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„Fate of nanopesticides in soil“

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Patrick Machinski, BSc

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Zusammenfassung

Nanopestizide decken verschiedene Produktbereiche ab und können nicht in eine einzige Kategorie eingeordnet werden. Viele Nanoformulierungen setzen sich aus verschiedenen Tensiden, Polymeren und Metall-Nanopartikeln im nm Bereich zusammen. Nanopestizide dienen im Allgemeinen dem selben Zweck wie herkömmliche Pestizide, erhöhen aber die Löslichkeit von schwer löslichen Wirkstoffen, setzen die Wirksubstanz langsam bzw. gezielt frei und/oder bieten einen Schutz gegen frühzeitigen Abbau.

Zum ersten Mal wurden reguläre Protokolle der OECD Richtlinien verwendet um (i) die Verhaltenseigenschaften von Nanopestiziden in der Umwelt zu quantifizieren und (ii) sie mit ihren Wirkstoffen, in zwei verschiedenen Agraroberböden, zu vergleichen.

Es gab keinen Unterschied zwischen Abbauparameter der Nanoformulierung und der Wirksubstanz (Atrazin) aber wichtige neue Erkenntnisse wurden gewonnen. Die Ergebnisse zeigen, dass das Freiwerden von Atrazin aus seinem Nanoträger schneller sein könnte als das Einsetzen des Abbaus durch Mikroorganismen oder durch abiotische Prozesse, falls die Nanoträger nicht auch abgebaut wurden.

Soprtionskoeffizienten erhielt man durch die klassische Batch Methode und ein Zentrifugier-Verfahren. Letztere erlaubt das Bestimmen der Sorption bei realen Boden/Lösung Verhältnissen. Bei der Batch Methode zeigte die Nanoformulierung höhere Sorptionskoeffizienten, aber die Ergebnisse sollten mit Vorsicht betrachtet werden, da diese Methode nicht entworfen wurde, um Nanoformulierungen zu untersuchen. Das Zentrifugier-Verfahren erwies sich als sehr nützlich für die Bestimmung der Langlebigkeit von Nanopestiziden und die Zeitspanne, in der ein Einfluss auf das Verhalten des Wirkstoffes zu erwarten ist.

Es sind noch weitere Untersuchungen notwendig und die Entwicklung neuer Methoden, um die Bioverfügbarkeit und die Langlebigkeit von Nanopestiziden nach ihrer Aufbringung zu bestimmen.

Abstract

Nanopesticides cover a wide variety of products and cannot be considered to represent a single category. Many nanoformulations combine several surfactants, polymers and metal nanoparticles in the nm size range. The aims of nanopesticides are generally common to other pesticide formulations, these being to increase the apparent solubility of poorly soluble active ingredients, to release the active ingredient in a slow/targeted manner and/or to protect against premature degradation.

For the first time regulatory protocols of the OECD guidelines were used (i) to quantify the environmental fate properties of a nanopesticide, and (ii) to compare them to those of its active ingredient in two different agricultural top soils.

Degradation parameters for the nanoformulation and the pure active ingredient (atrazine) showed no difference but important knowledge was earned. The result indicates that the release of atrazine from its nanocarriers could be faster than its onset of degradation by microorganisms or abiotically if the nanocarriers were not degraded too.

Sorption coefficients were obtained from a classical batch method and a centrifugation technique. The latter allows measuring sorption at realistic soil to solution ratios. For the batch method the nanoformulation showed higher sorption coefficients but this results should be considered carefully since the classical batch method was not designed to investigate nanoformulations. The centrifugation technique turned out to be useful for determining the durability of nanopesticides and the period of time during which an influence on the fate of the active ingredient may be expected.

Further investigations are needed and new methods must be developed for determining the bioavailability and the durability of nanopesticides after their application.

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INDEX

1. Introduction

Pesticides are applied over large areas and thus are an important source of environmental pollution, which is hard to control. To protect the environment, it is necessary to know the fate properties of a pesticide, sorption and degradation being the two most important (Fenner et al., 2013).

Pesticides may be lost during and after their application in the field. Low or moderate sorption onto soil organic matter and clays can lead to leaching to ground water. Prolonged persistence in soils, together with many other factors lead to large amounts of pesticides in the environment.

In the last decade, the possibility to use nanotechnology to reduce pesticide losses became recognised. Not all nanopesticides have been studied in terms of their impact on the agricultural surroundings. In the last decade the amount of patents for nanopesticides increased (Kah et al., 2012) and some new formulations seem to have a benefit for our environment. However, there is still a need to perform an overall risk assessment of these nanoformulations.

There are some standardised protocols to study the fate of pesticides, but they were not designed to investigate nanoformulations. It is not known if standardised protocols can be useful for this issue. It would be very beneficial if they are suitable. This would avoid the need to design completely new protocols. In the case protocols are only partially useful, they may be adapted or improved.

A new design of atrazine as a nanoformulation is now becoming more relevant because quantities applied on the field are promised to be much lower in comparison with the pure active ingredient. Losses during application and leaching should thus be reduced.

In this study the main fate properties of atrazine, sorption and degradation, as active ingredient and as a nanoformulation were investigated. The goals were to test and evaluate the applicability of regulatory protocols to define the fate of a polymer-based nanopesticide. The nanoformulation should sorb more on the soil particles compared to its active ingredient and release it with time. This would reduce the input in the environment, its pollution and could be a great benefit for human and environmental health. To achieve these goals, degradation and sorption parameters were determined in two different soils. Sorption data were derived from a batch set up and a centrifugation technique, the latter with one and seven days of incubation.

This master thesis starts with some background information about the nanoformulation and its active ingredient, followed by a description of the methods applied in this study. Finally the results are discussed in terms of mechanisms and placed into the context of the environmental exposure assessment of pesticides to evaluate priority for future research.

1.1. Atrazine

Atrazine is a herbicide from the family of the triazines, which inhibits photosynthesis in target weeds. Triazines are one of the most commonly used classes of herbicides, and are applied to control weeds in maize, sorghum, and sugar cane plantations (Grillo et al., 2012). They consist of an aromatic ring with 3 nitrogen atoms. The main characteristics are listed in table 1. Other pesticides from this family are cyanazine, propazine and simazine.
Atrazine is a white crystalline substance that is sold under a variety of trade names for primary usage as a selective herbicide to control broadleaf and grassy weeds in corn and sorghum. Its water solubility is very low but it is comparatively soluble in many organic solvents.

1.1.1. Atrazine in the U.S. and other countries

The application of atrazine is forbidden since 1991 in Germany and since 1995 in Austria. In contrast to the U.S. approach of allowing pollution to occur until there is scientific evidence of its risks, the European Union has a uniform limit of 0.1 ppb for the residue of any pesticide in drinking and ground water. While scientists representing Syngenta characterize this standard as neither health-based nor scientifically supported, it appears that the E.U. generally adopts the position that it is unhealthy to drink pesticide-contaminated water, which represents a health-based and scientifically-supported position. Based on the inability to keep water contamination below this level, European regulators announced a ban on atrazine use in October 2003, one week before the U.S. EPA approved its continued use (Sass, Colangelo, 2006).
Atrazine is the most heavily used agricultural pesticide in North America and is applied for controlling weeds in numerous crops including corn, sorghum, sugarcane, soybeans, wheat, pineapple and various range grasses. Agricultural use of atrazine has also been reported in South Africa, Australia, New Zealand, Venezuela, Canada and Brazil. Global use is estimated at 70-90 million kg annually. Resistance to atrazine has developed in various strains of weeds typically present in crop fields, sometimes in less than two generations. Atrazine has been detected in lakes and streams at levels ranging from 0.1 to 30.3 µg/L. In runoff waters directly adjacent to treated fields, atrazine concentrations of 27.0-69.0 µg/L have been observed and may reach 1000 µg/L. Some of these concentrations are demonstrably phytotoxic to sensitive species of aquatic flora.
Atrazine is usually applied in a water spray at concentrations of 2.2-4.5 kg/ha before weeds emerge. The chemical is available as a technical material at 99.9 % active ingredient and as

a manufacturing-use product containing 80 % atrazine. Although annual use of atrazine in the U. S. is about 35 million kg, atrazine concentrations in human foods are negligible. Monitoring of domestic and imported foods in the human diet by the U.S. Food and Drug Adminstration between 1978 and 1982 showed that only 3 of 4500 samples analysed had detectable atrazine residues.

1.1.2. Degradation and Metabolites of atrazine

There are three major atrazine degradation pathways: hydrolysis at carbon atom 2 in which the chlorine is replaced with a hydroxy group. N-dealkylation at carbon atom 4 (loss of the ethylpropyl group) or 6 (loss of the isopropyl group) and splitting of the triazine ring (Eisler 2007).

Atrazine is not usually found below the upper 30 cm of soil in detectable quantities. Atrazine persistence in soils is extremely variable. The typical DT50 is about 75 days but ranges from 28 to 150 days (University of Hertfordshire, 2013). The degradation is faster in soils with high organic carbon and high clay content and high microbial density. Microbial action, usually by way of N-dealkylation and hydrolysis to hydroxyatrazine, probably accounts for the major breakdown of atrazine in the soil, although non-biological degradation pathways of volatilization, hydroxylation, dealkylation and photodecomposition are also important.

The major atrazine metabolite in soil and aquatic systems is hydroxyatrazine. Atrazine may be converted to non-phytotoxic hydroxyatrazine by chemical hydrolysis, which does not require a biological system. Bacterial degradation proceeds primarily by N-dealkylation. There is general agreement that atrazine degradation products are substantially less toxic than the parent compound and not normally present in the environment.

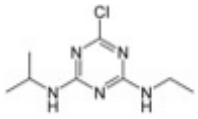
1.1.3. Toxicity of atrazine to humans and animals

Atrazine effectively inhibits photosynthesis in target weeds by blocking the electron transport during Hill reaction of photosystem II. Most authorities agree that atrazine could induce some loss in aquatic vegetation and animals like frogs (Eisler 2007).

Studies showed that atrazine adversely affects amphibian larval development. Another study demonstrated that adult amphibian males exposed to atrazine were both demasculinized (chemically castrated) and completely feminized as adults (Hayes et al., 2010).

Atrazine hardly degrades in water and moderately degrades in soils. Approximately 20 – 30 % of applied atrazine settle down in sediments which function as a sink. Atrazine in soil can leach in the groundwater and therefore is a risk for the benthic community and to humans. Symptoms by humans are a ZNS-Depression and a weak eye and or skin irritation. Atrazine can be dangerous to humans and animals because it acts as endocrine disruptor. Atrazine is thought to be carcinogenic und animal tests showed a breast and testicle cancer. The IARC ("International Agency of Research on Cancer"), a department of the WHO, classified atrazine as "possibly carcinogenic to humans".

Table 1: Characteristics of atrazine

atrazine	Structural formula	Chemical name	Empirical formula	Molecular weight
		2-chloro-4-ethylamino-6-isopropylamin-1,3,5-triazine	$C_8H_{14}ClN_5$	215,7 g/mol
	Melting point	**Henry's law constant**	**Physical state**	
	173-175 °C	6.13 x 10^{-8} to 2.45 x 10^{-7} atm-m^3/mol	white, crystalline, non-combustible, noncorrosive substance	
	Solubility		**Log K_{ow}**	**DT50**
	Water Acetone Methanol	32.0 mg/L at 25 °C 31.0 g/L at 25 °C 18.0 g/L at 27 °C	2.71	Typical 75, lab studies range 28-150 d
	K_{oc}	**Kd**		
	100 L/kg	Literature data: Kf range 1.3-6.3 mL/g, kfoc range 70-429 mL/g, 1/n range 1.04=1.10, Soils = 13		

1.2. Nanoformulation

The usage of authorised active ingredients is now being optimised more than ever before. The increasing regulatory pressure indirectly want to amplify the effect on the target organisms and at the same time to keep the impact in the environments as small as possible. For this purpose, nanoformulations have received increasing attention. Nanoformulations are

widely used in pharmaceutical, medical and personal care products but within the agrochemical sector they are just emerging. More than 3000 patent applications, 60 peer-reviewed papers and 25 reports and reviews confirm the growing significance of these new bearers of hope (Kah et al., 2013).
After Kah et al., 2012 nanoformulations can be classified in

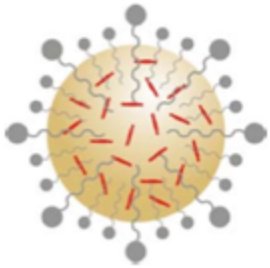
1. Nanoformulations primarily aiming at increasing solubility,
   they can be subclassified in micro-emulsions, nano-emulsions and nano-dispersions.

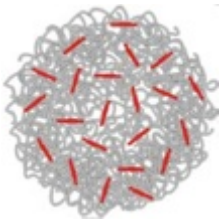
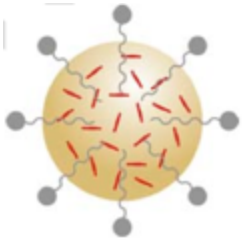
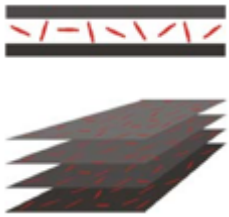
2. Nanoformulations aiming at slow/controlled release
   and can be divided in polymer-based, solid lipid, porous hollow silica nano-particles, layered double hydroxides (LDH) and clays.
   Further, there are two types of nano-particles: nanocapsules and nanospheres. Nanocapsules consist of a polymeric shell and an oily nucleus and nanospheres consist of a polymeric matrix without any oil (Grillo et al., 2011). The primary aim of the polymer-based nanoformulations is to have a controlled release of active ingredient and act as a protective reservoir. The location of the active ingredient is not specified for the nanospheres, whereas nanocapsules have a core-shell structure, which saves the active ingredient in the centre (Kah et al., 2012).

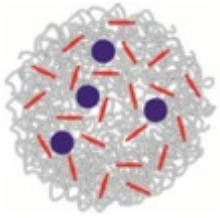
3. Nanoformulations containing nano-metals or oxides.
   These ones can be associated with another active ingredient or are alone (table 2).

The nanoformulation used in this study consists of polymer-based nanocapsules, which belong to the nanoformulations aiming at slow/controlled release (see point 2.). The nanocapsules were made after the “Interfacial deposition of pre-formed polymer” method, first described by Fessi et al., 1988. The nanoformulation considered here is made of a polymer that is biodegradable poly(ε-caprolactone) (PCL). The characteristics of this polymer are a slow degradation rate in aqueous systems, insolubility in water and harmless to the environment.

Table 2: Classification of nanoformulations, after Kah et al., 2012

Increasing the solubility of poorly water-soluble a.i.		
Micro-emulsion (6-50 nm)	Nano-emulsion (20-200 nm)	Nano-dispersion (50-200 nm)
		

Slow/controlled release and protection against premature degradation			
Soft matrix		Hard matrix	
Polymer-based (10-300 nm)	Solid lipid (200 nm – 100 µm)	Porous hollow silica (100-200 nm)	LDH and clays (µm range)
			

Containing nano-metal or oxides	
Associated with another a.i. (µm range)	Alone (1-30 nm)
	

1.3. Sorption

Sorption and degradation parameters are the most important features to predict the environmental fate of pesticides. Sorption experiments show the distribution of pesticides in the soil/water environment. Hence they determine the amount of pesticides available in the soil solution for degradation and uptake by plants. It is necessary to determine sorption as accurately as possible (Kah, Brown, 2007).

1.3.1. Batch Equilibrium Method

The Batch Equilibrium Method is an Adsorption/Desorption study useful for determining the distribution of chemicals between soil and water (distribution coefficient, Kd). Sorption data can be used to predict the availability of a pesticide for degradation, transformation and uptake by organisms and is paramount for modelling purposes. The distribution of a chemical between soil and aqueous phases is a complex process depending on many influencing factors occurring in the natural compartment (nature of substance, characteristics of the soil, climate). All this factors cannot be considered in this study but it provides valuable information on the environmental relevance of the adsorption of a chemical (OECD, 2000).
A disadvantage of the Batch Equilibrium Method is that some features are not standardised (temperature, type of vessel, type of shaking, centrifugation speed and soil to solution ratios). It is therefore sometimes difficult to directly compare results from different studies. For instance, the different soil to solution ratios produce different results (Kd's). Soil to solution ratios should be achieved with an adsorption at least more than 20 % but the optimum would be more than 50 % and the concentration must be above the limit of detection. The advantages of the Batch Equilibrium Method are a good separation between soil and solution, enough solution for analyses and it is easy to use in the lab (Kah, Brown, 2007).

1.3.2. Centrifugation Method

The Centrifugation method is also a adsorption/desorption study but performed at realistic soil to solution ratios. This method generates lower Kd's for some pesticides, probably due to shaking (Kah, Brown, 2007) and soil type. It is a simple, cheap and fast method and also easy to use in the laboratory. The solution is separated from the soil by centrifugation forces and immediately filtered before it is collected at the bottom of the test vessel. Shaking is not

necessary for this method. The disadvantage is the relative small amount of solution extracted from the soil.

1.4. Degradation

A degradation test is important to see if a pesticide can be degraded, how long this process will take, and to evaluate further decisions on application rates etc. The degradation is typically represented by DT50's: the timespan to degrade half of the initially spiked amount. It is usually given in days.

Degradation can be divided into biotic and abiotic degradation. Biotic transformation processes are driven by microorganisms or plants and abiotic by chemical and/or photochemical reactions. Which process will take place is dependent on the structural affinity of the pesticide and the environmental conditions like sorption, leaching and transport behaviour (Fenner et al., 2013).

Through the degradation process pesticides are transformed in metabolites, which may accumulate in a sink, such as sediments in a lake or a river, in which the runoff of the field crops flows to. Metabolites can be harmful to the environment and should be also taken into account for an overall risk assessment. Metabolites are however not considered in this study as they fall out of the scope of the question addressed.

1.4.1. Bioactivity

Bioactivity was measured in this study to see if the microorganisms activity in soils decrease during the degradation test. The bioactivity depends on the organic carbon content of a soil and microorganisms are the main driver of degradation of pesticides.

Biotic processes generally show higher rates than abiotic reactions, owing to enzyme catalysed reactions. A comparison of these rates for atrazine dechlorination makes it highly likely that biotic atrazine degradation dominates in the environment. Nevertheless metabolites of atrazine like for example hydroxyatrazine or desethyl-atrazine are still found in the environment. The enzyme producing hydroxyatrazine acts faster than the enzyme consuming it and so a steady state of hydroxyatrazine accumulation is the consequence. This means that hydroxyatrazine is not a metabolite. It is a metabolic intermediate that can accumulate to high levels (Fenner et al., 2013).

2. Materials and Methods

2.1. Chemicals

Pestanal analytical-grade of atrazine (6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine, CAS RN 1912-24-9) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

The PCL nanocapsules were prepared according to the interfacial deposition of pre-formed polymer method as described in details by Grillo et al. (2012). In brief, the organic phase is a mixture of 100 mg of polymer (PCL), 30 mL of acetone, 200 mg of oil (triglycerides of capric and caprylic acids, in the form of Miglyol 810), 40 mg of sorbitan monostearate surfactant (Span 60), and 10 mg of atrazine. Poly(ε-caprolactone) (PCL) is a biodegradable polymer which is mostly used in controlled release systems and consists of a aliphatic polyester which forms micro- and nanoparticles. The aqueous phase was made with 30 mL of a solution containing 60 mg of polysorbate 80 surfactant (Tween 80). The components of the two phases were dissolved. With magnetic stirring the organic phase was put slowly into the aqueous phase. The whole solution was shaken for 10 min. After Shaking the organic solvent was evaporated under reduced pressure using a rotary evaporator. The nanoparticle solution was evaporated to a final volume of 10 ml. That gave a final atrazine concentration of 1 mg/ml. The formulation was stored in amber flasks at room temperature (25 °C) in the dark. All experiments were carried out with the same nanoformulation (i. e. from a single batch).

Organic solvents used were HPLC gradient grade. Acetone for cleaning purposes was analytical grade and methanol for extraction performed for the bioactivity test was also analytical grade.

Sorption experiments were carried out at 1.5 mg/kg, and degradation experiments were carried out at 10 mg/kg (mass of active ingredient per kilogram of dried soil) due to the typical atrazine application rates in the field (2.2 – 4.5 kg/ha) (Eisler 2007) and incorporation in the upper 2.5 cm of the soil profile with a density of 1 g/cm^3. Sorption isotherms of atrazine to soils are typically very close to being linear (University of Hertfordshire 2013). Therefore non-linearity is unlikely to be the reason for discrepancies observed between the batch and centrifugation methods.

2.2. Soils

Two different agricultural top soils were used, sampled in March 2013 from the region Rheinland-Pfalz (Rhineland-Palatinate) in Germany. The soils were sampled at about 20 cm depth and 3 °C air temperature. The sand (ID-number 2.1) was uncultivated, the loam (ID-number 2.4) was taken from a meadow with apple trees. No fertilisers or pesticides were applied for at least the last 4 years before sampling. The soils where dried after sampling at room temperature until they were sieveable (1 day). The soils were first sieved with a 10 mm mesh and afterwards with a 2 mm mesh. Sampling, sieving at 2 mm, and analyses were performed following ISO standards and Good Laboratory Practice by LUFA-Speyer (Germany). The preparation and storage was performed at 20 °C.

The soil organic carbon content was calculated as the difference in carbon content (determined by heat-conductivity detector after combustion at 950 °C) before and after combustion of the soil samples at 425 °C. The maximum water holding capacity (MWHC) corresponds to the residual water remaining in saturated soil samples, left to drain for 2 h onto a saturated sand bath (Schlichting et al. 1995). The main soil characteristics are summarized in Table 1.

Table 3: Main properties of the two soils studied

ID	Texture (USDA)	pH[a]	Clay (%)	Silt (%)	Sand (%)	OC (%)	CEC (meq/100g)	MWHC (g/100g)	Bioactivity (0)[b] (mg_{TPF}/kg_{soil})	Bioactivity (55) (mg_{TPF}/kg_{soil})
2.1	Sand	5.1 ± 0.3	2.8 ± 1.1	10.2 ± 1.8	87.0 ± 1.5	0.65 ± 0.10	4.3 ± 0.5	31.1 ± 2.1	35 ± 2	28 ± 3
2.4	Loam	7.2 ± 0.2	25.9 ± 2.1	40.5 ± 1.0	33.6 ± 1.8	2.26 ± 0.5	31.4 ± 4.6	44.1 ± 1.2	170 ± 41	157 ± 6

CEC cation exchange capacity, MWHC maximum water holding capacity, TPF 1,3,5-triphenylformazane

[a] Measured on 10 g of soil in 25 mL 0.01 M $CaCl_2$ background solution

[b] Bioactivity measured on soils at the beginning and after 55 days of incubation

2.3. Characterisation and analytical methods

Grillo et al. (2012) described the characteristics of the nanoformulation of atrazine by atomic force microscopy, transmission electron microscopy, attenuated total reflectance infrared spectroscopy, two genotoxicity assays tests and a release kinetics test in deionised water.

Furthermore, the colloidal characteristics of the diluted nanoformulation (in deionised water or 0.01 M CaCl2 by a factor 100) were defined with (1) a particle size analyser based on the principle of laser obscuration (EyeTech, Ambivalue) and (2) a Zetasizer Nano (Malvern), combining dynamic light scattering and electrophoretic mobility measurement. Scattered light was analysed at a fixed backscattering angle of 173°. The particle diffusion coefficient with the Stokes–Einstein equation, using the cumulant method for fitting the autocorrelation function and using a refractive index of 1.5, yielded the hydrodynamic diameter of the particles.

Analysis of atrazine was performed by high-performance liquid chromatography (HPLC) equipped with a ZORBAX Eclipse XDB-C18 column (4.6 × 150 mm, 5-µm pore size, Agilent). The mobile phase was a mixture of acetonitrile and water, starting with 20 % acetonitrile and increasing up to 100 % acetonitrile within a 15-min run. The flow velocity was 1 mL/min and the column thermostat was set to 30 °C. Atrazine was quantified at 230 nm. Calibration curves were made of seven standards (0.1–15.0 mg/L). Retention time was about 8 min. Based on the signal-to-noise ratio, the limit of detection was 0.05 mg/L and the limit of quantification was 0.09 mg/L.

To quantify potential losses of atrazine recovery tests were performed (e.g. sorption to filters and tubes). No corrections were made due to losses beneath 13 %. The concentration of atrazine in the nanoformulation was measured after dilution in acetonitrile, which breaks down polymer molecules and releases the total amount of active ingredient loaded (as described by Grillo et al. 2012).

To specify the total amount of atrazine present in soil (used in the degradation and centrifugation methods) extraction efficiency was tested in both soils. For both atrazine formulations recoveries ranged from 95 to 112 %.

SigmaPlot 12.0 for Windows was used for all statistical analyses.

2.4. Degradation kinetics

The fresh soils were preincubated after OECD guideline 307 (2002). Studies with soils freshly collected from the field are strongly preferred, but if the collected and processed soil has to be stored prior to the start of the study, storage conditions must be adequate and for a limited time only (4 ± 2°C for a maximum of three months) to maintain microbial activity (OECD 307). In this study the soils were adjusted to a moisture content just below 50 % of the MWHC and were preincubated for 5 days at 20°C in the dark to let the microorganisms get used to the future conditions.

The moisture content of the soils was determined by drying in the oven at 105 °C for 12 hours. After the soils were taken out of the oven, they were allowed to cool down in an exsicator. The moisture content was determined by weighing the soil samples before and after drying and calculation of the mass difference.

The water content was adjusted again to 50 % of the MWHC and the soil samples were spiked dropwise with 10 mg/kg atrazine. Atrazine was used as active ingredient and as a nanoformulation. The active ingredient was diluted in acetone and 0.5 mL of the pesticide solution was applied dropwise to the soil with a 0.5 mL syringe (Agilent Technology). 3 mL of the nanoformulation stock solution were directly applied dropwise to the soil and the soils were mixed thoroughly. Both concentrations were chosen to reach an initial concentration of 10 mg atrazine / kg soil to 300 g soil dry weight. For each soil and pesticide triplicates were made and one blank with the soil 2.1 and 2.4 without atrazine (AI and NF).

The samples were incubated at 20 °C in the dark in an incubator (Binder GmbH) and samples were taken 10 times over a period of 120 days. The moisture content was held at 50 % of the MWHC and controlled once a week. The best way to keep the moisture content on a constant level was to cover the glass flasks with aluminium foil without holes.

On the sampling days about 22 g of soil samples were weighed in 125 mL glass jars, closed with Teflon coated screw caps and immediately frozen at -20 °C for a minimum of 12 hours to stop degradation. Atrazine was extracted by adding 40 mL of acetonitrile (soil to solution nearly 1:2) to the glass jars and shaking for one hour at 150 rpm on a side to side shaker (GFL GmbH). The soil was allowed to settle for a minimum of one hour. The supernatant was transferred to glass vials (1,5 mL) and analysed for atrazine by HPLC.

Standards were made for the HPLC with 0.1, 0.5, 1.0, 2.5, 5.0, 10.0 and 15 mg/L. The standards were measured for every extraction. The standards are so chosen that the expected concentrations will be within the analytical range. After extraction the concentrations were plot over a range of 120 days and the DT50's were calculated by curve fitting.

Three kinetic models were fitted to the degradation curves: a simple first-order equation, a first-order multicompartment (Gustafson and Holden) model, and a first-order sequential (Hockey-Stick) model. Parameters were optimized according to recommendations by FOCUS (2006) using the least-squares method with Microsoft Excel Solver.

During the degradation experiment the bioactivity was measured by estimating the dehydrogenase activity at the first day and after 9 and 18 weeks of incubation for each soil and pesticide in triplicates as described in Kah et al. (2007) and references therein. At first a tris buffer solution was prepared with a concentration of 0.1 mol/L. To prepare the tris buffer solution 3,0285 g tris(hydroxymethyl)aminomethane (general purpose grade, Merck Science) was dissolved in 150 mL of Milli-Q water and the pH-value was adjusted to 7.6 with 1 mol/L hydrochloric acid. Then the solution was made up to 250 mL with Milli-Q water.
The substrate solution (0.5 % TTC solution) was prepared by dissolving 250 mg of 2,3,5-triphenytetrazoliumchloride (98%, Avocado Research Chemicals Limited, 0.5% by weight) in 50 mL tris buffer solution. This solution can be stored at 4 °C for one week in the dark.
A Triphenylformazane (TPF, Sigma-Aldrich Co. Ltd.) solution was made for the standards. For the stock solution 50 mg TPF were dissolved in methanol and then made up to 500 mL to a resulting concentration of 100 mg/L. Six calibration solutions were made with concentrations of 1 mg/L, 2.5 mg/L, 5 mg/L, 10 mg/L, 20 mg/L and 30 mg/L. The standards were measured with a Perkin Elmer Lambda 35 UV/VIS-Spectrometer and a calibration curve was generated.
On the first day approximately 5.00 g soil with a water content of 50 % of the MWHC were sampled in centrifugation tubes. 5 mL of substrate solution (TTC) were added to the sample tubes and 5 mL of tris buffer solution were added in the blank tubes. The tubes were covered properly, shaken and incubated at 30 °C in the dark for 24 h. Now the hydrolysis of TTC into formazan is induced by the enzyme Dehydrogenase, which is active in every living microorganism.
On the second day the product of the hydrolytic reaction formazan was extracted with 25 mL of methanol. Afterwards the tubes were agitated for one hour at 150 rpm. Then the samples were centrifuged at 4 °C and 2500 rpm and for 10 min. The absorbance of the samples and blanks was determined at 485 nm with a Perkin Elmer Lambda 35 UV/VIS Spectrometer.

2.5. Measurement of sorption parameters by the batch method

Atrazine was used as active ingredient and as a nanoformulation. Sorption coefficients (Kd, L/kg) were determined with triplicates using a standard batch equilibrium method (OECD, 2000). The moisture content of the soils was estimated like above in the 2.3. Degradation section. 10 g portions of naturally moist soil were weighed in 50 mL PTFE centrifugation tubes (Semadeni) and 0.01 M $CaCl_2$ was added to reach a soil to solution ratio of 1:2. The samples were pre-equilibrated for 14 h on a side-to-side shaker at 150 rpm. The suspensions were spiked with the analytical grade standard or the nanoformulation to reach a concentration equivalent of 1.5 mg atrazine / kg soil. To avoid co-solvent effects the volume spiked was kept below 0.1 % of the total volume. The samples were shaken again for 24 h until a pseudo equilibrium was reached. The period of 24 hours was chosen on a test measuring adsorption between 6 and 48 h. To avoid degradation the samples were kept in the dark during the procedure. After shaking the soils were centrifuged at 3000 g and 30 min. Afterwards the concentration of atrazine was analysed from the supernatant with HPLC to determine Kd's. Subfractions of the supernatant were filtered with a 0.22 µm nylon filter (25 mm diameter, Yeti Syringe Filters, Merz Brothers GmbH) before HPLC analysis to determine the behaviour of atrazine molecules loaded onto the nanospheres, from those released within the time interval of the experiments.

As reference for the initial concentration replicate controls without soil were used. Blanks (triplicates for each soil) confirmed the absence of background.

With the aim of assessing the effect of the background solution, the same protocol was repeated with Milli-Q water instead of 0.01 M $CaCl_2$.

The solutions were filtered after the sorption batch method using a 0.22 µm nylon filter (25 mm diameter, Yeti Syringe Filters, Merz Brothers GmbH).

2.6. Measurement of sorption parameters by the centrifugation method

Atrazine was used as active ingredient and as a nanoformulation. The moisture content was estimated like above in the 2.3. Degradation section. The moisture content of the soils was adjusted to 50 % of MWHC. Two times 150 g of each soil were incubated at 4°C in the dark for three days. On the third day the soils were spiked with each pesticide to get a concentration of 1.5 mg/kg. The active ingredient was dissolved in acetone and the nanoformulation diluted in Milli-Q water. 1.0 mL of the pesticide solution was applied dropwise to the soil. The soils were again brought to a moisture content of 50 % of the MWHC. After stirring the soils were incubated at 4°C in the dark. After one and seven days the moisture content was readjusted by weighing and 10 g samples (triplicates) were given into the inserts of a 50-mL centrifuge tube (VectaSpin 20, PVDF, Whatman International Ltd., Maidstone, UK). Before the soils were inserted a 1.6 µm filter (Whatman, GF/A, 1.6 µm, 25 mm diameter, Cat No 1820-025) was put on the bottom of the insert of the centrifugation tube. The filter was prewetted with 100 µL Milli-Q water. The samples were then centrifuged at 1500 g and for 30 min. An aliquot of soil solution was put afterwards in a 1.5 mL glass screw vial with an 200 µL insert using a syringe to determine the exact volume for pesticide concentration by HPLC. The centrifugation force applied was such that the soil was subjected to a pressure of 200 kPa. The pressure of 200 kPa has been proposed as the boundary between ‘‘mobile’’ and ‘‘immobile’’ water (Addiscott 1977). The total concentration of pesticides present in the soil after one and seven days (used as initial concentration for the calculation of Kd) was determined by extracting soil samples (nearly 22 g, triplicates per soil and pesticide) with 30 mL of acetonitrile. After shaking for 1 h and 150 rpm on an end-over-end shaker, the samples were allowed to stand until the soil had settled (min. 1 h). Pesticide concentrations in the clear supernatant were quantified directly by HPLC.

3. Results and discussion

3.1. Characteristics of the nanoformulation

Release of atrazine from the polymer occurred by relaxation of polymeric chains after the Korsmeyer-Peppas model and not by diffusion (Fick) and depends on the amount of CH_3-groups and hydrophobicity of the active ingredient (Grillo et al. 2012). The active ingredient exits the nanocapsule in two phases. In the first one 64 % of the herbicide is released after 2 days. Then the release rate decreases and after 5 days 72 % were released. Similar observations were previously reported for the release of ametryn from polymer microspheres (made of poly-hydroxybutyrate or poly-hydroxybutyrate-valerate) (Grillo et al. 2011).

The nanoformulation showed in deionised water a very good colloidal stability, a narrow size distribution based on measured zeta potential (-41.7 ± 0.6 mV), hydrodynamic diameter with distribution centred on 293 ± 5 nm and a relatively low polydispersity index (0.3). When colloidal stability was monitored over time it maintained at least for 270 days (Grillo et al. 2012). No large aggregates were present when the size distribution was measured with EyeTech, since all the nanocarriers were smaller than the physical lower limit measureable by laser obscuration approach (i.e. 633 nm).

The standard background solution for regulatory protocols is a 0.01 M $CaCl_2$ background solution (sorption batch set-up). If salts are present, especially with divalent cations, aggregation by charge screening and/or cation bridging is strongly promoted (Liu et al., 2013). The nanoformulation built aggregates immediately when it was dispersed in 0.01 M $CaCl_2$. The absolute value of the zeta potential decreased (-8.8 ± 1.4 mV) and it was not possible to measure the hydrodynamic diameter by dynamic light scattering because of the settling of aggregates larger than 1 µm. Laser obscuration yielded an average size of 1.1 ± 0.7 µm.

To determine the release rate of the active ingredient from a nanocarrier, tests are carried out in a diluted system like deionised water, which creates infinite sink conditions. The rate differs when these experiments are carried out in soils due to changes in aggregation status and physicochemical degradation processes. This rates are crucial to define the fate of a nanopesticide but no accepted techniques are known yet. Ford et al. 2007 proposed to derive release rates from soil degradation experiments. The applicability of degradation experiments to this nanopesticide is further discussed in the “degradation kinetics” section.

3.2. Degradation kinetics

The degradation curves shown in Fig. 1 were fitted by three models, a simple first-order equation, a first-order mulitcompartment model and a first-order sequential model. The simple-first order kinetic model described the data very well and was used to derive DT50 values (time required to degrade 50 % of the initial pesticide concentration).

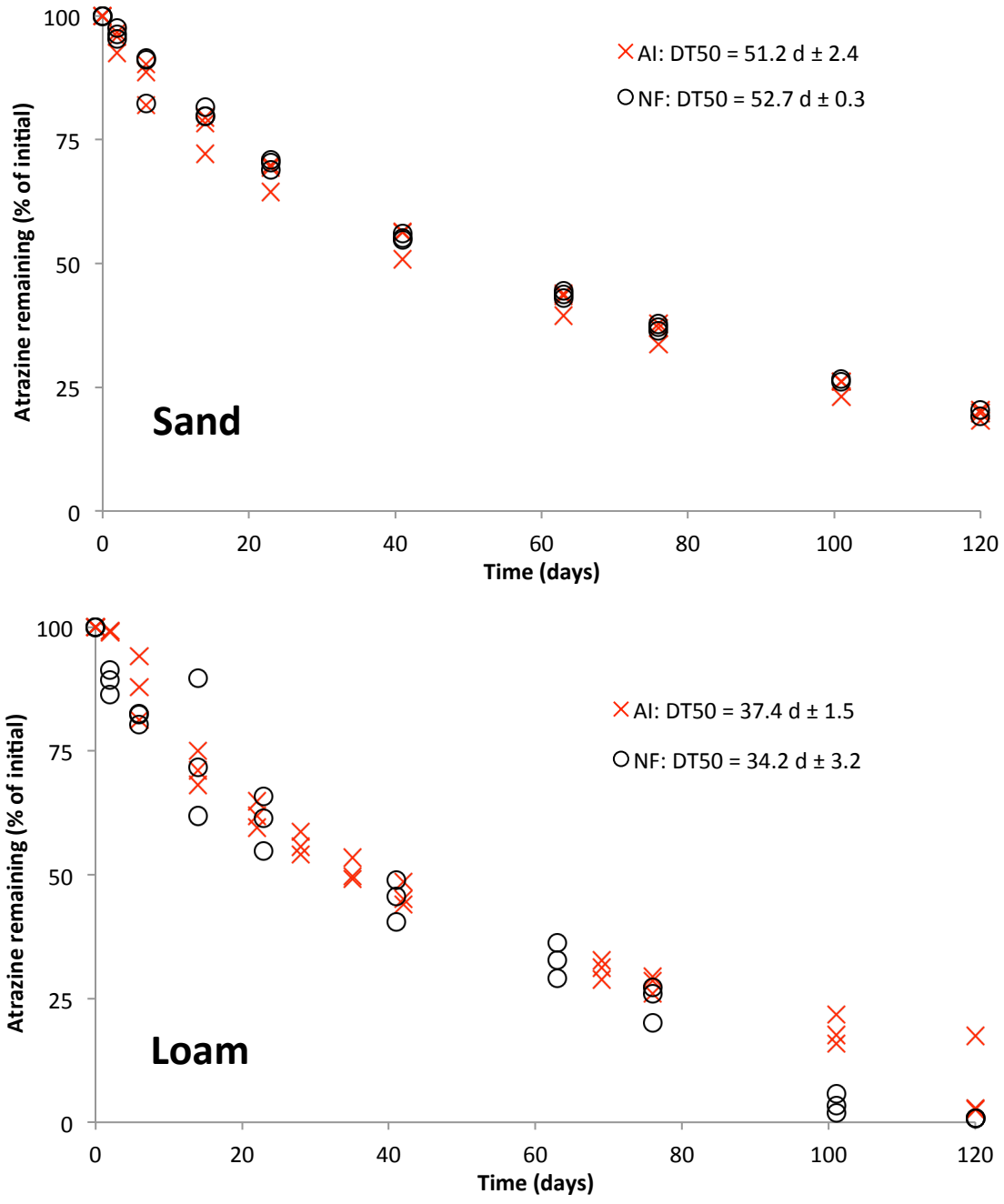


Fig. 1: Degradation curves for the sand and the loam with the technical grade standard (X) and for the nanoformulation (O)

DT50's in the sand are 51.2 ± 2.4 days for the active ingredient and 52.7 ± 0.3 days for the nanoformulation. DT50's in the loam are 37.4 ± 1.5 days for the active ingredient and 34.2 ± 3.2 days for the nanoformulation. The observed DT50's lie in the low range of reported DT50's. The range of reported DT50's is from 28 days to 150 days (http://sitem.herts.ac.uk).

DT50's (days ± stdev)	Active ingredient	Nanoformulation
Sand	51.2 ± 2.4	52.7 ± 0.3
Loam	37.4 ± 1.5	34.2 ± 3.2

Table 4: DT50's for the sand and the loam with active ingredient and nanoformulation

There is no significant difference in DT50's between active ingredient and nanoformulation in the sand and in the loam. This could be explained by release kinetics. After Grillo et al 2012 the release of active ingredient from the nanocapsule seemed to happen in two steps. In the first step nearly 60% of the active ingredient was released after 2 days and in the second 72% after 5 days. This would mean that the release kinetics of the nanoformulation are relatively fast compared to the degradation kinetics and the effect of release of active ingredient from the nanocapsule happens in a much shorter time span compared (e. g. 24 hours) to the degradation experiment.

Ford et al 2007 proposed to derive release rates for slow release from degradation experiments for a nanoformulation and the corresponding active ingredient. The release from the nanocarriers happens then in two steps. The first step, a lag phase or delay, before the nanocapsules are degraded, is attributed to the slow release. This slow release can be quantified with a two-compartment model. The applicability to nanopesticides has not been tested yet.

Like presented above, there was no significant difference between the nanoformulation and the active ingredient in the sand and the loam. This leads to the point, that there is no slow release or it is before the first sampling point before 24 hours.

If one compares the curves for the release kinetics from Grillo et al 2012, due to the release of 64 % in two days and no lag phase in this study, one can assume that this slow release could happen before the first sampling point after 24 hours or was not happening in this case at all.

Another explanation for the lack of difference between the formulations in the two soils could be, that the nanocapsules are easy to degrade, like it is reported in Eubeler et. al 2010. The microorganisms, which degrade the polymer, are very common in the environment. The PCL

is mainly degraded by the enzymes lipase and esterase. In addition the polymer is degraded faster in aerobic conditions than in anaerobic, like it is the case in this study. The microorganisms could had started to degrade the nanocapsules within hours so the active ingredient was set free and the microorganisms could choose between the nanocapsules and the active ingredient. It is also possible that they degraded the nanocapsules very fast completely and then the active ingredient, but this should have happened in a very short time span, approximately 20 hours. If it would be more than 24 hours no degradation of Atrazine after 24 hours could had been observed. So the release of Atrazine from the nanocapsules with a release rate of 2 days for 60 % of the total amount of Atrazine, like it is reported by Grillo et al 2012, shows that the degradation kinetic is faster then the release kinetic.

No lag phase was observed at this degradation experiment for the sand and the loam. This could be due to the preincubation period. It seems like the microorganisms got used to the future conditions in that time and were in some kind of a starting position. When the active ingredient and the nanoformulation were added to the soils, the microorganisms started to degrade it immediately.

But there is a significant difference between the DT50's in the sand and the loam. The higher organic carbon content of the loam and therefore higher microbial activity and faster degradation can explain this.

Links between degradation and sorption are important to assess for leaching processes because even a small correlation can cause big effects in the leaching process. Positive relationships have been observed between sorption coefficients Kd's and DT50's. This would mean that a higher sorption goes in hand with a slower degradation. Also some negative relations between this two were observed. In our case we have a negative relationship. When the sorption coefficient was higher the DT50's were less. The sorption coefficients were higher in the soil with the higher OC content. The OC content has opposite effects, higher sorption and higher bioactivity. In this study the sorption was to low to counterbalance the effect of degradation.

One should keep in mind that this protocol was not designed to investigate the fate of a nanopesticide. The applicability of this protocol is not very satisfying but gave good indications that there is a difference in the behaviour of a nanopesticide compared to its active ingredient in a relative much shorter timespan compared to its degradation kinetics.

Further experiments will be needed to determine, if a nanoformulation can delay a degradation compared to its active ingredient. This investigation showed that the protocol used for the degradation experiment (OECD guideline 307, 2002) is useful, but the sampling time should be modified according to the release rate of the nanoformulation (in this case for a shorter timespan, e.g. 72 h).

3.3. Analysis of the results between active ingredient and nanoformulation

Table 5: Sorption coefficients (Kd, L/kg ± standard deviation) for atrazine applied as pure technical grade (AI) or as nanoformulation (NF)

		Batch			Centrifugation	
		Non-filtered		Filtered	1 day	7 days
		$CaCl_2$	DI water	$CaCl_2$		
Sand	AI	0.32 ± 0.03	0.30 ± 0.03	0.31 ± 0.04	0.45 ± 0.01	0.61 ± 0.01
	NF	0.59 ± 0.01	0.65 ± 0.03	0.57 ± 0.03	0.54 ± 0.00	0.60 ± 0.02
Loam	AI	1.43 ± 0.02	1.38 ± 0.08	1.46 ± 0.05	0.60 ± 0.25	0.82 ± 0.13
	NF	1.96 ± 0.04	2.01 ± 0.02	1.94 ± 0.02	0.84 ± 0.03	0.98 ± 0.35

Measurements were performed with the batch method (with 0.01 M $CaCl_2$ or deionized water background, with or without filtration) and with the centrifugation method (after 1 and 7 days of incubation)

Table 5 shows the sorption coefficients (Kd, L/kg) achieved by the batch and centrifugation technique. For the batch method, described in the OECD guidelines (2000, 0.01 M $CaCl_2$ background, no filtration) no significant difference in Kd's was observed between the tests carried out in $CaCl_2$ solution and deionized water. The nanoformulation forms aggregates in the $CaCl_2$ solution but not in deionized water. In real nature conditions the nanoformulation is more likely to form aggregates (e.g. with natural colloids). Therefore the batch experiment was carried out in two different background solutions. In this study, there was no difference between the two background solutions, showing that the difference in aggregation status did not influence sorption measurements.

Kd's were always significantly higher for the nanoformulation than for the active ingredient in both soils ($p<0.001$). Due to the natural colloids and higher ionic strength in the soil suspensions, aggregation of the nanocapsules occurred, regardless of the background solution (0.01 M $CaCl_2$ or deionized water). These nanocarriers and soil colloids are hard to differentiate by currently available techniques because of similar chemical compositions and structure. Therefore it was not possible to measure the size and surface charge of the nanoformulation in the soil suspension. The nanoformulation much likely formed aggregates and probably settled down during the centrifugation process implicating that higher Kd's does

not directly mean higher sorption due to the interaction of nanoformulation with soil particles. Further consequences about potential transport should therefore be analyzed carefully.
At the end of the batch experiment the soil solutions were filtered. Filtering the soil suspension after the centrifugation step had no effect. In table 2 the results are shown only for the $CaCl_2$ solutions. They suggest that the nanoformulation formed aggregates and settled down during the centrifugation step so that no nanoformulation was left in the supernatant.

When measuring sorption with the centrifugation method Kd's for the active ingredient were again lower than for the nanoformulation after one day of incubation. This could be explained by higher sorption of the nanoformulation or the retention of aggregated nanocarriers at the filter beneath the soil sample.
After seven days no significant difference between the active ingredient and the nanoformulation was observed, much probably due to the total release of active ingredient from the nanocarriers. Grillo et al. (2012) showed that it takes 5 days to release 72 % of active ingredient from the nanocapsules. This timespan matches good together with the release observed here.
After the University of Hertfordshire (2013) sorption isotherms of atrazine to soils are typically very close to being linear. Non-linear isotherms are therefore unlikely to be the reason for discrepancies observed between the batch and centrifugation method.
These results suggest that the retention of the releasing atrazine by the nanoformulation will not exceed seven days. But a slight delay in the release can significantly influence transport patterns, especially in rainfall situations after pesticide application.
This study showed that the centrifugation method is a good protocol to investigate the sorption of a pesticide and to derive sorption coefficients. The advantage of this method is its realistic soil to solution ratio while the Batch Equilibrium Method is carried out in a solution and is useful to assess the period during which a nanoformulation can effect the fate of an active ingredient.

3.4. Comparison of the differences between soils and methods

Atrazine has a small dissociation constant (pKa=1.7) (University of Hertfordshire 2013) and therefore it is much unlikely for the soil pH-value to affect the sorption behavior of atrazine.

Normalizing the sorption coefficients with the OC content, clay content and cation exchange capacity (CEC) suggest that the difference in sorption between the two soils occurs because of the different OC content.

Differences between the batch and the centrifugation method are probably due to the shaking step in the batch method. The shaking process scatters the soil particles in the solution and more surface for sorption is available (Yazgan et al. 2005). Comparisons between the batch and the centrifugation technique are quite rare, and the results seem to depend on the soil-pesticide combination. For example, Kah and Brown (2007) showed, that although the batch technique gave significantly higher values of Kd than the centrifugation method for the more strongly sorbed molecules in the more sorptive soils, it tended to give lower sorption coefficients compared to the centrifugation method when sorption was weaker. In this study the same trend was observed. The loam showed higher Kd's by batch than by centrifugation method after 1 day ($p<0.01$) and the sand lower Kd's by batch than by centrifugation method. After Harper (1994) sorption coefficients increase with decreasing water content. This could be explained with a reduced competition by water molecules for sorption sites and an influence of solubility as the herbicide solution becomes more concentrated. The high soil to solution ratio in the centrifugation experiment (about six for the sand) could have caused a precipitation of atrazine due to its higher concentration in the soil solution. The concentration of atrazine in the soil solution would be the same order of magnitude like the solubility of atrazine in water (35 mg/L) (University of Hertfordshire 2013) if all the atrazine molecules would stay in the soil solution and dissolve in the total amount of water in the soil. Further, the soils were incubated at 4 °C additionally decreasing solubility but increasing any potential for precipitation at the soil particle surface. The sand had a lower moisture content and a weaker sorption compared to the loam. So the probability for precipitation was higher for the sand and could explain higher Kd's measured by centrifugation than by batch method.

Sorption of atrazine was stronger after seven days than after one day ($p<0.001$ in the sand for AI). This leads to the assumption of a time-dependent sorption like it was also shown by Mudhoo and Garg (2011).

The importance for suitable protocols to assess a time-dependent sorption and for higher tier assessment procedures is growing. The centrifugation and the batch method are good candidates. The batch technique can overrate the amount and rate of a time-dependent sorption but has the advantage to be easy to reproduce and to be more consistent than the centrifugation method. The centrifugation method has a number of advantages over the batch method like the realistic soil to solution ratio and the ability to assess the release of an active ingredient of its nanocarrier while the dilution and shaking at the batch technique can

be unfavorable for the structure of the nanocarriers and its interactions with the active ingredient.

3.5. Conclusion and consequences for the environmental exposure assessment of nanopesticides

For the first time regulatory protocols were applied to investigate the fate of a nanopesticide compared to its active ingredient in two different soils.

At the degradation experiment no difference between the nanoformulation and the active ingredient was observed. That suspects a relatively fast release of atrazine from its nanocarrier to the degradation kinetics and/or the nanocapsules could have been biotically or abiotically degraded. More precise investigations on the bioavailability and stability of the nanopesticide should be carried out in the future for realistic release profiles to compare the nanocarrier to its active ingredient (for example the degradation test could be carried out in less days with the focus on the first days and with more sampling units).

The sorption coefficients were derived by the classical batch set up and the centrifugation method whereby the latter one represents realistic soil to solution ratios.

In both cases higher Kd's were observed for the nanoformulation, but the results should be examined carefully. The protocols must be modulated for the nanopesticides since these protocols are designed for organic solutes and not for nanocarriers. Differences in Kd's should be considered carefully as they not directly represent retention in the soil. Another important point is the aggregation status of the nanopesticides. During their lifecycle nanocarriers dwell in different media (e.g. storage, tank preparation prior to spraying or accidental discharge into surface water bodies). Differences in the water chemistry, for instance ionic composition, pH or presence of dissolved organic matter, can affect the aggregation status with consequences on the behaviour in the environment. Therefore the aggregation properties should be determined in expected water chemistry environments.

The centrifugation method, which is useful to derive sorption coefficients at realistic soil to solution ratios, turned out to be a very beneficial method to study the timespan in which the nanocarriers have an effect on the active ingredient. On the other side, this method provides sparse information about the background processes and mechanisms but has the advantage to be easy combined with degradation experiments. This method is therefore useful to determine nanopesticides with a relatively short lifespan after application in the field and for which current environmental risk assessment procedures may be sufficiently protective.

The mobile and persistent active ingredient atrazine was released from its nanocarrier within some days. Sorption and degradation parameters remained in the range of previously

reported values although significant differences were observed between the active ingredient and the nanoformulation. Combinations of active ingredients and nanocarriers representing a range of scenarios for possible impact on transport and degradation processes like mobile active ingredient on immobile nanocarriers and slow release of poorly persistent active ingredients, should be investigated in future assessments.

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CURRICULUM VITAE

BILDUNGSWEG

Okt. 2012 - Feb. 2015	Masterstudium der Erdwissenschaften Universität Wien Masterarbeit: Fate of Nanopesticides in Soil Betreuer: Univ. Prof. Dr. Thilo Hofmann, Dr.Melanie Kah
Okt. 2008 – Juni 2012	Bachelorstudium der Erdwissenschaften Universität Wien Bachelorarbeit: Kinematik der Freiner Störung westlich von Naßwald, Betreuer: Dr. Kurt Decker
Okt. 2006 – Juni 2008	Vorbereitung für die Studienberechtigungsprüfung für das Studium der Erdwissenschaften
Jän. 2001 – Sept. 2003	Lehre als Werkzeugmaschineur mit Lehrabschluss Fa. Schoeller-Bleckmann in Ternitz
Sept. 1996 – Juni 2000	Htl Automatisierungstechnik Wiener Neustadt Zwei Jahre abgeschlossen
Sept. 1992 – Juni 1996	Sporthauptschule Wiener Neustadt Moderner Fünfkampf
Sept. 1988 – Juni 1992	Volksschule Pestalozzi Wiener Neustadt

BERUFLICHER WERDEGANG

Okt. 2013 – April 2014	Aufsicht in der Kletterhalle Edelweiss, 1010 Wien, geringfügige Anstellung
Sept. 2013 – Aug. 2014	Bildungskarenz
April 2012 – Aug. 2013	Assistent des Leiters der Kletterhalle Edelweiss, 1010 Wien, administrative Tätigkeiten im Ausmaß von 20 Std./Woche

Okt. 2011 – März 2012	Verkäufer, Fa. Northland, 20 Std./Woche, 1210 Wien
April 2009 – Okt. 2009	Mathematik-Nachhilfelehrer, Fa. Team plus, 1050 Wien
Juni 2006 – Juli 2006	Werkzeugmacher, Fa. Ing. Anton Kornfeld, Günselsdorf
Nov. 2005 – Mai 2006	Werkzeugmaschineur, Fa. Schoeller-Bleckmann, Ternitz
Okt. 2005 – Nov. 2005	Werkzeugmaschineur, Fa. Dynacast, Wiener Neustadt
Sept. 2004 – Sept. 2005	Zivildienst bei der Rettung in Wiener Neustadt, Ausbildung zum Rettungsanitäter
Okt. 2003 – Aug. 2004	Werkzeugmaschineur, Fa. Schoeller-Bleckmann, Ternitz

PRAKTIKA

Mai 2014 – Aug. 2014	Fa. Geoexpert, Wien Kartierung v. Gefahrenzonenplänen, Steinschlagrisikostudien, Gutachtenerstellung
Aug. 2011 – Sept. 2011	Fa. Terra Umwelttechnik Wien Pumpversuche, Bohrkernaufnahmen
Juli 2010 – Aug. 2010	Universität Wien, Mitarbeiter bei einer archäologischen Ausgrabung im südlichen Wiener Becken
Juli 1998	JLC-Chemie Wiener Neustadt
Juli 1997	IPEG Förderanlagen

PUBLIKATION

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Analysing the fate of nanopesticides in soil and the applicability of regulatory protocols using a polymer-based nanoformulation of atrazine

WEITERE KENNTNISSE

Sprachkenntnisse	Englisch gut in Wort und Schrift Polnisch, Muttersprache
EDV-Kenntnisse	Microsoft Office Grundkenntnisse in AutoCAD Grundkenntnisse in ArcGIS Drill & Log RockFall 6.1
Weitere Qualifikationen	Führerschein Klasse B Kranschein für Flurkräne bis 5 Tonnen

HOBBY' S

Klettern

Rennradfahren

Laufen