the analysis of nitrohydrochloric acid (aqua regia) soluble ("total") microelement concentration (Hans et el., 1992, Hungarian Standards Institution 2006), 4.5 mL of concentrated hydrochloric acid (37% m/m), 1.5 mL of concentrated nitric acid (65% m/m) and 1 mL of hydrogen peroxide was added to 1 g of soil. Microwave digestion was carried out in a MLS-1200 Mega Lab station (Milestone Inc., Shelton, CT, USA). After digestion, the solution was cooled and filled up to 50 mL with bidistilled water. As the stock solution was completed, it was ready for the analysis. The calibration curves were based on four different sets of standard solutions, each containing 4 different concentrations. In the case of As, Hg, Se, Mo, Cr, Cd, Co, Ni and Sn, the concentrations ranged from zero to 50 nominal ppm, for Zn, Cu and Pb it was from zero to 100 nominal ppm. In these two cases, standards also contained cc. $HNO_3$, and Fe and Al as ballast, both in different quantities, according to the concentration level of the solution. As regards to Ba and B, nominal ppm of the

calibration solutions extended to 600 nominal ppm for Ba, while for B, this data was 60 nominal ppm. For these two elements, concentrated nitric acid and Ca 3% (nominal) was also added to the standards.

### 2.3 Instrumental analysis

#### *2.3.1 GC-MS*

GC-MS analyses were carried out on a Saturn 2000 workstation (Varian Inc., Walnut Creek, CA, USA), consisting of a Chrompack CP 3800 gas chromatograph and a Saturn 2000R ion-trap detector. The gas chromatograph was equipped with a Varian 1079 split/splitless injector and a CP 8200 autosampler. GC-MS determinations were carried out using electron impact (EI) or chemical ionization (CI) ion sources, detecting total ion count (TIC) in full scan mode or selected ion(s) in selective ion monitoring (SIM) mode. A capillary column CP-Sil 8 CB filled with 5% diphenyl polysiloxane and 95% dimethyl polysiloxane (30 m, 0.25 mm, film thickness 0.25 μm) (Chrompack, Middelburg, the Netherlands) was used. The carrier gas was helium 5.0 at a flow rate of 1.0 mL/min. The mode of injection was splitless (0-1.5 min), then the split ratio set to 50. Both isothermal injection (ITI) and temperature-programmed injection (TPI) were applied. During ITI, the injection temperature was set to 230°C. The injection volume was 1 μl. The corresponding column temperature, following an initial period of 120°C for 1 min, was increased to 270°C at 10°C/min, and kept at 270°C for 14 min. During TPI, the injection temperature was 60°C for 0.50 min, raised to 260°C at 200°C/min rate, held for 5 min, raised further to 60°C at 200°C/min rate, held for 20.00 min. The injection volume was 5 μl. Solvent venting was not applied. The corresponding column temperature, following an initial period of 70°C for 0.5 min, was increased to 100°C at 60°C/min, further increased to 240°C at 10°C/min and kept finally at 240°C for 20 min. The transfer line temperature was 270°C. The mass spectrometer was operated in electron impact (EI) or chemical ionization (CI) mode using methanol as reagent gas with CI storage level of 19.0 m/z. The temperature of the ion trap was 150°C. The maximum ionization time was 2000 μs, the maximum reaction time 40 ms, the ionization level 25 u, the reaction level 40 amu, reagent reaction time 9000 μs, scan time 0.60 s/scan, between 45 and 400 amu in full-scan mode. Chlorophenoxyacetic acid type herbicides (2.4-D, dichloprop, MCPA, etc.) were determined upon derivatization with trimethylsilyl *N*,*N*-dimethyl carbamate and t-butyldimethylsilyl *N*,*N*-dimethyl carbamate (Maloschik et al., 2010).

#### *2.3.2 ICP-AES*

ICP-AES measurements were carried out on a ULTIMA 2 ICP optical emission spectrometer (HORIBA JobinYvon, Longjumeau, France) equipped with a Meinhart cyclonic spray chamber nebulizer with a demountable quartz torch; operating with high purity (99.999%) argon gas, at plasma and nebuliser gas flow rates of 12.0 and 0.70 L/min, respectively, under 300 kPa nebulizer pressure, sample uptake rate of 1.0 mL/min with 30 sec sample uptake delay. The temperature of the plasma was about 10000 K. The analysis was helped by an autosampler, with automata sample switch. Injection of the samples was carried out by continuous pumping, at a volume of 1 mL/min. Measurement time was approx. 20 sec/element. Reference wavelength for the AES was Ar, and the different wavelengths for analysis were chosen from the lowest to the highest.

With this high resolution sequential ICP-AES, 14 elements were measured at wavelengths (nm) given in parentheses: As (189.042), B (208.858), Ba (455.403), Cd (228.802), Co (228.616), Cr (267.716), Cu (324.754), Hg (194.163), Mo (202.030), Ni (231.604), Pb (220.353), Se (186.080), Sn (188.988), Zn (213.856). Evaluation of contamination was carried out with compliance of legal contamination regulations using the “B” limit threshold value specified in 10/2000 and 6/2009 regulations (Hungarian Ministries 2001, 2009).

#### *2.3.3 Enzyme-linked immunosorbent assay (ELISA)*

The determination of herbicide active ingredient glyphosate was carried out by the validated commercial immunoassay (PN 500086 by Abraxis LLC, Warminster, PA, USA) using antibodies specific for glyphosate (Mörtl et al., 2013). Measurements were carried out in 96-well microtiter plates according to manufacturer instructions. Acyl-derivatized samples or analytical standards were incubated with glyphosate-specific antibodies immobilized on the walls of the microtiter wells, and an enzyme conjugate of glyphosate was added. Upon washing, the bound enzyme quantity was determined by a colorimetric reaction providing optical signals at 620 nm and 450 nm wavelengths. Glyphosate concentrations were determined using standard calibration curves of linear or sigmoid fit.

### 2.4 Toxicity tests

#### *2.4.1 Soil microbiology*

Microbial enzymatic activity in the soil was measured by fluorescein diacetate (FDA) hydrolysis

(Schnürer & Rosswall 1982, Adam & Duncan 2001), optimised for soil samples. Samples were stored at the temperature of 4°C until analysis. FDA reagent (stock solutions at 1 and 10 g/L FDA in acetone) was added to 1 g of soil per replication in 15 mL phosphate buffer (pH 7.6). Upon shaking for 2 hours at 30°C. Then, the reaction was terminated by acetone (1:1 suspension in the solvent), applying a 1.5 hour long glass bead pre-shaking step to reach a proper level of suspension. Upon centrifugation of the suspensions at 3000 rpm), the amount of fluorescein developed was measured from the supernatant of each sample on a spectrophotometer at 490 nm. Statistical analyses of FDA data have been performed using one-way analyses of variances (ANOVA), effects of pesticides residues, soil humus content and soil texture have been analysed by multiple regression.

#### *2.4.2 Immobilization test on* Daphnia magna

Aquatic biotests using the giant water flea (*Daphnia magna* Straus) were carried out on soil and water samples with highest contamination rates detected. Immobilization tests were performed according to the ISO 6341:1996 standard (International Organisation for Standardisation 1996). Test animals were kept in 16/8 hr light/dark photoperiod with the testing atmosphere kept at 20-22°C and free from poisonous vapours or dusts. The breeds and the controls were kept in aqueous solution containing $CaCl_2$, $MgSO_4$, $NaHCO_3$ and KCl at concentrations of 220.5, 61.6, 64.8 and 5.75 mg/L, respectively. The bioanalytical accuracy of the test was assessed in potassium dichromate test: the mortality caused by $K_2Cr_2O_7$ was measured at 0.25, 0.5, 0.75, 1 and 1.25 mg/L concentrations, and the sensitivity of the test animals was considered proper according to the standard protocol if the $EC_{50}$ value obtained for potassium dichromate fell between 0.6 and 1.7 mg/L. Water samples and aqueous soil extracts were applied directly to the test in volumes of 10 mL per replication. Floating matter when occurred was removed from the water samples by centrifugation for 5 min at 3000 rpm. For extraction of soil samples 300 g of soil was extracted with 500 mL of distilled water, the mixture was ultrasonicated for 10 min, and filtered in a suction filtration apparatus through MN 640W filter paper. Tests were carried out at the first larval stage (6-24 hours) for 48 hours, when the immobilization of the subject animals was recorded (10 animals per test) in quadruplicates. Mortality (immobilisation) rates were calculated by the Henderson-Tilton formula (Henderson & Tilton 1955), correcting the measured mobility inhibition with that detected for untreated control and eliminating the effect of varying number of test individuals applied at the repetitions. Therefore, percentage mortality/immobilisation refers to values corrected with the Henderson-Tilton formula. $EC_{50}$ values were calculated using probit transformation and log-linear regression, the data were statistically evaluated by one way ANOVA.

### 2.5 Computing accurate sample sizes

Reliability of estimates depends on both accuracy and precision. Accuracy is about how close the estimate is to its true value on average. Precision is about how similar repeated estimates are to each other. Percentage relative precision (PRP) of the estimation at heavy metal and pesticide residues contamination, i.e. was used to estimate precision of the measurements. PRP is the difference between the estimated mean of the measurements and its 95% confidence limits, expressed as a percentage of the estimate. However, because confidence limits are sometimes asymmetrically distributed around the estimate, the mean difference between them and the estimate was used. Estimation of sample sizes needed to attain a fixed percentage relative precision has been calculated on the basis of the following equation (Morris 2003):

$$m_0 = \left(\frac{200}{Q}\right)^2 \left(\frac{s}{\bar{N}}\right)^2$$ where

Q: the required percentage relative precision
$\bar{N}$: mean value in the sample unit
s: standard deviation in the sample unit
$m_0$: sample size required for there to be a 95% chance of obtaining a PRP of Q or less.
PRP: percentage relative precision = 50 x ($CL_2$-$CL_1$)/$\bar{N}$, where $CL_2$ and $CL_1$ are the 95% upper and lower confidence limits, respectively. If $m_o < 25$, than the calculated sample size must be increased by two samples.

## 3. RESULTS AND DISCUSSION

### 3.1 Examination of sampling effort

For reliability assessment, percentage relative precision of the pollution level estimation was calculated at each site. As expected, precision was highly influenced by the heterogeneity of the sites and thus directly depended on the variance of the data. To show the consequences of small-scale heterogeneity of sites, contamination characteristics of a homogeneous and a heterogeneous site are presented on figure 2 showing the curves of nickel

concentration, pesticide residue levels and soil texture with soil depth. Similar slopes in saturation percentage indicated identical soil textures among the five samples at the homogeneous site (Fig. 2, A), whereas soil textures differed substantially in the heterogeneous set (Fig. 2, B), probably due to complex sedimentation. Sample sizes needed for 10% and 20% precision in nickel concentration varied between 3-4 drillings and 3-16 drillings at the homogeneous and heterogeneous sites, respectively. For pesticide residues, appropriate sample sizes have been determined between 3-8 drillings, considering the higher percentage values of the precision (50% and 100%). 100% percentage relative precision actually indicates only the occurrence of the contamination. This result pointed out that the level of site heterogeneity highly influences the required sample sizes for a given precision, and also indicated the extreme importance of the composite sampling design and homogenisation in the course of sample preparation in environmental monitoring.

### 3.2 Chemical analysis

#### *3.2.1 Pesticides*

Thirty-three active ingredients and metabolites (acetochlor, alachlor, aldrin, atrazine, butylate, carbofuran, carbofuran phenol, DDD, DDE, DDMU, DDT, diazinon, dibutilphtalate, dieldrin, endrin, endrin ketone, EPTC, α-, β- and γ-HCH, heptachlor, heptachlor epoxide, isodrin, metolachlor, phenkapton, phorate, prometryn, propachlor, sulfotep, TBP (tributil phosphate), terbutryn, trifluralin) and 14 related compounds (AMPA, 2,4-D, 2,4-DB, dichloprop, dimetachlor, fenoxycarb, glyphosate, MCPA, MCPB, mecoprop, metribuzine, propisochor, simazine) or compound groups (camphechlor) were monitored by GC-MS.

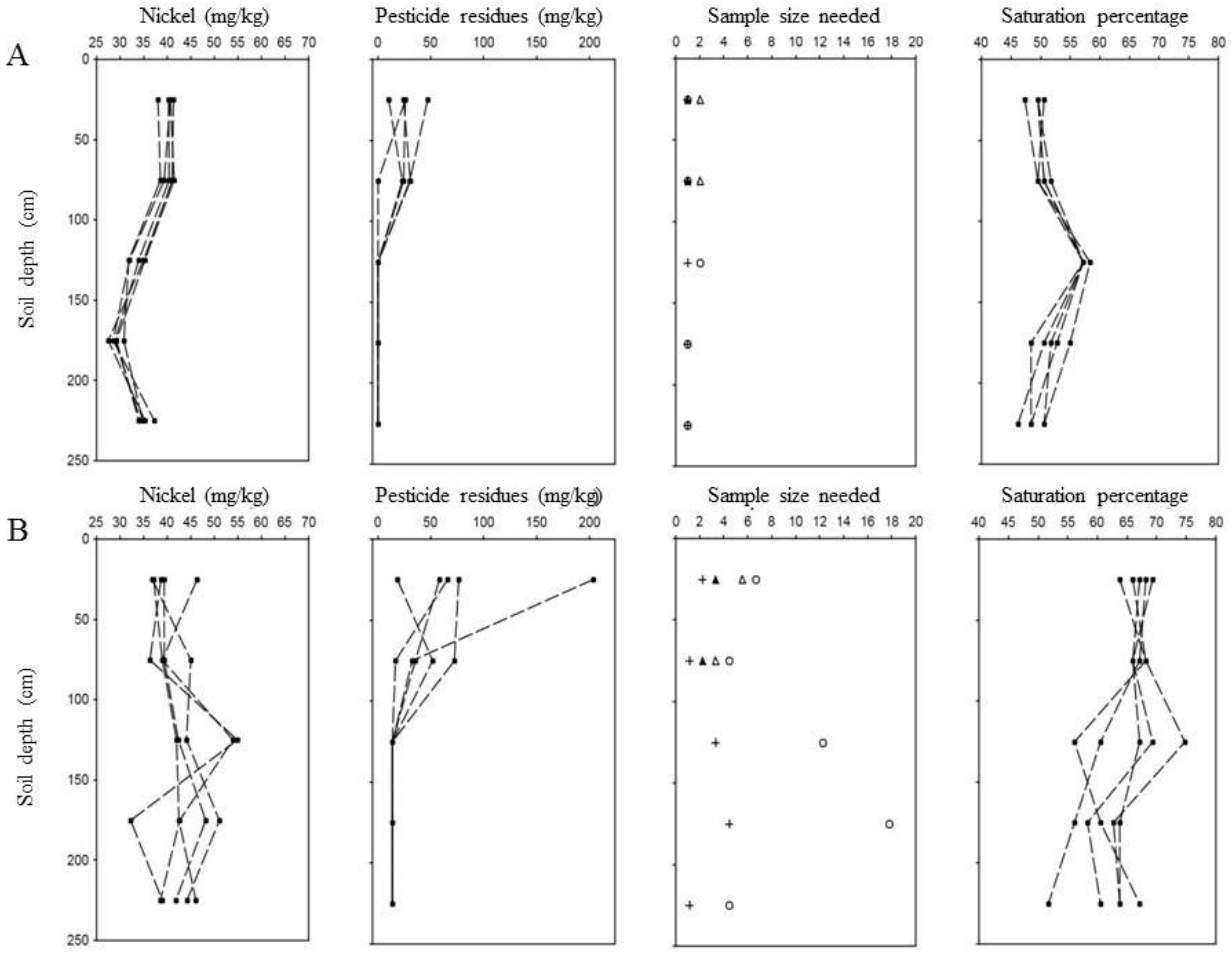


Figure 2. Contamination profiles at a homogenous (site *A*, Battonya 1) and a heterogeneous (site *B*, Köröstarcsa 2) sampling site. Nickel concentration was proven to exceed the "B" limit value (40 mg/kg) in both cases. The amount of total pesticide residues at site *B* is regarded to be significant;10% PRP (percentage relative precision) at nickel (open circle); 20% PRP at nickel (cross); 50% PRP at pesticide residues (open triangle), 100% PRP at pesticide residues (closed triangle) are shown in the last column.

Of the 423 soil samples analysed, 77 samples contained detectable contamination by one or more target compounds (contamination was marginal in four cases). Therefore, contamination rate found was 17-19%. Of the 202 water samples analysed, 76 samples contained detectable contamination by one or more target compounds (contamination was marginal in 11 cases). Therefore, contamination rate found was 18-67%. The most common soil contaminants appeared to be atrazine (10-580 ng/g), trifluralin (3-200 ng/g), acetochlor/metolachlor (5-80 ng/g), as well as DDT/DDE (38-460 ng/g) and lindane/HCH (7-103 ng/g); the most common water contaminants were acetochlor (0.02-3900 µg/L), atrazine (0.5-100 µg/L), metolachlor (0.001-56 µg/L), trifluralin (0.8-9 µg/L) and diazinon (0.001-0,85 µg/L). The found contamination levels are in certain cases alarming as the corresponding harmonised EC Directive (European Parliament and Council 2006) effective in Hungary as well sets the maximum residue limit of 100 ng/L for a given pesticide compound and 500 ng/L for all pesticide residues in subsurface water.

The herbicide active ingredient glyphosate wasdetected as water contaminant at concentrations of 0.54-0.98 ng/mL by a commercial ELISA method, at very high or high levels in 5 and 16 cases, respectively (relative to the substantial background signal level of the immunoanalytical method).As the reported cross-reactivity of the commercial ELISA kit used with the main glyphosate metabolite aminomethyl phosphonic acid (AMPA) is reported to be below 0.0002% (Mört et al., 2013), the method only detected the parent compound and not its degradation product. Frequent occurrence of glyphosate is of major concern due to the high water contaminating potential of glyphosate, and due to its known ecotoxicological (cytotoxic, endocrine disruptive and mutagenic/teratogenic) effects, particularly when exerted in co-exposure or synergy with polyethoxylated tallowamine often used as adjuvant for this herbicide active ingredient (Székács & Darvas 2012).

#### *3.2.2 Heavy metals and other microelements*

From the total amount of soil samples (322 samples from 67 drillings) microelemental pollution was detected in 59 samples. Regarding soil samples taken from agricultural lands, 16 drilling points (42 soil samples) out of 45 points proved to be contaminated. Contamination was caused prevailingly by Ni, followed by As and Ba, the first two classified as readily available toxic microelements, the latter as a toxic but practically insoluble or rare microelement. Ni contamination showed a correlation with soil depth and soil texture as seen from its 3D surface plot (Fig. 3), having peak Ni contamination in heavy clay soil at depths of 150 cm. Sampling from industrial sites was carried out in 22 drilling points (which produced 67 soil samples). The result of the measurement showed 11 points to be contaminated, mostly by Ni (all 11 points), and also by As, Ba and Se.

Water samples added up a total amount of 85 samples (45 contaminated), 39 of them from agricultural lands. These were taken mostly from the soil drillings or from groundwater monitoring wells, while nine of them from nearby surface waters (irrigation channels). As for highest contamination, eleven samples appeared to top the limit values, mostly by B, and also by Se. Water samples originating from industrial sites occurred to be highly contaminated: none of the five sites proved to be clean, and out of the 46 samples, contamination was found in 34 of them. Contamination was caused not only by B and Se, as in the case of agricultural fields, but also by As and Cr, all classified as bioavailable toxic microelements.

### 3.3 Toxicity testing

#### *3.3.1 Soil microbial activity*

Soil microbial activity on arable lands (nine sites) and industrial locations (four sites) were measured by using FDA analysis with 5 replicates per site (65 drillings). Soil microbial activity differed significantly between arable lands and industrial sites ($F_{(1, 63)} = 74.5$, $p < 0.001$), arable soils showed 14 times higher microbiological activities than industrial ones ($F_{(1, 38)} = 0.39$, $p > 0.05$). Such pattern can be explained by the more favourable ecological conditions for the soil microflora occurring in arable lands than those of industrial sites. FDA activity correlated with humus content in the upper soil layer ($R^2 = 0.6$), constituting another sign for the effect of biotic conditions on soil microbiological activity. However, agricultural land use practice (intensive vs. organic farming) did not affect FDA activity. The reason for such phenomenon may be the fact that the overall duration of organic farming practices at these locations after decades of intensive agriculture was too short for the spontaneous development of a mature microbiological community with higher biomass. Soil microbiological activity is influenced by numerous biotic and abiotic environmental factors, of which contamination is only one driving force. We examined how abiotic soil factors and contamination affected soil microbiological activity.

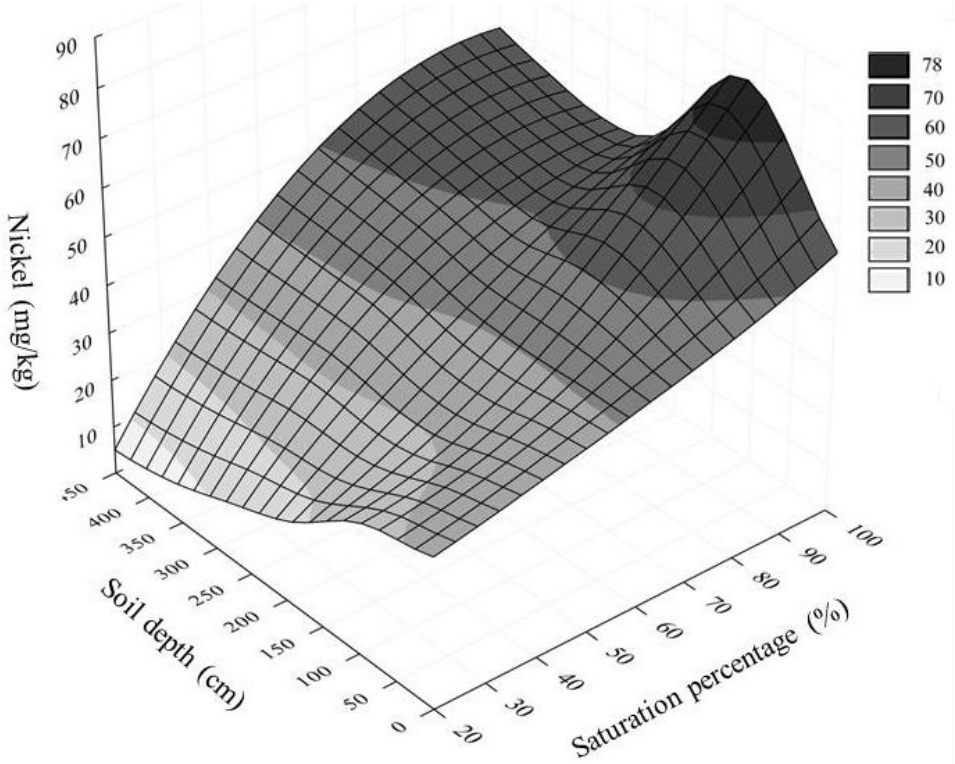


Figure 3. Nickel concentration according to soil depth and soil texture. The 3D surface plot was produced for nickel concentration by using distance weighted least squares estimation.

By using multiple linear regression modelling humus content, soil texture and soil pesticide residues were set as independent variables against FDA, as a dependent variable. The partial regression coefficients were obtained respectively -0.08 at herbicide and insecticide residues, 0.4 at soil texture and 0.84 at humus content, ($R^2$ = 0.7). This result showed a statistically not significant, weak negative effect of herbicide and insecticide pollution on FDA, whereas humus content and soil texture did influence microbial activities in soils. Therefore, such a general microbiological activity pattern generated from FDA analysis alone cannot be regarded as a predictor for examined soil contamination.

### *3.3.2 Aquatic toxicity detected on Daphnia magna indicator organism*

There apply strict regulations in pesticide registration regarding aquatic toxicity of the candidate compounds. If the pesticide preparation is dangerous for aquatic organisms, specific protective distances (200, 50 or 20 metres) apply from water courses (European Parliament and Council 2000, Hungarian Ministries 2004). As a result, toxicity exerted on *D. magna* is required to be determined for each registered pesticide active ingredient and is listed among the chemical and biological features of pesticides (Tomlin 2000). Zooplankton, *Daphnia* is widely used as a test organism in order to evaluate the toxicity of several contaminants as well as their mixture (Barata et al., 2007). A recent report of the effects of herbicides on zooplanktons gives a comprehensive overview of the highly varying $EC_{50}$ values on *D. magna* and other daphnids, revealing possible deviation patterns (Rico-Martínez et al., 2012). Organisms in the environment are permanently exposed to complex mixtures of low concentrations of contaminants from mainly anthropogenic sources (Cedergreen et al., 2008). Particularly aquatic organisms are endangered by toxicants since they spend their life entirely or majorly in their aquatic media, and therefore, may suffer exposure to single or multiple water contaminants all over their lifetime. The evaluation of the additive effects of multiple contaminants (e.g. pesticides) in water at low concentrations has received great attention lately.Addition and synergism were observed among sublethal concentrations of diazinon, malathion and chlorpyrifos on coho salmon (*Oncorhynchus kisutch*) (Laetz et al., 2009). A recent review reports a number of combined toxicological interactions of pesticide mixtures such as pyrethroids, carbaryl and triazine herbicides at molecular level (Hernández et al., 2013).

Exposure to pesticides at sublethal doses not only exert combined toxitity to affected organisms, but may also induce their increased sensitivity to other stress factors such as predator stress, parasite

infection or UV-radiation (Kelly et al., 2010, Beketov et al., 2011, Janssens & Stoks 2013). As reported in the scientific literature, toxic effects of low concentration pesticide mixtures on zooplanktons (including *D. magna*) and on algae are typically close to the sum of the effects for each pesticide compound applied independently, therefore, overall toxicity levels are estimated on the basis of toxicity exerted by single compounds. Such phenomenon has been reported not only for insecticides, but for herbicide (acetochlor, metolachlor, glyphosate, 2,4-D, atrazine) mixtures as well (Relyea 2009).

As expected, no toxic effect was observed in the *D. magna* immobilization test in the case of the vast majority of the water samples. In contrast, significant or salient aquatic toxicity was detected in all soil and water samples heavily contaminated with pesticide residues and/or toxic heavy metals (Table 1), indicating that these contaminants do cause toxicity on *D. magna*. Nonetheless, a clear superposition of the individual toxicity of the contaminants tested was not seen in the statistical analysis of the aquatic toxicity results. Among pesticide type organic micropollutants, mostly insecticides are expected to be considered toxic to *D. magna*: compounds designed to have toxic effects on insects are more likely to cause similar effects on other arthropods, than substances optimized for their effects on plants or on fungi. This is well reflected among the target analyte pesticides in the present study by the outstandingly low $EC_{50}$ value of diazinon on *D. magna* (0.96 µg/L). The toxicity of microelements on *D. magna* is highly dependent on element speciation, therefore, toxicity values reported in the literature commonly refer to the most abundant forms of the given elements. Besides, the toxicity of metals in aquatic environment varies widely, depending both on environmental conditions and on the sensitivity of the exposed organisms (Kozlova et al., 2009). Most prevalently found contaminating microelements in this study (As, B, Ni and Se) exert minor toxicity of *D. magna*. As a result, significant toxicity was expected only from the most contaminated surface water samples or aqueous soil sample extracts, particularly from those contaminated with insecticides. Dibutyl phthalate, commonly reported as ubiquitous water contaminant (Fromme et al., 2002), has been found in certain water samples, in some cases at concentrations as high as 100 ng/mL (e.g. W4A1, W5D1), yet no toxicity on *D. magna* was observed, in accordance with the reported marginal toxicity of the compound on *D. magna* ($EC_{50}$ = 3.0-5.2 mg/L) (Staples et al., 1997).

Samples W1E1 and W1G1 heavily contaminated with acetochlor and atrazine and containing elevated levels of boron caused full mortality in the *D. Magna* biotest, when applied undiluted. These two water samples were measured in 5-, 10- and 25-fold dilutions as well, and it was found that 50% mortality ($EC_{50}$) was reached when the samples were applied at dilutions of 6.4- and 13.3-fold, respectively. The strong mortality caused by these samples was a clear result of the synergistic effect of the individual contaminants, as the actual (although apparently high) levels were far below of the individual $EC_{50}$ values. Sample W1D1 represented a similar case with slight diazinon and acetochlor, and considerable metolachlor contamination (the latter still not reaching even 1% of the $EC_{50}$ value of metolachlor) and causing 65% mortality on *D. magna*. As toxicity of that high magnitude would not be expected on the test animal neither from the pesticide residue, nor from the microelement contamination detected in the sample, the observed biological effect is either due to an unidentified component or caused by synergistic interactions among the detected contaminants. In contrast, a case of low or no toxicity, when significant effect on *D. magna* was expected, was also recorded: sample W2F1 caused no immobilisation of *D. magna* larvae. This was rather intriguing, because the measured diazinon content of the sample was close to the $EC_{50}$ value of the compound on the test animal. In such case at least limited mortality would have been expected to be observed. To test whether the *D. magna* population used in these experiments shows sufficient sensitivity to diazinon the $EC_{50}$ value of the compound was experimentally determined and was found to be 0.34 µg /L (0.27 to 0.39 µg /L).

Diazinon was spiked into water sample W2F1 at this concentration, verified to cause substantial mortality, yet mortality still not appeared in the *D. magna* immobilisation test. This observation indicates a clear antagonist effect among contaminants such as sub-lethal concentrations of diazinon and copper. Similar antagonistic patterns observed when crustacean *Ceriodaphniadubia* or mayfly *Ephoronvirgo* were exposed to a mixture of copper and diazinon (Banks et al., 2003 and Van der Geest et al., 2000). Another critical water sample (W3A0) of high boron content and of limited (40%) toxicity on *D. magna* was also spiked with diazinon at 0.34 µg/L concentration, and resulted in full (100%) mortality in the *D. magna* immobilization test. This verified assay sensitivity to diazinon, and indicated a slight synergism between diazinon and the boron content of the sample.

Table 1. Toxicity of water and soil samples contaminated with pesticides and heavy metals on *Daphnia magna* as indicator organism

| **Samples** w-water s-surface | **Pesticide contamination** (µg/L or mg/kg) | | | | | | | **Element content** (µg/L or mg/kg) | | | | **Mortality (*D. magna*)** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | acetochlor | atrazine | diazinon | metolachlor | terbutryn | trifluralin | glyphosate | As | B | Ni | Se | |
| W1D1 | 0.18±0.04 | - | 0.008±0.001 | 55.9±4.9 | – | – | – | 8.0 | 145 | 5.0 | – | + |
| W1E1 | > 1000 | ~ 100 | < 0.001 | 1.66±0.22 | 0.18±0.03 | 0.8±0.1 | – | – | 609 | 6.0 | 12.6 | +++ |
| W1F1 | 35.9±3.68 | 1.0±0.008 | 0.011±0.003 | 0.039±0.007 | – | – | _ | 40.5 | 69.5 | – | – | – |
| W1G1 | > 1000 | > 100 | < 0.001 | 0.56±0.13 | 0.35±0.07 | 9.0±1.2 | – | 9.6 | 360 | 15.9 | 23.2 | +++ |
| W2F1 | – | – | 0.84±0.008 | – | – | – | 0,75±0,08 | 1.8 | 367 | 2.5 | – | – |
| W2F1[a] | – | – | 1.18±0.05[b] | – | – | – | 0,75±0,08 | 1.8 | 367 | 2.5 | – | – |
| W3A0 | – | – | < 0.001 | – | – | – | – | – | 1544 | 0.9 | – | + |
| W3A0[a] | – | – | 0.34±0.01[b] | – | – | – | _ | – | 1544 | 0.9 | – | +++ |
| W4A1 | – | – | – | – | – | – | _ | – | 846 | – | – | – |
| W4A0 | – | – | – | 0.004±0.001 | – | – | – | 2.0 | 121 | 3.0 | – | – |
| W5A1 | – | – | – | – | – | – | _ | – | 183 | – | 5.3 | – |
| W5D1 | – | – | – | – | – | – | _ | – | 152 | – | 3.7 | – |
| W5A0 | – | – | – | < 0.001 | – | – | – | 2.6 | 111 | 5.0 | – | – |
| W6B1 | 1.54±0.28 | 0.50±0.11 | – | 0.044±0.005 | – | – | 0,60±0,05 | – | 700 | 2.0 | – | – |
| W6A0 | – | – | – | – | – | – | – | 12.9 | 127 | 2.6 | – | – |
| W7F1 | 0.26±0.005 | – | 0.012±0.004 | – | – | – | _ | – | 816 | 2.7 | – | – |
| S1E3 | 0.005±0.001 | 0.20±0.04 | – | 0.011±0.002 | – | 0.011±0.01 | – | 7.81 | 22.7 | 40.1 | – | ++ |
| S1A2 | 0.014±0.002 | – | – | – | – | – | – | 10.8 | 22.6 | 35.4 | – | – |
| S1D3 | 0.011±0.002 | – | – | 0.019±0,004 | – | – | 0,56±0,26 | 16.6 | 49.2 | 53.1 | – | – |
| S2A1 | – | – | – | 0.081±0,008 | – | – | – | 16.3 | 40.5 | 51.2 | – | – |
| S3A0 | – | – | – | – | – | 0.20±0.03 | – | 15.4 | 30.8 | 37.1 | 0.742 | +++ |
| *D.magna* $EC_{50}$ | 9000 [c] | 87000 [c] | 0.96 [c] | 25000 [c] | 2660 [c] | 250 [c] | 780000 [c] | 7500-15040 [d] | 56000-141000 [f] | 7300 [g] | 430-4070 [h] | |

[a] Fortified with diazinon at $EC_{50}$ value obtained in laboratory *D. magna* colony / [b]Diazinon level specified as the sum of the measured and spiked concentration. / [c] Reported for the parent compound (Tomlin 2000) / [d] Measured as As(III), $As_2O_3$ (Lilius et al. 1995, Guilhermino et al., 2000) / [f] 141000 µg/l for B(III), tetraborate (Maier & Knight 1991), 56000-66000 µg/L for elemental B nanoparticles (Strigul et al., 2009) / [g] Measured as Ni(I), $NiCl_2$ (Pedersen & Petersen 1996, Ferreira et al., 2010) / [h] 430-3000 µg/L for Se (IV), selenite and 550-5300 µg/L for Se (VI), selenate (Martins et al., 2007)

Sample S1E3 contained various pesticide and microelement contaminants, primarily nickel at a substantially high level of 40.1 mg/kg. The aqueous extract of this soil sample caused 95% immobilisation on *D. magna*. Soil sample S3A0,containing (along with other microelements) high level (15.4 mg/kg) of arsenic, the aqueous soil extract caused 100% immobilisation, and required a 2.54-fold dilution to reach $EC_{50}$.

Detectable toxicity to *Daphnia magna* has not been observed on water samples with detected content of glyphosate residues. This is in accordance with the known toxicity of glyphosate and AMPA to *D. magna* (780 and 690 mg/L, respectively) (Tomlin 2000, European Commission 2002), escalated by polyethoxylated tallowamine detergents used as formulating agents (Brausch et al., 2007). Nonetheless, recent literature data indicate sublethal effects of glyphosate and its formulations on aquatic organisms (Pérez et al., 2012).They may cause reduction of juvenile size and affect the growth, fecundity and abortion rate of daphnids (Cuhra et al., 2013) and inhibit cholinesterase activity of mussel and fish (Sandrini et al., 2013) as well.

## 4. CONCLUSIONS

The present study combines chemical analysis of pesticide residues and microelements from topsoil and subsoil, as well as surface and ground water samples with biotests on total soil microbiological activity using fluorescein diacetate (FDA) hydrolysis and on aquatic toxicity using the ISO 6341:1996 standard immobilisation protocol on *Daphnia magna* Straus. Contamination by organic micropollutants, mainly pesticide residues occurred more frequently in surface water (18-67%), than in soil (17-19%); the most contaminated samples arrived from an identified illegal contamination site scheduled for remediation. Residues of herbicide active ingredients atrazine, acetochlor/metolachlor and trifluralin were found both as water and as soil contaminants at various concentrations up to 3900 ng/mL and 580 ng/g, respectively. In addition, residues of the insecticide active ingredients diazinon also occurred as water contaminant below 1 ng/mL. Of the 14 microelements monitored, 18% and 53% contamination frequencies above the legal threshold value was detected for soil and water samples, respectively, with Ni, As and Ba as most common soil contaminant microelements, and B and Se as major water contaminant microelements. While a clear correlation between detected soil contamination and microbiological activity determined by FDA analysis could not be established, toxicity tests with *D. magna* showed substantial toxicity in 6 cases. The survey indicated that biotests are worthwhile to be carried out even if analytical measurements reveal sublethal level contamination to the given test organism, because contaminant interactions may result in lethal effects.Interactions may appear synergistic, antagonistic and additive.


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