UNIVERSITY OF NOVA GORICA
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# ENVIRONMENTAL STABILITY AND TOXICITY ASSESSMENT OF CHLORANTRANILIPROLE AND ITS DERIVATIVES

DISSERTATION

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# TABLE OF CONTENTS

# ABSTRACT

This study investigated the stability and in particular, the photostability of a newly developed insecticide chlorantraniliprole (CAP, 3-bromo-N-[4-chloro-2-methyl-6-(methylcarbamoyl)phenyl]-1-(3-chloro-2-pyridine-2-yl)-1H-pyrazole-5-carboxamide) in water, as well as its toxicity to selected non-target organisms. A stability study in acetonitrile-water media with different pH values showed that CAP is not stable in basic solutions. In acetonitrile and tris buffer solution of pH 9 (1:4), the concentration of CAP (20.6 μM) dropped by 27% after three weeks of incubation in the dark at room temperature. Further studies revealed that CAP in such conditions degrades to one specific degradation product **H** (2-(3-bromo-1-(3-chloropyridin-2-yl)-1H-pyrazol-5-yl)-6-chloro-3,8-dimethylquinazolin-4(3*H*)-one). CAP was also found to be photoactive. The half-life of CAP at an initial concentration of 17 μM in acetonitrile-tap water (1:4) continuously irradiated in a solar simulator at 750 W/m$^2$ was 2.12 days. Three main photodegradation products (**A** (2-((2-bromo-4H-pyrazolo[1,5-d]pyrido[3,2-b][1,4]oxazin-4-ylidene)amino)-5-chloro-N,3-dimethylbenzamide), **B** (2-(3-bromo-1-(3-hydroxypyridin-2-yl)-1H-pyrazol-5-yl)-6-chloro-3,8-dimethylquinazolin-4(3H)-one) and **C** (2-(3-bromo-1H-pyrazol-5-yl)-6-chloro-3,8-dimethylquinazolin-4(3H)-one)) of CAP were identified and characterized with several spectroscopic techniques. The difference in the course of their formation was dependent on media composition. In deionized water with a slightly acidic pH of 6.1 the degradation of CAP halted at its first transformation product **A**, while in tap water at pH 8 the degradation proceeded to compound **B**, and subsequently to the main degradation compound **C**. This shows that the transformation of CAP in water is a result of chemical and photochemical reactions, greatly influenced by the pH and the bases present in the water.

Our toxicity tests showed that CAP is highly toxic to the water flea *Daphnia magna*, with acute and chronic $LC_{50}$s of 9.35 μg/L and 3.71 μg/L, respectively. No effect was observed on the reproduction of the daphnids. CAP was highly toxic also to springtail *Folsomia candida*, with an $LC_{50}$ for effects on survival of 5.14 μg/g dw and an $EC_{50}$ for effects on reproduction of 0.20 μg/g dw, after 28 days exposure in natural Lufa 2.2 soil. A toxicity assessment study on *F. candida* using soils with different organic matter contents revealed that CAP is less toxic in high organic soils

compared to the low organic soils. An avoidance test with *F. candida* suggests that CAP is affecting the animals in a very prompt way, making their locomotive ability to dysfunction. CAP was not toxic to the survival and reproduction of the enchytraeid *Enchytraeus crypticus*, the oribatid mite *Oppia nitens* and the benthic worm *Lumbriculus variegatus*, as well as on the survival, consumption rate and body mass of the isopod *Porcellio scaber* even at the CAP concentrations as high as 800-1000 µg CAP/g dw. CAP degradation products **B**, tested at nominal concentrations up to 1 mg/L, and **H** (c = 0.14 mg/L nominal) did not show any adverse effects on water flea *D. magna* and no effect of degradation product **H** (with maximal tested concentration 800 µg/g dw) was observed also on *L. variegatus*.

***Keywords:*** chlorantraniliprole, degradation, transformation products, toxicity, *Daphnia magna*, soil invertebrates

# POVZETEK

V sklopu disertacije smo raziskovali stabilnost novo razvitega insekticida klorantraniliprola (CAP, 3-bromo-N-[4-kloro-2-metil-6-(metilcarbamoil)fenil]-1-(3-kloro-2-piridin-2-il)-1H-pirazol-5-karboksamid) v vodi in njegovo strupenost na izbrane netarčne nevretenčarske organizme. Raziskava stabilnosti v acetonitril-vodnih medijih z različnimi pH vrednostmi je pokazala, da CAP ni stabilen v bazičnih raztopinah. V acetonitrilu in pH 9 tris pufru (1:4) je koncetracija CAPa (20.6 μM) po treh tednih inkubiranja v temi pri sobni temperaturi padla za 27%. Nadaljnje študije so pokazale, da se CAP v takšnih pogojih razgradi v en specifičen razgradni produkt, spojino **H** (2-(3-bromo-1-(3-kloropiridin-2-il)-1H-pirazol-5-il)-6-kloro-3,8-dimethilkinazolin-4(3*H*)-on). Ugotovili smo, da se CAP v prisotnosti svetlobe razgrajuje. Njegova razpolovna doba v acetonitril-pitni vodi (1:4, 17 μM) je po neprekinjenem obsevanju v sončnem simulatorju intenzitete 750 W/m$^2$ znašala 2.12 dni. Identificirali in okarakterizirali smo tri glavne fotorazgradne produkte: spojino **A** (2-((2-bromo-4H-pirazolo[1,5-d]pirido[3,2-b][1,4]oksazin-4-iliden)amino)-5-kloro-N-3-dimetilbenzamid), spojino **B** (2-(3-bromo-1-(3-hidroksipiridin-2-il)-1H-pirazol-5-il)-6-kloro-3,8-dimetilkinazolin-4(3H)-on) in spojino **C** (2-(3-bromo-1H-pirazol-5-il)-6-kloro-3,8-dimetilkinazolin-4(3H)-on)). Pokazali smo, da se ti razgradni produkti tvorijo različno glede na lastnosti medija. V deionizirani vodi z rahlo kislim pH (6.1) je razgradnja CAPa potekla le do prvega razgradnega produkta, spojine **A**. V pitni vodi s pH 8 pa se je razgradna pot CAPa nadaljevala v spojino **B** in nadalje v glavno spojino **C**. To kaže, da je razgradnja CAPa v vodi posledica kemijskih in fotokemijskih reakcij, močno odvisnih od pH in baz, prisotnih v vodi.

Strupenostni testi so pokazali, da je CAP zelo strupen za vodno bolho *Daphnia magna*, z akutno $LC_{50}$ vrednostjo 9.35 μg/L in kroničnim $LC_{50}$ 3.71 μg/L. Nobenega strupenostnega učinka ni bilo opaženega pri razmnoževanju vodnih bolh. CAP se je izkazal kot zelo strupen tudi za skakače *Folsomia candida*, z $LC_{50}$ vrednostjo 5.14 $\mu g/g_{suhe\ zemlje}$ in $EC_{50}$ vrednostjo za reprodukcijo 0.20 $\mu g/g_{suhe\ zemlje}$ po 28-dnevni izpostavljenosti CAPu v Lufa 2.2 zemlji. Študija strupenosti CAPa na skakače *F. candida* v zemlji z različnimi vsebnostmi organske snovi je pokazala, da ima CAP manjši strupenostni učinek v zemlji z večjim odstotkom organske snovi v primerjavi

z zemljo, kjer je ta vsebnost organskih snovi manjša. Test izogibanja s skakači *F. candida* pa nakazuje, da CAP vpliva na gibalne sposobnosti izpostavljenih živali na zelo hiter način. Strupenostnih učinkov CAPa na preživetje in razmnoževanje pri črvih *Enchytraeus crypticus*, oribatidnih pršicah *Oppia nitens* in bentičnih črvih *Lumbriculus variegatus*, kot tudi ne na preživetje, stopnjo porabe hrane in telesno maso kopenskih enakonožcev *Porcellio scaber,* tudi ob izpostavljenosti zelo visokim koncetracijam CAPa (800-1000 µg CAP/$g_{suhe\ zemlje}$) nismo zaznali. Prav tako nismo zaznali nobenih negativnih učinkov na vodno bolho *D. magna* v primerih izpostavitve le-te razgradnim produktom CAPa, spojini **B**, testirana pri koncetraciji do 1 mg/L in spojini **H** pri koncentraciji 0.14 mg/L. Spojina **H** prav tako ni bila strupena za bentične črve *L. variegatus*, pri maksimalni testirani koncetraciji 800 µg/$g_{suhe\ zemlje}$.

***Ključne besede:*** klorantraniliprol, razgradnja, razgradni produkti, strupenost, *Daphnia magna*, zemeljski nevretenčarji.

# LIST OF TABLES

# LIST OF FIGURES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| **a.i.** | active ingredient |
| **ACR** | acute to chronic ratio |
| **AOP** | adverse outcome pathway |
| **APVMA** | Australian Pesticides and Veterinary Medicines Authority |
| **BWC** | body weight change |
| **CAP** | chlorantraniliprole |
| **CDOM** | colored dissolved organic matter |
| **CEC** | cation exchange capacity |
| **CI** | confidence interval |
| **CR** | consumption rate |
| **CRO** | cumulative reproductive output |
| **CV** | coefficient of variation |
| **DAD** | diode array detector |
| **dd$H_2O$** | double deionized water |
| **DMSO** | dimethyl sulfoxide |
| **DOC** | dissolved organic carbon |
| **DOM** | dissolved organic matter |
| **$Dt_{50}$** | degradation half life |
| **dw** | dry weight |
| **$EC_{10}$** | 10% effective concentration |
| **$EC_{50}$** | median effective concentration |
| **EFSA** | European Food Safety Authority |
| **EPA** | Environmental Protection Agency |
| **ESI** | electro spray ionization |
| **HA** | humic acids |
| **HPLC** | high-performance liquid chromatography |
| **HRMS** | high-resolution mass spectrometry |
| **IR** | infrared |
| **ISO** | International Organisation for Standardization |
| **IUPAC** | International Union of Pure and Applied Chemistry |
| **Kd** | distribution coefficient |
| **Koc** | soil organic carbon-water partition coefficient |

| | |
|---|---|
| **Kow** | octanol-water partition coefficient |
| **LC/MS/MS** | liquid chromatography coupled with mass spectrometry |
| **$LC_{50}$** | median lethal concentration |
| ***m/z*** | mass to charge ratio |
| **NMR** | nuclear magnetic resonance |
| **OECD** | Organisation for Economic Co-operation and Development |
| **OM** | organic matter |
| **pKa** | acid dissociation constant |
| **RyR** | ryanodine receptors |
| **SAM** | standard addition method |
| **TN** | total nitrogen |
| **TOC** | total organic carbon |
| **TOF MS** | time of flight mass spectrometry |
| **$t_R$** | retention time |
| **UV** | ultraviolet light |
| **VIS** | visible light |
| **WHC** | water holding capacity |

# 1 INTRODUCTION

Modern agricultural practice inevitably involves the large scale application of pesticides for crop protection. Because of the environmental hazard of pesticides, such as the organochlorinated and organophosphate insecticides, used in the past, there is an ongoing need to develop new, less hazardous and more selective insecticides. One of these new insecticides is chlorantraniliprole (CAP), belonging to the anthranilic diamides, designed and marketed by DuPont. Positive experiences on pest control made CAP widely used. Its formulated products became registered in many agricultural areas around the globe (Bassi et al., 2009) and are allowed for treating an increasing number of crop species (DuPont, 2011). However, although CAP and its formulated products are fairly new, only few studies on fate of CAP in the environment and ecotoxicological risks are available.

Once pesticides are released into the environment, their movement across different compartments and transformations that they are compelled to can hardly be controlled. This also counts for the risks they may impose to non-target species. For this reason, understanding their behavior and impacts is imperative before these compounds are widely used on the fields. Pesticides in the environment can be degraded biologically or by chemical reactions. Depending on the source initiating the transformation, physical-chemical properties of the compound and conditions in the environment, different parts of the insecticide molecule can be altered or broken down. Due to that, several degradation pathways can exist for a single compound (Roberts 1998, Roberts and Hutson, 1999). When the insecticide is applied to arable land, it is expected that its concentration is decreased after a certain period of time; firstly due to its dissipation and secondly, due to its transformation. However, before the insecticide is completely mineralized (completely degraded to inorganic compounds) it is first transformed in a cascade of different transformation products. These transformation products can possess properties that are very different from those of the parent compounds; as a consequence they can differ in the mobility, persistence and toxicity to target and non-target species (Boxall et al., 2004). Because of this, it is essential to identify the main degradation products, characterize

their properties, perform model or practical experiments to understand their behavior and toxicity and include them in environmental monitoring.

In this study we focused on the stability of the insecticide chlorantraniliprole (CAP) in different aqueous solutions and we characterized its main chemically- and photo-induced degradation products. For some pesticides, the chemical breakdown initiated by the exposure to light is a prominent way of degradation in water. As there is a considerable lack of information on its photostability, a wide range of experiments was performed with CAP using different sources of light. In the photo-induced degradation pathway, three main degradation products were characterized and their course of formation was studied in detail.

The second part of our work was dedicated to ecotoxicological studies of CAP. One of the most important features of CAP is its high toxicity to a wide range of insect pests (Cordova 2006, Lahm et al. 2007, 2009, Sattelle et al. 2008). The death of an insect occurs due to the permanent muscle contraction which is caused by binding of CAP to the insect ryanodine receptors that regulate the release of $Ca^{2+}$ from the intracellular calcium deposit stores (Cordova 2006, 2007, Lahm et al. 2007, 2009, Sattelle et al. 2008). Comparing its affinity to insect and mammalian ryanodine receptors revealed that CAP features a remarkably low toxicity to mammals. CAP was shown to be 300-fold less potent to mammalian ryanodine receptors compared to the insect ones (Lahm et al., 2007). Generally, CAP was characterized to have very little toxicity to terrestrial and aquatic vertebrates, but was found to be toxic to selected terrestrial and aquatic invertebrates (EPA, 2008).

The high selectivity and toxicity of CAP towards insects raised concerns about its effect on non-target insects, but scientific studies on its toxicity to non-target organisms are scarce. Some studies (for example Brugger et al. 2010, Larson et al. 2012, Gradish et al. 2010, Dinter et al. 2009) indicate that due to no or very low adverse effects to non-target terrestrial species, CAP would be a suitable tool for integrated pest management. On the other hand, CAP appeared to be highly toxic ($LC_{50}$ 951 µg/L) to non-target crayfish in an acute (96 h) toxicity test (Barbee et al., 2010). We aimed to continue on this by investigating the ecotoxicity of CAP to several aquatic and soil invertebrates. The species tested included *Daphnia magna, Lumbriculus variegatus, Folsomia candida, Porcellio scaber, Oppia nitens* and *Enchytraeus crypticus.*

Overall, our research was dedicated to a holistic study of CAP degradation in the aquatic environment and its ecotoxicological risk to several non-target aquatic and terrestrial invertebrate species.

# 2 THEORETICAL BACKGROUND

## 2.1 Introduction to pesticides

A pesticide can be defined as a chemical or mixture of chemicals used to kill, attract, repel, regulate or interrupt the growth and mating of pests, or to regulate plant growth (Randall et al., 2007).

There are several ways to classify pesticides (Saravi and Shokrzadeh. 2011), however most often we classify pesticides according to the type of the pest they target. According to this classification, one can distinguish insecticides (targeting insects and other arthropods), herbicides (controlling weeds and other unwanted plants), fungicides (killing fungi), avicides (controlling pest birds), bacteriocides (controlling bacteria), disinfectants (targeting microorganisms), miticides (targeting mites), rodenticides (controlling rodents), nematicides (killing nematodes), molluscicides (controlling snails and slugs), predacides (control predatory vertebrates), piscicides (control pest fish), repellents (repelling insects, related invertebrates, birds and mammals), defoliants (for defoliation of plants), desiccants (used for drying plant tissues) and growth regulators (substances that alter the growth or development of a plant or animal) (Randall et al., 2007).

The use of pesticides was found to be economically favorable. It was estimated that pesticide use in US arable systems returns about $4 per $1 invested for pest control (Pimentel, 2005). However, the use of pesticides imposes external costs that are not reflected in the market. These external costs are carried by society (effects on human health) and the environment (environmental degradation).

Most of the times, pesticides are applied to agricultural fields by spraying in form of a formulation (Randall et al., 2007), which consists of a certain percentage of active ingredient together with several other, often inert, ingredients. The latter ingredients have different functions: they can serve as a carrier of the active ingredient to facilitate easier application by enabling dissolution or dispersion in water, modify surface activity or can act as stabilizing agent and pesticide activity enhancers, for example (Randall et al., 2007). However, the type of formulation usually is designed based on the physical-chemical properties of the active ingredient, and aims at increasing the efficacy of the pesticide, but also to mitigate unwanted effects the

pesticide can cause to the environment (Katagi, 2008). On the other hand, as summarized by Katagi (2008), the surfactants that are quite abundantly used in pesticide formulations may also have negative biological effects on non-target organisms in the environment. Therefore, apart from the active ingredient, also adjuvants contained in the formulated products are under investigation to understand their environmental fate and ecotoxicological risks.

## 2.2 Pesticides in the environment

When pesticides in the form of formulated products enter the environment after being applied to the crop fields, their physical-chemical properties, the properties of soil and sediment, the way of application, climate, geographical area and presence and dimensions of the water bodies nearby fields further dictate their fate (Katagi, 2008). They can degrade chemically or biologically, dissipate via spray drift, volatilization, run-off, and leach to deeper soil layers, they can be taken up by the crop or non-target organisms or undergo other processes (Randall et al., 2007). All these processes are summarized in the Figure 1.

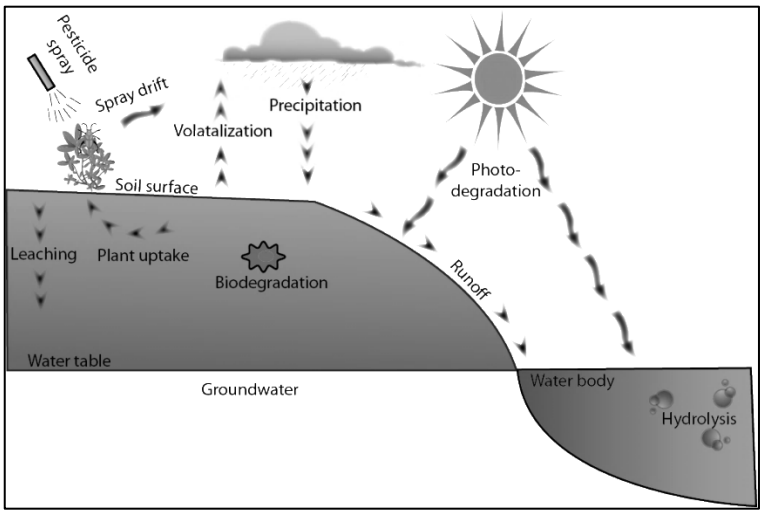



***Figure 1: Degradation and dissipation of pesticides in the environment.***
***Adapted from Randall et al. (2007).***

Pesticide regulation authorities, such as the United States Environmental Protection Agency (EPA), are responsible for reviewing laboratory and field studies to

determine the environmental fate of pesticides. These studies include the mobility and degradation of pesticides with the identification of transformation products and their accumulation in the environment (EPA, 2014). For a pesticide to be registered, it also has to meet ecological safety criteria. Toxicity data are, according to EPA's requirements, provided by the producer of the pesticide and are further on reviewed and evaluated for its risk by EPA itself (EPA, 2014). In Europe, national authorities are acting in the same way, while registration of pesticides is increasingly coordinated at the European Union level with active involvement of the European Food Safety Agency (EFSA).

Although the information that governmental agencies require from the pesticide manufacturers is nowadays extensive, but the description of the studies found in the reports sometimes is rather limited. In addition, assessment of pesticide fate in the environment often is based on modeling (EPA, 2012). This calls for independent research addressing the environmental fate and risks assessment of the new emerged pesticides, such is chlorantraniliprole.

## 2.3 Key pesticide physical-chemical properties affecting their environmental fate

The way pesticides behave in the environment is ruled predominantly by their physical-chemical properties and the properties of the environment (Katagi, 2006). The key physical-chemical properties determining the fate of pesticides are water solubility, octanol-water partition coefficient, soil adsorption coefficient, vapor pressure, Henry's law constant and molecular structure, which determines their susceptibility to transformation or degradation and transportation processes (Zacharia, 2011).

- *Water solubility* is of great importance as it is affecting the mobility, reactions and degradation pathways of chemicals. Highly polar and therefore well water-soluble compounds will not accumulate in the soil and are prone to degrade via hydrolysis, which is a favored reaction in water (Zacharia, 2011).

- *Vapor pressure* determines the dissipation of the pesticide into the air due to vapor drift, which consequently causes air pollution. Pesticides with a high vapor pressure therefore must be handled with special care to prevent losses and dispersion into the atmosphere (Zacharia, 2011).

- *Henry's law constant* is the ratio of the chemical concentrations in air and in water and it therefore expresses the tendency of the chemical to volatilize from water into the air (Zacharia, 2011). Chemicals with a high Henry's law constant will easily volatilize from water into air and can be distributed over large areas. As for pesticides with high vapor pressure, they need to be handled in such a way to prevent their vapors to escape into the atmosphere. Chemicals with a low Henry's law constant tend to persist in water and may adsorb to soil (REACH, 2008).

- *Octanol-water partition coefficient (Kow/Log Kow)* is defined as the ratio of the chemical concentrations in n-octanol and water, when these phases are in equilibrium, at a specified temperature (Pontolillo and Eganhouse, 2001) (Equation 1).

$$Kow = \frac{c_{octanol}}{c_{water}}, \quad \text{(eq. 1)},$$

where $Kow$ stands for octanol-water partition coefficient, $c_{octanol}$ stands for the concentration of the compound in the octanol phase (mg/L) and $c_{water}$ is its concentration in the water phase (mg/L), at equilibrium.

- *Soil adsorption coefficient* is determining the tendency of pesticides to adsorb to soils and sediments. Especially less polar pesticides tend to bind to the non-polar organic matter fraction in soils and sediments, which is determining their further environmental fate and degradation pathways (Zacharia, 2011). Soil adsorption can be expressed as a distribution coefficient ($Kd$ in mL/g, Equation 2), which is defined as the ratio of

compound concentrations in soil and water, without considering the fraction of the organic matter the soil contains (Zacharia, 2011):

$$Kd = \frac{c_{soil}}{c_{water}}, \quad \text{(eq. 2)},$$

where $c_{soil}$ is the concentration of a compound in soil (mg/g) and $c_{water}$ is its concentration in water (mg/mL).

The sorption isotherm, describing the relationship between $c_{soil}$ and $c_{water}$ over a range of concentrations generally is linear in case of low concentrations. At higher concentrations, however, either the soil or water phase may become saturated with compound. Most common sorption isotherms applied to cope with this non-linearity are the Freundlich and Langmuir isotherms (Goldberg, 2005).

Since it has been shown that the organic matter fraction of the soil plays a main role in determining the sorption behavior of organic compounds (Delle Site, 2001), the distribution coefficient ($Kd$) is often related to the organic fraction of the soil and expressed as soil organic carbon-water partitioning coefficient, $Koc$ (mL/g, Equation 3) (Zacharia, 2011):

$$Koc = \frac{Kd*100}{\%\, organic\ carbon}, \quad \text{(eq. 3)},$$

where $Kd$ is the distribution coefficient (see Equation 2). The $Koc$ values for many chemicals are reasonably well predicted from the octanol-water partition coefficient ($Kow$) (see for example Brown and Flagg, 1981).

## 2.4 Pesticide degradation

Pesticide degradation can occur by chemical reactions (photolysis, hydrolysis, reduction and oxidation) or biologically by microbial action (Andreu and Picó, 2004). The chemical breakdown of pesticides usually occurs in water and in the atmosphere, while the biological degradation processes are most prominent in soil (Zacharia, 2011). The degradation pathways of a pesticide are often complex, involving several reaction steps, before it is completely mineralized (Roberts 1998, Roberts and Hutson 1999). With each transformation step, a new transformation

product is formed, possessing unique properties. Compared to the parent compound, degradation products can differ in persistence, mobility, as well as toxicity (Sinclair and Boxall, 2003). Several evidences indicate that pesticide transformation products can be even more toxic to non-target organisms than their parent compounds (see for example Belfroid et al., 1998 and Sinclair and Boxall, 2003). Therefore there is an increasing concern regarding the formation of pesticide transformation products and these products are often being studied on their own to assess their environmental stability and toxicity (Žabar, 2012).

In the following subchapters the degradation processes of organic chemicals are described. Since our study was focused mainly on the photodegradation of CAP in water, this degradation route is described in more detail.

### 2.4.1 Hydrolysis of pesticides

One of the most important pathways of pesticide degradation in water is via hydrolysis. An extensive review on this topic was published by Katagi (2002), who concluded that, generally, degradation driven by hydrolysis is following first order kinetics and can be catalyzed by the acids or bases present in surface water. Water pH was found to be an important factor for the chemical degradation of some pesticides. Pyrethroids and carbamates, for example, were found to be stable under neutral environmental conditions, but hydrolyzed when the pH increased (Katagi, 2002). On the other hand, a drop of pH to acidic values increases the hydrolysis rate of triazine and sulfonylurea herbicides (Katagi, 2002). Apart from the pH, factors influencing pesticide hydrolysis include dissolved organic matter, clay minerals, metal ions and oxides (Katagi, 2002).

### 2.4.2 Photodegradation of pesticides

Light-induced degradation is a very important naturally occurring process of pesticide elimination in the environment. Photodegradation also is adapted widely as

a technique for fast and efficient remediation of waste waters in so-called advanced oxidation processes (Burrows et al., 2002). The photolysis of organic compounds in this process is induced by using light of the proper wavelength and by applying photosensitizers and techniques that generate reactive species that in turn react with the pollutants present in the contaminated water (Burrows et al., 2002).

One can distinguish direct and indirect photolysis. Direct photolysis occurs by the absorbance of visible or UV light by the chemical itself, while upon indirect photolysis a sensitizer absorbs the light in the first step and transfers this energy to the pollutant or produces reactive species that react with the pollutant and in this way induces its transformation (Méallier, 1999).

The degradation of a pesticide can follow a very different way whether it is induced by direct or indirect photolysis (Wayne and Wayne, 1996). A well-described example of that are the photodegradation studies of atrazine. In a direct photolysis experiment, the first step of atrazine degradation was dechlorination, followed by hydroxylation (Chen et al., 2009). On the other hand, Torrents et al. (1997) found that the indirect photolysis (the irradiation of aqueous solutions of atrazine containing nitrate as a sensitizer in which $^{\bullet}OH$ is readily generated) is leading to alkyl oxidation and and/or removal of the alkyl moiety. Dealkylated and sometimes oxygenated products, still containing chlorine, are therefore the main products of photo-initiated hydroxyl radical reactions in the indirect photolysis process.

### *2.4.2.1 Direct photolysis*

Photo-induced degradation of pesticides can occur by direct photolysis, initiated by the absorption of light that excites the reactant molecule. Here, the absorption of visible or ultraviolet light by a molecule introduces sufficient energy to break or reorganize most covalent bonds (Wayne, 2005). After the absorption of a photon, the excited molecule may then undergo a variety of subsequent reactions to form products (Wayne, 2005). In the direct photolytic process, photoproducts from the excited state of the pesticide can be formed in two different ways (Méallier, 1999):

Pesticide + h$\nu$ → Pesticide*

Pesticide* → Photoproducts

Pesticide* + X → Photoproducts,

where Pesticide* stands for the pesticide in the excited state and X stands for the solvent or other molecules.
Most pesticides absorb light in the UV region between 250 and 300 nm (Méallier, 1999). In the natural environment the absorption of light by pesticides is limited by additional factors including (Méallier, 1999):

- The transparency of the natural water
- The molecular extinction coefficient
- The water solubility
- The solar spectrum – wavelengths of the solar spectra reaching the soil surface may not be efficient for the direct photolysis of the pesticides
- The pH value of the water – may cause a shift of the absorption maximum and may affect the hydrolysis constant.

### *2.4.2.2 Indirect photolysis*

The other way of pesticide photodegradation in the environment is via indirect photolysis. Here, a molecule, in the role of a so-called sensitizer, which is other than the pesticide of interest, absorbs the light (Burrows et al., 2002). The degradation of the pesticide occurs by the energy transferred from the excited sensitizer or reactions with other reactive species that are formed after their interaction with the excited sensitizer (Méallier, 1999).

Indirect photolysis is especially important when the pesticide poorly absorbs light in the UV-A and visible part of the solar spectra and is then unable to be efficiently degraded by direct photolysis (Richard and Canonica, 2005). Sensitizers are often chromophores, absorbing the UV-A and/or visible part of the solar spectra.
After the excitation by a photon, sensitizers such as colored dissolved organic matter in natural waters undergo conversion to triplet states and react with substrates (Boreen, 2006). Most important are reactions with triplet oxygen and water, forming

highly reactive species including singlet oxygen ($^1O_2$), superoxide radical anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide ($H_2O_2$), solvated electron ($e^-_{(aq)}$) and other radicals ($^{\bullet}OOR$, $^{\bullet}R$ and $CO_3^{\bullet -}$, Boreen, 2006). The sensitizers in triplet excited states themselves and the formed reactive species can subsequently react with the pollutants in the water and in this way cause their transformation (Boreen, 2006).
The general mechanism of the indirect photolysis of a pesticide was described by Méallier, (1999) and is as follows:

$Y + h\nu \rightarrow Y^*$
$Y^* + Pesticide \rightarrow Pesticide^* + Y$
$Y^* + Pesticide \rightarrow Pesticide^{\bullet} + Y^{\bullet}$
$Pesticide^* \rightarrow Photoproducts$
$Pesticide^* + X \rightarrow Photoproducts$
$Pesticide^{\bullet} + X \rightarrow Photoproducts,$

where Pesticide* is the pesticide in the excited state, Y is the sensitizer or radical initiator, Y* is the sensitizer or radical initiator in the excited state, $Pesticide^{\bullet}$ and $Y^{\bullet}$ are pesticide and a sensitizer or radical initiator in a radical form, respectively and X represents all molecules present in the solution.
Synthetic sensitizers that are used in laboratory studies include dyes (methylene blue, rose Bengal, riboflavin etc.) and often ketones (acetone, acetophenone), undergoing conversion to triplet states (Boreen, 2006). The most important naturally occurring substances that are taking a major part in the indirect photolysis of the pesticides in waters are:

- colored dissolved organic matter,
- nitrates and nitrites,
- iron species,
- carbonates and hydrocarbonates (Méallier, 1999).

### *2.4.2.3 The role of dissolved organic matter in the photodegradation of organic pesticides*

In surface waters, dissolved organic matter (DOM) is composed of dissolved organic substances derived from living organisms as a product of their metabolism or of their decomposition (Richard and Canonica, 2005). Two major fractions of DOM are humic and fulvic acids, which give a yellowish or brown color to natural waters (Richard and Canonica, 2005). This colored dissolved organic matter (CDOM) with its absorption of natural light plays a crucial role in the photochemistry in natural waters (Richard and Canonica, 2005). The excitation of CDOM can lead to the formation of multiple reactive species that could react with pesticides and initiate their transformation. Figure 2 summarizes the major photochemical processes of CDOM and possible reactions of the generated reactive species with pollutants (Richard and Canonica, 2005).

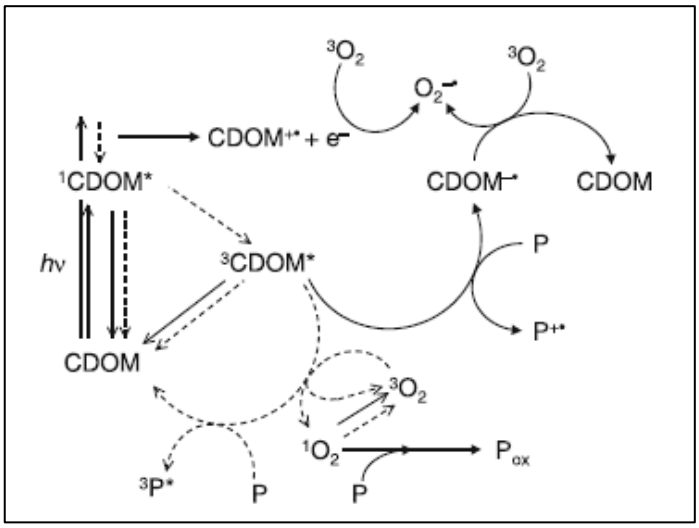



***Figure 2: Photochemical processes of colored dissolved organic matter, involved in the transformation of pollutants in natural waters.***

***Source: Richard and Canonica (2005).***

***Abbreviations: CDOM = colored dissolved organic matter, $^1$CDOM* = CDOM in excited singlet state, $^3$CDOM* = CDOM in excited triplet state, P = pollutant, $^3$P* = pollutant in an excited triplet state, $P_{ox}$ = oxidation product of P formed upon reaction of P with singlet oxygen. Arrows: full arrow heads = chemical reaction, open arrow heads = energy transfer/loss processes, continuous arrows = radiation process, dashed arrows = radiationless processes.***

Main reactive species involved in the transformation of pollutants upon irradiation of CDOM are excited triplet states of CDOM, hydrated electrons, hydroperoxyl radicals and superoxide radical anions, singlet oxygen, hydroxyl radicals, carbonate radicals and other DOM-derived radicals (Richard and Canonica, 2005). Despite its important role as a sensitizer, DOM can also inhibit the phototransformation of organic chemicals or stay chemically inert. No effect of DOM on photodegradation was for example found for anthracene (Bertilsson and Widenfalk, 2002), while photolysis was inhibited for carbofuran in the presence of humic substances (Bachman and Patterson, 1999). Humic acids enhanced the photodegradation of the fungicides carboxin and oxycarboxin (Hustert et al., 1999). The possible reason for the inhibition effect of DOM could lie in the binding of the pollutant to DOM, shortening the lifetime of excited states.

#### *2.4.2.4 The role of nitrite and nitrate in the photodegradation of organic pesticides*

Nitrites and nitrates present in neutral natural waters absorb solar light between 290 and 400 nm and in reaction with water they generate highly reactive hydroxyl radicals (Méallier, 1999):

$NO_2^- + H_2O \rightarrow NO + \bullet OH + OH^-$

$NO_3^- + H_2O \rightarrow NO_2 + \bullet OH + OH^-$

Enhanced photodegradation with nitrate, for example, was found for the ß-blocker drug atenolol (Ji et al., 2012) and the phenylurea herbicide monolinuron, which photodegradation was induced also by nitrite (Nélieu et al., 2004). The enhancement of photodegradation was suggested to be a result of the formed hydroxyl radicals. Many examples summarized by Remucal (2014) show that nitrites and nitrates can be an important source of OH radicals and therefore play a significant role in the elimination of pollutants in nitrate rich natural waters.

### *2.4.2.5 The role of iron in the photodegradation of organic pesticides*

In natural aquatic systems, both inorganic and organic iron complexes can be photoactive and their role in photolytic processes is described by Waite (2005). Among inorganic ferric iron species, $FeOH^{2+}$ occurring in low pH environments (pH of 3-5) was found to be the most photoactive, especially in the UV region. The absorption of light results in a formation of $Fe^{2+}$ and OH radicals, which can be responsible for oxidizing the contaminants.

However, as most natural waters have pH ranging from 6-8, organic iron complexes play a more important role in pollutant transformation. Upon absorption of light, organic complexes may undergo redox transformations within which reactive species can be formed and react with the pollutants. An example of iron-organic complexes, widely used in photolytic studies, is Fe(III)-oxalate, a basis for the ferrioxalate actinometer. In a sequence of reactions, hydrogen peroxide can be formed and its reaction with $Fe^{2+}$ generates •OH radicals in a so-called Photo-Fenton reaction. In natural waters, also DOM can strongly bind to iron and influence the redox transformations of iron. Reactions with such ligands initiated by light induce the production of multiple reactive species which may significantly promote the degradation of organic compounds in water.

Photocatalytic reactions with iron complexes were for example found effective for the degradation of atrazine using the Fe(III)-citrate complex (Ou et al., 2008) and 4-chlorophenol using iron complexed with nitrilotriacetic acid (Fe(III)-NTA) (Abida et al., 2004).

### *2.4.2.6 The role of carbonates and hydrocarbonates in the photodegradation of organic pesticides*

Carbonates and hydrocarbonates are known as radical scavengers and can therefore act as inhibitors of pesticide photodegradation processes (Méallier, 1999). Such effect on the $Mn^{II}$ catalysed ozonation was for example investigated for the herbicide atrazine, where bicarbonate greatly reduced its degradation rate by quenching produced radicals (Ma and Graham, 2000).

### *2.4.2.7 Natural photochemistry in water vs. experimental photochemistry*

In a complex environment, such as natural water bodies, there are many factors that influence the photochemistry of a compound. In the laboratory, on the other hand, in order to understand photochemical reactions, the number of parameters that may influence the photolysis of a compound is greatly reduced. Most of the laboratory studies first focus on direct photolysis of a compound, performing experiments in pure distilled or deionized water. However, there is evidence that the photodegradation pathway in such pure waters can differ substantially compared to natural waters, and therefore caution is needed for extrapolation of such results to the natural environment (Lavtižar et al., 2014).

The first main difference between natural and experimental photochemistry is the light source. Photochemistry in natural waters is driven by solar light with wavelength spectra ranging from 290-800 nm (Clark and Zika, 2000), however the percentage of each spectral range reaching the surface waters is very different (Table 1). Much of the UV spectra is filtered out by the atmosphere (Gibson, n.d.). The spectral range most important for natural photochemistry lies in the region of visible light, UV-A and higher wavelengths of UV-B solar spectra.

***Table 1: Distribution of the irradiance energy in natural sunlight over different wavelengths.***

| Spectral region | Wavelength | % Total energy |
|---|---|---|
| IR | >700 nm | 49.4 |
| Visible | 400–700 nm | 42.3 |
| UV-A | 320–400 nm | 6.3 |
| UV-B | 290–320 nm | 1.5 |
| UV-C | <290 nm | 0.5 |

***Adapted from Gibson (n.d.).***

For experimental photodegradation studies, different light sources can be applied. Germicidal UV-C lamps are often used due to their high energy, and therefore fast reactions. To simulate the degradation in natural environments, UV-A lamps and lamps with wider spectra in UV-A and visible regions are often used. Xenon lamps with filters for abstraction of the low-wavelength UV spectra and solar simulators are gaining popularity in environmental photochemistry, as their emitted light spectra are

approaching the one of sunlight. A comparison of solar spectra with the spectra of the solar simulator apparatus Suntest (Atlas), used also in our photodegradation studies is presented in Figure 3.

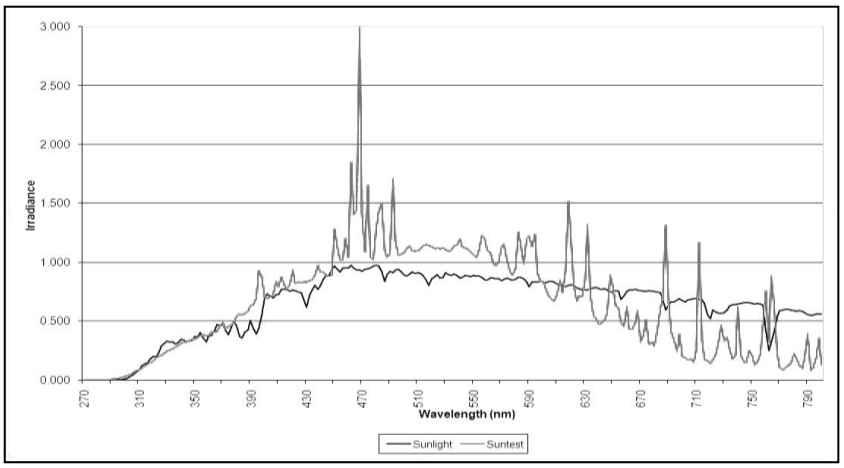



***Figure 3: Comparison of natural sunlight spectrum with the spectrum emitted by the solar simulator used in our photodegradation experiments on chlorantraniliprole.***

***Graph was provided by Atlas Material Testing Solutions.***

In natural waters, photochemistry is governed by numerous sensitizers and radical scavengers dissolved in water. This is making the photochemistry of pollutants rather variable and complex. On the other hand, in experimental settings indirect photolysis is usually studied using a specific sensitizer and sometimes in combination with one specific radical scavenger. The lifetimes of reactive species formed in indirect photolytic processes in natural waters are also very diverse, and so are the reactions they undergo with organic substances in water (Boreen, 2006). Experimental photolysis approaches more to static, simplified and controlled processes. As many organic pollutants have low solubility in water, their solubility is in such studies often increased by adding organic solvents. If the study aims to approach the environmental conditions a small amount of a solvent that is transparent in the UV and VIS region, and highly polar and electrochemically stable should be used.

Experimental studies, although very simplified, are crucial for understanding the photochemical processes taking place in the environment. They also give us the opportunity to study the mechanism of photolytic processes. Photolytic reactions are rapid, however, the use of the laser flash photolysis technique employed by lasers

with pulse widths in the nano- or even subfemto- second range allows us to record the short-lived intermediates of photochemical transformations.

### 2.4.3 Biological degradation of pesticides

Biological degradation or biodegradation is a transformation or alteration of the chemical, carried by the metabolic or enzymatic actions of microorganisms, and is considered an important route of removal of organic pollutants in the environment (Porto et al., 2011). The ability to degrade the xenobiotics present in the environment was developed by some native microorganisms and is often a complex process (Porto et al., 2011), however it can be enhanced by proper genetic modifications of the microorganisms (Schroll et al., 2004), as well as creating optimal conditions for microbes to degrade the pollutants, such are aeration, fertilization and increasing temperature of the soil (Doelman et al., 1988). A great influence on biodegradation has the aging of contaminants in soil. Several laboratory and field studies show that organic compounds that persist in soil for longer periods become less available to microorganisms (Alexander, 1995).

Microbial degradation of pesticides if often studied using pure cultures, where the culture is usually isolated from soil that is contaminated with the particular pesticide (Porto et al., 2011). Biodegradation, especially the one catalyzed by the enzymatic activity of the microorganisms, was found to be a rewarding method for the remediation of soil polluted with several insecticides. This even includes the most notorious ones belonging to the groups of organochlorines, organophosphates and carbamates (Porto et al., 2011).

## 2.5 Ecotoxicology – investigation of the adverse effects of chemicals on living systems

In the late 1970s, due to the increasing awareness of the negative effects of chemicals in the environment to species other than human, ecotoxicology as a science was officially born. The basis of ecotoxicological studies are bioassays and laboratory toxicity tests on single species exposed to a range of concentrations of a chemical of

interest (Walker et al., 1996). To evaluate the toxicity of a chemical, different endpoints can be monitored and results thus obtained can be extrapolated to the population or community level. Therefore, such laboratory tests provide an essential rapid evaluation of the toxicity of a chemical and allow comparison of results obtained with different tests, test organisms and chemicals.

The field of ecotoxicity has improved significantly over the last decades. While first ecotoxicity tests were using only mortality as an end point and were mainly short termed, nowadays more consideration is given to long-term tests using reproduction and other sublethal effects which have more ecological relevance (van Gestel, 2012). Also endpoints which are more sophisticated, such as ones occurring at the biochemical level, are increasingly used as they may provide more insight into the mode of action of a chemical and may act as early warning indicators of higher-level effects (Walker et al., 1996). To understand and provide the linkage between molecular effect and adverse outcome at different levels of biological organization, new tools have been developed and became sophisticated in a so called adverse outcome pathway (AOP) tool. AOP can provide information of adverse outcomes initiated on macro-molecular and cellular level up to population level for various xenobiotics, possessing different modes of action (Ankley et al., 2010). With further development, AOP ensures a key role in predictive (eco)toxicology (Ankley et al., 2010).

To identify and characterize potential hazards of new and existing chemical substances, organizations such as the Organization for Economic Co-operation and Development (OECD) and the International Organization for Standardization (ISO) started to develop guidelines for testing new and existing chemicals on selected species. These guidelines are standardized and internationally accepted for the generation of toxicity data for the purpose of the registration of pesticides and all other new chemicals. The OECD guidelines for the testing of chemicals include a wide collection of most relevant toxicity test methods prepared to study the potential hazards of chemicals to organisms representative of aquatic and terrestrial ecosystems, including fishes, honey bees, algae, cyanobacteria and other selected microorganisms, plants, non-biting midges, earthworms, water black worms, enchytraeids, predatory mites, water fleas, amphibians, collembolans, dipteran dung

flies and birds (OECD, n.d.). If available, all our toxicity tests were performed following OECD or ISO guidelines.

In the field as well as in laboratory studies, indicator species are used to assess the possible effects of the chemicals in soil and water. Edwards et al. (1996) identified as indicators terrestrial species or taxonomic groups that play an important role in ecosystem functioning, are present in a wide range of soil ecosystems, exist in large and dominant populations, are testable under natural conditions, employing methods of assessing their populations that are efficient, readily-available and non-laborious. Analogous to terrestrial indicator species, the same characteristics can be considered for the aquatic indicator organisms as well.

The principle of the interaction between xenobiotic chemicals and living organism is presented in Figure 4. The interaction can be described by two main steps (Walker et al. 1996, Katayama et al. 2010). In the first one, the compound is absorbed by the organism from its environment (chemical uptake). The second step consists of processes that govern the fate of the chemical within the organism. The combination of two determines the ecotoxicity of a chemical (Katayama et al., 2010).

- First step: The chemical uptake. The uptake of a chemical can occur via different routes, the most usual one is by passive diffusion through the skin, cuticle and membranes (Walker et al., 1996). Highly lipophilic organic chemicals also have a higher affinity for this process, since membranes also consist of lipids - usually phospholipids (Katayama et al., 2010). The potential of a chemical to enter through natural barriers into the organism is indicated by the term bioavailability and depends on the type of organism, route of entry, time of exposure and the matrix containing the compound (Anderson et al., 1999).
- Second step: The transportation of the absorbed chemical from the environment to the sites of action in the body, the rate of uptake and its internal distribution and processing in the body (toxicokinetics). After the uptake of the chemical by an organism, four types of sites are identified: sites of action, metabolism, storage and excretion (Walker et al., 1996). In the first process the chemical in the body

interacts with the action sites, causing toxicity. In the second process, the chemical is metabolized by enzymes produced by the organism in order to make it less toxic and enhance its excretion. In some cases, metabolism leads to the production of metabolites that are more toxic than the parent compound. This is for instance the case for the organophosphate insecticide parathion, which by itself is not very toxic compound, but in the animal liver is metabolized to the extremely toxic para-oxone (Philp, 2013). Chemicals can be also stored in the body in a form that in a toxicological sense is inert. In this case the chemical neither reacts with action sites, nor does it induce the organism to increase metabolism and excretion processes. The last process is excretion; the chemical is excreted either in its original state (unchanged) or more often in form of metabolites.

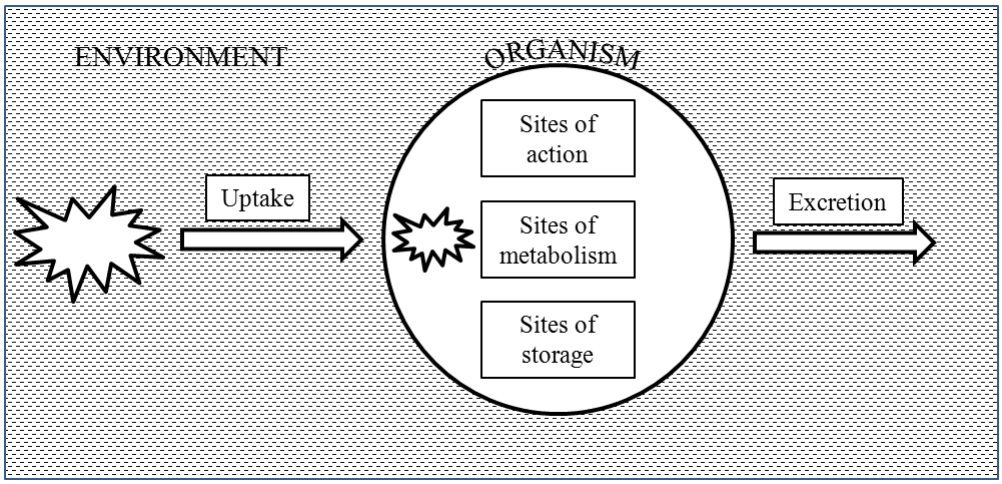



***Figure 4: A model describing the exposure of an organism to a xenobiotic chemical in the environment and the fate of the chemical within the organism.***
***Adapted from Walker et al., 1996 and Katayama et al., 2010.***

## 2.5.1 Parameters influencing the bioavailability of compounds

Whether an animal will suffer from toxic effects of a pesticide depends on the combination of the nature of the substance and the organism itself in terms of the effectiveness of its mode of action to that specific organism. However the first

condition is that the chemical is available to interact and be absorbed by the living organism.

Bioavailability is greatly affected by the combination of the physical-chemical properties of the pesticide, biological factors and physical-chemical conditions of the environment in which the pesticide and organism interact (Katayama et al., 2010). However, considering the model of the organism – chemical interaction (Figure 4), chemicals that are bioavailable to the organism may not necessarily cause adverse effects if they don't reach the right target organs that could be affected by the chemical (which depends on its mode of action).

To predict the bioavailability of a compound to biota, three parameters - water solubility, octanol-water partition coefficient and organic carbon sorption coefficient of the compound are important.

- Water solubility is a key factor governing the bioavailability of a pesticide to organisms – not only to aquatic but also to terrestrial ones, as the pore water is the main route of exposure for the soil-dwelling organisms (van Gestel and Ma 1988, 1990, van Gestel 1997, Smit and van Gestel 1998, Didden and Römbke 2001).
- Related to the water solubility, the bioavailability can be predicted by determining the octanol-water partition coefficient (Kow) (see Equation 1) (Walker et al., 1996). Kow is a measure of the hydrophobicity of the chemical and is widely used to predict the bioaccumulation of pesticides in organisms. Organic chemicals with a high Kow value have an increased tendency to pass the lipophilic natural barriers of the organism, accumulate in the tissues of living species and be transferred across the food chain (Zacharia, 2011).
- As the organic matter fraction in the soil as well as dissolved organic matter in the water can sorb organic compounds to a great extent and with that influence their bioavailability, the organic carbon sorption coefficient (Koc) (see Equation 3) also is an important parameter determining bioavailability. The Koc is also related to Kow and inversely related to water solubility. The intensity of sorption to the soil is further enhanced by pesticide aging – with increasing contact time of the pesticide with the soil, sorption is increased, as was demonstrated for instance for atrazine

by Park et al. (2003, 2004). This shows that aging can lead to a significant reduction of the bioavailability of a chemical, which was proven by several studies (Regitano et al. 2006, Ahmad et al. 2004, Morrison et al. 2000), with the first evidences reported in the review of Alexander (1995).

In the aquatic environment, Farrington (1991) summarized that most important parameters governing the bioavailability are solubility of the compound, its partitioning between solid surfaces, colloids and soluble phases, sorption and desorption rates and the physiological status of the test organism. Additionally, parameters such as salinity, pH, water temperature, types as well a quantity of dissolved organic carbon and particulate matter can influence these relationships (Pritchard, 1993).

The bioavailability of pesticides to soil organisms is highly dependent on the characteristics of the soil that determine the sorption of the pesticides. These characteristics are soil organic matter content and properties, soil texture, soil acidity, Fe- and Al- oxide content and clay mineralogy (Johnson and Sims, 1993). Besides organic matter, the surface area of the soil, ruled predominately by the type of clay and proportion of small clay particles, also influences the sorption of organic chemicals (Katayama et al., 2010).

Cation exchange capacity is another parameter that may influence the sorption of xenobiotics and is especially important for metals but also for ionic organic chemicals (Katayama et al., 2010).

Among the most important parameters affecting the sorption of chemicals to the soil is soil pH, which becomes important when the chemicals dissociate at the normal soil pH range (Katayama et al., 2010). An example for this is pentachlorophenol for which sorption to the soil decreases when the soil is alkaline (as the compound dissociates at pH above its pKa of 4.74 (Howard et al., 1991)) compared to the acidic soils, where it remains in the non-dissociated, more lipophilic form (Katayama et al., 2010).

### 2.5.2 Toxicity assessments with selected non-target aquatic and terrestrial organisms

In laboratory ecotoxicity tests, a chemical of interest is usually tested with selected species. Many tests have been designed and standardized for aquatic and terrestrial invertebrates and plants. For testing the toxicity of CAP, two aquatic and four terrestrial invertebrates have been selected to achieve the aims of our research.

Aquatic toxicology has its roots in 1940s, when the adverse effects of - at that time - widely used insecticide DDT on fish and wildlife were becoming too obvious to be ignored (Pritchard, 1993).

Among the standardized toxicity tests on aquatic organisms, the water flea *Daphnia magna* and the sediment dwelling annelid *Lumbriculus variegatus* were used as test organisms in our study. With *L. variegatus* a reproduction test was performed, while *D. magna* was used in acute and (chronic) reproduction tests. The acute test allowed us to rapidly assess toxicity of compounds by determining the number of immobilized animals as a final endpoint. On the other hand, the chronic test is long term and gave us the possibility to monitor other parameters of toxicity such as reproduction, animal growth, and behavioral changes. Reproduction as an endpoint is especially relevant for extrapolating the results to the population level (van Gestel, 2012).

The development of ecotoxicity tests on soil invertebrates has been summarized by van Gestel (2012). The first OECD guideline using soil invertebrates appeared in 1984 and is describing an acute toxicity test with the earthworm *Eisenia fetida*. Several other tests, with survival, reproduction, avoidance and growth as the endpoints, were developed and standardized by OECD and ISO, using species representative of the most prominent groups of soil invertebrates. Toxicity tests using other species, such as oribatid mites and isopods, are not standardized, but are already commonly used in ecotoxicity studies (for a summary of some, see Laskowski et al. 1998, van Gestel and Doornekamp 1998).

The exposure routes can be different for different organisms. Because soft-bodied organisms need a constant contact with the soil pore water to remain hydrated, the main uptake route of the chemical is by absorption through the skin from the soil solution and also by feeding (Katayama et al., 2010). On the other hand, hard-bodied

organisms with tracheal systems can take the chemicals up from contaminated food, pore water and the soil atmosphere, if the chemical is volatile (Katayama et al., 2010).

To achieve the aims of our research, reproduction tests were performed with the potworm *Enchytraeus crypticus*, the oribatid mite *Oppia nitens* and the springtail *Folsomia candida*. The latter specie was also used in an avoidance test, which is a rapid and sensitive indicator for unfavorable conditions caused by chemicals present in the soil. The main endpoints of the test with the isopod *Porcellio scaber* were survival, body weight change and consumption rate.

### *2.5.2.1 Water flea* Daphnia magna *as a test organism*

*Daphnia magna* is a planktonic crustacean, widely distributed in small to medium sized freshwater ponds and pools of the Holeartic region (De Gelas and De Meester, 2005). By feeding on phytoplankton as a main food source on one hand, and being an important food source for fish and some aquatic invertebrates on the other hand, they play a significant ecological role in freshwater ecosystem food webs (Miner et al., 2012). Because of their high sensitivity to toxicants, they serve as a good indicator of pollution (Adema, 1978). For this reason they became widely used in toxicity tests for single chemicals or mixtures of chemicals as well as in bioassays to assess the toxicity of waste waters and polluted natural waters. Among the already mentioned ecological importance, its parthenogenetic reproduction, short life cycle, high fecundity and ease of culturing are other main benefits that are making daphnids very favored in aquatic ecotoxicology (Adema, 1978), with currently several test guidelines being available (OECD 2004a, OECD 1998, ISO 2012).

### *2.5.2.2 Blackworm* Lumbriculus variegatus *as a test organism*

For strongly adsorbing chemicals and those that bind to sediment with covalent bonds, the ingestion of contaminated sediment can be a significant route of exposure (OECD, 2007). Organisms that are often used to test the possible negative impacts of sediment-bound substances are aquatic oligochaetes. They play an important role in the sediment of aquatic systems. With their moving and ingesting the substrate, they

importantly contribute to the bioturbation of the sediment (OECD, 2007). As prey to other organisms they can be carriers of the compounds to higher trophic levels (OECD, 2007), which can further accumulate in their tissues.

In our test with CAP, the aquatic oligochaete *Lumbriculus variegatus* was used as a test organism. *L. variegatus* can be found in sediments worldwide (Egeler et al., 2005). It is reproducing asexually by fragmentation, after which the fragments regenerate by morphallaxis (Drewes and Fourtner, 1990).

### *2.5.2.3 Woodlouse* Porcellio scaber *as a test organism*

Woodlice are terrestrial crustaceans, belonging to the order Isopoda. They in fact are the only crustaceans living on land. They are mainly found feeding on dead plant material and thus acknowledged as ecologically important macro-decomposers in the detritus food chain (Laskowski et al., 1998). Their ability to cope with different environmental conditions allows them to populate most of the terrestrial habitats in many regions of the world (Warburg et al., 1984). The first laboratory ecotoxicological tests using the woodlice species *Porcellio scaber* and *Oniscus asellus* determining sublethal effects were developed in 1994 by Drobne and Hopkin (1994). Since then, toxicity studies with isopods have still not yet been standardized. There is high potential for its standardization due to the ecological relevance of isopods, the broad knowledge of their life history, and the rich past experiences using isopods as test animals (van Gestel, 2012). Tests can be designed using a variety of different endpoints to assess the toxicity of chemicals. Most often survival, reproduction, growth rate, and food consumption are followed, where isopods are exposed to the test chemical either through food or in contaminated soil (van Gestel, 2012).

### *2.5.2.4 Potworm* Enchytraeus crypticus *as a test organism*

Enchytraeids are short, white colored soil-dwelling annelids. As decomposers of organic matter and due to their high abundance, enchytraeids have a great environmental importance (Didden, 1993). However, despite their crucial ecological role, enchytraeids were generally neglected as test organisms (Römbke, 2003). Now, standardized tests on enchytraeids are available from OECD (2004b) (guideline 220)

and ISO (2004) (guideline 16387) and they nowadays are also regularly used in soil quality assessments (Römbke, 2003). Didden and Römbke (2001), reviewing field and laboratory studies on the effects of various chemicals on enchytraeids, concluded that these organisms are in general quite sensitive to chemical stressors. In our toxicity test, the enchytraeid *Enchytraeus crypticus* was used due to its sensitivity, relatively high reproduction rate and short generation time (van Gestel et al. 2011, Castro-Ferreira et al. 2012).

### *2.5.2.5 Springtail* Folsomia candida *as a test organism*

Collembolans or springtails are one of the most abundant groups of soil arthropods on the Earth, occupying a large diversity of ecosystem types (Hopkin, 1997). Especially in soils that are rich in humus, they can be found in highest densities, feeding on fungi in soil and leaf litter (Hopkin, 1997). Being considered to be the oldest existing hexapods (Whalley and Jarzembowski 1981), their origin is widely studied to explain the evolution of insects (see for instance: Engel and Grimaldi, 2004).

Before the standardization of some of the laboratory toxicity tests using springtails, they were already widely used as bioindicators of environmental pollution (Wiles and Krogh, 1998). This was due to their high sensitivity to chemicals, which in turn made them very favorable in laboratory toxicity testing. In ecotoxicology, *Folsomia candida* is most commonly used among collembolans. This is due its wide distribution, ecological importance, parthenogenetic reproduction, relatively short life cycle, high reproduction rate and easy culturing (Wiles and Krogh, 1998). In toxicity tests the main end points are survival, reproduction, growth and avoidance. Prior to toxicity experiments, animals are in most cases age-synchronized and tests are started with juveniles or adults of similar age.

### *2.5.2.6 Oribatid mite* Oppia nitens *as a test organism*

As for isopods, there is also no standardized test guideline using oribatid mites as a test species. However, using mites, in particular *Oppia nitens* in laboratory toxicity tests is fairly new. A reproduction test with this species was first proposed in 2010 by

Princz et al., followed by an avoidance tests one year later (Owojori et al., 2011). Like springtails, mites also represent a highly diverse and abundant group of soil arthropods. Their environmental importance is very much in favour for using them in ecotoxicological studies as their role in the mineralization of dead plant material is significant. This role of *O. nitens* is especially important for the boreal regions. There they serve as a valuable indicator of environmental disturbances due to their high sensitivity to xenobiotics (Princz et al., 2010). Lebrun and van Straalen (1995) also concluded that oribatid mites hold a great potential for their use in stress ecology studies.

## 2.6 Ryanodine receptors and its activators: A new group of insecticides

Anthranilic diamides are a recently emerged group of insecticides with a very specific and distinctive mode of action when compared to other insecticide groups. They act as activators of ryanodine receptors (RyR), intracellular non-voltage calcium channels present in the sarcoplasmic reticulum (SR) of muscles and the endoplasmic reticulum (ER) of non-muscle cells. Their role is critical for muscle contraction (Sattelle et al., 2008). However, with the binding of the diamide insecticide to the RyR, the calcium channel remains in an open state. This causes a depletion of the entire calcium depot, leading to impaired regulation of the muscle excitation, contraction and relaxation cycle. This eventually continues to complete muscle contraction and paralysis, ensuing death of the insect (Lahm et al., 2007).

Mammals express three different RyR channels (RyR1, RyR2, RyR3), showing 65% homology at the amino acid level (Ogawa et al. 1999). In contrast, birds, amphibians and fish possess only two types of RyRs (RyRA and RyRB) (Ogawa et al. 1999), where RyRA shows homology with mammalian RyR1, while RyRB most closely resembles the RyR3 isoform (Oyamada et al., 1994). Insects express more types of RyR, but comparison between them, made by Sattelle et al. (2008), shows that insect RyRs are very similar to each other in amino acid sequence. However, from their comparison, it is evident that they are functionally very different from their mammalian homologues (Takeshima et al., 1994). Takeshima et al. (1994) suggest

that comparison of the amino acid sequence of the fruit fly (*Drosophila melanogaster)* with the mammalian RyR subtypes reveals around 45% overall homology.

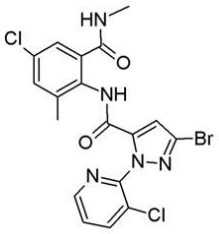
The knowledge about the activation of the RyR and the idea to use it as a mode of action for pest control is not new. It was studied intensively by using a plant metabolite called ryanodine, produced by the trees and shrubs of the genus Ryania in order to defend themselves from harmful pests (Lahm et al., 2009). However, attempts to exploit the natural insecticide for commercial use have proven unsuccessful since ryanodine was found not only to be toxic to pests but also to mammals (Cordova et al., 2006). The efforts to develop an insecticide with an identical mode of action but with higher selectivity seemed to be paid off by the discovery of the phthalic diamide called flubendiamide and the anthranilamide chlorantraniliprole. Recently, new diamide pesticides have been developed and their properties were described by Gnamm et al. (2012). Novel diamide insecticides also contain sulfur groups such as sulfoximines, sulfonimidamides and other sulfonimidoyl derivatives.

## 2.7 Selected insecticide: chlorantraniliprole

Chlorantraniliprole (CAP) was synthesized by DuPont with the trade name Rynaxypyr® and was first registered in 2007 in the Philippines (Lahm et al., 2009). Since 2010 its formulated product Coragen is available for plant treatment also in Slovenia and it is the only registered representative of this class of pesticides (mainly used on fruit trees, vines and potatoes) in the country so far ("Seznam registriranih"…, 2014). Data show that CAP has an exceptional insecticidal activity on a range of Lepidopteran pests. For example, when CAP solution was applied to soybean leaves with which selected test insects in the toxicity test were fed, the following $EC_{50}$ values were obtained for larvae: 0.01 mg/L for the diamondback moth (*Plutella xylostella*) and the fall armyworm (*Spodoptera frugiperda*) and 0.05 mg/L for the tobacco budworm *(Heliothis virescens*) (Lahm et al. 2007, 2009).

In Table 2, the physical-chemical properties of chlorantraniliprole are summarized. They can be used for the prediction of the environmental fate of CAP and its bioavailability to the test organisms, discussed in the further chapters.

***Table 2: Nomenclature and physical-chemical properties of chlorantraniliprole.***

| | |
|---|---|
| Chemical formula | $C_{18}H_{14}N_5O_2BrCl_2$ |
| IUPAC name | 3-bromo-4′-chloro-1-(3-chloro-2-pyridyl)-2′-methyl-6′-(methylcarbamoyl)pyrazole-5-carboxanilide |
| Structural formula |  |
| Water solubility (20°C) | pH 4: 0.972 mg/L |
| | pH 7: 0.880 mg/L |
| | pH 9: 0.971 mg/L |
| Vapor pressure | 6.3 x $10^{-12}$ Pa @ 20°C |
| | 2.1 x $10^{-11}$ Pa @ 25°C |
| Henry's law constant (20°C) | 3.2 × $10^{-9}$ Pa $m^3$ $mole^{-1}$ |
| Dissociation constant, pKa, (20°C) | 10.88 ± 0.71 |
| Soil:Water Coefficients (Average Koc), (mL/g) | 153-loam sand |
| | 509-silty clay loam |
| | 272-sandy loam |
| | 526-loamy sand |
| | 180-loam |
| Octanol-water partition coefficient, log Kow (20°C) | pH 4: 588 |
| | pH 7: 721 |
| | pH 9: 654 |

***Source: EPA (2008), FAO (2008)***

### 2.7.1 Degradation of chlorantraniliprole in the environment

From the EPA report (2008) we can learn that CAP is persistent in soil and moderately persistent in water. The transformations it may go through are of chemical, photochemical or biological nature.

- Chemical degradation of CAP

According to FAO (2008), the chemical degradation of CAP in water is mostly caused by hydrolysis, catalyzed by acids or bases with which the compound comes into contact. From the same report we can learn that CAP (0.6 mg/L) at 25 °C in the dark was stable at pH 4 and 7 for at least 30 days but not at pH 9. In this buffer solution (borate buffer, with added acetonitrile (1%) as a co-solvent) CAP underwent dehydration to form one degradation product. The half- life of CAP was approximately 10 days.

- Photochemical stability of chlorantraniliprole

FAO (2008) reports that in water CAP is degraded to three major photodegradation products. In sterile natural water, the photolytic half-life ($Dt_{50}$) of CAP was 0.31 days under continuous irradiation (Xe arc lamp, 300-800 nm, UV filter). However, in FAO (2008) the degradation pathways as well as the experimental and analytical procedures are poorly established and transformation intermediates are inadequately described. So far, only two scientific studies investigated the photochemical stability of CAP in water. The first one (Lavtižar et al., 2014) provides a complete description of chemical and photochemical degradation of CAP with the identification and characterization of the main transformation products. In that study, both the direct and indirect photolysis of CAP were investigated. The second study (Sharma A.K. et al., 2014) confirmed the results of Lavtižar et al. (2014) and added (photo)stability studies of CAP in soil. They found that chemical and photodegradation of CAP in soil (pH = 7.5, 3.5% organic matter) is not significant.

- Biodegradation of CAP

Although biodegradation plays an important role in the degradation of chemicals in the environment, for CAP the major transformation is via abiotic transformations in terms of dehydration and rearrangements of subsequent products. Two biotic

transformation products were found in soil but in minor concentrations (FAO, 2008). According to FAO (2008), CAP degrades in soil but its degradation is sometimes limited by the sequestration in the soil.

### 2.7.2 Dissipation of chlorantraniliprole

A field study performed on 5 soils originating from the US and Europe, indicated a moderate sorption of CAP to soil, with an average $K_{OC}$ value of 329 mL/g, with a range of 152-535 mL/g (APVMA, 2008). Malhat et al. (2012) investigated the dissipation of CAP in tomatoes and soil. In their study, plants were sprayed with the CAP formulation Coragen (containing 20% a.i.) at the recommended rate of application (60 mL / 4200 $m^2$). They found that the half-life of CAP in the soil under the treated plants was 3.6 days, with absence of rain during the experimental period and at average temperatures ranging from 17-26 °C. In a rice field system, the half-life of CAP was 16 days in soil, with fast degradation of CAP in the first week and further dissipation at a slower rate during the next three weeks of the study (Zhang et al., 2012). Here the soil pH was 6.2 and OM content was 2.52 %. In this study, CAP residues were found also in water. The initial CAP concentration measured in water was 0.028 mg/L, and dropped over the time with a half-life of 0.85 days. Sharma N. et al. (2014) studied the dissipation of CAP applied as a granulated formulation to a sugarcane field. The half-lives in soil with pH 8.0 and 0.30 % organic carbon were 8.36 and 8.25 days for the application dosages of 100 and 200 g a.i. /ha, respectively. APVMA (2008) suggests that CAP may reach aquatic habitats through spray drift or runoff. According to Health Canada (2013), CAP is expected to leach through the soil profile beyond 60 cm and may therefore reach the groundwater. This statement, however, seems to disagree with the low water solubility and high Koc values measured for CAP, which suggest that drift and runoff will be more important sources of CAP in surface water than leaching. In surface waters there is a risk of CAP accumulation due to its sorption to sediments. CAP residues are expected to accumulate in soil from year to year, when the use of CAP on the fields is extended (EPA, 2008). A study on the accumulation of CAP in agricultural fields showed that

up to approximately 48% of applied CAP was expected to carry over to the following growing season (Health Canada, 2013).

The low vapor pressure and low Henry's law constant (Table 2) indicate that CAP is non-volatile in the environment. Therefore, according to this information reported by Health Canada (2013), no CAP residues are expected in the atmosphere, which also prevents the long-range transport of CAP.

Since CAP was assigned to be persistent in soil (EPA, 2008), it is expected to reach the groundwater through leaching and surface water mainly through runoff and spray drift unchanged, and undergo possible transformations in the water compartment.

### 2.7.3 Impacts of chlorantraniliprole and its transformation products on non-target organisms

The EPA (2008) reports very low toxicity of CAP to terrestrial vertebrates but they do report its potential to cause direct adverse effects on some non-target terrestrial insect species. From the toxicity data reports of CAP to non-target organisms it can be concluded that the sensitivity to the pesticide is quite variable among the tested invertebrates. According to EPA (2008) the lethal concentrations for selected freshwater fishes are in all cases above the CAP solubility in water, however this data concerns mainly for acute, short-term exposures. Very high acute toxicity was however reported for aquatic invertebrates such as the mayfly *Centroptilum triangulifer* ($LC_{50}$ = 0.0116 mg CAP/L), the caddisfly *Chimarra atterima* ($LC_{50}$ = 0.0117 mg CAP/L), the midge *Chironomus riparius* ($LC_{50}$ = 0.0859 mg CAP/L), the water flea *Daphnia magna* ($LC_{50}$ = 0.0116 mg CAP/L) and the amphipod *Gammarus pseudolimnaeus* ($LC_{50}$ = 0.0351 mg CAP/L) (EPA, 2008). Among the estuarine and marine invertebrates, a high toxicity of CAP to the eastern oyster *Crassostrea virginica* was observed, with an acute $EC_{50}$ of 0.0399 mg CAP/L. The CAP concentrations causing adverse effects on water and terrestrial plants are reported to be below the EPA's screening levels of concern (EPA, 2008). Reviewing the scientific literature on the toxicity to aquatic organisms also showed that CAP was highly toxic ($LC_{50}$ = 0.95 mg/L) to the crayfish *Procambarus clarkii* in an acute (96 h) toxicity test (Barbee et al., 2010). An $LC_{50}$ of 14.4 mg/L was found for the fresh

water fish *Channa punctatus* by Nagaraju and Venkata Rathnamma (2013) in a 96 h exposure test and a 96 h $LC_{50}$ of 11.0 mg/L was reported for the grass carp *Ctenopharingodon idella* (Venkata Rathnamma and Nagaraju, 2013). However the values of the last two studies most likely correspond to the concentrations tested with the CAP formulated product (18.5% SC) as a whole and not to CAP as an active ingredient of the formulation.

Some data on the ecotoxicity of CAP to non-target terrestrial organisms can also be found in EPA reports. For the toxicity of CAP formulated product to hoverflies a lethal rate 50 ($LR_{50}$) of 4.64 g CAP/ha was found. CAP was also shown to be toxic to the springtail *Folsomia candida*, with a reproduction $EC_{50}$ of 0.48 μg/g dry soil.
Apart from the mentioned reports, scientific studies on the toxicity of CAP to non-target organisms are scarce. Brugger et al. (2010) summarized toxicity data of CAP, the technical product as well as its formulated products on seven species of parasitic wasps. In the 24h acute tests, no effect was observed when applying worst case scenarios, so testing above crop-relevant exposure concentrations. Little or no effect was also observed on soil invertebrates, bumblebees (Gradish et al., 2010) and honeybees (Dinter et al., 2009) and four species on turf–inhabiting beneficial insects: *Harpalus pennsylvanicus*, *Tiphia vernalis*, *Copidosoma bakeri*, and *Bombus impatiens* (Larson et al., 2014). Lefebvre et al. (2011) evaluated contact and residual effects of CAP to eggs, larvae, adults and female fecundity of the predatory mite *Galendromus occidentalis*. Test petri dishes containing apple leaves, prey (*Tetranychus urticae*) and test predators were treated with CAP formulation to mimic a worst case laboratory exposure. The concentration of CAP was 350 g/kg WG (wettable granule, Altacor® 35 WG; 1.667 g a.i./L). The authors concluded that CAP is non-toxic to the predatory mites considering all endpoints studied, except for the larvae, where CAP was assigned to be marginally toxic. Martinou et al. (2014) investigated lethal effects of CAP to the predatory bug *Macrolophus pygmaeus* via three routes of exposure: contact with treated leaf surface, oral ingestion of treated food and direct contact to spray droplets. Bugs were exposed to CAP at the highest label rates (40.0 mg a.i./L) for 72 h. CAP caused less than 25% mortality to the *M. pygmaeus* nymphs, and was classified as harmless. In their test of sublethal effects the authors showed that CAP can cause a decrease in plant feeding, while other behaviors (walking, time needed for the arrival to the egg patch, feeding on the egg

patch, preening and resting) were not affected. Additionally, CAP had no effect on predation rate. On the other hand, CAP was toxic to the larvae of the aphidophagous predators *Harmonia axyridis* and *Coleomegilla maculate* via contact with CAP residues (dry Petri dish previously dipped into CAP solution prepared at field application rate: 50.75 g a.i./ha), where complete mortality occurred after 6 days of exposure. Though indicative of CAP toxicity, it is very hard to translate these data to field exposure conditions.

This brief summary of available data shows there is a great need for a more systematic study of the toxicity of CAP to different organisms from soil and water.

There is also no scientific literature about the possible chronic effects of CAP degradation products on organisms in the environment. While it is beneficial that chemicals have decreased persistence, there are also concerns about a possible higher toxicity of degradation products, compared to parent compounds (Sinclair and Boxall, 2003). Since it is essential to be aware of the possible ecological risks of these compounds in the environment, we included two CAP degradation products in acute and chronic tests with the water flea, *D. magna*. One degradation product of CAP was also included in our tests for its toxicity to the sediment dwelling annelid *L. variegatus.*

# 3 RESEARCH GOALS

The general goal of this work was to contribute to increasing the scientific knowledge on the environmental fate of CAP in aquatic environments and its potential risk to selected non-target organisms. More specifically, our research goals were as follows:

1. To investigate the stability of CAP in aqueous solutions at different pH values.
2. To investigate the stability of CAP in water when exposed to different light sources.
3. To identify and characterize CAP main degradation products formed spontaneously in water solution or upon the solar irradiation.
4. To investigate the course of CAP degradation, using different aqueous media (deionized vs. tap water).
5. To investigate the stability of CAP degradation products and to approach the mechanism of their formation and degradation.
6. To investigate the influence of humic acids and nitrate present in water on the photodegradation of CAP.
7. To investigate the possible toxicity of CAP and two of its main degradation products to the water flea, *Daphnia magna* in acute and chronic toxicity tests.
8. To investigate the possible toxicity of CAP and one of its degradation products to the fresh water blackworm *Lumbriculus variegatus*.
9. To investigate the possible toxicity of CAP to selected terrestrial invertebrates: *Folsomia candida*, *Porcellio scaber*, *Oppia nitens* and *Enchytraeus crypticus.*
10. To investigate the influence of soil organic matter content on the toxicity of CAP to *Folsomia candida*.

# 4 MATERIALS AND METHODS

## 4.1 Materials

Analytical standards used:

- Chlorantraniliprole (CAP, 3-bromo-N-[4-chloro-2-methyl-6-(methylcarbamoyl)phenyl]-1-(3-chloro-2-pyridine-2-yl)-1H-pyrazole-5-carboxamide); (99.5 % purity), from Dr. Ehrenstorfer
- Pyridine from Sigma Aldrich

Materials used in the stability studies in different water media:

- Acetonitrile
- Citric acid for buffer preparation of pH 4.0, 5.5 and 7.2 (0.1 M)
- Tris (hydroxymethyl)aminomethane for buffer preparation of pH 7.2, 8.0 and 9.0 (0.2M)
- NaCl, KCl, $KH_2PO_4$, $Na_2HPO_4$ for preparing the phosphate buffer solution (10 mM)
- Double deionized water ($ddH_2O$, < 18 MΩcm), prepared with a NANOpure water system (Barnstead, USA)
- Dutch tap water of pH 8.2 and a hardness of 1.49 mmol/L

Materials used in the photodegradation studies:

- Acetonitrile
- Double deionized water ($ddH_2O$, < 18 MΩcm), prepared with a NANOpure water system (Barnstead, USA)
- Slovenian tap water with total hardness of 1.43 mmol/L, pH 8.0, organic matter content (TOC) = 667 ± 10.8 µg/L and total nitrogen (TN) = 0 mg/L
- Humic acid (Sigma Aldrich, technical grade)
- $KNO_3$

Materials used in the HPLC analysis:

- Acetonitrile
- Double deionized water (dd$H_2O$, < 18 MΩcm), prepared with a NANOpure water system (Barnstead, USA)
- Formic acid

Chemicals needed for the NMR analysis of compounds:

- acetone $d_6$
- $CD_2Cl_2$
- $CDCl_3$

Chemicals used for the preparation of degradation products:

- dichloromethane
- heptane
- $SiO_2$
- diethyl ether
- petroleum ether
- acetic acid
- $Na_2CO_3$

Chemicals used in the toxicity tests with *Daphnia magna*:

- Dimethylsulfoxide (DMSO)
- Salts and vitamins for the preparation of ISO medium (OECD, 2004a) and Elendt M4 medium (OECD, 1998)

Materials used in the toxicity tests with *Lumbriculus variegatus:*

- Acetone
- Dimethylsulfoxide
- Salts ($NaHCO_3$, $KHCO_3$, $CaCl_2 \times 2H_2O$, $MgSO_4 \times 7H_2O$) in demineralized water for the preparation of the reconstituted Dutch standard water)
- $CaCO_3$ for pH adjustment of the artificial sediment

- Materials for reconstitution of the sediment
    - quartz sand (grain size 0.5 – 1 mm, Sibelo MV, Mol, Belgium)
    - kaolin clay (Keramikos, Haarlem, the Netherlands)
    - cellulose (Sigma Aldrich, ST. Louis, MO)

Materials used in the toxicity tests with soil invertebrates:

- Acetone, technical grade
- $CaCl_2$ for soil pH determination
- Lufa 2.2 standard soil (LUFA, Speyer, Germany)

All chemicals were of analytical or technical grade provided by Sigma Aldrich, Fluka or Merck, except where stated differently.

## 4.2 Analytical and characterization procedures

### 4.2.1 HPLC analyses in degradation studies

Degradation of CAP and formation of degradation products in the different test solutions (See Chapter 5.1) was followed by analyzing test solutions using an HP 1100 HPLC-DAD employed with Luna C18 column, Phenomenex (4.6 × 250 mm, particle size 3 μm, pore size of 100 Å) with a constant temperature of 22 °C. The mobile phase consisted of acetonitrile and dd$H_2O$ acidified with 0.1 % formic acid with isocratic elution at a ratio of 60:40 (v/v). Flow rate was 1 mL/min and injection volume 30 μL.

All the transformation products – compound **A** (2-((2-bromo-4H-pyrazolo[1,5-d]pyrido[3,2-b][1,4]oxazin-4-ylidene)amino)-5-chloro-N,3-dimethylbenzamide), **B** (2-(3-bromo-1-(3-hydroxypyridin-2-yl)-1H-pyrazol-5-yl)-6-chloro-3,8-dimethylquinazolin-4(3H)-one), **C** (2-(3-bromo-1H-pyrazol-5-yl)-6-chloro-3,8-dimethylquinazolin-4(3H)-one)) and compound **H** (2-(3-bromo-1-(3-chloropyridin-2-yl)-1H-pyrazol-5-yl)-6-chloro-3,8-dimethylquinazolin-4(3*H*)-one) (See in Chapter 4.3) were isolated and separately injected into the HPLC, to obtain the corresponding

retention times and peak area - concentration relations. This enabled us to monitor their formation and disappearance during the degradation of CAP.

### 4.2.2 UV-VIS absorption spectra determination

UV-VIS absorption spectra were determined for CAP and its phototransformation products **A**, **B** and **C**. The compounds were dissolved in acetonitrile and diluted with dd$H_2O$ to obtain a solvent ratio of 1:4. UV-VIS spectra were determined also for humic acids (HA), at concentrations 10, 30 and 100 mg/L, dissolved in acetonitrile-dd$H_2O$ (1:4) that was used in the photodegradation experiment. Humic acid absorption spectra were taken for comparison with the absorption spectra of CAP and the emitted light spectra of the solar simulator to explain the possible shielding effect. All UV-VIS absorption measurements were obtained using a Hewlett Packard HP 8453 spectrometer.

### 4.2.3 $^{1}$H and $^{13}$C nuclear magnetic resonance spectroscopy of transformation products

NMR spectroscopy was used to obtain an indication of the chemical structures of the CAP transformation products. $^{1}$H and $^{13}$C NMR spectra of compounds were obtained using a Bruker Avance III 500 NMR spectrometer. After isolation, the transformation products were dissolved in 650 µL of the most appropriate solvent, depending on the solubility of the compounds: $CD_2Cl_2$ was used for transformation products **H** and **B**, $CDCl_3$ for compound **A**, and acetone $d_6$ for compound **C**.
Chemical shifts were reported against the tetramethylsilane standard.

### 4.2.4 Elemental analyses of transformation products

The mass fractions of carbon, hydrogen and nitrogen (%) of the pure transformation products were determined using Perkin Elmer Series II, CHNC/O Analyzer 2400. The obtained results were compared with the calculated content of each element for each transformation product.

### 4.2.5 LC-MS-TOF analyses of transformation products

The mass spectra of transformation products were obtained on an Agilent 6224 Accurate Mass TOF LC/MS system with double electro spray ionization (ESI) source at atmospheric pressure, operating in positive mode. Since the compounds were pure, no chromatography was needed and compounds dissolved in acetonitrile were directly injected into the MS system. The results were compared with the calculated molar mass of the compounds.

### 4.2.6 IR spectroscopy of transformation products

IR spectroscopy was used as a one of the supporting methods to suggest the structure of compounds, based on the presence of the main functional groups. The characterization of transformation products based on their IR spectra was determined with a Perkin-Elmer Spectrum BX spectrometer, using an attenuated total reflectance sampling technique. Samples were examined in solid state.

### 4.2.7 Melting point determination of transformation products

Melting point of transformation products was determined using an OptiMelt EZ (Stanford Scientific) Automated Melting Point System.

### 4.2.8 X-Ray Crystallography of transformation products

X-Ray Crystallography data were collected on a Nonius Kappa CCD diffractometer using graphite monochromated Mo-$K_\alpha$ radiation. Data reduction and integration were performed with the software package DENZO-SMN (Otwinowski and Minor, 1997). Averaging of the symmetry-equivalent reflections largely compensated for the absorption effects. The coordinates of some or all of the non-hydrogen atoms were found via direct methods using the structure solution program SHELXS (Sheldrick 1997a, b). The positions of the remaining non-hydrogen atoms were located by use of a combination of least-squares refinement and difference Fourier maps in the SHELXL-97 program (Sheldrick, 1997a, b). Non-hydrogen atoms were refined anisotropically. The amide hydrogen atom of **A** and the hydroxyl hydrogen atom of **B**, located in the final stages of the refinement from the different Fourier maps, were refined with isotropic displacement parameters. The remaining hydrogen atoms were included in the structure-factor calculations at idealized positions. All the calculations were performed using the WinGX (Farrugia, 1999). Figures depicting the structures were prepared by ORTEP-3 (Farrugia, 1997).

### 4.2.9 Total organic carbon and total nitrogen determination

Total Organic Carbon (TOC) and Total Nitrogen (TN) content of tap water were determined on an Analytic Jena Multi C/N 3100 analyzer. Prior to the analysis, samples were acidified to pH 2-3 with hydrochloric acid.

### 4.2.10 Analyses of the test solutions from the acute and chronic toxicity tests with *Daphnia magna*

Tests solutions in the polypropylene tubes that were dedicated for concentration measurements were stored in the freezer (-20°C) prior analysis. All samples were

filtered prior to analyses with a polypropylene syringe filter (13 mm diameter, 0.22 μm pore size, Acrodisc, VWR, Amsterdam, the Netherlands).
To quantify, individual stock standards were prepared by dissolving the pure compound in acetonitrile, from which external calibration standards were prepared and used before the analyses of the test solutions.

Samples from the acute tests were measured using UHPLC system (Nexera UFLC, Shimadzu, Den Bosch, the Netherlands) coupled to a high resolution Time of Flight Mass spectrometer (Q-TOF; maXis 4G, Bruker Daltonics, Wormer, the Netherlands). Compounds were retained on a Waters X-Bridge C18 stationary phase (100 × 2.1 mm; 3.5 μm particle size, 100 Å pore size). The elution was isocratic and consisted of 55% A (80:20 $H_2O$/acetonitrile with 5 mM ammonium formate and 0,016% formic acid) and 45% B (acetonitrile) at a flow rate of 0.4 mL/min. Injection volume was 10 μL.
The LC effluent was ionized with an IonBooster source set in positive mode. The ionization settings were as follows: vaporizer temperature 250°C, dry heater temperature 200°C, dry gas flow 3 L/min, nebulizer gas flow 4.1 bar, capillary voltage 1000V, end plate offset -400V and charging volt 300V. Nitrogen was used as ionization and collision gas.
The mass spectrometer was upgraded with a HD collision cell. The settings used were as follows: collision cell RF 350 Vpp, transfer time 50 μs and pre puls storage time 10 μs.
Mass spectra were recorded in MS mode and MS/MS from *m/z* 50 – 1200 *m/z* at a rate of 2 Hz. The former were used for quantitation, while two product ions from MS/MS scans were used to confirm the identity of CAP (*m/z*: 452.9336 and 285.9199) and TP2 (*m/z*: 416.9571 and 354.9777).

The analyses of test solutions from the chronic test with CAP was performed using an HPLC system (Nexera Prominence XR, Shimadzu, Den Bosch, the Netherlands) coupled to a tandem mass spectrometer (QTRAP 4000, Applied Biosystems, Toronto, Canada). The chromatography was conducted same as on Q-TOF, except from the flow, which was here 0.2 mL/min.

Electrospray ionization (ESI) was set to positive mode to ionize the LC effluent. The ionization settings were as follows: ionization temperature 500°C, ion spray voltage 5500 V, curtain gas 10, nebulizer gas 40, heater gas 50 and collision gas 6 (all arbitrary units). Nitrogen was used as ionization and collision gas. Multiple reaction monitoring was used for MS detection and two transitions were measured to quantify CAP (Table 3), using a scan time of 60 ms.

***Table 3: Transitions measured for chlorantraniliprole quantification on LC-MS/MS spectrophotometer.***

| Transition | Collision energy | Collision cell exit potential |
|---|---|---|
| 484 → 453 | 16 | 20 |
| 484 → 286 | 17 | 23 |

The mass spectrometer was automatically calibrated during every measurement by injection of 20 µl of a 2 mM sodium formate solution dissolved in 1:1 isopropanol – water. Mass errors of the MS and MS/MS signal were checked to be below 5 mDa.

The actual concentrations of TP2 from the chronic toxicity test were measured by direct injection of the sample into a Shimadzu Prominence HPLC-DAD system. The mobile phase, elution and the column were the same as for the measurements of CAP from the chronic toxicity test, except from the injection volume, which was here 20 µL.

The concentrations from the acute toxicity test with CAP were measured in the test media at the beginning and at the end of the toxicity test (24h) in 6, 5, 3, 3 and 2 replicas for CAP concentrations 2, 5, 10, 20 and 50 µg /L, respectively. TP2 was in acute test measured in one replica in the beginning and two replicas at the end of the test, for all concentration range. The concentrations from the chronic toxicity test with CAP were measured in one replicate per concentration from three renewal events in freshly prepared and old test media (3 days) for 1 and 3 µg /L and from one renewal event for higher test concentrations. TP2 was in test solutions from the chronic toxicity test measured in four renewal events in new and old media (3 days), one replica per concentration.

To control for matrix effects in test solutions with CAP, the standard addition method (SAM) was applied for each sample. To the 500 µL sample 0, 5, 10, and 15 µL standard CAP solution (100 µg/L) was added and topped to 1 mL with acetonitrile.

The CAP concentration was calculated by dividing the intercept with the slope from the regression line obtained from the SAM dilution series.

## 4.3 Preparation of chlorantraniliprole transformation products

In our experiments four main degradation products of CAP were formed, indicated as compounds **A** (2-((2-bromo-4*H*-pyrazolo[1,5-d]pyrido[3,2-b][1,4]oxazin-4-ylidene)amino)-5-chloro-*N*,3-dimethylbenzamide) , **B** (2-(3-bromo-1-(3-hydroxypyridin-2-yl)-1*H*-pyrazol-5-yl)-6-chloro-3,8-dimethylquinazolin-4(3*H*)-one), **C** (2-(3-bromo-1*H*-pyrazol-5-yl)-6-chloro-3,8-dimethylquinazolin-4(3*H*)-one ) and **H** (2-(3-bromo-1-(3-chloropyridin-2-yl)-1*H*-pyrazol-5-yl)-6-chloro-3,8-dimethylquinazolin-4(3*H*)-one). To characterize these products, they were produced in higher amounts applying the following techniques:

- To prepare compound **A**,100 mg CAP, dissolved in 500 mL acetonitrile-dd$H_2O$ (1:4 v/v), was irradiated with four low pressure Hg lamps (Philips UV-C, 15 W, $\lambda_{max}$ =254 nm) in a quartz cell (10 mm × 10 mm × 40 mm). After the total degradation of CAP, the solvent was evaporated under reduced pressure and the reaction mixture was separated by radial preparative chromatography ($SiO_2$, ethyl acetate-petroleum ether-acetic acid (1:1:0.05)). After crystallization (dichloromethane, heptane), 58 mg (63 %) of compound **A** was obtained.
- To obtain degradation product **B**, 170 mg of CAP, dissolved in 500 mL of acetonitrile-dd$H_2O$ (1:1 v/v) was irradiated as described above. Crude **A** was then dissolved in a mixture of 100 mL acetonitrile and 100 mL 0.05 M pH 8 phosphate buffer. The reaction mixture was left for six days at room temperature and the solvent evaporated. The

remaining solid residue was dispersed in water and filtered. After crystallization (acetonitrile, water), 55 mg (35 %) of pure compound **B** was obtained.

- To prepare compound **C**, 12 mg of compound **B**, dissolved in acetonitrile-dd$H_2O$ (1:1 v/v) was irradiated with 6 UV-A (broad spectrum with maximum at 352 nm) lamps (15 W black-light, FL15BLB, Sankyo Denki, Japan). The degradation was monitored by HPLC and the irradiation was stopped when compound **B** was completely degraded (approximately 10h). The solvent was evaporated and the reaction mixture was subjected to radial preparative chromatography ($SiO_2$, diethyl ether-petroleum ether-acetic acid (1:1:0.1)) and 1.6 mg (17 %) of reasonably pure compound **C** was obtained. Complete characterization of **C** could not be performed due to its low yield. Nevertheless the data obtained by LC-MS-TOF and $^1$H NMR were evident enough to suggest its chemical structure.
- To obtain compound **H** in higher amounts, 100 mg of CAP was dissolved in acetonitrile-dd$H_2O$ (1:4 v/v, 250 mL) with added $Na_2CO_3$ (250 mg). The solution was placed in the refrigerator for 4 days, until compound **H** precipitated. After filtration, 67 mg (70 %) of pure **H** was obtained.

The isolated compounds were used for the characterization by LC-TOF-MS, elemental analysis, IR, $^1$H and $^{13}$C NMR spectroscopy and X-Ray Crystallography. The melting point of the chemicals was also determined. Compounds **A**, **B** and **H** were further used in the chemical and photochemical stability studies while compounds **B** and **H** were also included in the toxicity assessments.

## 4.4 Stability of chlorantraniliprole and transformation product A in different aqueous media

### 4.4.1 Stability of chlorantraniliprole in aqueous media

Stability of CAP was studied in different aqueous media: in Dutch tap water with pH 8.2 and with dd$H_2O$ prepared buffer solutions of pH 4.0, 5.5, 7.2, 8.0, using citric acid, and of pH 7.2, 8.0 and 9.0 using tris (hydroxymethyl)aminomethane. All solutions contained also 20 v% of acetonitrile. The initial concentration of CAP was 9.94 mg/L (20.6 μM). The solutions were stored in duplicate glass vials and kept in the dark at the room temperature (20 °C) for 21 days. Aliquots were taken for the HPLC analysis on days 0, 1, 2, 4, 7, 14 and 21.

### 4.4.2 Stability of transformation product A

Stability of degradation product **A** was studied in 1:4 v/v mixture of acetonitrile and 10 mM phosphate buffer solution with pH 4.0, 7.0 and 9.0, prepared in dd$H_2O$. The initial concentration was 50 mg/L. Samples were incubated at room temperature (20 °C) in the dark. Immediately after preparation of the solutions, HPLC measurements were performed that ran continuously for 5 hours and at longer intervals afterwards (2, 6, 50, 90 and 100 h from the start of the experiment). Before the samples were analyzed, a standard solution of CAP in acetonitrile with a known concentration was injected into the system as a reference.

## 4.5 Photochemical degradation of chlorantraniliprole and its transformation products

Photodegradation of CAP was studied using different light sources. For the purpose of characterization of CAP transformation products, preparative photochemical reactions were carried out with low pressure Hg lamps (Philips UV-C, 15 W). For the pilot CAP degradation experiments and for the photolysis of degradation product

**B**, an LTD MLU18 photochemical reactor was used, equipped with a black-light blue lamp (FL15BLB, Sankyo Denki, Japan, 15 W) emitting a broad UV-A light spectrum. In order to simulate natural sunlight conditions, a solar simulator (Suntest CPS+, Atlas MTT) operating at a light flux of 750 W/m$^2$ was used to study the photostability of CAP in different aqueous media: dd$H_2O$, tap water, tap water amended with humic acids and with the addition of nitrate.

### 4.5.1 Photodegradation of chlorantraniliprole using low pressure Hg lamps (254 nm)

Illumination of the CAP solution with UV-C light was predominantly used for the preparation of CAP photodegradation products. However, one experiment was intended to follow the UV-C photodegradation of CAP over time. For this experiment, a solution of CAP was prepared in acetonitrile-dd$H_2O$ (1:1 v/v), with a concentration of 50 mg/L. Fifty mL of solution was poured into the quartz cell (10 mm × 10 mm × 40 mm) and irradiated with 4 UV-C lamps (Philips UV-C, 15 W). The aliquots for HPLC analysis were taken after approximately 5, 10, 20, 30, 40, 50, 60, 80,100, 120, 150 and 180 seconds of irradiation. The pH of the solution was measured before the irradiation started and after 2 minutes of irradiation. The irradiated solution was, wrapped in the aluminum foil, standing over night and sampled for HPLC analysis again the next morning.

### 4.5.2 Photodegradation of chlorantraniliprole in tap and deionized water under UV-A light

For the CAP photodegradation study under UV-A light, two solutions were prepared, one in acetonitrile-dd$H_2O$ (1:4 v/v) and one in tap water instead of dd$H_2O$. CAP concentration in both solutions was 19 mg/L (39 μM). Two replicates of each solution were made. The solutions in quartz cells were then irradiated with six UV-A light emitting lamps. The average intensity, measured with PCE-UV34 UV light

meter, PCE group, working in a range of 0-2 $mW/cm^2$, was 0.1362 $mW/cm^2$. Aliquots for HPLC measurements were taken at the start of the experiment and daily during the continuous irradiation for 10 days. Additionally, at the beginning and end of the experiment, the pH of the samples was determined.

### 4.5.3 Photodegradation of chlorantraniliprole in tap and deionized water under simulated solar light

In a simulated solar (Suntest) apparatus, irradiating at the intensity of 750 $W/m^2$, two final experiments were conducted. In the first experiment CAP was tested at a concentration of 31 μM (15 mg/L) in acetonitrile-dd$H_2O$ (1:4 v/v). In the second, dd$H_2O$ was replaced with tap water and CAP concentration was 17 μM (8 mg/L). Solutions (20 mL) in borosilicate glass vessels were continuously irradiated for 6 days and aliquots were collected daily for HPLC analysis. The pH of the solutions was measured at the start and end of the test. Dark controls, consisting of CAP solutions wrapped in aluminum foil, were also included. The average temperature in the Suntest chamber was 26.3 °C.

### 4.5.4 Photodegradation of chlorantraniliprole under simulated solar light in the presence of humic acids and nitrate

To determine the possible influence of dissolved organic matter (DOM) on CAP photodegradation rate, CAP solutions were prepared in acetonitrile-tap water (1:4 v/v) with addition of 0, 10, 30 and 100 mg/L humic acid. To study the influence of nitrate on CAP photodegradation, the solution was prepared containing 10 mg/L nitrate. The CAP concentration was in all cases 8 mg/L (17 μM). Twenty mL of each solution was added into identical borosilicate erlenmeyers, which were properly closed to avoid evaporation of the solvent. Samples were then irradiated continuously for 6 days in a Suntest chamber at 750 $W/m^2$, and degradation was

monitored daily by HPLC. Corresponding dark controls (samples wrapped in aluminum foil) were also included.

### 4.5.5 Photodegradation of compound H in tap water under UV-A light

To study the photostability of compound **H**, a solution of **H** was prepared in tap water-acetonitrile mixture in 1:3 (v/v) ratio (150 mg/L). As compound **H** appeared to be less water soluble than CAP, a higher volume of organic solvent was needed. Solution (100 mL) in a quartz cell was then irradiated by 6 UV-A lamps with emission peak of 352 nm and intensity of 0.1362 $mW/cm^2$. The aliquots were taken each hour for an HPLC analysis.

### 4.5.6 Laser flash photolysis study of chlorantraniliprole and transformation product B

With an aim to track the short-lived intermediates in the photolysis process of CAP and compound **B**, short-term laser photolysis was performed with an Applied Photophysic LKS 60 instrument, equipped with a Nd:YAG laser (Brilliant B, Quantel) using the $4^{th}$ harmonic (266 nm, laser pulse width ≈ 8 ns). The equipment, besides the laser used to monitor the absorbance consisted of a 150 W pulsed xenon lamp, monochromator and a photomultiplier. The signal was digitalized using an oscilloscope (Agilent infiniium DSO8064A). Pro-K software from Applied Photophysics was used for data analysis.

CAP solutions (10 μM) saturated with air in the first, and argon in the second experiment, were prepared in acetonitrile-dd$H_2O$ (1:9 v/v). Traces were recorded at wavelengths between 280 and 400 nm. Solution was renewed after every third measurement.

To study whether compound **B** forms any short term intermediates, a stock solution was firstly prepared by dissolving 3 mg in 10 mL acetonitrile and the solution was

diluted to obtain the maximum absorption of compound **B** around 0.2 AU. Prior to the measurements, solutions were saturated with argon. Absorption spectrum was taken at the wavelengths from 340-620 nm.

### 4.5.7 Photochemical degradation of transformation product B under UV-A light

To study the stability of CAP transformation product **B**, 4.6 mg of pure compound **B** was dissolved in 50 mL of acetonitrile-dd$H_2O$ (1:1). The solution was placed in a quartz cell and irradiated with six UV-A lamps, emitting light with its spectral maximum at 352 nm (average intensity was 0.1362 mW/cm$^2$). To monitor degradation over time, aliquots for HPLC analysis were taken at the beginning of the experiment and every hour, for 10 hours.

## 4.6 Toxicity tests with the water flea *Daphnia magna*

### 4.6.1 Test organism

We selected the fresh water crustacean *Daphnia magna* Straus to determine the acute and chronic toxicity of CAP and its transformation products **B** and **H**. *D. magna* neonates (younger than 24h, clone 4) for the toxicity tests were obtained from Grontmij Aquasense (Amsterdam, the Netherlands). The cultures were maintained in a glass aquarium in Elendt M4 medium (OECD, 2004a); the volume of medium was sustained at a minimum of 30 mL per adult, with a total volume of 4-4.5 L. The pH of the medium was 7.8 ± 0.5 and the conductivity 50–80 μS/mm. To keep the concentration of dissolved oxygen high, the medium was kept under continuous aeration. Cultures were maintained at a 16:8 h light-dark photoperiod with a twilight zone of 30 min, and a temperature of 20 ± 1 °C. The medium was renewed twice per week with the simultaneous removal of neonates. New cultures were started by isolating daphnid neonates, younger than 24 hours, from the culture cultivated for

three weeks. The daphnids were fed five days a week with a suspension of the alga *Scenedesmus subspicatus* originating from a batch culture in CP-medium (NPR 6505, 1994; Waaijers et al., 2013). The algal culture was kept in a climate room at 20 ± 1 °C under continuous light and aeration. Every two weeks, algae were harvested by filtration (0.45 µm). The supernatant was removed and the algae were resuspended in Elendt M4 medium (stored at 4 °C in the dark until feeding). The cell density was verified with a spectrophotometer (Hachlange Dr2800) and total organic carbon (TOC) with a TOC analyzer (TOC-V cph, Shimadzu). The density of the food suspension corresponded to $3 \times 10^9$ cells/L and about 65 mg carbon/L. The new daphnid culture was fed 69 mL (days 1-2), 102 mL (days 3-7) and 139 mL (day 8 and further) of algae suspension per day (Waaijers et al., 2013). Acute toxicity tests with the reference compound $K_2Cr_2O_7$ were performed on a regular basis, in order to test whether the sensitivity of *D. magna* cultures was within the limits ($EC_{50}$ 24h = 0.6-2.1 mg/L), as set by the guideline (OECD, 2004a).

### 4.6.2 Acute toxicity tests

To determine the acute toxicity of CAP and its two transformation products **H** and **B**, daphnids were exposed in 48h immobility tests, according to OECD guideline 202 (OECD, 2004a). The following nominal concentrations were tested: 0, 2, 5, 10, 20, 50 µg/L CAP; 0, 0.05, 0.1, 0.2, 0.5 and 1 mg/L compound **B** and 0.14 mg/L compound **H** (the limit of water solubility, EFSA 2013). All solutions were prepared in ISO medium (OECD, 2004a). Because of the low solubility of the compounds in water (CAP: 0.88 mg/L; FAO 2008, EPA 2008) dimethylsulfoxide (DMSO) was used as a carrier solvent and therefore a solvent control was also included. DMSO was chosen because of its low toxicity to *D. magna* compared to other organic solvents often employed in toxicity tests (Barbosa et al., 2003). A DMSO concentration of 0.0006 v/v % was used in the tests with CAP and **H** and 0.0024 v/v % for **B**, with all treatments per toxicity test containing the same solvent concentration.

Per test concentration, four replicates were prepared. Each replicate consisted of a polypropylene tube containing 40 mL of test solution into which five daphnid

neonates, younger than 24h, were placed using a disposable transfer pipette. The test tubes were randomly distributed in a climate-controlled fume hood (20 ± 1 ºC), with a 16:8 h light-dark regime.

After 24 and 48 h the daphnids were checked for immobility. The daphnids that were not able to swim after gentle stimulation by tapping the tubes were considered immobilized. Physical-chemical parameters (temperature, oxygen level, pH, hardness and conductivity) were determined at the beginning and at the end of test for all test concentration, as recommended by the guideline (OECD, 2004a).

### 4.6.3 Chronic toxicity tests

To determine the chronic toxicity of CAP and its two transformation products, **H** and **B**, 21-day daphnid reproduction tests were performed following OECD guideline 211 (OECD 1998, 2012), except where noted. Nominal test concentrations were: 0, 1, 3, 6, 9, 12 µg/L CAP, 0, 0.05, 0.1, 0.2, 0.5 and 1 mg/L compound **B** and 0.14 mg/L compound **H**. Test solutions were prepared in Elendt M4 medium (OECD 1998, 2012) and DMSO was used as a carrier solvent (0.00012 % (v/v) in the test with CAP and **H** and 0.0025 % (v/v) in the test with **B**. Per test concentration, fifteen replicates were prepared. Each replicate consisted of a 50 mL polypropylene tube containing 40 mL of test solution. The tubes were randomly distributed in a controlled fume hood (20 ± 1º C) with a light-dark photoperiod 16:8 h. The experiment was started by introducing one daphnid neonate younger than 24 h into each test tube. The test solutions were renewed three times a week using freshly prepared stock solutions. Before and directly after renewal, oxygen concentration, temperature and pH were measured, as recommended by OECD guideline 211 (OECD 1998, 2012). Daphnids were fed daily with a concentrated alga suspension (*S. subspicatus*) obtained from Grontmij Aquasense, Amsterdam. The density of algal suspension was 2850 cells/µL and the daily aliquots per daphnid were: day 0-2: 450 µL, day 3-5: 700 µL, and days 6-21: 900 µL. Daily, the daphnids were checked for immobility and mortality was recorded if no movement of the daphnid was noticed after a gentle stimulus. When reproduction started, the offspring was counted and removed from the tubes on a daily basis.

## 4.7 Toxicity test of chlorantraniliprole and compound H with the sediment worm *Lumbriculus variegatus*

This toxicity test was carried out to assess the possible effects of prolonged exposure to CAP and compound **H** on the sediment-ingesting endobenthic oligochaete *Lumbriculus variegatus* (Müller). The endpoints of interest in this test were the mortality of adults and the reproductive output. The test followed OECD guideline 225 (OECD, 2007). Because the ingestion of contaminated food can be a significant exposure route, stinging nettle was used as a food source and homogeneously mixed in with the freshly prepared sediment at the beginning of the test.

### 4.7.1 Test organism

The culture of *L variegatus*, used in the toxicity test, was originally obtained from Utrecht University, the Netherlands and further cultured at the University of Amsterdam (León Paumen et al., 2008). Prior to the toxicity test, the animals were maintained in glass aquaria (32 × 17 × 18 cm) filled with demineralized water and a layer of cellulose as a substrate, which was covering the bottom area of the aquaria. The aquaria were kept in a climate room, with a constant temperature of 20 °C and a 16:8 light:dark photoperiod. The aquaria were washed on a regular basis to prevent bacterial and fungal growth with renewal of overlaying water and sediment. Cultures were fed weekly with commercially available fish food, Tetraphyl® (Tetra Werke, Melle, Germany).

### 4.7.2 Preparation of the media and sediment spiking procedure

For the toxicity test, a static sediment-water system was assembled using an artificial sediment and reconstituted water as test media following the procedure of Marinković et al. (2011).

The formulated sediment was prepared by mixing quartz sand (grain size of 0.5-10 mm), kaolin clay and α-cellulose in a ratio of 75, 20 and 5 %, respectively. $CaCO_3$

was used to adjust the pH of the artificial sediment to 7.0 ± 0.5.The sediment was prepared in bulk, well mixed and moistened with demineralized water to obtain a water content of 34 % (w/w).

Selected compounds were tested in the following concentrations: 0, 25, 50, 100, 200, 400 and 800 µg/g dw CAP and 800 µg/g dw compound **H**. Since CAP and transformation product **H** have a low solubility in water, the spiking was accomplished according to León Paumen et al. (2008), using acetone as the solvent. Solvent controls were also included in the test. A portion of 10 % (60 g per treatment) of the total amount of dry weight reconstituted sediment, dedicated for the spiking of one treatment, was added into the 1L glass bottle. A defined amount of CAP and compound **H** was dissolved in 150 mL acetone that was added to the soil and the mixture was rolled on a roller bank (20 rpm) overnight. The next day the bottles were opened and placed in the fume hood to allow for evaporation of the solvent. When no traces of acetone were left, the spiked sediment was moistened with demineralized water to 34 %, after which the remaining wet sediment was added. At that point 0.5 % dw of stinging nettle was added to the formulated sediment as a food source for the entire duration of the test (28 days). To homogeneously mix the sediment-food-compound mixture, the bottles were placed on the roller bank for 24h (20 rpm). For the overlying water, Dutch standard water was used and prepared freshly by dissolving $NaHCO_3$, $KHCO_3$, $CaCl_2 \times 2H_2O$ and $MgSO_4 \times 7H_2O$ in demineralized water to concentrations of 100, 20, 200 and 180 mg/L, respectively.

### 4.7.3 Toxicity test set up and procedure

Eight replicates were prepared per treatment, four for the toxicity test, while the other four were sacrificed for chemical analysis and did not contain animals. Prepared sediment was distributed into 150 mL glass test jars, each jar containing 60 g of sediment. On top of the sediment, 120 mL of reconstituted Dutch standard water was slowly poured. The test beakers were placed in a climate-controlled fume hood (20 ± 1 ºC), with a 16:8 h light-dark regime and covered with plastic foil to limit water evaporation. However, to allow aeration, a glass pipette, connected to a constructed

aeration system, perforated the foil and was dipped into the overlying water. This allowed continuous and gentle aeration of the static water-sediment system. While aerating, the test jars were conditioned for seven days to allow the test compounds to equilibrate with the sediment-water compartments.

After equilibration, ten intact and complete worms of similar size, that were actively swimming upon a gentle mechanical stimulus, were introduced into each test beaker in a random order. The animals were exposed to the static system for a period of 28 days without renewal of the overlying water and without any further addition of food. However, due to the continuous aeration, the water level had to be sustained by replenishing the water loss on a daily basis. Once a week, temperature, pH, oxygen concentration, water hardness and ammonia content were measured in the overlying water. Sediment, water and pore water samples for chemical analysis were taken at the beginning of the test and at days 7, 14, 21 and 28.

After 28 days of exposure, the worms were collected from the test beakers. Sediment was gently sieved through 250 µm mesh, allowing the sediment to pass the sieve with the worms remaining on the sieve. Recovered worms from each replicate were washed into a broad glass vessel where they were counted and examined. The numbers of juveniles and adults distinguished into complete, incomplete (recently fragmented) and dead worms were recorded. Missing and non-responsive (after gentle mechanical stimulus) adult worms were considered dead.

The reproductive output was calculated, using the following equation (Equation 4):

$$Y(\%) = \frac{n_{t28} - n_{t0}}{n_{t0}} \times 100 \quad \text{(eq. 4)}$$

where $Y$ is the average reproductive output, $n_{t0}$ is the initial number of worms (T = 0; 10 worms), and $n_{t28}$ is the number of worms found in a jar at the end of the test (28 days).

## 4.8 Toxicity tests of chlorantraniliprole with soil invertebrates

The mean distribution coefficient of CAP between water and soil phase ($K_d$) was found to be 3.18 mL/g (range 0.8-7.88) and the corresponding average $K_{OC}$ was 329 mL/g (range 152-535); values obtained by testing on 5 soils from the US and Europe (APVMA, 2008). These results indicate that CAP can be moderately adsorbed to soils. Due to the risk that the accumulation of CAP in the soil could harm soil dwelling terrestrial animals, the toxicity of CAP was assessed for several soil invertebrates: springtails, isopods, oribatid mites and enchytraeids.

### 4.8.1 Soil spiking procedure

For all toxicity tests, except where noted, a natural loamy soil Lufa 2.2 (LUFA Speyer, Germany) was used. It contains 3.74 ± 0.26 % organic matter (OM) and has a $pH_{CaCl2}$ of 5.5 ± 0.2 and a water holding capacity (WHC) of 42.5 ± 3.2 g/100g (Lufa, 2013). Before the start of the toxicity tests, the soil was pre-dried in the oven at 50 °C overnight.

For each treatment, a specified amount of soil was weighed, from which 10 or 25 % was used for spiking. Acetone was used as a carrier solvent of CAP; all the treatments received the same and sufficient amount of the solvent, assuring that the insecticide was homogeneously distributed over the whole portion of the soil. Corresponding solvent controls were prepared along. After spiking, the glass beakers with the spiked soil were closed and placed in the fume hood overnight. The next day, the jars were opened to let the acetone evaporate completely. Spiked soil was then merged with the remaining portion of the soil and mixed thoroughly with a spoon. Demineralized water was added to the soil, while constantly stirring, until a soil moisture content equivalent to 50 % of the soil's WHC was achieved, except where noted differently.

### 4.8.2 Soil pH determination

The pH of the soils was measured at the beginning and end of the tests following ISO guideline 10390 (ISO, 2005) with slight modifications. pH was measured in duplicates for every treatment in the toxicity test. For this measurement, 6 g of moist soil was placed in a plastic bottle and 25 mL of a 0.01 M analytical grade $CaCl_2$ solution was added. The bottles were tightly closed and the suspension was shaken for 2h at 200 rpm. The bottles were left to stand for few hours until the soil settled, after which the pH of the overlying was measured with a pH meter. Two blanks containing only $CaCl_2$ were also measured.

## 4.9 Toxicity test of chlorantraniliprole with the woodlouse *Porcellio scaber*

In the test, isopods were exposed to soil contaminated with CAP, and endpoints measured included survival, food consumption and behavior of the isopods.

### 4.9.1 Test organism

Adult isopods *Porcellio scaber* were brought to the laboratory from an uncontaminated area in Bilthoven, the Netherlands. Animals were placed in a glass terrarium with a thick layer of Lufa soil covered with natural occurring leaves (mainly maple and poplar) obtained from the same site as the isopods. Prior to the experiment, the animals were kept in a climate room at 15 °C with a 16/8 h light/dark regime for approximately one month to acclimatize. Twice a week, the inside of the terrarium was sprayed with water to sustain the moisture content and fresh leaves were provided on a regular basis.

### 4.9.2 Toxicity test procedure

As no information about the toxicity of CAP to isopods was available in the literature, a range-finding toxicity test with widely spaced concentrations was designed to test in which concentration range toxicity would occur. Soil was spiked with nominal concentrations of 10, 100 and 1000 μg CAP/g dw, and a control and solvent control were included. A portion of 25 % (25 g) of the total amount of dry soil was spiked, using 45 mL of acetone. After evaporation of the acetone, the spiked soil was mixed with the rest of the soil and moistened to 45 % WHC. Well homogenized soil was then placed in clean plastic beakers, so that the bottom of the beakers was completely covered with soil (approximately 25 g moist soil per beaker). The beakers were covered with plastic lids, which were perforated to allow for air circulation. Per treatment, four replicates were made, three for the toxicity test and one for the chemical analysis at the end of the test. A portion of prepared soil was also taken at the beginning of the test and stored in the freezer for further chemical analysis.

Prior to the experiments, healthy and active isopods of similar size, which were not in the process of molting, were selected from the culture and placed into a large glass container. From there, three isopods were collected for each replicate, examined, cleaned from soil particles and weighed both individually and together. The weight of individual animals varied from 35 to 67 mg. The selected isopods were then added to the test beakers, each beaker containing three animals. No distinction was made regarding to sex of the animals. The mass of the beakers was recorded in order to follow water loss during the experiment. Test beakers were randomly placed in the test incubator set at 20 °C, 75 % relative humidity and a 16/8 h light/dark photoperiod.

At the start of the test and every 7 days, the animals were fed alder leaves (*Alnus glutinosa*). Leaves were washed, cut into identical round pieces, dried overnight in an oven at 50 °C and their mass was recorded before being placed in the test beakers. Leaf residuals were at the same time removed from the test containers, dried and weighed. Care was taken that the amount of food provided was always in abundance. Evaporated water was replenished weekly and animal condition was monitored daily, and any behavioral changes and deaths were recorded. After 32 days the test was finished with the final weighing of the surviving animals and leaf left overs. This

data allowed us to calculate the body weight changes (BWC) and determine the consumption rate (CR).

The body weight change (BWC) was determined as (Equation 5):

$$BWC = \frac{Bw_{t32} - Bw_{t0}}{Bw_{t0}} \times 100 \quad \text{(eq. 5)},$$

where $Bw_{t32}$ is fresh body weight (g) of the isopods in one replica at the end of the toxicity test and $Bw_{t0}$is fresh body weight (g) of the isopods in one replica at the start of the toxicity test.

The consumption rate (CR, g food/g isopod/day) was calculated using the Equation 6:

$$CR = \frac{Fc}{Bw_{t32} \times t} \quad \text{(eq. 6)}.$$

In this equation, $Fc$ stands for the total amount of food (g) the isopods in one replica consumed over the toxicity test period $t$ (32 days) and $Bw_{t32}$stands for fresh body weight (g) of the isopods at the end of the toxicity test.

## 4.10 Toxicity test of chlorantraniliprole with the potworm *Enchytraeus crypticus*

The oligochaete *Enchytraeus crypticus* was exposed to three widely spaced CAP concentrations. The procedure of the toxicity test followed OECD guideline 220 (OECD, 2004b), where the main toxicological endpoint of interest is the reproductive output. Enchytraeids were interesting for our study especially due to fact that they can be exposed to CAP by contact with and ingestion of the contaminated soil and the via the pore water.

### 4.10.1 Test organism

The species *E. crypticus* used in our test has been cultured for several years at the VU University, Amsterdam in climate rooms at 16 °C, 75 % relative humidity and a 16:8 h light dark regime. The enchytraeids were cultured in an agar substrate, prepared with aqueous soil extract and fed twice a week with a mixture of oat meal, dried yeast, yolk powder, and fish oil (He et al., 2014).

### 4.10.2 Toxicity test procedure

The basis of our toxicity test design was OECD guideline 220 (OECD, 2004b). *E. crypticus* was exposed to three CAP concentrations: 10, 100 and 1000 µg CAP/g dw with three replicates per treatment, two for the toxicity test and one for chemical analysis at the end of the test. A portion (25 %) of Lufa soil was spiked with CAP dissolved in 33 mL of acetone; a solvent control was included. After equilibration and acetone evaporation, the spiked soil was combined with the rest of the soil, moistened to 50 % of the maximum WHC and well homogenized. From this, a sample was taken for chemical analysis, the rest was distributed into glass test jars, each jar receiving 30 g of moist soil. Ten adult enchytraeids with similar size and clearly visible white clitellum were then placed on the surface of the soil of each jar using a metal hook. Grinded oat flakes were provided as a food source and jars were loosely closed with a screw cap to avoid extensive water evaporation and still allow air circulation. The randomly positioned test jars were placed in climate room at 20 °C, 75 % relative humidity and 16/8 h light/dark photoperiod. When the test proceeded, food and water were replenished when necessary.

After 21 days, the test was terminated. Soil samples from the additional jars not containing animals were used for chemical analysis. Test samples with the animals were firstly fixated by adding 10 mL 97 % ethanol. After 2 min, 100 mL of water was added and the samples were transferred to plastic containers. To stain the animals, 300 µL of Bengal rose dye was added to each sample after which the containers were tightly closed and the content was agitated vigorously for few seconds. Dyed samples were then incubated overnight at 4 °C. The next day, samples

were sieved though 160 μm mesh to remove the majority of the soil particles. Adults and juveniles from each jar remained on the sieve and were subsequently collected in a white tray and counted under a magnifying glass.

## 4.11 Toxicity test of chlorantraniliprole with the oribatid mite *Oppia nitens*

Oribatid mites of the species *Oppia nitens* were exposed to five wide ranged CAP concentrations. With this test we sought to get more information about the possible effects of CAP on the survival and reproduction of oribatid mites.

### 4.11.1 Test organism

*O. nitens* was cultured at the VU University, Amsterdam, at 20 ± 2 °C and 16/8 h light/dark regime with a light intensity of 400-800 lux. They were maintained in plastic containers with a bottom of plaster of Paris and fed weekly, adding few grains of granulated dry yeast. The culture substrate was moistened with demineralized water once a week. When the culture became overpopulated, a fraction of the population was transferred to a new container for culturing using a dry paint brush. The culture substrate was renewed every 3-6 months, or as necessary. For the test, non-synchronized adults, with dark brown color shade and good physical health were used.

### 4.11.2 Toxicity test procedure

For the toxicity test, five CAP spiked treatments were prepared in three replicates, with nominal concentrations of 10, 33.3, 100, 333 and 1000 μg CAP/g dw, including a control and a solvent control. One replicate per treatment was dedicated for chemical analysis. The publication of Princz et al. (2010) served as a guideline for

the test. CAP was introduced to Lufa 2.2 soil by spiking a 25 % portion of the soil dedicated for one treatment using 33 mL acetone as a carrier solvent (see 4.8.1). The soil was moistened to 50 % WHC and distributed to plastic test containers, each containing 25 g of moist soil. The test used containers with a gauze bottom to enable easy extraction of the mites at the end of the test.

Mites of similar color were first isolated from the culture into a separate container with a plaster of Paris bottom. The selection of mites for the toxicity test was conducted under a stereomicroscope - 20 visually healthy mites with same color shade were collected from the detached selection and randomly placed in the test containers. Containers were equipped with a lid with a hollow center, but covered with tightly woven mesh that prevented mites from escaping and also limited water condensation in the containers. Evaporated water had to be replenished daily. At the beginning and after 14 days of exposure, the mites were fed with a few grains of dry baker's yeast. Test containers were incubated at 20 °C, 75 % relative humidity and 16/8 h light/dark regime and their position was regularly changed. After 35 days the test was terminated and the mites were recovered from the test soil via heat extraction. The extraction itself lasted for 5 days and mites were collected on a plaster of Paris bottom, placed under the test containers which had perforated bottom. During the extraction period, the plaster bottoms were moistened twice to prevent drying of the collected animals. Finally, the number of collected adults and juveniles was recorded by counting them under the microscope.

## 4.12 Toxicity tests of chlorantraniliprole with the springtail *Folsomia candida*

Several toxicity tests with CAP were performed on the soil dwelling springtail *Folsomia candida*. First, a standard chronic toxicity test was conducted, where in addition to survival of the exposed animals also their fecundity was determined. The second research question we aimed to answer with laboratory toxicity tests was whether springtails were able to avoid soil contaminated with CAP. Lastly, we wanted to study if and to what extent soil properties influenced the toxic effect of

CAP on collembolan reproduction. Toxicity tests were designed following the available international test guidelines (OECD 2009; ISO 2011, ISO 1999).

### 4.12.1 Test organism

*F. candida* (Berlin strain) has been cultured for about 20 years at the VU University, Amsterdam in containers with a bottom of charcoal-amended plaster of Paris, placed in a control climate room at 20 ± 1 °C, 12/12 h light/dark cycle and 400-800 lux illumination. Cultures were maintained by keeping the relative humidity of the air within the containers at 100 % and with frequent aeration and hygiene (removing dead individuals from the cultures as well as mouldy food). They were fed *ad libitum* with dr. Oetker dry baker's yeast.

### 4.12.2 *Folsomia candida* age synchronization

All performed reproduction toxicity experiments with springtails were initiated with juveniles of the same age: 10-12 days old. To achieve their age synchronization, a selection of adults from the cultures was randomly spread in plastic boxes having a moistened black-colored plaster of Paris base. Each box received approximately 30 adults and few grains of dry baker's yeast. The containers were placed in a climate room together with the cultures. After two days, all adults were carefully removed from the boxes, leaving only the freshly laid eggs. Boxes, placed back in the climate room, were regularly moistened and aerated. When the springtails hatched (usually after 8-10 days) they were fed a few grains of dry baker's yeast twice a week, until they were used in the toxicity tests.

### 4.12.3 Procedure of the reproduction toxicity test over two generations

For this study, first the standardized reproduction toxicity test was performed. When the test was finished, a second toxicity test was started using the juveniles produced and collected from the first test. This was done to test for possible increase of toxicity upon exposure over different generations.

The reproduction test followed OECD guideline 232 (OECD, 2009) using natural Lufa 2.2 soil as a substrate. Soil (10 % total dw of the total amount for the test concentration) was spiked with CAP in acetone (15 mL) to obtain the following nominal concentrations: 0.1, 0.254, 0.64, 1.6, 4, 10 and 25 μg CAP/g dw, control and solvent controls were prepared along. See 4.8.1 for the spiking procedure. Soils were moistened to 50 % WHC and portions of 30 g per replicate were distributed into 100 mL glass test jars. Five test replicates and an additional one for chemical analysis were prepared. Springtails (10 ± 1 days old) were examined under a microscope and only visually healthy individuals in good physical condition were used for the experiment. The toxicity test started by randomly placing ten springtails onto the surface of the soil of each test jar, using a suction device. Animals were fed with dry baker's yeast (dr. Oetker) at the beginning and again after 10 days of the test and evaporated water was replenished once per week. Test jars were incubated at 20 ± 1 °C and a light/dark regime 12/12 h.

After four weeks the test was finished and animals from each replicate were extracted by flotation. Water was slowly poured into the test jar to form a 2 cm layer over the top of saturated soil, the suspension was gently stirred with spatula and emptied into a 500 mL beaker. Walls of the test jars were rinsed to assure that the whole content was collected in the beaker. The beaker was then gently swirled and the mixture stirred with a spatula allowing the springtails to float on the water surface. To determine the springtail survival, the number of adults was recorded on the spot and the surface of the solution was photographed using a digital camera (Nikon Coolpix P510). Two pictures per sample were taken. Photoshop's Count Tool was applied to count the number of juveniles per test replicate and a mean value of every sample was calculated. The pH of the soil was measured and samples were stored in the freezer for chemical analysis.

To continue the test with the next generation, juveniles produced in the first toxicity test were transferred from the surface of the water to containers consisting of plastic rings with a thick bottom layer of plaster of Paris, using a spoon. The water excess was absorbed by the plaster. Juveniles from all replicates per treatment were collected together. The containers were closed with a perforated lid and left in a climate room overnight. The next day, 10 juveniles per replicate were distributed into five test jars containing freshly spiked soil of the same concentration range as used in the first test. The procedure of the test was identical to the previous toxicity test, except for the test duration. Because the newborn juveniles the test was started with were younger than 10 days, we ended the second test after five weeks, instead of four, using same procedure as described before. The extension was made to ensure juvenile production also in the second test.

### 4.12.4 Procedure of the reproduction toxicity tests in different soil types

To determine the influence of soil type on CAP toxicity to springtails, toxicity tests were performed as described above for the first generation reproduction toxicity test (4.12.3), following OECD 232 guideline (OECD, 2009). Four identical tests were conducted, however, for each test a different soil was used. All tested soils were natural and were brought to the laboratory from clean areas in Germany (Lufa 2.2), Portugal (Coimbra), The Netherlands (grassland) and United Kingdom (North Wales), here assigned as LF, CO, DG and NW, respectively. Their characteristics: organic matter (OM) content, pH, cation exchange capacity (CEC) and maximum water holding capacity (WHC) are reported in Table 4 (Waalewijn-Kool, 2013).

***Table 4: Properties of the test soils Coimbra (CO), Lufa 2.2. (LF), Dutch grassland (DG) and North Wales (NW) used in the toxicity tests on chlorantraniliprole with* Folsomia candida.**

| Soil type | OM(%) | $pH_{CaCl_2}$ | CEC (mval/100 g) | WHC (g/100g) |
|---|---|---|---|---|
| CO | 2.37 ± 0.06 | 5.9 | 5.17 ± 2.47 | 32 |
| LF | 3.09 ± 0.04 | 5.7 | 6.34 ± 0.81 | 45 |
| DG | 10.6 ± 0.31 | 6.8 | 20.0 ± 0.8 | 73 |
| NW | 14.7 ± 0.18 | 5.0 | 11.8 ± 2.47 | 96 |

***OM = organic matter, CEC = cation exchange capacity WHC = water holding capacity. Data taken from Waalewijn-Kool (2013).***

All soils were pre-dried overnight at 50 °C, and subsequently spiked with CAP to obtain the following nominal concentrations: 0.0256, 0.064, 0.16, 0.4, 1 and 2.5 µg CAP/g dw for Lufa and Coimbra soil and 0.064, 0.16, 0.4 1, 2.5 and 6.25 µg CAP/g dw for North Wales and Dutch grassland soil. Moisture content of the different spiked soils and corresponding controls was brought to 50 % of the respective WHC. For the test, glass jars of 100 mL were filled with 20-30 g of moist soil. Five replicates were made for each treatment, and two extra jars for measuring CAP concentration and soil pH at the end of the test. A Lufa 2.2 control was included in every test in order to secure that the outcome was due to the properties of the soils and not due to a poor health status of the test animals. DG and NW soils had same Lufa control, as they were run simultaneously. Lufa controls were treated in the same way as the controls of the test soils.

Experiments started by adding ten (10-12 days old) springtails into each test jar and a few granules of baker's yeast were added. Exposure took place in a climate room at 20 ± 1 °C and 12/12 h light/dark photoperiod. During the test the jars were aerated twice a week, moistened to their initial weight with deionized water once a week, and more food was added after half of the test period. After 28 days of exposure, the toxicity tests were terminated by flotation as described above (4.12.3).

### 4.12.5 Procedure of the avoidance test

The aim of the avoidance experiment was to determine whether the springtails avoid soil (Lufa 2.2) contaminated with CAP and whether this avoidance behavior shows a dose-related trend. To test this, springtails were placed in a plastic round test container that contained an equally split dividing it in two halves with uncontaminated soil (control) on one side and contaminated soil on the other side. Controls (C), solvent controls (SC) and five CAP concentrations were prepared in bulk Lufa 2.2 soil and moistened to 50 % WHC as described above. The halves of the test container were divided using a metal divider that was placed along the marked lines, splitting the container into two equal parts. With the metal plate positioned, 15 g of moist clean soil was always added to one side and same amount of test soil to the other side. With that, seven combinations were achieved: C-C, C-SC, C-1, C-3.3, C-10, C-33, C-100 µg CAP/g dw, each combination consisting of 5 replicates for the test and 2 additional ones for chemical analysis. The divider was then removed and the surface was smoothened. Subsequently, twenty synchronized healthy and physically active adult springtails (approx. 30 days old) were placed on the center of the soil. The containers were closed with a lid and placed randomly in a climate room (20 ± 1 °C, 75 % humidity, 16:8 h light:dark regime). No food was provided and no disturbances were made during the course of the test, allowing the animals to choose and stay at the preferred site. After two days, the test was finished. Already in the climate room, the soil in the test container was divided rapidly into two parts, using a metal divider and only after the splitting, the jars were taken out for counting. With that we prevented that animals would change their position due to our disturbance. The number of springtails on each side of the test container was assessed by floatation. Soil from one side was gently scooped out into a glass beaker and topped with glass of water. The same was done with the second portion. In both glasses of water, the springtails were floated and the number of animals was then recorded for each side as described above (see 4.12.3). The mean avoidance was calculated using the following equation (Equation 7):

$$A = \frac{C-T}{N} \times 100 \quad \text{(eq. 7)}$$

Where $A$ stands for Avoidance (%), $C$ is the number of collembolans counted in the control soil, $T$ stands for the number of collembolans counted in the test soil and $N$ is total number of springtails recovered.

A positive net response indicates avoidance to the tested compound, while a negative net response would indicate attraction to the compound.

## 4.13 Data analysis

If not stated differently, all statistical analyses were performed using SPSS 20 for Windows or GraphPad Prism 5.03.

### 4.13.1 Data analysis in the stability studies

In the stability study of CAP in different aqueous media, degradation patterns were compared to each other using one-way analysis of variance (ANOVA), followed by post hoc Tukey's multiple comparison test or unpaired two tailed t-test for comparing two-column data.

The degradation kinetics was tested by plotting ln ($c/c_0$; where c is the concentration and $c_0$ the initial concentration) versus time for the first order kinetics. Obtained $R^2$ was compared to the $R^2$ obtained for second order kinetics, so when plotting $1/(c/c_0)$ versus time.

The degradation of CAP and its studied transformation products was in all cases following the first order kinetics, so it could be described by Equation 8:

$$c = c_0\, e^{-kt} \quad \text{(eq. 8).}$$

In this equation, $c$ is the concentration of compound (μM) at a particular time $t$ (days), $c_0$ is the concentration of the compound (μM) at time $t$ = 0, and $k$ represents

degradation rate coefficient ($day^{-1}$). When the data allowed fitting this model, half-life ($Dt_{50}$) values for the degradation of CAP and its transformation product **A** were calculated using the Equation 9:

$$DT_{50} = \frac{\ln(2)}{k}, \quad \text{(eq. 9)},$$

where $k$ is the degradation rate coefficient ($day^{-1}$ for CAP, $hours^{-1}$ for compound **A** in pH 7 and $minutes^{-1}$ for compound **A** in pH 9 solution).

The significance of differences between the photodegradation of CAP in tap water amended with different concentrations of humic acids was determined using ANOVA, followed by Tukey's post hoc test. To determine whether half-lives differed significantly, a Chi-squared test was applied. All statistical analyses were for CAP stability studies performed using SPSS Statistics for Windows.

## 4.13.2 Data analysis in toxicity studies

### *4.13.2.1 Comparison of controls and solvent controls and treatments*

Controls and solvent controls were in toxicity tests compared using an unpaired Student's t-test. When they did not differ significantly, the controls were pooled. The dataset from different treatments within the test were tested for significance using one way analysis of variance (ANOVA), followed by post hoc Tukey's multiple comparison test. To compare treatments with the controls, Dunnett's multiple comparison test was used.

Grubbs' test was used to detect possible outliers among the replicates. Significant outliers were removed when $P < 0.05$.

### *4.13.2.2 Dose-response analysis*

Where possible, $LC_{50}$, $EC_{50}$ and $EC_{10}$ values were calculated according to Haanstra et al. (1985). To determine $LC_{50}$ and $EC_{50}$, the data of the toxicity end point were fitted

against the actual (where obtained) or nominal concentration of CAP in the test solution by a logistic curve (Equation 10):

$$Y(x) = Y_{max}/(1 + (\mathrm{c}/XC_{50})\text{^}\mathrm{b}) \quad \text{(eq. 10)}.$$

Here, *Ymax* is the maximum response (average survival/reproduction of the controls), *c* is CAP concentration (µg/L or µg/g dw, nominal value), $XC_{50}$ stands for $LC_{50}$ or $EC_{50}$ of the toxicity test and *b* represents the steepness of the curve.

The $EC_{10}$ for effects on reproduction was derived by rewriting this equation to read (Equation 11):

$$Y(x) = Y_{max}/(1 + (10/90)(\mathrm{c}/EC_{10})\text{^}\mathrm{b}) \quad \text{(eq. 11)}$$

The 95 % confidence intervals (CI) for the $EC_{50}$ and $EC_{10}$ values were calculated using the statistical program GraphPad Prism software. The fitted $EC_{50}$ were statistically compared using the F-test.

### *4.13.2.3 Specific analysis*

In the chronic toxicity tests with *D. magna*, the fecundity was followed as an additional toxicity end point, expressed as cumulative reproductive output (*CRO*). *CRO* was calculated as follows (Equation 12):

$$CRO = \sum_{i=0}^{\Omega} m_t \quad \text{(eq. 12)}$$

where *CRO* is the cumulative reproductive output per surviving parent animal for a specific treatment (control or exposure concentration), *t* is the time of experiment in days with Ω as the last day of the experiment (21 days) and $m_t$ is the number of living offspring per adult at time *t.*

CRO data set was tested as described above for survival data.

The acute to chronic ratio (ACR) was calculated using the following equation (Equation 13):

$$ACR = \frac{\mathrm{LC_{50}}\ acute}{\mathrm{LC_{50}}\ chronic} \quad \text{(eq. 13)}$$

In the avoidance test with *F. candida*, the differences in the number of springtails recovered from the treated and untreated sides of the test beakers in the five replicates of each CAP concentration was tested for significance by two-tailed Wilcoxon's matched pairs test ($P < 0.05$). This test was also used to compare the difference in avoidance between each halves of the untreated controls, as well as between control/solvent control combinations.

# 5 RESULTS AND DISCUSSION

Because of the very low solubility of CAP (0.88 mg/L) (FAO 2008; EPA 2008) and its degradation products in water, all solutions for chemical and photochemical experiments were prepared with addition of acetonitrile to avoid the precipitation of compounds during the experiments. For the degradation studies with simulated solar light, we aimed to obtain aqueous media with low organic solvent content, but still enabling to test as high CAP levels as possible to obtain higher sensitivity of the method. For that reason we worked with a water-acetonitrile ratio of 4:1 v/v. Since acetonitrile is transparent in UV and VIS regions, highly polar and electrochemically stable, it is a suitable organic solvent for photochemical experiments (Hirakawa, 2012). It is expected not to affect the outcome of photochemical degradation and was therefore applied as a co-solvent in our experiments.

## 5.1 Chlorantraniliprole degradation pathways in water

Four main transformation products were observed in the degradation experiments with CAP, which were further on identified and characterized by several analytical techniques. These transformation products are:

- Compound **H** (2-(3-bromo-1-(3-chloropyridin-2-yl)-1H-pyrazol-5-yl)-6-chloro-3,8-dimethylquinazolin-4(3*H*)-one): it is formed directly from CAP in a chemical process, regardless of presence of light. The reaction takes place in basic aqueous media.
- Compound **A** (2-((2-bromo-4H-pyrazolo[1,5-d]pyrido[3,2-b][1,4]oxazin-4-ylidene)amino)-5-chloro-N,3-dimethylbenzamide): it is formed directly from CAP in a photolytic process. It is the first photodegradation product of CAP, formed regardless of the pH of the aqueous media.
- Compound **B** (2-(3-bromo-1-(3-hydroxypyridin-2-yl)-1H-pyrazol-5-yl)-6-chloro-3,8-dimethylquinazolin-4(3H)-one): it is a second product in a photodegradation pathway of CAP. It is formed from compound **A**, however, its formation is not of photochemical nature. The spontaneous transformation from **A** to **B** is driven by basic media.

- Compound **C** (2-(3-bromo-1H-pyrazol-5-yl)-6-chloro-3,8-dimethylquinazolin-4(3H)-one)): it is the third product in the photolytic pathway of CAP. It is formed from compound **B**, when exposed to the light.

The degradation pathway of CAP is presented in Figure 5. This scheme is based on mechanism proposed by FAO (2008) and confirmed by the results of the present study. To facilitate an easy following of the reaction changes, atoms or functional groups have been numbered in the scheme.

In the following chapters, the pathways of the formation of CAP transformation products are presented and discussed. Each transformation product is also described by its stability and characterization data, by which their chemical structure was suggested.

This degradation pathway has been later on verified also by Sharma A.K. et al. (2014).

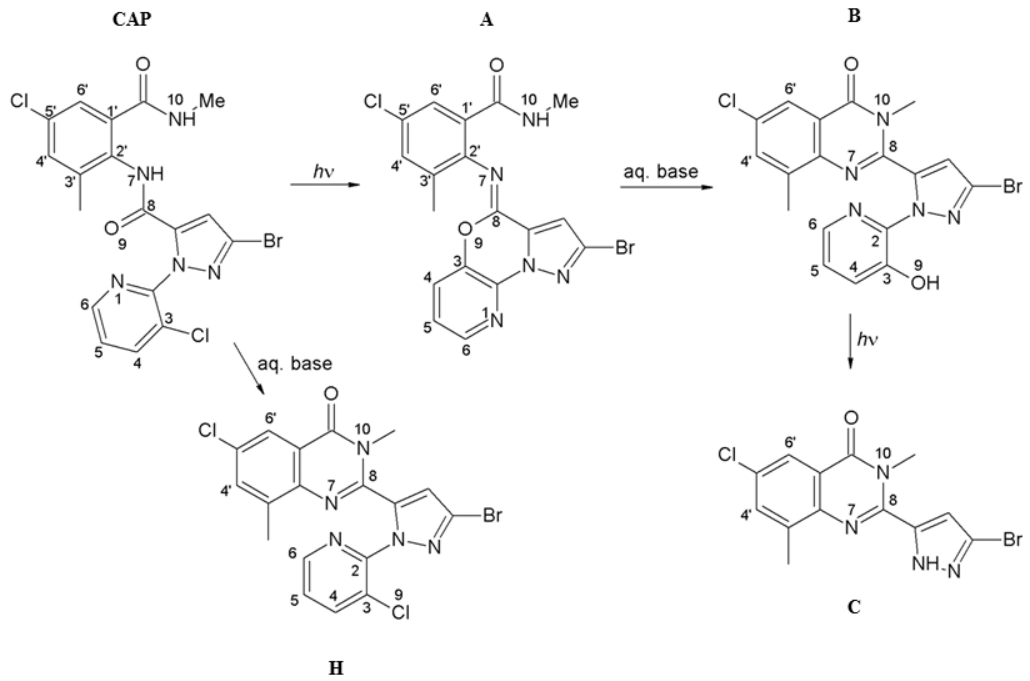



***Figure 5: Degradation pathway of chlorantraniliprole in water.***

***Shown are the different products (A, B, C and H) formed upon chemical and/or photochemical degradation; aq. base indicates conversions taking place in the dark, while hν denotes a photochemical transformation. This scheme is based on FAO (2008) and confirmed by the results of the present study.***

## 5.1.1 HPLC analysis of chlorantraniliprole and its transformation products

The degradation of CAP and the formation of its transformation products were followed by HPLC, where the separation was achieved as seen in Figure 6. The retention times ($t_R$) at the fixed settings were 3.9 min for CAP, 4.2 min for compound **A**, 8.6 min for compound **B**, 6.2 min for compound **C** and 12.8 min for compound **H**.

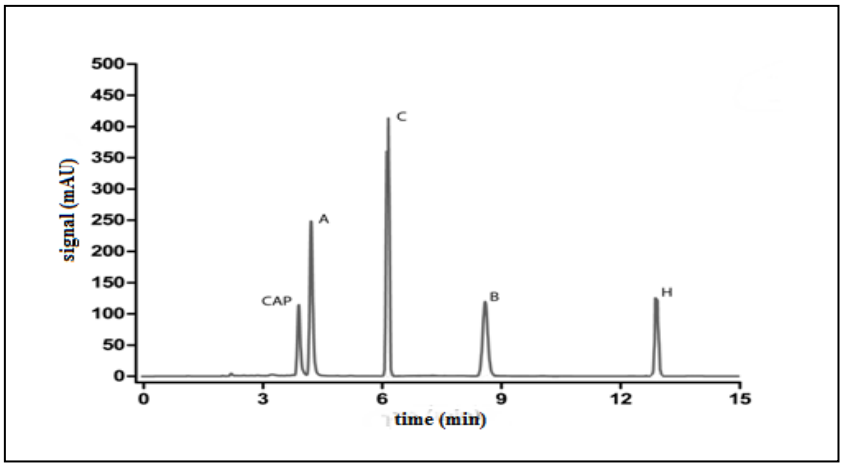



***Figure 6: HPLC-DAD chromatogram of chlorantraniliprole and its transformation products: A, B, C and H.***

The calibration curves of CAP and its two main transformation products **B** and **H** are shown of Figure 7 with enclosed information of calibration curve quality ($R^2$).

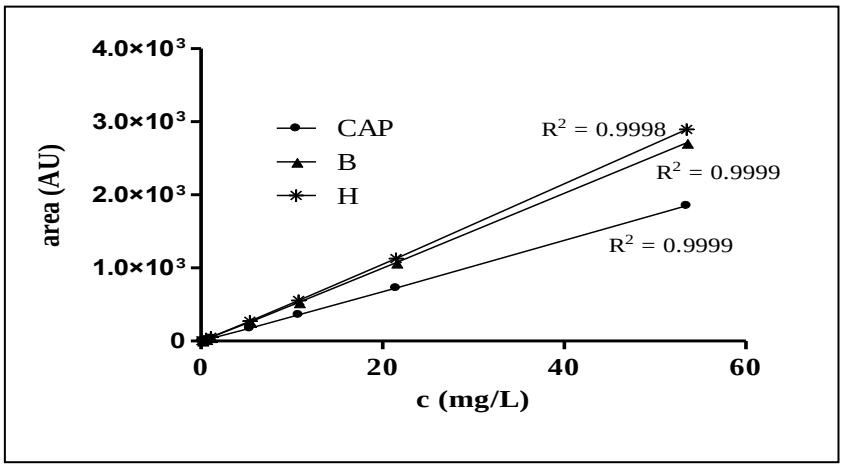



***Figure 7: Calibration curves for standard solutions of chlorantraniliprole and compounds B and H in acetonitrile-water solution (1:1), obtained by HPLC-DAD analysis.***

A separate calibration curve had to be made for CAP MS/MS analysis and is shown in Figure 8. This calibration curve was prepared to measure concentrations of test solutions after they were spiked with a CAP standard applying the standard addition method (SAM).

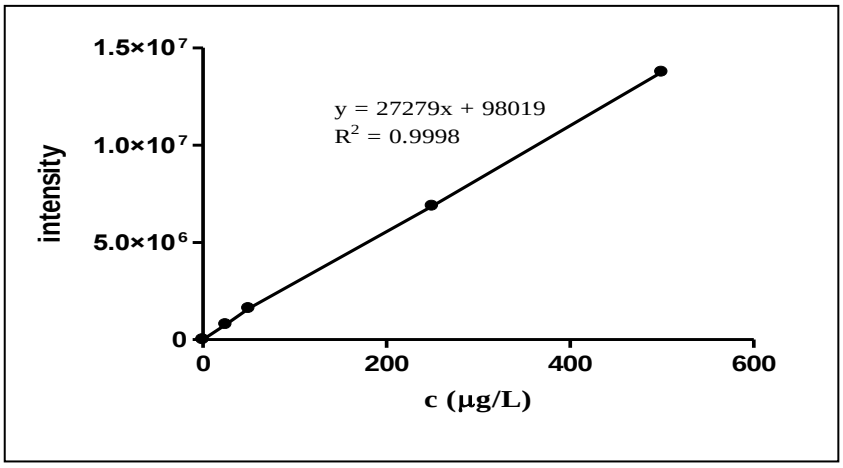



***Figure 8: LC-MS/MS calibration curve for chlorantraniliprole in acetonitrile-water solution (1:1).***

## 5.1.2 UV-VIS absorption spectra of chlorantraniliprole and its photodegradation products

In Figure 9, absorption spectra of CAP and its photodegradation products **A**, **B** and **C** are presented.

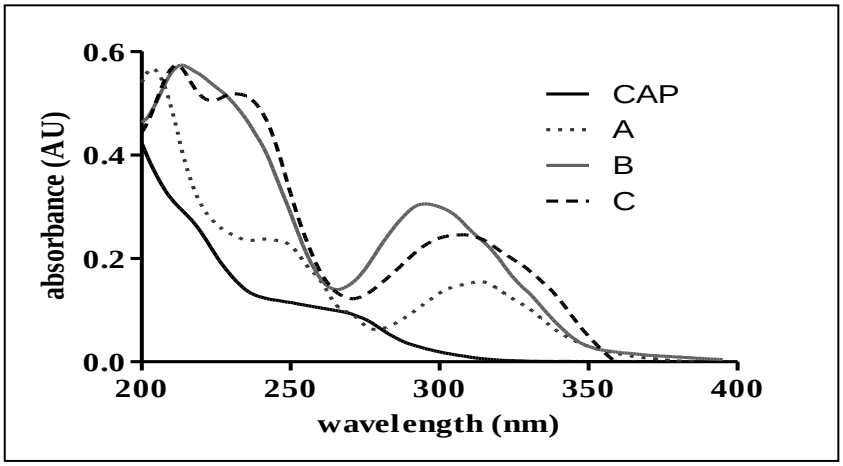



***Figure 9: UV absorption spectra of chlorantraniliprole and its transformation products A, B and C.***

CAP was found to absorb strongly in the UV-C region, with only a weak band from 280 to 310 nm overlapping with the solar and the simulated solar (Suntest) spectra (see Figures 3 and 9). Therefore, the wavelengths longer than 310 nm, such as UV-A

and visible light, are not absorbed enough to cause chemical transformation of CAP. From this we could already speculate that CAP in the environment is photochemically quite stable, as the only fraction of the solar spectra that could cause the chemical reaction of CAP is in UV-B region (290-320 nm), which is reaching the earth surface in only a small fraction.

Similar to CAP, its phototransformation products **A**, **B** and **C** have a major absorbance peak in the UV-C region, but they exhibit an additional absorption peak in the area of 270-350 nm, thus absorbing also in the UV-A region of the solar spectrum.

### 5.1.3 Stability of chlorantraniliprole in different aqueous media

The influence of pH on CAP degradation (at 20.6 μM) was determined at room temperature in acetonitrile-buffer solutions of pH 4.0, 5.5, 7.2, 8.0 and 9.0 and in Dutch tap water at pH 8.2., incubated in the dark. The results of the HPLC analysis, performed daily during the incubation of CAP in the dark are shown in Figure 10. The type of the buffer used (citric acid and tris (hydroxymethyl)aminomethane buffer) had no influence on the stability of CAP, as may be concluded from the comparison of the results for both buffer solutions at pH 7.2. From Figure 10, it can be seen that CAP remained stable at pH values lower than 7.2 while after three weeks in the dark, the concentration of CAP at pH 9.0 dropped by 27 % and by 17 % in tap water at pH 8.2. Degradation was slower in a pH 8.0 buffer, with 5.5 % CAP loss after three weeks. This difference in degradation rate between tap water and buffered solutions might suggest that the electrolytic composition of the tap water have an effect on CAP degradation rate. The difference in stability of CAP was in all test solutions significant ($P < 0.05$) only when compared to pH 9.

It should be remarked that the degradation of CAP resulted in the simultaneous formation of a single transformation product, which was not recorded and monitored at that time. However, according to the several independent studies on CAP transformation we performed, it can be suggested that the transformation product

formed in this experiment is compound **H**, due to the conditions in which compound **H** is formed (spontaneous transformation, in the dark and basic pH).

From the results obtained, it can be concluded, that in the dark CAP is a very stable compound when dissolved in aqueous media of acidic or neutral pH. Significant transformation can however, be expected in basic water. The presented stability test demonstrates that CAP transformation may occur also in natural waters with alkaline pH, higher water hardness and buffer capacity. As stated also by EPA (2008), alkaline-catalyzed hydrolysis is expected to be one of the major routes of CAP dissipation.

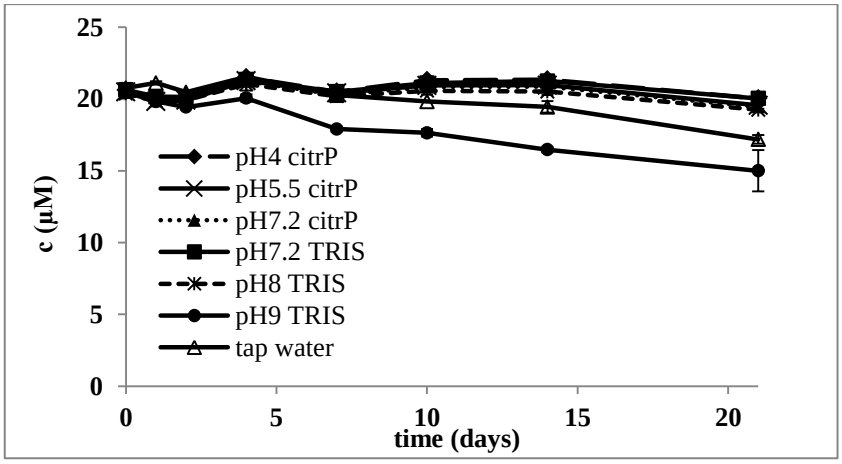



***Figure 10: Stability of chlorantraniliprole in a mixture of acetonitrile and different aqueous media: tap water and buffer solutions of pH 4.0, 5.5, 7.2, 8.0 and 9.0, incubated in the dark at room temperature.***

## 5.1.4 Photodegradation of chlorantraniliprole in deionized water using low pressure Hg lamps (254 nm)

The irradiation of a CAP solution (50 mg/L, acetonitrile-dd$H_2O$ 1:1 v/v) with UV-C light resulted in a rapid degradation (Figure 11). After three minutes of UV-C light exposure, only 2 % of CAP remained in the solution. Figure 11 also shows that along with CAP degradation, one distinctive transformation product was formed: degradation product **A**. Its concentration was increasing constantly with declining

CAP concentration, suggesting that compound **A** in such experimental setup is photochemically stable. No other transformation products were observed in the irradiated solution analyzed by HPLC-DAD. No significant difference in peak areas of the two compounds was observed also when analyzed after overnight incubation in the dark at room temperature.

The pH of the solution was 4.2 at the start of the irradiation while after irradiation the value dropped to 3.6 ($T$ = 20.6 °C), therefore with the CAP degradation, the solution became slightly more acidic.

The nearly exclusive formation of compound **A** enabled its isolation and characterization.

On Figure 11, the areas of the chromatographic peaks are shown instead of their molar concentrations. The reason is that the peak area of compound **A** could not be converted to its concentrations, as the compound was at that time not yet isolated and its peak-concentration relationship therefore not yet determined.

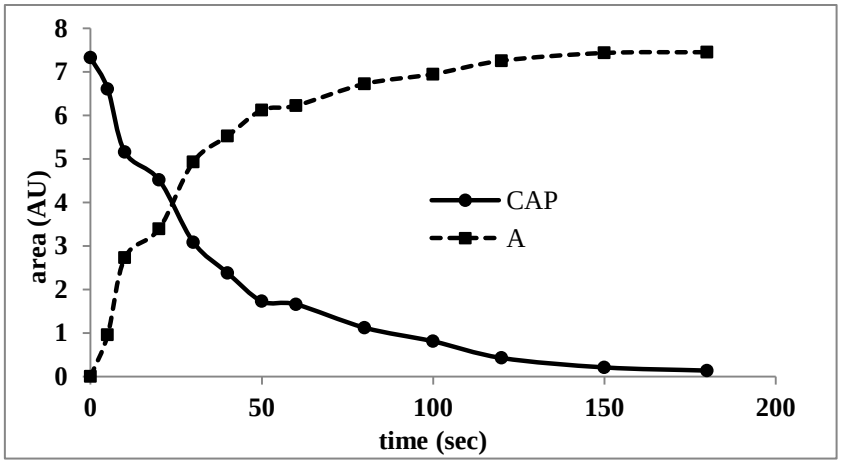



***Figure 11: Degradation of chlorantraniliprole in acetonitrile-dd$H_2O$ (1:1 v/v) solution of pH 4.2 at room temperature under UV-C light and formation of transformation product A.***

### 5.1.5 Photodegradation of chlorantraniliprole in deionized water and tap water under UV-A light

Compared to the rapid CAP degradation when irradiated with the UV-C light source, the CAP photolysis in deionized water under UV-A light was much slower. Starting with a concentration of approximately 39 μM (19 mg/L, two replicates), 26 % of CAP still remained after 10 days of constant irradiation. Figure 12 shows the degradation curves of CAP in deionized and tap water when irradiated with UV-A light (broad spectrum). In deionized water, CAP was degraded with $Dt_{50}$ values of 5.2 days (95 % CI: 4.9-5.5 days) and 4.2 (95% CI: 4.1-4.3) days in tap water. When applying a generalized likelihood ratio test, the difference between these $Dt_{50}$ values, though not large, was significant ($X^2_{df=1}$ = 41.4; P < 0.001). Even more striking difference in degradation rates between two different aqueous solutions was later observed by Sharma A.K. et al. (2014). They performed the photolysis of CAP (c = 0.6 mg/L) in buffer solution of pH 7 (0.01 M maleic acid buffer solution) as well as in natural water of pH 7.4 using an artificial sunlight (456 $W/m^2$ intensity) as a source of irradiation. By comparing the degradation rates researchers concluded that the reaction was in natural water 4-fold faster than in a buffer solution. Results from both studies suggest that substances, such as dissolved organic matter and ions, present in tap and natural water may influence CAP photodegradation. To test this hypothesis, we irradiated CAP solutions in acetonitrile-tap water (1:4 v/v) with 0, 10, 30 and 100 mg/L humic acid in a solar simulator. Results are discussed in the Chapter 5.1.8.

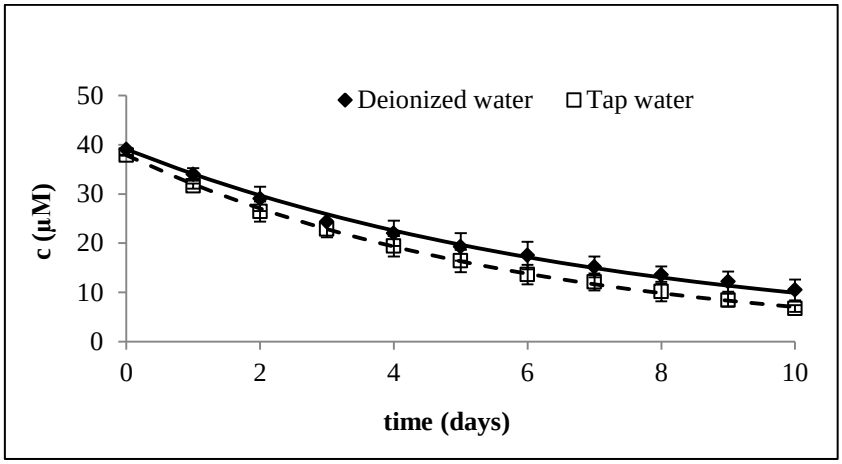



***Figure 12: Degradation curves of chlorantraniliprole (39 µM) in acetonitrile-tap water and acetonitrile-dd$H_2O$ under continuous irradiation of UV-A light.***

## 5.1.6 Photodegradation of chlorantraniliprole in deionized water under simulated solar light

Irradiation of CAP dissolved in acetonitrile-deionized water solution (1:4 v/v, 31 µM) in a solar simulator (750 W/$m^2$) resulted in a degradation of CAP with the $Dt_{50}$ of 4.7 (95% CI: 4.6-4.8) days. After 6 days of continued irradiation, still 36 % of the initial CAP concentration was present in the solution. From the Figure 13 (left panel) it can be seen that upon photolysis of CAP in dd$H_2O$-acetonitrile one principal degradation product was formed, compound **A**. The formation of compound **A** was accompanied with minor amounts of two other transformation products in the photolytic pathway of CAP - compounds **B** and **C**. The concentration of **A** gradually increased, indicating that, despite the continuous irradiation, it is a very stable compound. This was already confirmed by the irradiation with UV-C light, where compound **A** was also the principal and stable degradation product.

Compound **H** was not detected in this test solution. As compound **H** is formed spontaneously from CAP but only under alkaline conditions, this was an expected result since the pH of the solution at the beginning of the irradiation experiment was too low (6.1) and even much lower (3.8) at the end of experiment. The drop of the pH of irradiated solution is in agreement with the 3-min UV-C light irradiation experiment, where the pH dropped from 4.2 to 3.6.

The SUM curve in Figure 13 (left panel) shows the sum of the concentrations of all compounds monitored during CAP degradation. Overall the sum was staying constant, showing that the majority of the transformation products were included in our monitoring.

In the dark control (Figure 13, right panel), incubated along the test solutions exposed to irradiation, no changes in CAP concentration were observed, demonstrating that CAP is stable in deionized water at 26 °C. This stability can again be explained by the acidity of the solution (pH of 6.1 at the beginning of the test). Clearly, no compound other than CAP was observed in the dark test solution.

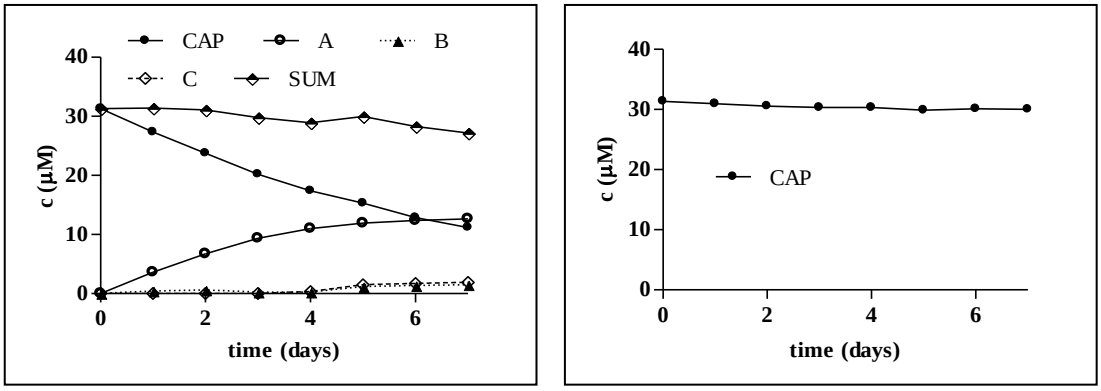



***Figure 13: Left panel: Degradation of chlorantraniliprole (31 µM) in $ddH_2O$-acetonitrile (4:1) when irradiated with simulated artificial sunlight. Right panel: Dark control of chlorantraniliprole (31 µM) in $ddH_2O$-acetonitrile (4:1) when incubated in a solar simulator.***

### 5.1.7 Photodegradation of chlorantraniliprole in tap water under simulated solar light

To simulate degradation processes in natural waters, the photolysis experiments with artificial sunlight were further continued using tap water. With an initial concentration of 17 μM (8 mg/L), 10 % of CAP remained in the solution after 6 days of high intensity irradiation (750 W/m$^2$). The calculated half-life was 2.2 (95% CI: 2.1-2.4) days.

A striking difference in degradation pathway was observed in tap water (Figure 14, left panel), compared to deionized water (Figure 13, left panel). First, due to higher pH of the medium (8.0), compound **H** was formed. Moreover, photodegradation product **A** was now detected only in traces. While the concentration of compound **B** remained low, concentration of compound **C** increased substantially making it the main degradation product in this photolytic reaction. The SUM curve indicates that compounds **A**, **B**, **C** and **H** form the majority of the transformation products in the CAP degradation pathway, however some losses can be observed. The sum of concentrations of all compounds followed during the irradiation process was 8 % lower on the last day than at the beginning of the irradiation experiment. This loss could be due to the formation of other minor degradation products. From the differences in the degradation pathways in dd$H_2O$ and tap water it is apparent that bases present in tap water greatly influence the CAP photodegradation pathway. In the dark control, the concentration of CAP dropped to 77 % after six days of incubation in the operating Suntest apparatus. It is evident from Figure 14 (right panel) that compound **H** was simultaneously formed along with CAP degradation. This can again be explained by the basic pH of the solution (pH = 8.0). However one can notice the difference between the Dutch tap water at pH 8.2, incubated in the dark, at room temperature (Chapter 5.1.3, Figure 10), compared to the Suntest dark controls in Slovenian tap water at pH 8.0 (17 % in 3 weeks compared to 23 % in 6 days). The slower degradation in Dutch tap water might be due to the difference in temperature, which was higher in the Suntest dark controls running along with the photodegradation experiment (up to 27 °C compared to 20 °C).

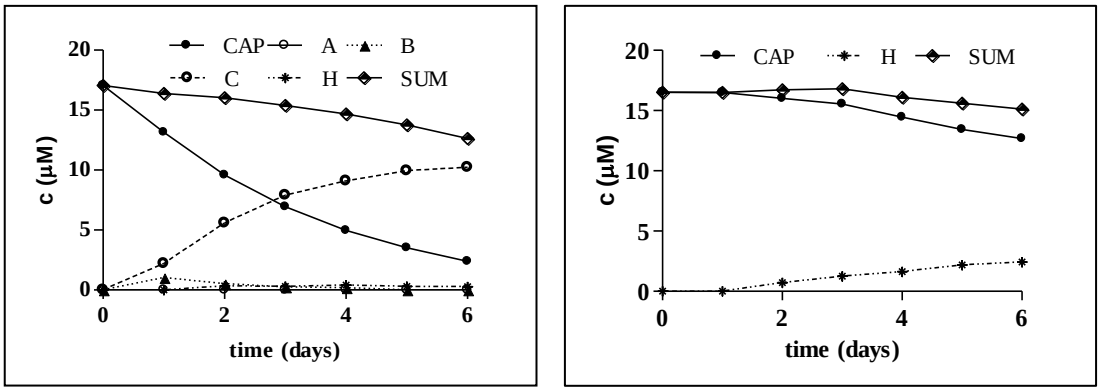



***Figure 14: Degradation of chlorantraniliprole (17 µM) in tap water-acetonitrile (4:1) when irradiated with simulated artificial sunlight (left panel) and when kept in the dark at 22-27 ºC (dark control) (right panel).***

***Error bars indicate standard deviations (n = 2).***

The results of Sharma A.K. et al. (2014) in many ways coincide with our findings. The course of degradation product formation in their photodegradation experiment in natural water (pH 7.4) matched with the results of our study using tap water (pH = 8.0), where compound **B** was formed and rapidly transformed into compound **C** and compound **A** was not detected (it was detected only in traces in our study). When Sharma A.K. et al. (2014) irradiated CAP in pH 7 buffer, compound **A** was formed but its concentration started to decline after it reached its peak at day 1. With degradation of **A**, compound **B** was forming and gradually degrading into product **C**. By comparing the results of both studies (Sharma A.K. et al., 2014 and the present study), the influence of the pH of the water media on CAP photolysis becomes even more evident. While compound **B** was well observed by Sharma A.K. et al. (2014) in natural water, it was observed only in traces in tap water in our study. This could be attributed to the higher light intensity used in our photolysis experiments (750 W/m$^2$ compared to 450 W/m$^2$). Compound **A** was a main degradation product at more acidic pH (deionized water, pH = 6.1), it was clearly seen in pH 7 buffer solution but it degraded in time (Sharma A.K. et al., 2014) and it was in natural waters with more basic pH (7.4 and 8.0) not detected or was observed in traces. It seems that even a small difference in pH greatly influences the course of CAP transformation products. These differences could also be due to the chemical composition of water (the presence or absence of electrolytes and organic matter) in natural and pure buffer/deionized water or combination of both – pH and chemical composition. We

aimed to investigate further whether humic acids and nitrates, common in natural waters affect the photolytic degradation pathway of CAP. Another good way to investigate this would be CAP irradiation in acidic and alkaline water, both obtained from the natural source and analyzed for its physical and chemical parameters.

### 5.1.8 Simulated solar photodegradation of chlorantraniliprole in tap water, amended with humic acids and nitrate

To simulate natural environmental conditions, a $NO_3^-$ concentration of 10 mg/L was used in the experiment, which corresponds with levels in low to moderately polluted surface water. There was hardly any difference in the photodegradation under simulated sunlight between CAP without ($Dt_{50}$ = 2.12 days; 95 % CI: 2.17-2.43) and with $NO_3^-$ ($Dt_{50}$ = 2.20 days; 95 % CI: 2.05-2.37) solutions, confirming the absence of an effect of $NO_3^-$ on CAP photodegradation (Figure 15).

To test if humic acids present in water have an influence on CAP photodegradation, CAP solutions in acetonitrile-tap water (1:4 v/v) with 0, 10, 30 and 100 mg/L humic acid were irradiated in a solar simulator. While the half-life of CAP degradation in the control solution was 2.12 days (95 % CI: 2.17-2.43), the addition of 10 mg/L humic acids slightly accelerated photolysis ($Dt_{50}$ = 1.84 days; 95 % CI: 1.78-1.90). Higher concentrations of 30 mg/L and 100 mg/L, however, increased the half-life to 2.28 (95 % CI: 2.17-2.40) and to 3.07 days (95 % CI: 2.89-3.29), respectively (Figure 15).

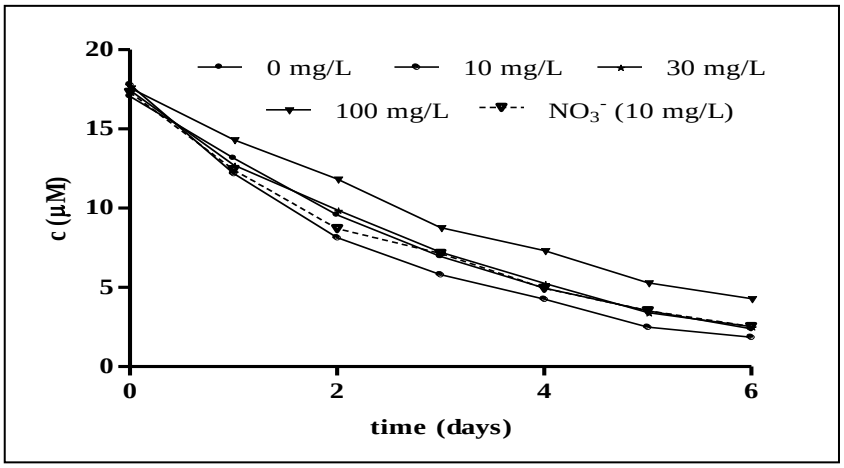



***Figure 15: Effect of dissolved organic matter and nitrate on the degradation of chlorantraniliprole (17 µM) in tap water-acetonitrile (4:1) amended with 0, 10, 30 and 100 mg/L humic acid (HA) or 10 mg/L NO3- and irradiated with simulated artificial sunlight.***

It therefore seems that humic acids have only a very weak influence on CAP photodegradation. A possible reason for the slight inhibition of degradation could lie in a shielding effect of humic acids on UV light penetration through the solution. This seems to be confirmed by our measurement, which showed that solutions of humic acids in tap water/acetonitrile (4:1) exhibited moderate to high absorbance in the photochemically active UV-B region (Figure 16).

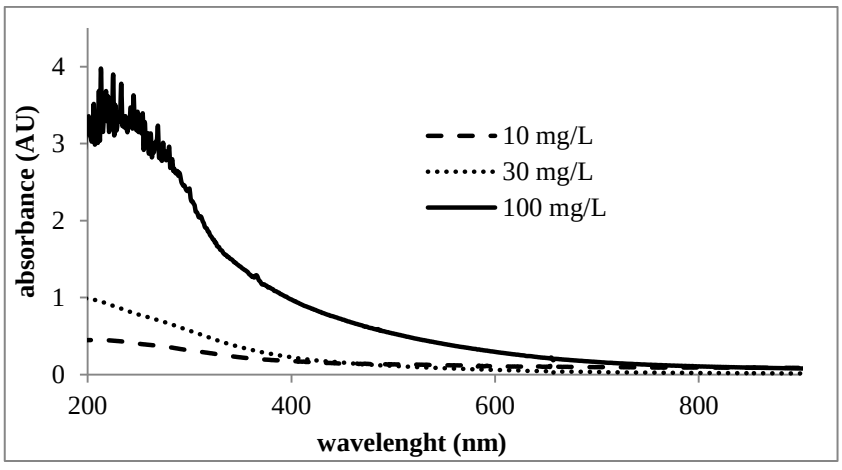



***Figure 16: UV absorption spectra of humic acids at three different concentrations: 10, 30 and 100 mg/L in tap water/acetonitrile (4:1).***

The photodegradation pathway of CAP in tap water amended with humic acids and nitrate was very similar to the one of CAP in tap water only, showing that the humic acids as well as nitrate did not influence the course of transformation product formation. In Figure 17 (left panel), the average CAP and transformation product concentrations during the photodegradation are plotted for tap water without and with different amounts of added humic acids and nitrate (10 mg/L). As the average concentration of compound **A** was zero during the whole period of irradiation, it is not included in the graph.

On the right side of Figure 17, the average CAP concentrations are given for the exposures without and with added nitrate and humic acids in all tested concentrations incubated in the dark (dark controls). The standard deviations of the different solution measurements show that there is hardly any difference in CAP degradation between the samples with and without different concentrations of humic acids or $NO_3^-$. Compound **H** was the only transformation product observed.

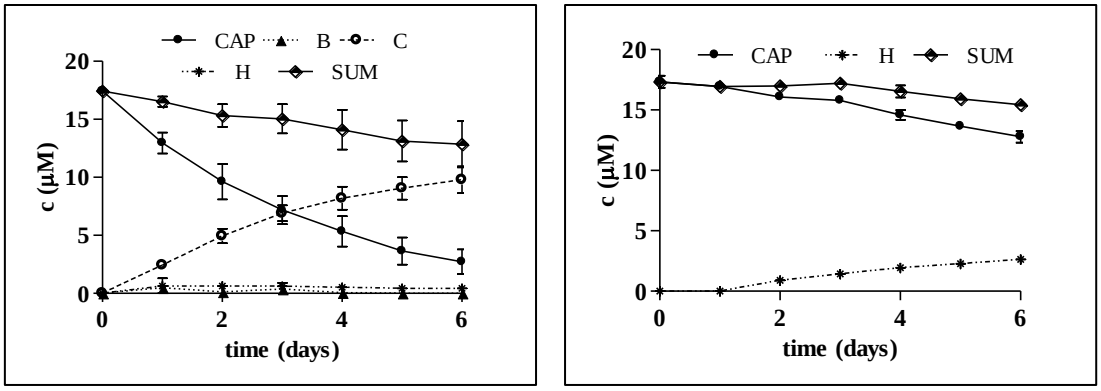



***Figure 17: Degradation of chlorantraniliprole (17 µM) in acetonitrile-tap water (1:4) without and with added nitrate (10 mg/L) and different concentrations of humic acids (10, 30 and 100 mg/L) when irradiated in the solar simulator (left) and when kept in the dark (right).***

***Error bars indicate standard deviations (all samples together; n=5).***

### 5.1.9 Degradation kinetics

Plotting ln ($c/c_0$) versus irradiation time in all cases resulted in a linear relationship suggesting first-order degradation kinetics. Therefore half-life ($Dt_{50}$) values for the degradation of CAP were calculated applying a first order degradation model. An example of CAP photodegradation under simulated light in acetonitrile-tap water (1:4, 17 µM) is given in Figure 18. The $R^2$ of 0.998 is showing a good linearity and therefore a good fit to the first order degradation model, while in the case of the second order degradation model, the fit was lower ($R^2$ = 0.907). First order kinetic of degradation of CAP and each of the degradation products was verified later on also by Sharma A.K. et al. (2014).

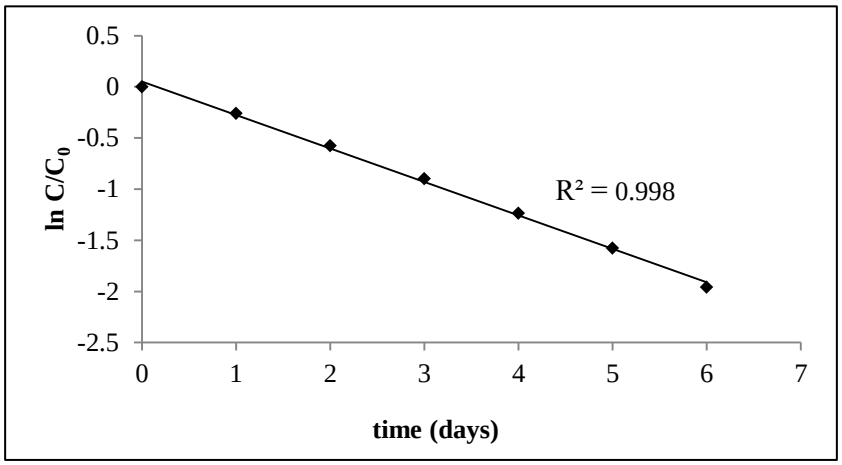



***Figure 18: First order degradation rate of chlorantraniliprole in acetonitrile-tap water (1:4, 17 µM), irradiated with simulated solar light.***

### 5.1.10 Identification, characterization and stability of chlorantraniliprole transformation products

Separate step-by-step experiments with the isolated transformation products were performed to understand the CAP degradation processes. We aimed to define the course of formation of CAP degradation products, as well as the conditions promoting their occurrence – especially as striking differences in their formation were observed between deionized and tap water. In the following text, the process of the formation of each transformation product is described as well as their detailed

characterization data. It is referred to the Figure 5 to follow the transformation processes.

## 5.1.11 Transformation product A

### *5.1.11.1 Formation of transformation product A*

From the CAP photodegradation experiments in acetonitrile-deionized water it was reasonable to assume that **A** is the first photodegradation product as no other transformation products were observed (UV-C irradiation, Figure 11), or only observed in traces (Suntest, Figure 13). Our hypothesis was additionally confirmed when we performed the laser flash photolysis (266 nm) of CAP on a nanosecond time frame. The result was the formation of a compound exhibiting a UV spectrum in the range 280-400 nm, identical to the spectrum of compound **A** (Figure 19, for the comparison of the absorption spectra, see Figure 9). This reveals that the transformation of CAP into compound **A** is instantaneous, and that the life span of possible CAP intermediates, such as CAP excited state, is shorter than $10^{-8}$ s. It cannot be excluded that CAP undergoes a triplet state. No differences were observed whether solutions of CAP were saturated with air or argon.

The stability of compound **A** in the laser flash photolysis experiments can be demonstrated by the trace presented in Figure 19 (insert), where no decay in absorbance was observed. This result is consistent with other photodegradation experiments performed in deionized water (see Figures 11 and 13).

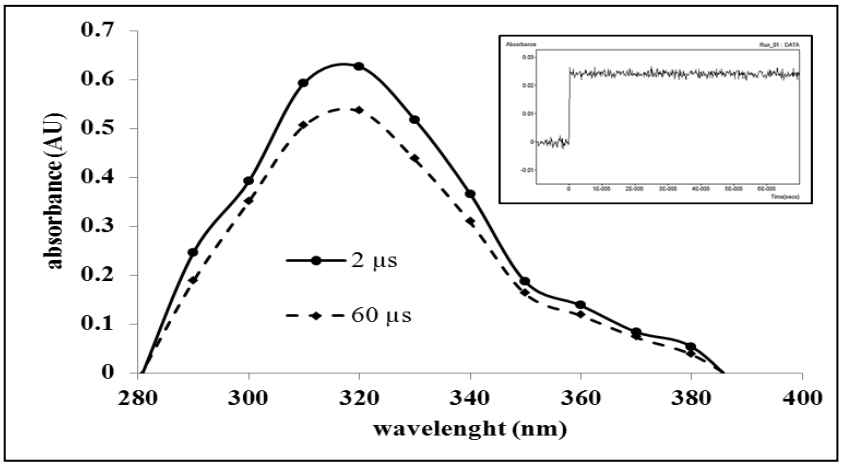



***Figure 19: Absorption spectra of a solution of chlorantraniliprole in deionized water-acetonitrile (4:1), after being exposed to nanosecond laser pulses (266 nm). The UV spectrum observed is identical to that of the CAP transformation product A, suggesting that the transformation is instantaneous as no short-lived intermediates could be observed. The insert shows the absorption trace of CAP, after being photolysed to its stable degradation product A by laser flash photolysis.***

Laser flash photolysis as well as irradiation studies of CAP in acetonitrile-dd$H_2O$ confirmed that compound **A** is a primary photochemical product which was therefore isolated and characterized first. Since compound **A** was stable in irradiated acetonitrile-dd$H_2O$ at pH 6.1, but was detected only in traces in tap water at pH 8.0, it is suggested that it reacts rapidly with bases in tap water to form compound **B**.

### *5.1.11.2 Stability of transformation product A*

To determine that compound **A** reacts with bases in tap water to form the subsequent compound **B**, the acetonitrile solution of compound **A** was diluted (1:4) with phosphate buffer solutions of pH 4, 7 and 9, and continuously monitored by HPLC. Dramatic differences between the samples were observed.

- Stability in pH 4 buffer solution

As anticipated, virtually no change in concentration of compound **A** incubated in pH4 buffer solution was noticed, not even after 6 days of incubation. On the last measurement day, the concentration of compound **A** was 99.3 % of its initial value.

The compilation of HPLC peaks in different measurement times is plotted in Figure 20.

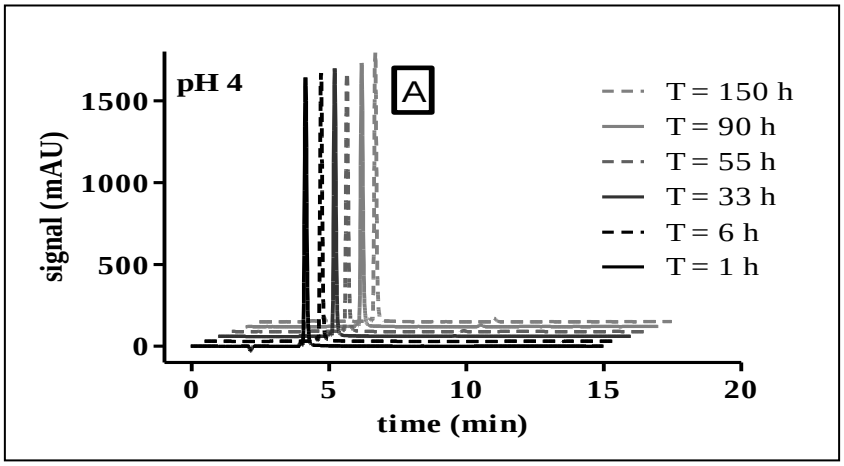



***Figure 20: HPLC chromatograms of compound A dissolved in acetonitrile and pH 4 phosphate buffer solution (1:4) incubated in the dark at 20 °C and monitored for 150 h.***

- Stability in pH 7 buffer solution

Contrary to results obtained in pH 4 solution, in neutral media a clear transformation of compound **A** into its subsequent transformation product **B** was noted. During the 67 h incubation, the concentration of compound **A** dropped by 95 %. The degradation was first order and the corresponding kinetics model, plotted in Figure 21, was applied. The degradation half-life was 8.3 h (95% CI = 8.2-8.4 h).

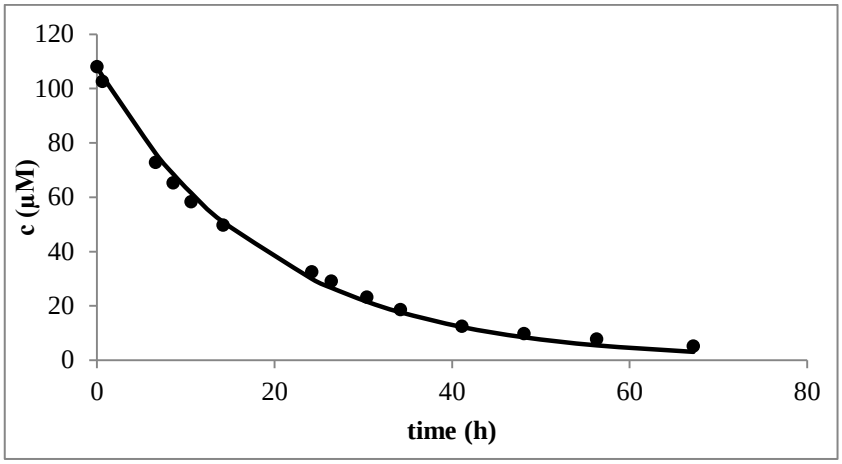



***Figure 21: Spontaneous transformation of compound A, dissolved in acetonitrile and pH 7 phosphate buffer solution (1:4, 50 mg/L) when incubated in the dark at 20 °C.***

- Stability in pH 9 buffer solution

As in pH 7 solution, also in basic medium compound **A** degraded into one single transformation product – compound **B**, however here, the process was found to be much faster. Already after 15 minutes the concentration of **A** had dropped to about 68 % of its initial level. After 5 hours of incubation, **A** was no longer detected and no other peak, except for the one of its transformation product **B**, was observed. The transformation was following first order kinetics. The time-dependent disappearance of compound **A** is shown in Figure 22. The calculated $Dt_{50}$ was 25.9 min (95% CI = 22.0-31.5min). However, a slight caution should be here addressed – the time of the analysis was 15 min, therefore the initial concentration of compound **A** at time $T = 0$ min could not be assessed. By the time the analysis was finished, part of compound **A** was already degraded. For the starting point at Figure 22, the initial concentration of compound **A** in the sample of pH 4 was taken, as all samples were made from same stock solution and diluted with same amounts of the different buffer solutions immediately before the analysis. The initial concentrations should therefore not differ between each other. The fit of the first order degradation model to the data (Figure 22) seems to confirm the validity of this approach.

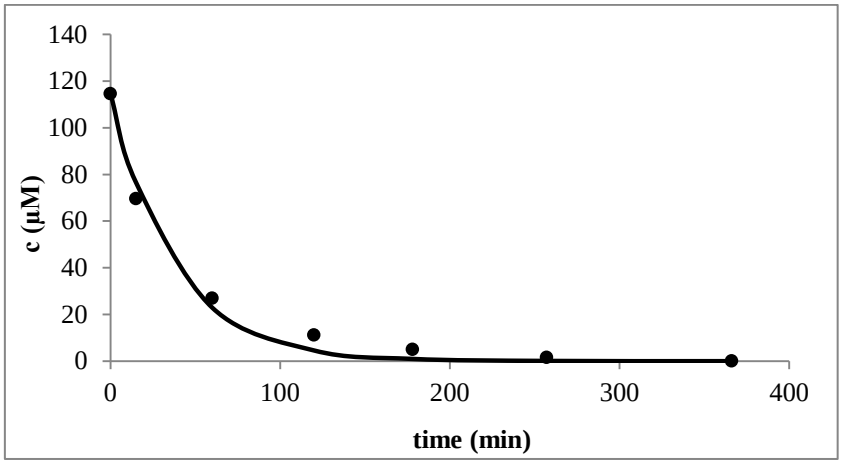



***Figure 22: Spontaneous and rapid degradation of transformation product A when dissolved in acetonitrile and pH 9 phosphate buffer solution (1:4, 50 mg/L) and incubated in the dark at 20 °C.***

### *5.1.11.3 Characterization of transformation product A*

Compound **A** has been characterized by HRMS, $^{1}H$ and $^{13}C$ NMR and IR spectroscopy, as well as X-ray diffraction.

HRMS mass measurement of compound **A** ($MH^+$ 446.0022, Figure 23) showed that its molecular mass is 36 atomic units lower than the mass of **CAP** ($MH^+$ 481.9779). This could indicate an elimination of an HCl group from the CAP molecule. The dramatic drop in pH of the acetonitrile-dd$H_2O$ CAP solution upon irradiation, from 6.1 to 3.8, strongly supports the proposed HCl elimination during the photolysis of CAP. Since HRMS is able to distinguish isotopes, three main fragments (M+2) can be observed in the HRMS spectrum of **A** that correspond to combinations of $^{35}Cl$, $^{37}Cl$, $^{79}Br$ and $^{81}Br$. The peak with the highest mass corresponds to the combination of the heaviest isotopes ($^{37}Cl$ and $^{81}Br$), however the intensity is lower due to the lower abundance of $^{37}Cl$ isotope (three times lower than $^{35}Cl$), while the ratio between the bromine isotopes is approximately 1:1. Fragments of smaller intensity with M+1 correspond to the $^{13}C$ isotope. Based on such isotopic patterns and mass, the molecular formula of compound **A** was suggested to be $C_{18}H_{13}BrClN_5O_2$.

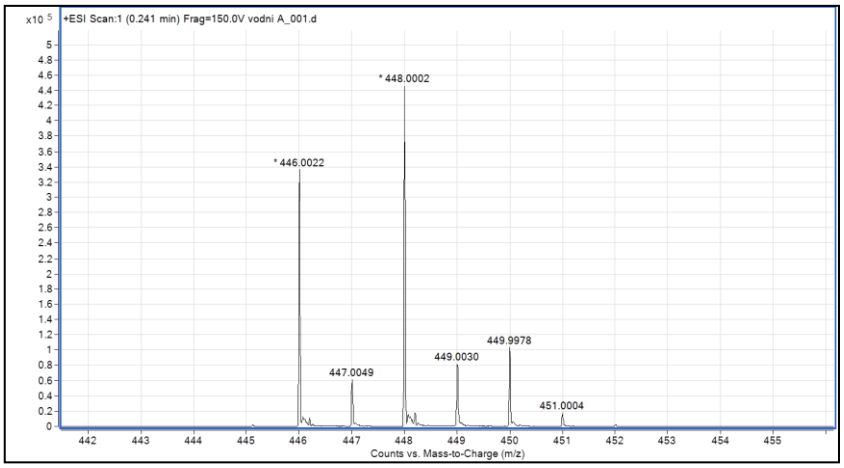



***Figure 23: The HRMS spectrum of chlorantraniliprole degradation product A.***

In the IR spectra of CAP (Figure 24) two peaks are seen in the region above 3200 $cm^{-1}$, one at 3258 $cm^{-1}$ and one at 3379 $cm^{-1}$, which could suggest two secondary amide groups. However in the IR spectrum of compound **A** (Figure 25), only one peak of moderate intensity was observed at 3309 $cm^{-1}$, corresponding to a secondary amide. Two peaks can be observed in a region above 1600 $cm^{-1}$. It can be suggested that the peak at 1659 $cm^{-1}$ is corresponding to C=O stretching vibration while the one at 1694 $cm^{-1}$ could be related to C=N stretching probably shifted to higher values due to the nearby oxygen.

$^{1}H$ NMR also showed the disappearance of NH proton of the type Ar-NH-CO, however, the methyl peak of a CO-NH-$CH_3$ remained as a doublet. Pyridine protons, particularly H4 (Figure 5), exhibit a moderate up-field shift, which could be induced by substitution of a chlorine atom on the pyridine ring by a less electron-attracting atom or group.

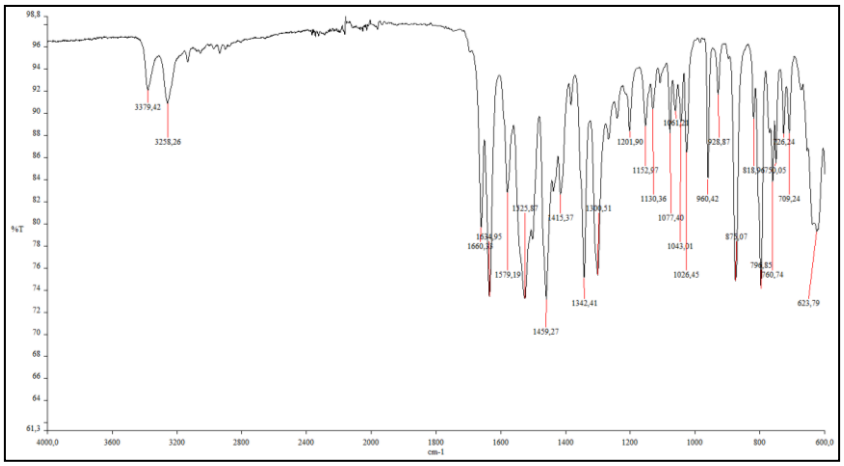



***Figure 24: IR spectrum of chlorantraniliprole.***

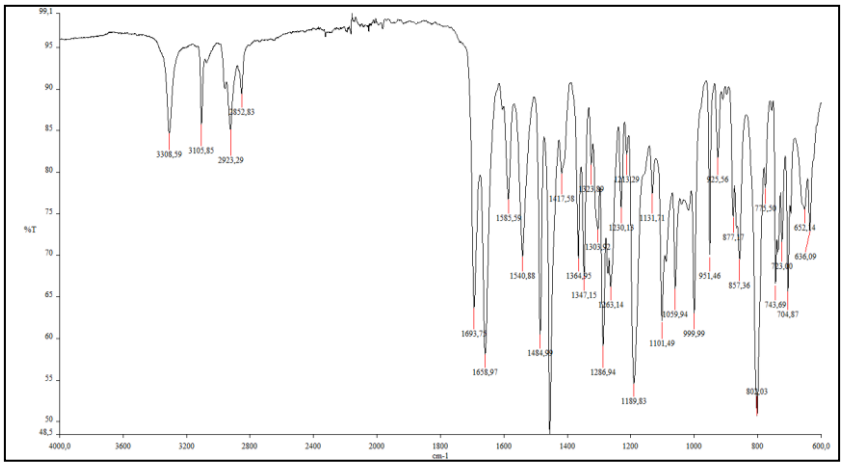



***Figure 25: IR spectrum of chlorantraniliprole degradation product A.***

The photochemical nucleophilic substitution of halogens in aryl halides is a well-documented reaction (Chen et al. 2009, Klán and Wirz 2009, Turro 1978), in aqueous solution mostly leading to the corresponding hydroxy derivatives. As evident from our spectroscopic data, in this case the chlorine atom in CAP molecule was not replaced by a hydroxy group, but rather by carbonyl oxygen atom(9) in an intramolecular nucleophilic substitution (Figure 5). This process seems very probable since the oxygen atom(9) is in a suitable position to form a six-membered oxazine ring. Characterization data of transformation product **A** are collected in Table 5.

The characterization of compound **A** became complete when its structure was determined by the X-ray diffraction (structure solved by dr. Barbara Modec). An ORTEP drawing with 50 % probability ellipsoids for compound **A** is presented in Figure 26.

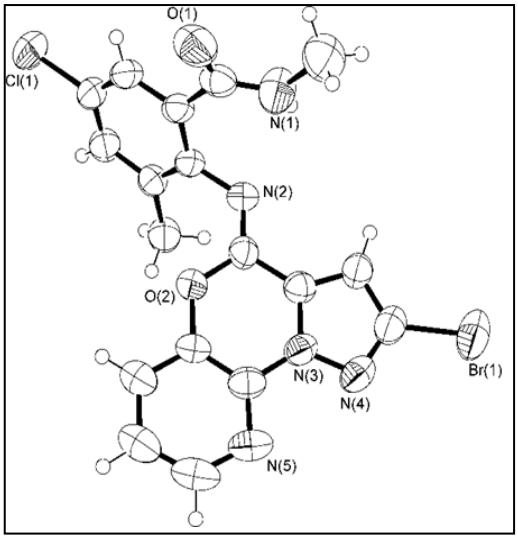
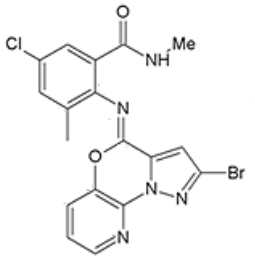



***Figure 26: ORTEP drawing with 50 % probability ellipsoids for chlorantraniliprole degradation product A.***

***See Figure 5 for the degradation pathway of chlorantraniliprole.***

***Table 5: Characterization data of chlorantraniliprole degradation product A.***

| Compound **A** | |
|---|---|
| IUPAC name | 2-((2-bromo-4*H*-pyrazolo[1,5-d]pyrido[3,2-b][1,4]oxazin-4-ylidene)amino)-5-chloro-*N*,3-dimethylbenzamide |
| Molecular formula | $C_{18}H_{13}BrClN_5O_2$ |
| Structural formula |  |
| Melting point (°C) | 133.8-135.7 |
| $^{1}$H NMR ($CDCl_3$) $\delta$/ppm: | 2.18 (s, 3H); 2.96 (d, $J$ = 5.0 Hz, 3H); 6.17 (br s, 1H); 7.09 (s, 1H); 7.23 (d, $J$ = 2.3 Hz, 1H); 7.26 (d, $J$ = 2.3 Hz, 1H); 7.38 (dd, $J$ = 4.7; 8.0 Hz, 1H); 7.85 (dd, $J$ = 1.6; 8.0 Hz, 1H); 8.46 (dd, $J$ = 1.6; 4.7 Hz, 1H); 10.06 (s, 1H) |
| $^{13}$C NMR ($CDCl_3$) $\delta$/ppm | 18.2 ($CH_3$); 26.8 ($CH_3$); 111.4 (CH); 123.5 (CH); 125.4 (CH); 127.5 (CH); 127.9 (C); 130.1 (C); 131.1 (C); 132.3 (C); 132.5 (CH); 132.8 (C); 134.8 (C); 138.4 (C); 139.6 (C); 140.0 (C); 144.9 (CH); 166.5 (C) |
| IR (ATR) $cm^{-1}$ | 1658(s), 1693(s), 3308(m) |
| HRMS (ESI, $MH^+$) | calcd for $C_{18}H_{14}BrClN_5O_2$: 446.0019, measured: 446.0022 |

## 5.1.12 Transformation product B

### *5.1.12.1 Formation of transformation product B*

The stability test of photodegradation product **A** in buffer solutions of pH 7 and 9 clearly showed that **A** is transformed into one single transformation product, which was further characterized as compound **B**. This transformation is indicated in Figure 27. In both solutions, compound **B** appeared to be stable for at least one week. Based on the obtained results, it can be concluded that compound **B** is formed by base catalyzed reaction; its formation is therefore not a photochemical process. This principle was used to produce compound **B** in higher amounts – CAP was first irradiated to compound **A**, which was then dissolved in pH 8 phosphate buffer solution and incubated in the dark for a few days. The formed compound **B** could then be isolated and characterized.

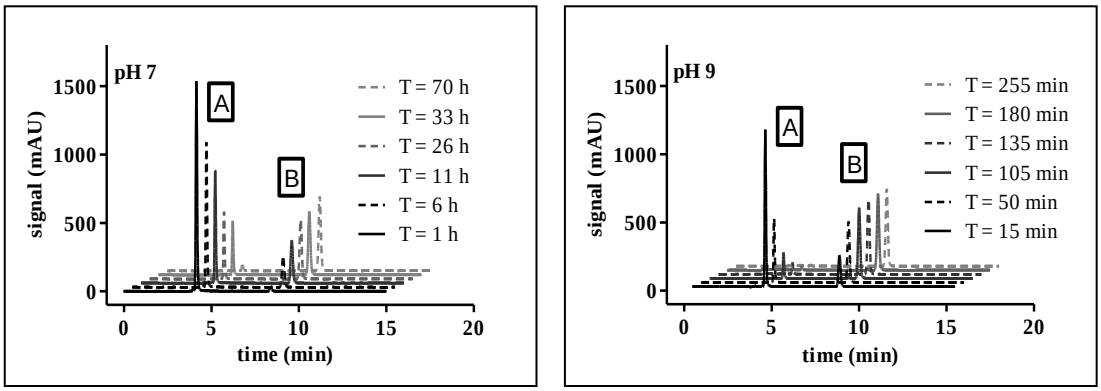



***Figure 27: HPLC chromatograms showing the formation of chlorantraniliprole transformation product B ($t_R$ = 8.6 min) out of compound A ($t_R$ = 4.2 min) in acetonitrile with pH 7 phosphate buffer solution (left) and with pH 9 phosphate buffer solution (right) incubated in the dark at 20 °C.***

### *5.11.12.2 Nanosecond photolysis of transformation product B*

Laser excitation (266 nm) of a degassed compound **B** solution in acetonitrile led to the formation of a transient product which showed an absorption maximum at 400 nm (Figure 28). The absorption spectrum of the transient product therefore is very different from the photodegradation product of **B** – compound **C** (Figure 5, for the comparison of the absorption spectra, see Figure 9). This gives us the evidence that

compound **B**, unlike CAP, during the photolysis transforms into an intermediate compound, that is long lived enough to be observed on a nanosecond time scale.
Unfortunately, we were not able to perform further studies to elucidate the nature of this intermediate and the reaction pathway leading to compound **C**.

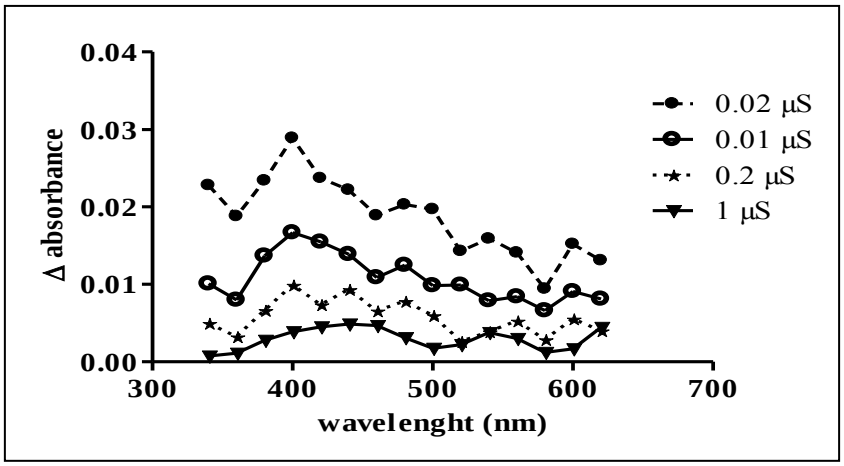



***Figure 28: Transient absorption spectra of the chlorantraniliprole transformation product B, obtained by laser flash photolysis at 266 nm.***

### *5.1.12.3 Characterization of transformation product B*

Purified **B** was characterized by HRMS, $^{1}H$, $^{13}C$ NMR and IR spectroscopy, elemental analysis and X-ray diffraction.
The HRMS spectrum of compound **B** is presented in Figure 29, with well distinguished peaks corresponding to chlorine and bromine isotopes. Comparing the spectra of compound **B** with the one of compound **A** (Figure 23), one can notice that they exhibit the same molecular mass (446.0030 for compound **A** and 446.0013 compound **B**). This reveals that compounds **A** and **B** are isomers.

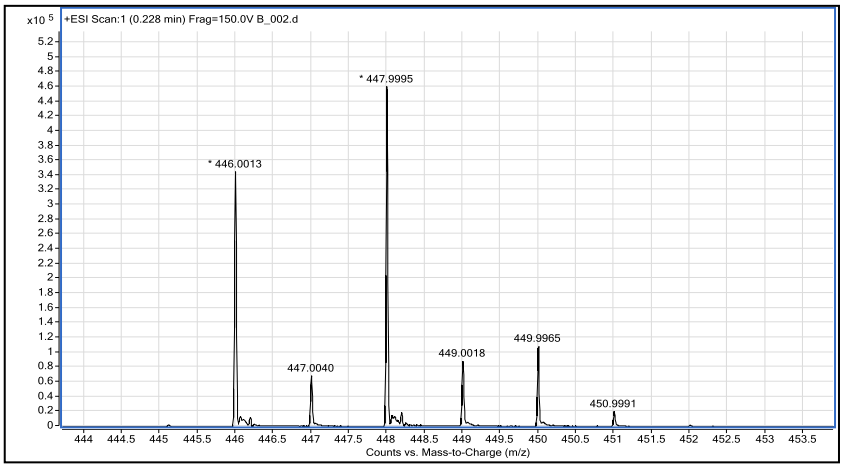



***Figure 29: The HRMS spectrum of chlorantraniliprole transformation product B.***

The IR spectrum of compound **B** is very distinct from the spectra of CAP and compound **A**. Here, a distinctive broad peak in the area of 3000-3200 $cm^{-1}$ appeared (Figure 30), characteristic for the OH group forming hydrogen bonds. Secondly, no sign of an NH group can be noticed in the area around 3300 $cm^{-1}$, suggesting that the nitrogen atom present in compound **B** is tertiary. Unlike for compound **A**, only one peak at 1670 $cm^{-1}$ is present, most probably due to the C=O group.

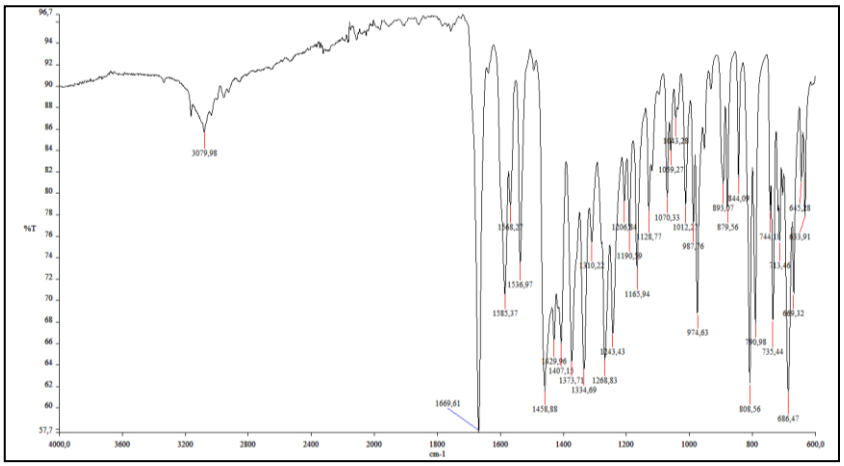



***Figure 30: IR spectrum of chlorantraniliprole transformation product B.***

In the $^{1}H$ NMR spectrum of compound **A** as well as its parent CAP, one of the methyl groups is attached to the NH group, resulting in a splitting of the methyl signal into a doublet. In the spectrum of compound **B**, the corresponding methyl group exhibits a singlet, which indicates that the proton on the adjacent N atom is absent. The signals of protons on phenyl ring are shifted to higher δ, those on

pyridine ring to lower δ. This can be rationalized by the formation of a bond C8-N10 and of a new quinazolinone ring with a mutual scission of a former oxazine. The cleavage of the oxazine ring results in the formation of a hydroxy group on pyridine (C3) (Figure 5). The corresponding broad singlet was indeed observed in the $^{1}$H NMR spectrum of compound **B** and the result is in good agreement with the obtained results from the IR analysis. In Table 6, all the characterization data for compound **B** is collected.

The structure of compound **B** was additionally determined by X-ray diffraction (Figure 31). A plausible mechanism of this transformation is the initial deprotonation of amide (N10) by a base and the attack of the resulting anion on the imine carbon (C8) (Figure 5). An analogous reaction took place in the transformation of CAP to compound **H** (see Chapter 5.1.14.).

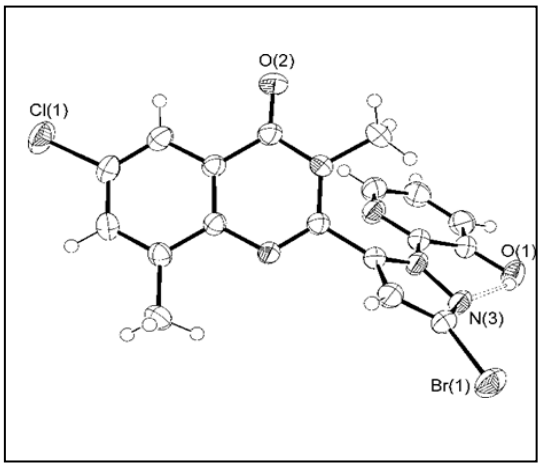
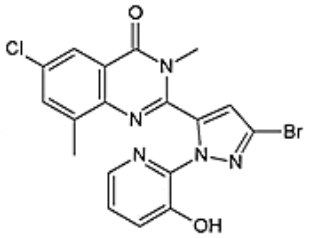



***Figure 31: ORTEP drawing with 50 % probability ellipsoids for chlorantraniliprole transformation product B.***

***Table 6: Characterization data of chlorantraniliprole transformation product B.***

| Compound **B** | |
|---|---|
| IUPAC name | 2-(3-bromo-1-(3-hydroxypyridin-2-yl)-1*H*-pyrazol-5-yl)-6-chloro-3,8-dimethylquinazolin-4(3*H*)-one |
| Molecular formula | $C_{18}H_{13}BrClN_5O_2$ |
| Structural formula |  |
| Melting point (°C) | 199-200 |
| $^{1}$H NMR ($CD_2Cl_2$) $\delta$/ppm: | 2.48 (s, 3H); 3.33 (s, 3H); 6.75 (s, 1H); 7.07 (dd, *J* = 4.6; 8.2 Hz, 1H); 7.42 (dd, *J* = 1.5; 8.2 Hz, 1H); 7.52 (dd, *J* = 1.5; 4.6 Hz, 1H); 7.55 (dd, *J* = 0.9; 2.5 Hz, 1H); 8.18 (dd, *J* = 0.6; 2.5 Hz, 1H); 10.38 (s, 1H) |
| $^{13}$C NMR ($CD_2Cl_2$) $\delta$/ppm | 17.3 ($CH_3$); 32.7 ($CH_3$); 112.6 (CH); 122.7 (C); 123.9 (CH); 124.4 (CH); 127.0 (C); 127.5 (CH); 132.9 (C); 135.2 (CH); 136.6 (C); 138.3 (C); 138.6 (CH); 139.2 (C); 145.0 (C); 145.1 (C); 146.8 (C); 161.4 (C) |
| IR (ATR) $cm^{-1}$ | 1668 (s), 2900-3200 (br) |
| HRMS (ESI, $MH^+$) | calcd for $C_{18}H_{14}BrClN_5O_2$: 446.0019, measured: = 446.0013 |
| Elemental analysis | calcd for $C_{18}H_{13}BrClN_5O_2$: C 48.43, H 3.16, N 15.70, found: C 48.01 %, H 2.72 %, N 15.36 % |

## 5.1.13 Transformation product C

### *5.1.13.1 Formation of transformation product C*

After the identification and isolation of compound **B**, our next goal was to see whether it is photoactive. We tested this by irradiating isolated compound **B**, dissolved in acetonitrile-dd$H_2O$ (1:1 v/v) (204 µM, 50 mL), with UV-A light. The progress of the reaction was monitored by HPLC and it was found that compound **B** degraded into one principal product, possessing the same retention time as compound **C** – the major photodegradation product of CAP when irradiated by simulated solar light in tap water media (Figure 14). After 10h of continuous irradiation, compound **B** completely degraded to compound **C** as a main product. An example of **B** degradation into compound **C** is shown in Figure 32.

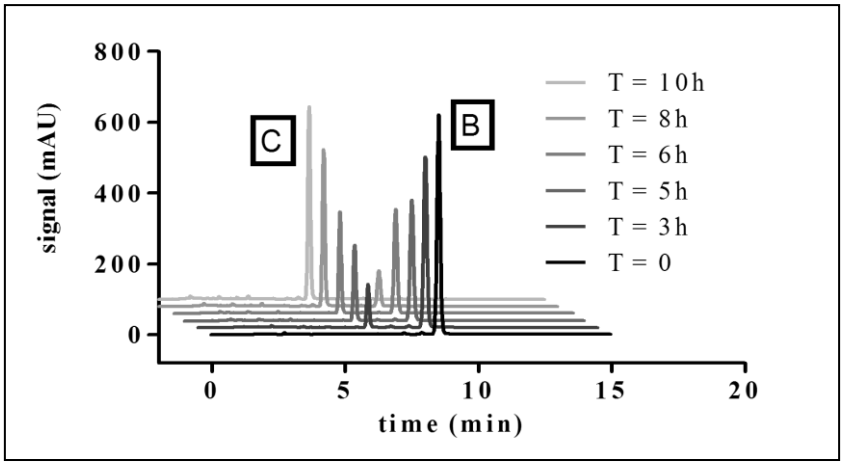



***Figure 32: HPLC chromatograms showing the formation of chlorantraniliprole transformation product C ($t_R$ = 6.2 min) from compound B ($t_R$ = 8.6 min) at different times of irradiation of compound B with UV-A light (366 nm) in acetonitrile-dd$H_2O$ (1:1) solution.***

### *5.1.13.2 Characterization of the transformation product C*

A HRMS measurement of compound **C** (Figure 33) yielded a molecular mass of 352.9802, 93 atomic units less than compound **B**. Main peaks correspond to the Cl and Br isotopes, and the ones in between (M+1) to $^{13}C$ isotope. In the samples, still some impurities were observed.

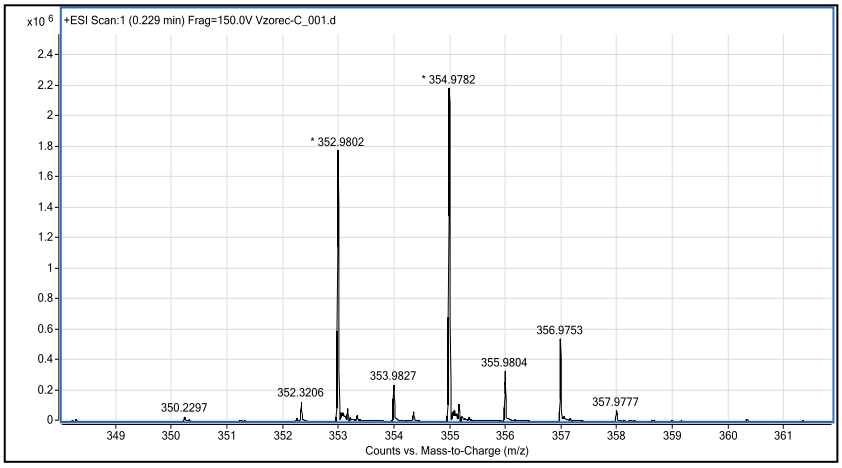
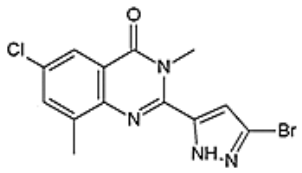



***Figure 33: The HRMS spectrum of chlorantraniliprole transformation product C.***

In the $^{1}H$ NMR spectrum, the pyridine protons vanished and a new broad singlet at δ = 13 appeared. Both techniques suggested the cleavage of the C2-N(pyrazole) bond and an expulsion of a pyridine moiety (Figure 5).

The structure of this compound was tentatively assigned as compound **C** (2-(3-bromo-1*H*-pyrazol-5-yl)-6-chloro-3,8-dimethylquinazolin-4(3*H*)-one). Its characterization data is summarized in Table 7.

***Table 7: Characterization data of chlorantraniliprole transformation product C.***

| Compound **C** | |
|---|---|
| IUPAC name | 2-(3-bromo-1*H*-pyrazol-5-yl)-6-chloro-3,8-dimethylquinazolin-4(3*H*)-one |
| Molecular formula | $C_{13}H_{10}BrClN_4O$ |
| Structural formula |  |
| $^1$H NMR (acetone $d_6$) $\delta$/ppm | 2.63 (s, 3H); 3.85 (s, 3H); 7.14 (s, 1H); 7.68 (d, $J$ = 2.5 Hz, 1H); 8.00 (d, $J$ = 2.5 Hz, 1H); 13.22 (br s, 1H). |
| HRMS (ESI, $MH^+$) | calcd for $C_{13}H_{11}BrClN_4O$: 352.998048 measured: 352.9802 |

## 5.1.14 Transformation product H

### *5.1.14.1 Formation of transformation product H*

The stability test of CAP in the dark in buffers with different pH as well as in the dark controls of photodegradation experiments revealed that the transformation product **H** is formed by base-promoted reactions. By dissolving CAP in a solution of $Na_2CO_3$ and keeping the solution in the refrigerator, the pure compound **H** dropped out in 70 % yield, which was further characterized by HRMS, $^1$H and $^{13}$C NMR, IR spectroscopy and elemental analysis. By analyzing the solution after **H** formation, no other degradation products could be observed.

### *5.1.14.2 Stability of transformation product H*

As compound **H** is probably one of the most important degradation products of CAP in natural water, we decided to study its stability.

When kept in basic aqueous solutions, compound **H** remained stable for at least one week. However, we speculated that exposed to the UV-A irradiation, compound **H** could transform into compound **B**. This assumption was based on the fact that compounds **H** and **B** have a similar structure (Figure 5) and due to the very common photo-induced nucleophilic substitutions of halogens in aryl halides with a hydroxyl group. The results of irradiation experiments showed that compound **H** is not transformed into compound **B**, but into a number of products, of which one appeared to be main (compound **I**). The retention time ($t_R$) of the newly formed main product was 2.7 min, showing it is much more polar than compound **H** (Figure 34). After continued irradiation however, compound **I** was degraded into a number of minor products. This disabled its isolation and characterization. The mass spectra, obtained by the HRMS analysis ($MH^+$), revealed the *m/z* of compound **I** to be 447.9987, with the suggested chemical formula $C_{18}H_{13}BrClN_5O_2$ - same as for compounds **B** and **A**. The HRMS spectrum suggests the expulsion of the chlorine atom from the molecule, therefore the process of formation is similar to formation of **A** from CAP. Since the HPLC retention time ($t_R$) of a molecule is very distinctive from the retention times of photoproducts **A** and **B**, we suggest different intramolecular rearrangements in the formation of compound **I**.

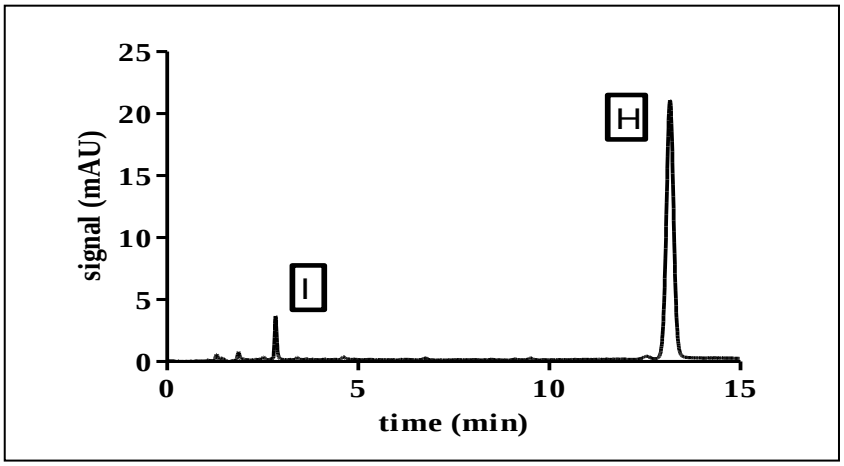



***Figure 34: HPLC chromatogram presenting degradation of compound H ($t_R$ = 12.8 min) into its main degradation product I ($t_R$ = 2.7 min), when irradiated wit UV-A light.***

### *5.1.14.3 Characterization of transformation product H*

In the ESI mass spectrum of compound **H** (Figure 35) an ($MH^+$) ion with *m/z* 463.9680 can be found. This is 18 atomic units lower than CAP (*m/z* 481.9779 $MH^+$), suggesting the loss of a water molecule during the transformation process. By comparing the HRMS spectrum of compound **H** with the spectra of the CAP photodegradation products, it can be seen that two chlorine atoms are still present in the molecule.

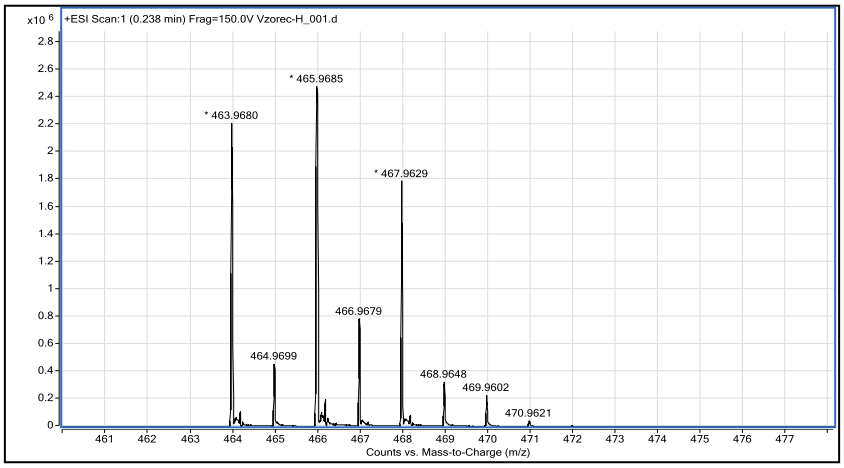
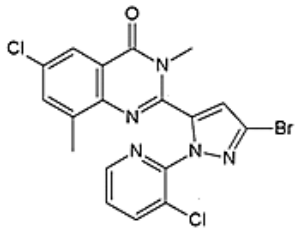



***Figure 35: The HRMS spectrum of chlorantraniliprole degradation product H.***

Overall the $^1$H NMR spectrum strongly resembled that of compound **B**, except for the pyridine part, which was similar to that of CAP. From the similarity of spectra and from the fact, that compound **H** was formed in a process analogous to that in which compound **B** was produced, we propose the structure of compound **H** to be identical to 2-(3-bromo-1-(3-chloropyridin-2-yl)-1*H*-pyrazol-5-yl)-6-chloro-3,8-dimethylquinazolin-4(3*H*)-one.

See Figure 35 and its characterization data in Table 8.

***Table 8: Characterization data of chlorantraniliprole transformation product H.***

| Compound **H** | |
|---|---|
| IUPAC name | 2-(3-bromo-1-(3-chloropyridin-2-yl)-1*H*-pyrazol-5-yl)-6-chloro-3,8-dimethylquinazolin-4(3*H*)-one |
| Molecular formula | $C_{18}H_{12}BrCl_2N_5O$ |
| Structural formula |  |
| Melting point (°C) | 215-216 |
| $^{1}$H NMR ($CD_2Cl_2$) $\delta$/ppm | 2.02 (s, 3H); 3.71 (s, 3H); 6.86 (s, 1H); 7.32 (dd, *J* = 4.7; 8.0 Hz, 1H); 7.43 (d, *J* = 2.4, 1H); 7.87 (dd, *J* = 1.6; 8.0 Hz, 1H); 8.01 (d, *J* = 2.4 Hz, 1H); 8.31 (dd, *J* = 1.6; 4.7 Hz, 1H) |
| $^{13}$C NMR ($CD_2Cl_2$) $\delta$/ppm | 16.7 ($CH_3$); 33.9 ($CH_3$); 112.3 (CH); 122.0 (C); 123.8 (CH); 125.8 (CH); 128.2 (C); 128.3 (C); 133.2 (C); 135.2 (CH); 139.0 (C); 139.2 (C); 140.0 (CH); 144.0 (C); 144.8 (C); 147.4 (CH); 148.7 (C); 161.6 (C) |
| IR (ATR) $cm^{-1}$ | 1584 (s), 1663 (s), 4303 (m), 3557 (m). |
| HRMS (ESI, $MH^+$) | calcd for $C_{18}H_{13}BrCl_2N_5O$: 463.9603 measured: 463.9680 |
| Elemental analysis | calcd for $C_{18}H_{12}BrCl_2N_5O$: C 44.58, H 2.89, N 14.45, found: C 44.71 %, H 2.87 %, N 14.30 % |

### 5.1.15 General discussion on chlorantraniliprole stability in natural waters

Based on our research, some important overall conclusions on CAP stability in water can be drawn. CAP dissolved in water undergoes two distinct transformations – spontaneous thermal transformation to compound **H** and photochemical transformation to products **A**, **B** and **C**. The irradiation of pure chlorantraniliprole in solid state (powder) for two days using the solar simulator at high intensity (750 $W/m^2$) did not initiate any transformation of CAP. Therefore, CAP needs to be dissolved in an aqueous solution in order to enable transformation. Besides chemical transformations, CAP can also be degraded biologically, by the enzymatic action of microorganisms. Although this process in especially important for degradation in soils and sediments (Zacharia, 2011), it is possible to take place also in water and therefore also in our non-sterile solution media. However, the influence of microbial degradation can in this study be neglected since photodegradation pathways were the same for each type of light used – including for germicidal UV-C lamps where all microorganisms are destroyed due to the high light intensity.

When CAP is applied to the fields, it is likely to bind to the soil, which limits its mobility to surface waters (APVMA, 2008). The most obvious way for its entering the water bodies is through runoff and spray drift, but even then its low solubility will make it likely to bind to and accumulate in sediments (Health Canada, 2013). However, the fraction that stays dissolved in the water is most susceptible for transformation but also can interact with aquatic organisms living in the water column.

We showed that the fate of CAP in water is highly dependent on the pH and bases present in the waters. Non-polluted rivers have a pH in a range of 6.5 to 8.5, depending on the concentration of $CO_2$ in the water, geology of the bedrock and watershed and other factors, such as oxidation of dissolved ferrous iron (Hem, 1985). The pH of a lake or river, especially those with poor buffer capabilities, may fluctuate substantially depending on the photosynthesis activity of the water body (Hem, 1985). In natural waters with basic pH, in the dark or exposed to the sunlight, CAP would be expected to transform to compound **H**, which would be in such waters among the most important degradation product of CAP. In pH 10 water solution the

half-life of CAP (0.6 mg CAP/L, at 25 °C) undergoing transformation to compound **H** would be around 10 days (FAO, 2008). Compound **H** was shown to be a very stable compound in the dark, but it tends to degrade when irradiated. If CAP would enter acidic waters, the transformation to compound **H** would be prohibited, but CAP could degrade photochemically to compound **A** if exposed to sunlight. Due to the shown stability of compound **A** in our experiments, there is a risk of accumulation of compound **A** in such environments. In natural waters with basic pH, the photodegradation of CAP would continue to compound **B**, a stable compound in the dark (at night), but photoactive when illuminated by sunlight. During sunny days, compound **C** would therefore be the main photodegradation product of CAP. Our study showed a high stability of compound **C**; it is therefore possible that this compound may persist also in natural environments.

Our study shows that CAP itself is not very stable in natural waters, however some of its transformation products may be more persistent. In acidic waters and in the presence of light, compound **A** is expected to accumulate, while in basic waters compounds **B** and **H** are suggested to be persistent in the dark and compound **C** also upon irradiation with sunlight. Resistance to further hydrolysis of compound **H** was reported by FAO (2008) and higher persistence of compound **H** compared to CAP was confirmed by Health Canada (2013). Colored dissolved organic matter and nitrates present in natural waters are not expected to enhance CAP degradation. It is therefore likely that complete mineralization of CAP will be a slow process in natural environments. The suggested environmental fate of CAP based on our study and information provided by EPA (2008), FAO (2008), APVMA (2008), Health Canada (2013) and the scheme from Randall et al. (2007) is presented in Figure 36.

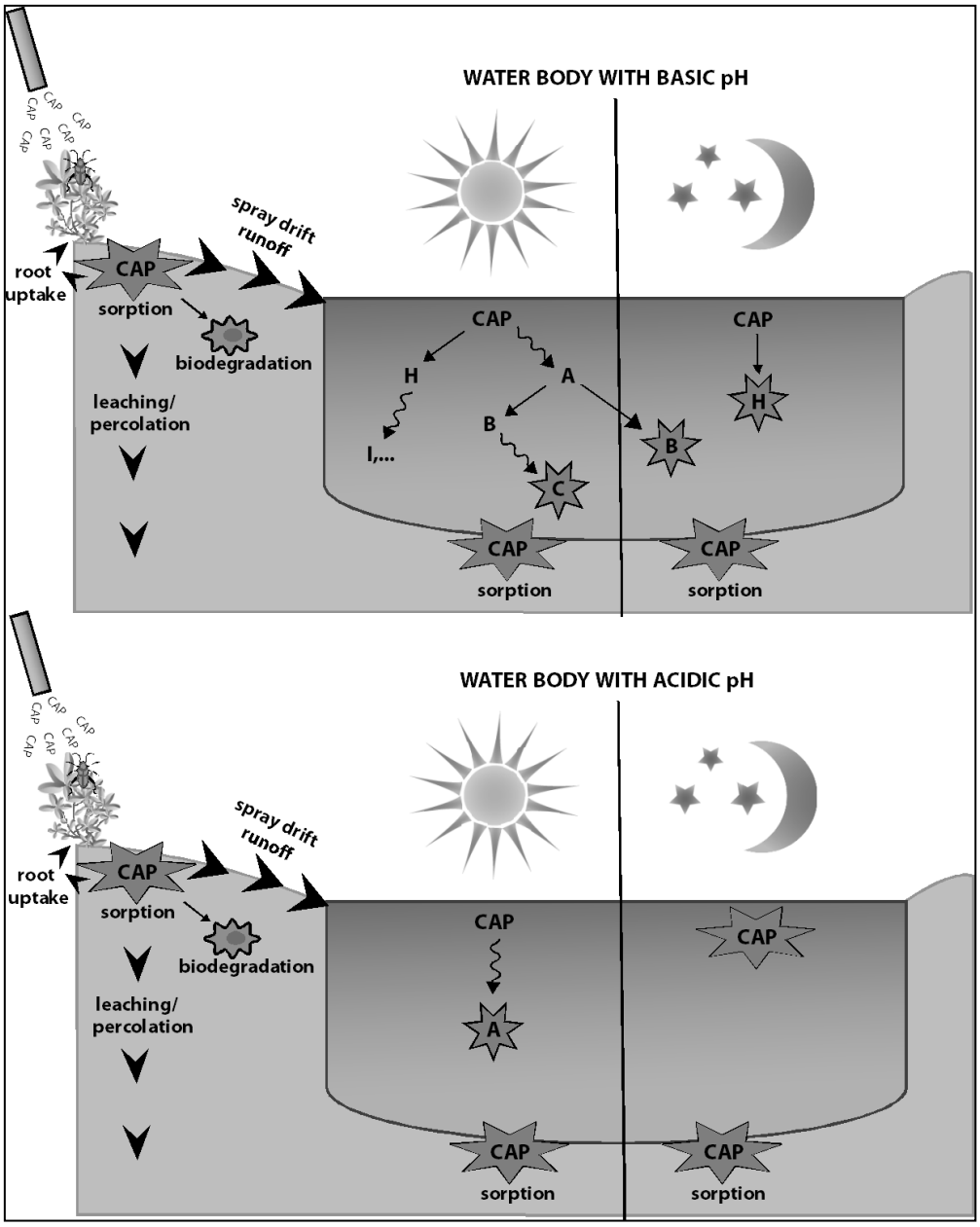



***Figure 36: A suggested fate of chlorantraniliprole in soil and water with (top) basic pH and (bottom) acidic pH.***

***Straight arrows represent chemical transformation and wavy arrows photochemical degradation. The names of compounds in star shapes suggest the persistence and possible accumulation of these compounds in the environment.***

## 5.2 Toxicity of chlorantraniliprole and its transformation products B and H to *Daphnia magna*

The sensitivity of the daphnids used in the acute and chronic tests to the reference toxicant $K_2Cr_2O_7$ ($EC_{50}$, 24 h = 1.1 mg/L, 95 % CI: 0.8-1.3 mg/L) was within the prescribed range ($EC_{50}$, 24 h = 0.6-2.1 mg/L) as set by the OECD guideline 202 (OECD, 2004a).

### 5.2.1 Acute toxicity tests of chlorantraniliprole and its transformation products B and H to *Daphnia magna*

#### *5.2.1.1 Physical-chemical parameters of the test solutions*

The physical-chemical parameters (temperature, dissolved oxygen concentration, pH and conductivity) of the test solutions at the beginning and end of the daphnid tests are summarized in Table 9. Water hardness was determined using test strips (working in the range of 0-425 mg/L $CaCO_3$); it was within in the recommended range of 140-250 mg/L $CaCO_3$ (OECD, 2004a). Other criteria and recommendations, set by the guideline were also met – these are pH between 6 and 9 with values not varying by more than 1.5 units in any one test, oxygen concentration above 3 mg/L and temperature between 18-22 °C, which should be constant within ± 1.

***Table 9: Physical-chemical parameters (pH, temperature (*T*) and concentration of dissolved $O_2$) of the test solutions at start and end of the test (48 h) of* Daphnia magna *exposure to chlorantraniliprole, compound B and compound H.***

| | CAP | | Compound B | | Compound H | |
|---|---|---|---|---|---|---|
| | T=0 | T=48 h | T=0 | T=48 h | T=0 | T=48 h |
| T (°C) | 20.9 ± 0.08 | 19.8 ± 0.11 | 20.9 ± 0.11 | 20.9 ± 0.07 | 20.7 | 21.2 |
| pH | 7.5 ± 0.02 | 7.9 ± 0.14 | 7.8 ± 0.03 | 7.8 ± 0.06 | 7.67 | 7.67 |
| $O_2$ (mg/L) | 8.9 ± 0.04 | 9.3 ± 0.08 | 9.2 ± 0.04 | 9.1 ± 0.02 | 9.17 | 9.12 |

***Values for chlorantraniliprole and compound B are the average (± SEM) of parameters along the tested concentration range.***

### *5.2.1.2 Actual concentration measurements of chlorantraniliprole and compound B*

Average actual concentrations of CAP and compound **B** from the acute toxicity tests are collected in Table 10. Values are mean concentrations measured in test samples collected at the start and end (48h) of the test. One measurement at CAP nominal concentration 5 µg/L had to be excluded from the data analysis, as it was recognized as an outlier (Grubb's test, $P < 0.05$). Generally, the concentrations of CAP and compound **B** were slightly higher at the end of the test than in the freshly prepared test solutions with which we started the tests. This is probably due to the evaporation of water during the 48h toxicity test.

The HPLC and MS analysis confirmed that control solutions were free of tested compounds.

***Table 10: Average measured concentrations of chlorantraniliprole and compound B in media used for the acute toxicity tests with* Daphnia magna.**

| CAP (µg/L) | | Compound B (mg/L) | |
|---|---|---|---|
| nominal | measured | nominal | measured |
| 2 | 2.84 ± 0.67 | 0.02 | 0.019 ± 0.00017 |
| 5 | 5.99 ± 2.65 | 0.05 | 0.048 ± 0.0016 |
| 10 | 9.76 ± 0.73 | 0.01 | 0.095 ± 0.0041 |
| 20 | 18.87 ± 1.03 | 0.2 | 0.21 ± 0.0039 |
| 50 | 50.71 ± 0.99 | 0.5 | 0.57 ± 0.0009 |
| | | 1 | 1.15 ± 0.055 |

***The ± values represent the standard deviation (n = 3 for compound B for all concentration range, and for CAP n = 6, 4, 3, 3 and 2 for 2, 5, 10, 20 and 50 µg CAP/L, respectively).***

We were unable to measure the concentrations of compound **H** in any of the test samples, also when the LC-MS/MS measurement with standard addition method (SAM) was applied to the test solutions. Since adsorption to the test containers might explain for this, we performed an adsorption test with compound **H**. A solution of compound **H** in pure $H_2O$ (triplicates) was poured into polypropylene test tubes (same tubes as used for the toxicity test) which were then incubated for 6 hours in the dark at room temperature. Samples were analyzed and results compared with freshly diluted samples. This test showed that compound **H** is adsorbing on the walls of the polypropylene test tubes with losses up to 25%. Despite that, we should still be able to measure the signal of compound **H** in the test tubes from the toxicity test, as the concentrations corrected for such loss due to adsorption still should have been above the detection limit of the analytical method. It is possible however, that adsorption was higher than expected from the short-term sorption test, as the vials were stored in the freezer for longer times. Another possibility is that the compound was rapidly degraded.

### *5.2.1.3 Acute toxicity of chlorantraniliprole to* Daphnia magna

The control and solvent control did not significantly differ from each other and were therefore pooled. The mean control survival was 93 % ($\sigma$ = 10 %), which meets the validity criteria (survival of the controls over 90 %), set by OECD guideline 202 (OECD, 2004a).

Until the end of the toxicity test, the survival of the daphnids remained high for the lowest two CAP concentrations (2 and 5 µg/L), but dropped considerably at 10 µg CAP/L ($survival_{48\ h}$ = 20 % of initial animals) and no daphnid was alive after 48h of exposure to 20 and 50 µg CAP/L.

The results show an extreme toxicity of CAP to *D. magna* with a clear concentration-related response (Figure 37), from which an $EC_{50}$ value of 9.4 µg/L (95 % CI: 9.1-9.6) was derived ($EC_{50}$ based on measured concentrations). The very high steepness of the concentration-response curve indicates a highly potent action of CAP on the exposed daphnids.

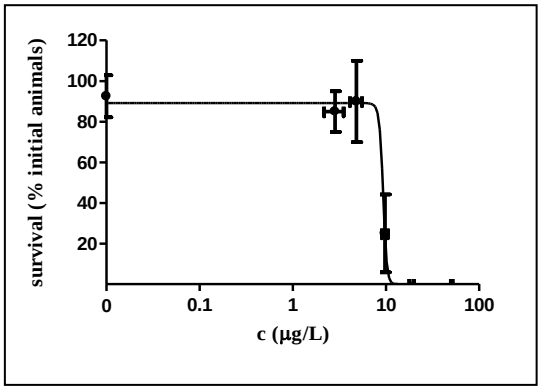



***Figure 37: Concentration – response curve for the acute (48 h) effect of chlorantraniliprole on* Daphnia magna *survival.***

***Error bars (in x and y) represent the standard deviation.***

The acute $EC_{50}$ for CAP (9.4 µg/L) obtained in our study was slightly lower than the value previously reported by the EPA (2008), which is 11.6 µg/L. Comparing the $EC_{50}$ value of CAP and other new era insecticides, such as imidacloprid ($EC_{50}$ = 84 mg/L, Daam et al., 2013), thiacloprid ($EC_{50}$ > 85.1 mg/L (FAO, 2010), and flubendiamide, an insecticide possessing the same mode of action as CAP ($EC_{50}$ > 60 µg/L, EFSA, 2013), it appears that CAP is one of the most toxic insecticides to *D. magna* used in current agricultural practice.

Comparing insects and crustaceans on molecular and morphological basis revealed that these two groups are closely related to each other (Boore et al., 1998), which could be a reason for the high toxicity of CAP to daphnids.

#### *5.2.1.4 Acute toxicity test of compounds B and H to* Daphnia magna

Daphnid survival in both controls was 100 %. The transformation product **H** showed no toxic effect on the daphnids as the mean survival was 95 % ($\sigma$ = 10 %) after 48 h of exposure. While the survival was high (95 % and higher) for the lowest four concentrations of compound **B**, it dropped to 65 % at 0.5 and 1 mg /L. From the acute test it appeared that compound **B** shows a toxic effect on survival of the daphnids at the highest concentrations tested.

### 5.2.2 Chronic toxicity of chlorantraniliprole and its transformation products B and H to *Daphnia magna*

#### *5.2.2.1 Physical-chemical parameters of the test solutions*

The temperature of the test solutions was in the recommended range (18-22 °C) and did not vary by more than 2 °C, so it was within the recommended limits (OECD, 2012). All criteria of physical-chemical parameters were met according to the OECD guideline: the dissolved oxygen concentration was in all cases above 3 mg/L at the beginning and during the test. The pH was within the recommended range (6-9), and did not vary by more than 1.5 units in any one test. Hardness was above 140 mg/L (as $CaCO_3$). The average values of the parameters measured in freshly prepared and old (3 days) media for CAP and compounds **B** and **H** are collected in Table 11.

***Table 11: Physical-chemical parameters (pH,* T *and concentration of dissolved O2) of the new and old media during the chronic exposure of* Daphnia magna *to chlorantraniliprole, compound B and compound H along the tested concentration range.***

| | **CAP** | | **Compound B** | | **Compound H** | |
|---|---|---|---|---|---|---|
| | new media | old media | new media | old media | new media | old media |
| T (°C) | 21.8 ± 0.3 | 20.4 ± 0.2 | 21.8 ± 0.2 | 21.4 ± 0.1 | 21.9 ± 0.5 | 20.5 ± 0.3 |
| pH | 8.0 ± 0.05 | 7.9 ± 0.08 | 8.0 ± 0.08 | 7.9 ± 0.04 | 8.0 ± 0.1 | 7.9 ± 0.1 |
| $O_2$ (mg/L) | 9.3 ± 0.13 | 9.2 ± 0.07 | 9.1 ± 0.08 | 9.1 ± 0.02 | 9.4 ± 0.2 | 9.1 ± 0.1 |

***All values represent the average (± SEM) along the concentration range (for CAP and compound B) each time the media was renewed.***

### *5.2.2.2 Actual concentration measurements of chlorantraniliprole and compound B*

In control test solutions, the MS analysis showed no signals that could correspond to the tested compounds.

Applying the standard addition method (SAM), actual concentrations for the chronic toxicity test with *D. magna* were measured for CAP and HPLC was used to measure the concentrations of its transformation product **B**. We were unable to detect compound **H** in any of the test solutions. Please see 5.12.1.2 for further information.

The actual concentrations of CAP and compound **B** measured in the chronic test are compared to the nominal values in Table 12. The actual concentrations (mean ± SD) are shown for renewed and old (3 days) media, taken at four separate media renewal events for compound **B**. Samples of CAP were analyzed from three renewal events at 1 and 3 µg/L and due to the complete mortality of the daphnids already at the beginning of the test, samples from one media renewal event (new and old media) were measured for CAP at 6, 9 and 12 µg/L. One significant outlier (Grubb's test, $P < 0.05$) at a CAP nominal concentration of 1 µg/L was identified, and therefore not considered in our calculations. Generally, measured concentrations of compound **B** were in all cases lower than the nominal values. Concentrations of compound **B** in old media, incubated in the climate control room for three days, were always lower than in the freshly prepared media. It seems that the media used for the toxicity test or other factors caused degradation of compound **B**. On the other hand, measured

concentrations of CAP were always higher than the nominal ones, except for 12 µg CAP/L. The measured CAP concentrations were in all cases higher in the old media (3 days old) than in the new media. This is excluding any degradation of CAP during the incubation period. As in the acute test, the higher measured concentrations of CAP in the old media could be due to the evaporation of water from the test tubes during the toxicity test. The fairly large deviations between the replicate measurements indicate that a matrix effect may be still present in the MS/MS CAP analysis.

***Table 12: Average measured concentrations of chlorantraniliprole and compound B in media used for the chronic toxicity test with* Daphnia magna.**

| **CAP (µg/L)** | | **Compound B (mg/L)** | |
|---|---|---|---|
| nominal | measured | nominal | measured |
| 1 | 0.86 ± 0.49 | 1 | 0.90 ± 0.08 |
| 3 | 3.02 ± 1.26 | 0.2 | 0.15 ± 0.03 |
| 6 | 8.02 ± 2.54 | 0.5 | 0.40 ± 0.05 |
| 9 | 9.50 ± 0.25 | | |
| 12 | 10.83 ± 1.58 | | |

***The ± values represent the standard deviation (n = 8 for B, n = 5 for 1 µg CAP/L, n = 6 for 3 µg CAP/L and n = 2 for 6, 9 and 12 µg CAP/L).***

### *5.2.2.3 Chronic toxicity of chlorantraniliprole to* Daphnia magna

The mean survival of the controls was 93 %, and met the validity criteria of at least 80 % survival set by the OECD guideline 211 (OECD, 2012).

Higher mortality, relative to the controls was observed already at the two lowest CAP nominal concentrations; 86.5 % for 1 µg CAP/L and 77.8 % for 3 µg CAP/L. CAP clearly affected daphnid survival at concentrations of 6 µg/L and higher. At 12 µg/L complete mortality occurred already after 2 days of exposure and after 4 and 6 days at 9 and 6 µg CAP/L, respectively (Figure 38, left panel).

From the survival data at the end of the experiment (21d) a clear concentration-response relationship was obtained (Figure 38, right panel), from which an $LC_{50}$ value of 3.7 µg/L (95 % CI: 3.2-4.2 µg/L) was derived, based on measured CAP concentration values. Like in the acute test, the very steep curve indicates a very prompt effect of CAP on daphnid survival.

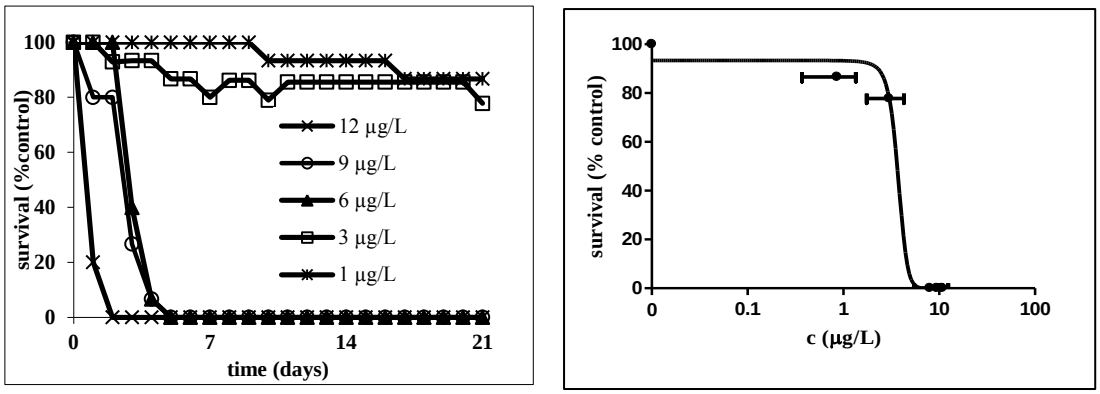



***Figure 38: Survival-time (left panel) and survival-concentration (right panel) relationships for* Daphnia magna *exposed to chlorantraniliprole for 21 days.***
***Concentrations shown on left panel are nominal ones. Error bars on the right panel represent the standard deviation (n =54 for 1 µg CAP/L, n = 6 for 3 µg CAP/L and n = 2 for 6, 9 and 12 µg CAP/L).***

The comparison between the acute (48h $LC_{50}$ value 9.4 µg/L) and chronic concentration response curves demonstrates that the toxicity of CAP increases with increasing exposure time, resulting in an acute (48h) to chronic (21d) ratio (ACR) of 2.5.

While CAP showed a clear effect on the survival of the exposed daphnids, no effect on reproduction was observed, as the cumulative reproduction output as well as the age at first reproduction of surviving animals did not differ between CAP concentrations and corresponding controls (Figure 39). The mean cumulative reproduction per female was 32.4 ($\sigma$ = 0.6), which is rather low for *D. magna*. The controls therefore failed to meet the validity criteria set by OECD guideline 211 (OECD, 2012), which is putting the limit at 60 juveniles/female. No specific explanation for this low reproduction can be found, as the survival of the controls was high and the sensitivity of the daphnids to the reference toxicant $K_2Cr_2O_7$ was well within the prescribed range. Nonetheless, higher juvenile production would not change the outcome of the test showing a clear absence of CAP effects on daphnid reproduction.

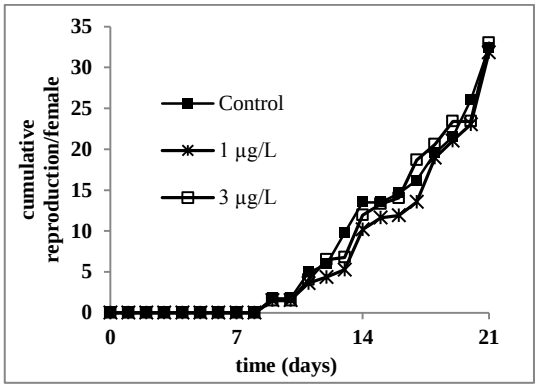



***Figure 39: Cumulative reproduction expressed as the number of juveniles per female of* Daphnia magna *exposed to chlorantraniliprole for 21 days.***
***Concentrations shown are nominal ones.***

### *5.2.2.4 Chronic toxicity of compounds B and H to* Daphnia magna

The survival of the controls remained 100 % until the end of the test.

In Figure 40, the survival (left panel) of *D. magna* exposed to compound **B** and compound **H** (0.013 mg/L nominal concentration) is shown as a function of time. Prolonged exposure to compound **H** showed no difference in survival of the daphnids, confirming the lack of effect observed in the acute test. In contrast to the slight mortality observed in the acute test at the highest test concentrations, during chronic exposure no significant effect on survival was observed ($P > 0.05$) for compound **B** at the same test concentrations.

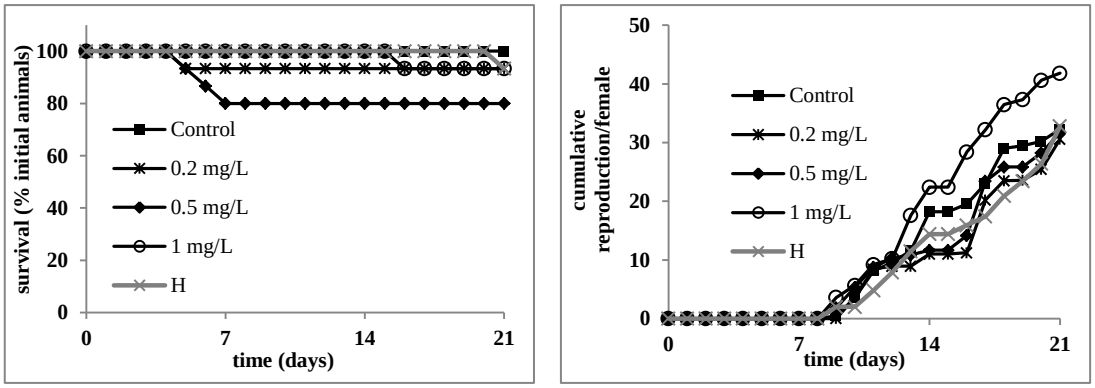



***Figure 40: Survival (left panel) and cumulative reproduction/female (right panel) of* Daphnia magna *exposed for 21 days to the transformation products B and H.***
***Concentrations shown are nominal values for compound B; compound H was tested at only one nominal concentration (0.013 mg/L).***

The right panel of Figure 40 indicates the reproductive performance of daphnids exposed to CAP transformation products. Firstly, no significant difference ($P > 0.05$) in age at first reproduction between controls, solvent controls and all tested concentrations of compounds **H** and **B** can be observed. The daphnids started reproducing after eight or nine days of exposure.

The cumulative reproduction after 21 days exposure to compound **B** was comparable to that of the controls, except for the highest test concentration (1 mg/L), where reproduction was stimulated, although the difference compared to the corresponding control was not significant ($P > 0.05$). The mean cumulative reproduction per female for the two lowest concentrations was 31.1 ($\sigma = 0.77$), while it was 32.3 for the control and 41.8 for the highest concentration. At the same time, the cumulative reproduction per female for compound **H** was 32.8. Like in the chronic toxicity test with CAP, even due to a generally poor reproduction of the daphnids, the results show a strong evidence of the absence of adverse effects of the compounds **B** and **H** on *D. magna* reproduction.

## 5.3 Toxicity of chlorantraniliprole and compound H to *Lumbriculus variegatus*

The pH and the temperature of the overlying water were in the recommended range throughout the test (pH 6-9, 20 ± 2 °C, OECD guideline 225; OECD, 2007). Due to the constant aeration of the test jars, the oxygen saturation was sufficient in all cases. Water hardness, measured with the indicator strip, varied between the test samples and was high, probably due to the $CaCO_3$ added in the reconstituted water. In all cases, ammonia, also measured with an indicator strip, was not detectable. The average values of the parameters with their standard error are summarized in Table 13. All concentrations are expressed as nominal values.

***Table 13: Physical-chemical parameters (pH,* T*, concentration of dissolved O2 and water hardness) of the overlying water in the* Lumbriculus variegatus *exposure tests with chlorantraniliprole and compound H.***

| C (µg/g dw) | T (°C) | pH | $O_2$ (mg/L) | Hardness (mg/L $CaCO_3$) |
|---|---|---|---|---|
| **C** | 20.2 ± 0.2 | 8.18 ± 0.11 | 8.91 ± 0.21 | 250-300 |
| **SC** | 20.2 ± 0.1 | 8.28 ± 0.09 | 8.92 ± 0.21 | 225-250 |
| **25** | 20.1 ± 0.2 | 8.42 ± 0.15 | 8.75 ± 0.25 | >375 |
| **50** | 20.2 ± 0.1 | 8.51 ± 0.12 | 8.49 ± 0.18 | >375 |
| **100** | 20.0 ± 0.1 | 8.54 ± 0.06 | 8.86 ± 0.19 | >375 |
| **200** | 19.9 ± 0.1 | 8.54 ± 0.05 | 8.14 ± 0.73 | 300-375 |
| **400** | 20.1 ± 0.2 | 8.47 ± 0.05 | 8.36 ± 0.45 | 300-375 |
| **800** | 19.9 ± 0.1 | 8.55 ± 0.04 | 8.44 ± 0.45 | >375 |
| **H 800** | 20.2 ± 0.2 | 8.43 ± 0.09 | 84.00 ± 0.27 | >375 |

***C (µg/g dw) is nominal concentration in sediment. All values are the average (± SEM) of replicates, measured once per week until the end of experiment (28 days).***

The number of worms counted per treatment at the end of the test is shown in Figure 41. The average number of living worms per replicate in the solvent controls increased by a factor of 4.2 at the end of test, compared to the initial number of worms that started the test. With this we met the criteria of the OECD guideline (OECD, 2007), where this factor was set to 1.8. A high variability between replicates and between treatments can be observed and one significant outlier was removed for compound **H** (Grubb's test, $P < 0.05$). From the present data, no concentration-response relationship can be observed for the effect of CAP. This was also the case when worms were distinguished between large worms without regenerated body regions (complete worms), complete worms with well visible regenerated, lighter-colored body regions (regenerated worms) and recently fragmented worms with non-regenerated body regions (incomplete worms) (Figure 41). A large number of regenerated and a small number of incomplete worms were observed also at the higher concentrations indicating that the regeneration capacity of the worms was not affected. The number of complete worms was similar among the treatments, which additionally suggests that the time of the start of reproduction was comparable between tested CAP concentrations and controls.

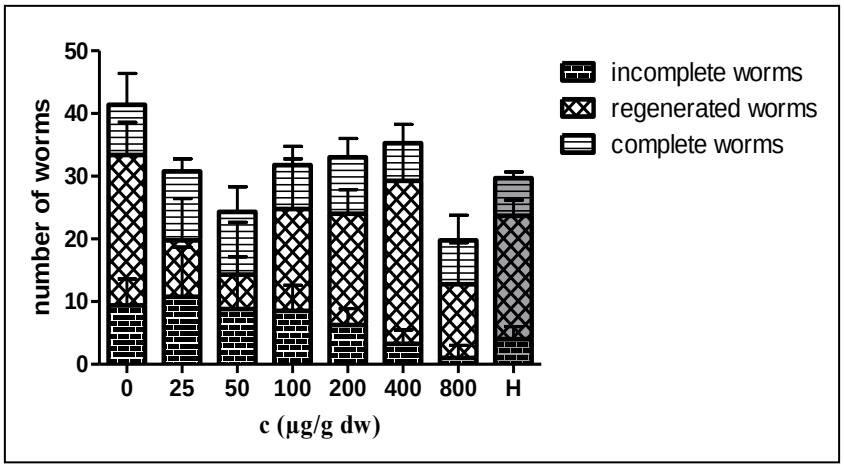



***Figure 41: Reproduction of* Lumbriculus variegatus *when exposed to chlorantraniliprole and compound H (800 µg/g dw, nominal concentration) for 28 days.***

***Worms are classified as complete, incomplete and regenerated. Error bars represent the standard deviation between the replicates (n = 4, except for compound H where n = 3). Concentrations are expressed as nominal values.***

In Figure 42, the reproductive output of *L. variegatus* exposed to the wide CAP concentration range and compound **H** (800 µg/g dw) is presented. Significant differences compared to the solvent control were found only for CAP concentrations of 50 and 800 µg/g dw ($P < 0.05$), therefore no concentration-response relationship could be detected. The horizontal dashed line in Figure 42 represents the validity criterion set by the OECD guideline (OECD, 2007), where the reproductive output of living worms per replicate in the controls should have increased by at least 80 % at the end of the test.

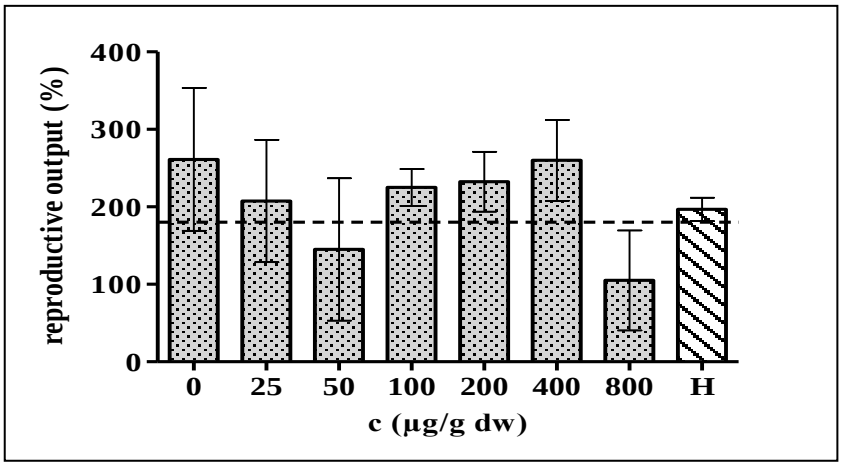



***Figure 42: Reproductive output of* Lumbriculus variegatus *when exposed to chlorantraniliprole and compound H (800 µg/g dw) for 28 days.***

***Error bars represent the standard deviation between the replicates (n = 4, except for control, where n = 8 and compound H, where one outlier has been removed; n = 3). Dashed line represents the validity factor (180 worms). Concentrations are expressed as nominal values.***

# 5.4 Toxicity of chlorantraniliprole to the woodlouse *Porcellio scaber*

## 5.4.1 Survival of *Porcellio scaber* exposed to chlorantraniliprole

The survival of isopods at the end of the test (32 days) was 100 % for the control and 88.9 % (σ = 19.2 %) for the solvent control, where one animal died in one replicate. The survival was identical to the solvent control at the two highest CAP concentrations tested (100 and 1000 µg/g dw), but much lower (66.7 %, σ = 57.7 %) at 10 µg/g dw, where during the test all three animals died in one replicate and an additional one in the second replicate on day 31. CAP therefore, even at the high concentrations tested did not affect the survival of the terrestrial isopods. The high mortality at the lowest tested concentration is most likely an artefact and not caused by CAP exposure. The animals that survived seemed to be in a good physical state and no behavioral changes between treatments compared to the control were observed.

## 5.4.2 Influence of CAP to the body weight change of *Porcellio scaber*

Since at the start of the test the weight was recorded for all three animals in each replicate, relative body weight change could be calculated only for the replicates with 100 % survival. Statistically the controls did not significantly differ from each other (Student's t-test; $P > 0.05$). Figure 43 represents the relative body weight changes per replicate, each containing 3 animals. In both controls, the weight of the animals was lower at the end than at the beginning of the test. This loss of the weight in the controls can be due to the stress caused by disturbances from the everyday observations of the animals' physical state, variations in soil moisture content or the act of replenishing moisture loss, and by transferring the animals. Weight loss was observed at all concentrations tested and was the lowest at the highest concentration tested. Statistical analysis showed no significant difference ($P > 0.05$) between the treatments, demonstrating that CAP did not affect weight change of the isopods.

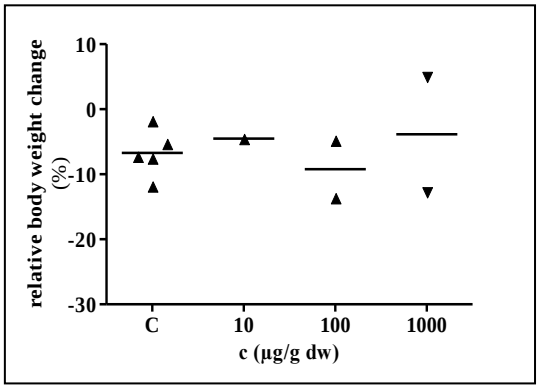



***Figure 43: Body weight change (in mg fresh weight of 3 animals/replicate) of adult* Porcellio scaber *after 32 days of exposure to chlorantraniliprole in Lufa 2.2 soil.***
***The lines represent the mean values of the replicates (n = 5 for control and n = 2 for the rest of the tested concentrations, except for 10 µg CAP/g dw, where n = 1). Concentrations are expressed as nominal values.***

### 5.4.3 Influence of chlorantraniliprole on the consumption rate of *Porcellio scaber*

*P. scaber* consumption rates (CR), calculated for 3 individuals together for a replicate, are plotted in Figure 44. The isopods' CR in the two controls was similar and interestingly lower compared to that for the CAP exposed animals. However, the difference was not significant, therefore implying that CAP did not affect the consumption rate of *P. scaber* within 32 days of exposure.

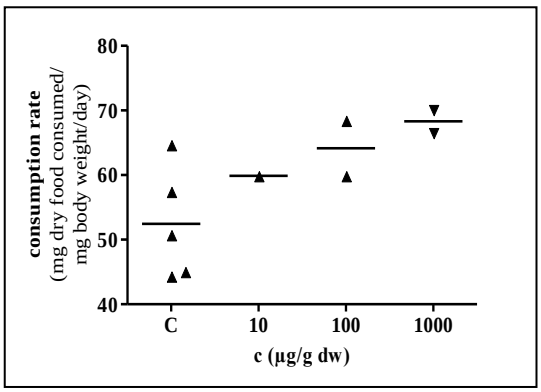



***Figure 44: Consumption rate (in mg dry food consumed/mg fresh body weight/day; 3 animals/replicate) of adult* Porcellio scaber *after 32 of exposure to chlorantraniliprole in Lufa 2.2 soil.***

***The lines represents the mean values of the replicates (n = 5 for control and n = 2 for the rest of the tested concentrations, except of 10 µg CAP/g dw, where n = 1). Concentrations are expressed as nominal values.***

The toxicity test showed no adverse effects of CAP on *P. scaber*, when looking at the usually more sensitive toxicity endpoints, such are animal fresh body weight change and consumption rate. For comparison, the organophosphate insecticide dimethoate affected the growth of *P. scaber* juveniles in a 4-week test with an $EC_{50}$ = 17.5 µg/g dw (Fischer et al., 1997) and also influenced food consumption ($EC_{50}$ = 38.2 µg/g dw; Rundgren and van Gestel, 1998) when exposed in Lufa 2.2. soil. In a food exposure experiment, conducted by Ribeiro et al. (2001), the insecticide endosulfan caused a significant decrease of food consumption and assimilation rates of the isopod *Porcellio dilatatus* at the highest concentrations tested (100, 250 and 500 µg/g of food). This eventually affected also the growth rate of the animals. In the

same study, feeding on the insecticide parathion (100-500 µg/g of food) caused a high mortality of the isopods. Differences in the route of exposure (via food or soil) can indeed influence the toxicity of the tested compound (Hornung et al., 1998), which would depend mainly on its partitioning between soil and food (Vijver et al., 2006). However Vijver et al. (2006), comparing the accumulation of Cd and Zn in *P. scaber* exposed to spiked soil and food, found no differences in uptake rates for Cd, and a lower uptake rate of Zn when applied to the food. Additionally, much higher toxicity of the insecticide dimethoate to *P. scaber* was observed when the isopods were exposed through soil than to contaminated food (Hornung et al., 1998). These data let us only roughly compare the toxicities between different exposure routes. Nevertheless, our results clearly show no adverse effects of CAP on *P. scaber* in laboratory toxicity tests.

## 5.5 Toxicity of chlorantraniliprole to the potworm *Enchytraeus crypticus*

To meet the validity criteria of the toxicity test on *E. crypticus*, defined by the OECD guideline 220 (OECD, 2004b), the survival in the controls should be above 80 %, the average number of juveniles counted per vessel at the end of the test at least 25 for the 10 adults that started the test, and the coefficient of variation (CV) of the mean number juveniles produced should be less than 50 %. As control and solvent control overlapped (Student's t-test, $P > 0.05$), they were pooled, and further analyzed as such. The average survival was 88 % ($\sigma$ = 13 %), the mean number of juveniles 585 ($\sigma$ = 63) per vessel and the CV (%) of reproduction was 11 %.

In Figure 45, the data on the survival and reproduction (number of juveniles) of *E. crypticus* exposed to CAP for 21 days are presented.

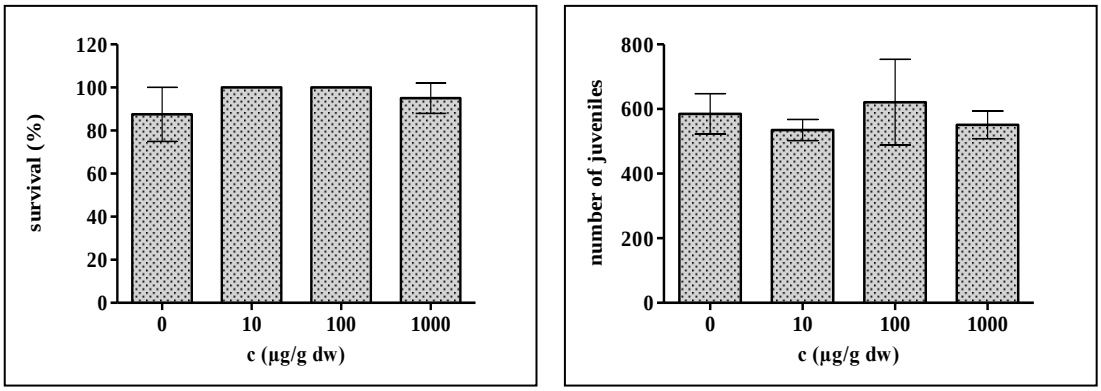



***Figure 45: The survival (%, left panel) and the reproduction (right panel) of* Enchytraeus crypticus *exposed for 21 days to chlorantraniliprole in Lufa 2.2 soil. Error bars represent the standard deviation (n = 2, except for the control, where n = 4). Concentrations are based on nominal values.***

As can be seen from the graph and was confirmed statistically (Dunnett's multiple comparison test, $P > 0.05$) CAP did not affect the survival and reproduction of the potworms. The survival of *E. crypticus* exposed to the different CAP concentrations was even higher than in the pooled controls. The average number of juveniles per replicate was for all treatments high (above 500). The lowest mean number of juveniles was found at a CAP concentration of 10 µg/g dw (551, $\sigma = 42$) and the highest at 100 µg CAP/g dw soil (621, $\sigma = 133$).

The absence of adverse effects of CAP on *E. crypticus* could be explained as follows. First, the ryanodine receptors in potworms are not susceptible to CAP binding and acting. Second, the bioavailability of CAP was lower. As the direct effects of pesticides are mainly caused by the uptake from the soil solution (Didden and Römbke, 2001), the bioavailability of CAP is proposed to be low due to its low solubility in water (0.880 mg/L, FAO 2008, EPA 2008) and therefore strong adsorption of CAP to the soil organic matter. It should however be mentioned, that effects could also occur via enchytraeid ingestion of the soil. On the other hand, even for soil ingesting animals, the main route of exposure to chemicals is still through the soil solution (Didden and Römbke, 2001).

CAP appears to be less toxic compared to some other insecticides tested on enchytraeids in laboratory studies. These examples (summarized by Jarratt and Thompson, 2009) include parathion, abamectin, pentachlorophenol, dimethoate, alpha-cypermethrin, lindane and others. However, referring to the example of the

insecticide parathion (Didden and Römbke, 2001) the sensitivity of exposed enchytraeid species was relatively low when tested in the laboratory tests, but the insecticide affected the abundance of enchytraeids already upon short-term exposure in the field. Therefore the results obtained in laboratory toxicity test cannot guarantee that CAP could not harm the enchytraeid community in the field, where its formulated products are applied.

## 5.6 Toxicity of chlorantraniliprole to the oribatid mite *Oppia nitens*

The mite survival and reproduction data of the controls and solvent controls overlapped (P > 0.05), so the average of both was used for further calculations and comparison of the effects.

The mean oribatid mite survival in the pooled controls was 80 % (σ = 15 %) and the average number of juveniles per replicate was 25 (σ = 11, CV = 44).

Figure 46 plots the survival (%, left panel) and number of counted juveniles (right panel) of *O. nitens* for the controls and the CAP concentration series.

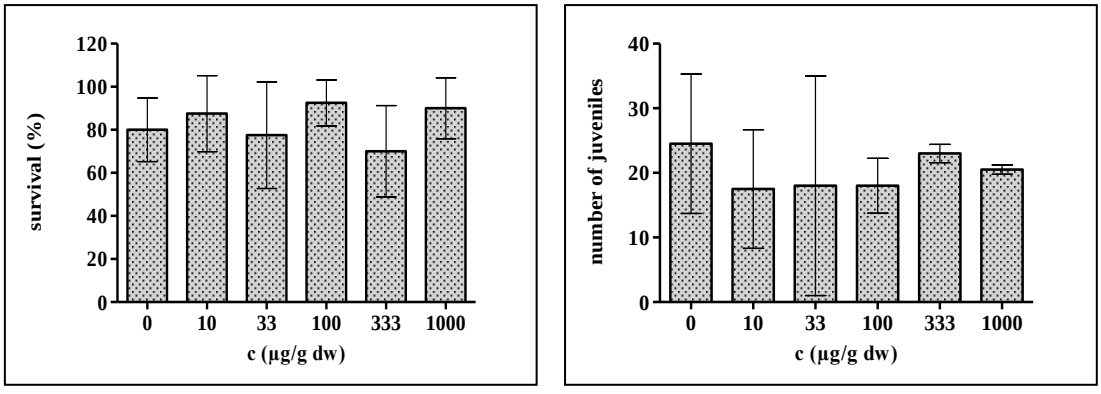



***Figure 46: The survival (%, left panel) and reproduction (right panel) of* Oppia nitens *exposed for 35 days to chlorantraniliprole in Lufa 2.2 soil.***

***Error bars represent the standard deviation (n = 2, except for the control, where n = 4). Concentrations are expressed as nominal values.***

Exposure to CAP did not cause any significant difference (P > 0.05) in survival and reproduction of *O. nitens*, compared to the pooled controls. Big variations in the

number of juveniles produced can be found between the replicates for some of the treatments. Overall, the number of juveniles produced (number of adults that stated the test = 20) was rather low compared to the results of Princz et al. (2010), who found up to 86 juveniles started in a test with 10 adults. However, their results varied significantly across different test soils. No information on *O. nitens* reproduction performance in Lufa 2.2 soil is available for comparison of our control data.

## 5.7 Toxicity of chlorantraniliprole to the springtail *Folsomia candida*

### 5.7.1 *Folsomia candida* reproduction toxicity test over two generations

The validity criteria for the untreated controls set by OECD Guideline 232 (OECD, 2009) and ISO Standard 11267 (ISO, 1999) mention that adult survival should be above 80 %, number of juveniles per test vessel higher than 100 and coefficient of variance of reproduction lower than 30 %. The first generation reproduction test on CAP with *F. candida* met all these validity criteria. The average survival, number of juveniles and CV were 88 %, 258 and 22 % for the untreated control and 94 %, 184 and 26 % for the solvent control, respectively. The control and solvent controls did not significantly differ from each other (Student's t-test, $P > 0.05$), they were therefore pooled.

The soil pH (Table 14) in all treatments in the first generation test was lower at the end compared to the start of the toxicity test, but appeared to be steady along the concentration range.

***Table 14: Soil pH at the start and end of the 28-day toxicity tests with chlorantraniliprole and* Folsomia candida *in Lufa 2.2 soil.***

| c (µg/g) | C | SC | 0.1 | 0.256 | 0.64 | 1.6 | 4 | 10 | 25 |
|---|---|---|---|---|---|---|---|---|---|
| **pH start** | 5.88 ± 0.11 | 6.03 ± 0.02 | 6.02 ± 0.02 | 5.86 ± 0.18 | 6.05 ± 0.02 | 6.05 ± 0.03 | 5.95 ± 0.07 | 6.02 ± 0.01 | 6.02 ± 0.0 |
| **pH end** | 5.43 ± 0.02 | 5.31 ± 0.01 | 5.21 ± 0.005 | 5.24 ± 0.01 | 5.20 ± 0.01 | 5.18 ± 0 | 5.28 ± 0.02 | 5.27 ± 0.03 | 5.26 ± 0.01 |

***All values presented are mean values (± SEM) including two replicates per treatment. Concentrations are expressed as nominal values.***

In Table 15, the average (± SD) survival (%) and the reproduction of *F. candida*, exposed to CAP concentrations up to 25 µg/g dw are collected. It can be seen that CAP severely affected springtail survival. Already at a CAP concentration of 1.6 µg/g dw the average survival decreased by more than 40 % compared to the pooled controls. The calculated $LC_{50}$, based on nominal concentrations, was 5.14 µg/g dw (95 % CI: 3.07-8.60 µg/g dw). Unlike in the toxicity test with *D. magna*, where CAP affected only the survival of the daphnids, an extreme effect on reproduction was observed. The average number of springtails counted at a CAP concentration of 0.64 µg/g dw was, for instance, 7 times lower compared to that in the pooled controls. The springtails surviving CAP concentrations of 10 µg/g dw and higher were not able to produce any instars.

***Table 15: Survival (%) and reproduction of* Folsomia candida *exposed for 28 days to chlorantraniliprole in Lufa 2.2 soil.***

| c (µg/g) | C | SC | 0.1 | 0.256 | 0.64 | 1.6 | 4 | 10 | 25 |
|---|---|---|---|---|---|---|---|---|---|
| **survival (%)** | 88 ± 13 | 94 ± 6 | 92 ± 8 | 96 ± 6 | 92 ± 13 | 54 ± 18 | 36 ± 17 | 44 ± 11 | 4 ± 12 |
| **reproduction** | 258 ± 57 | 184 ± 48 | 209 ± 106 | 64 ± 17 | 31 ± 6 | 8 ± 7 | 4 ± 3 | 0 ± 0 | 0 ± 0 |

***Survival and reproduction are mean values of five replicates (± SD). Concentrations are based on nominal values.***

For the effects on reproduction, a clear concentration-response relationship was obtained as shown in Figure 47. The calculated $EC_{50}$ for effects on reproduction was 0.20 µg/g dw (calculated using nominal concentrations) (95 % CI: 0.14-0.27 µg/g dw), showing that in case of *F. candida* reproduction is much more sensitive and therefore a more important indicator of CAP toxicity compared to survival. The reproduction $EC_{50}$ value for *F. candida*, reported by EPA (2008), is a factor of 2.4 higher – 0.48 µg/g dw. Since EPA (2008) does not provide any information about the

experimental setup, no fruitful discussion is possible of the reason for the differences between the two studies.

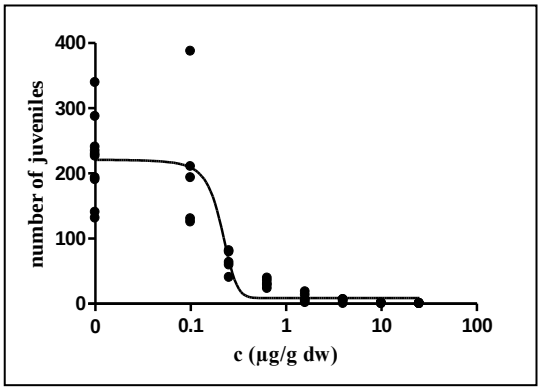



***Figure 47: Effect of chlorantraniliprole on the reproduction of* Folsomia candida *after 28 days of exposure in Lufa 2.2 soil.***

***Concentrations are expressed as nominal values*.**

When collecting the animals for the second generation test, it could already be observed that the springtails deriving from concentrations of 1.6 µg/g dw and higher were of poor physical condition. As not enough juveniles could be collected for the three highest concentrations, we continued the second generation test with controls and 0.1, 0.254, 0.64 and 1.6 µg CAP/g dw. The controls in the prolonged test (30 days) performed much worse compared to the first test. The average survival in the untreated control was low (52 %) and big variations in survival between the replicates were observed ($\sigma$ = 20 %). The survival in the solvent control was generally high (94 %, $\sigma$ = 6), but the reproduction was low. On average, 58 ($\sigma$ = 4) juveniles were counted which is below the validity criteria set by OECD and ISO (OECD 2009, ISO 1999). It is possible that floatation of the animals, transferring them to plaster of Paris, on which they stayed until the next day when the new test started, affected their viability. Nevertheless, some conclusions can be still drawn. First of all, no surviving animals were found at the higher concentrations tested. At the lowest tested concentration (0.1 µg/g dw) survival was only 18 % ($\sigma$ = 8 %). Reproduction in this treatment was also severely affected, with on average only 12 ($\sigma$ = 6) juveniles, representing 11.1 % compared to the solvent control. Although our trial to study the toxicity for the upcoming generation did not end as desired, our results still show that the second springtail generation was severely weakened due to

the exposure to CAP. This shows the importance of multigenerational toxicity studies to understand the long-term population effects of chemical compounds. In one study (León Paumen et al., 2008), where the toxicity of the organic compound phenanthrene was tested for 10 consecutive generations of *F. candida*, the effect on survival was similar for the first four generations. In the fourth generation, exposed to a concentration similar to the $EC_{50}$, the population became extinct as no juveniles were produced anymore. This probably has to do with the mode of the action of compound as well as the ability of the animals to adapt and metabolize the xenobiotics they are exposed to. At the concentrations tested in our study, it seemed that CAP caused toxicity to *F. candida* in a very potent way, as the reproduction was affected at a concentration a factor of two below the reproduction $EC_{50}$ obtained in the first test (0.20 µg/g dw, nominal concentration). It would be interesting to investigate more into this area with an attempt to find out whether a threshold concentration exists at which no adverse effects can be seen upon long-term, multigenerational exposure. And it would also be interesting to determine whether springtails are able to adapt to constant CAP exposures. A first suggestion would be to test CAP at lower concentrations than chosen in our study.

The $EC_{50}$ values of CAP and other pesticides for *F. candida* were compared. The reproduction $EC_{50}$ for the toxicity of the chlorinated insecticide toxaphene was 5.87 µg/g dw (Bezchlebová et al., 2007), meaning that CAP is almost 30 times more toxic to *F. candida* than toxaphene. Also abamectin was substantially less toxic to *F. candida* than CAP, with an $EC_{50}$ of 13 µg/g and an $LC_{50}$ of 67 µg/g dw in Lufa 2.2 soil (Kolar et al., 2008). On the other hand, abamectin was more toxic to *E. crypticus* ($EC_{50}$ 38 µg/g dw; Kolar et al., 2008), while CAP did not show any negative effects on this species. CAP was also less toxic than the organophosphorus insecticide profenofos, with an $EC_{50}$ for effects on the reproduction of *F. candida* of 0.10 µg/g dw (Liu et al., 2012). Considering this, profenofos is therefore twice more toxic than CAP, however the soil used in our experiments was different.

## 5.7.2 Reproduction toxicity of chlorantraniliprole to *Folsomia candida* in different soils

### *5.7.2.1 Control performance*

The adult survival of *F. candida* in Lufa controls in four soil types (LF = Lufa 2.2, CO = Coimbra soil, DG = Dutch grassland soil, NW = North Wales soil, for soil characteristics, see Table 4) was above 80 %, which is the threshold for the validity of the toxicity test set by the guidelines (OECD 2009; ISO 1999) (see Table 16). The validity criteria for control reproduction (a production of at least 100 instars per control replicate) and CV of reproduction (less than 30 %) were also met for the Lufa 2.2 controls. However big differences were seen between the reproduction of the animals in the different Lufa 2.2 controls. The reproduction in the Lufa 2.2 control was the highest in the test with the CO soil (mean number of juveniles = 325, $\sigma$ = 94), and lowest for LF soil (n = 182, $\sigma$ = 36). This significant difference indicates that the animal batch used in the toxicity test in LF soil was of lower quality, compared to the batches used in other test soils. But in this control the survival was the highest.

***Table 16: Control performance of* Folsomia candida *in the controls of tests with different soils; for the Lufa 2.2 controls of each test (left), and the pooled controls (the control and the solvent control) of the four test soils (right).***

| | Lufa Control | | | Pooled controls | | |
|---|---|---|---|---|---|---|
| Soil | Survival (%) | Reproduction | CV repr. (%) | Survival (%) | Reproduction | CV repr. (%) |
| **CO** | 96 ± 5 | 325 ± 94 | 29 | 91 ± 19 | 302 ± 36 | 32 |
| **LF** | 100 ± 0 | 182 ± 36 | 20 | 99 ± 26 | 184 ± 49 | 27 |
| **DG** | 84 ± 9 | 248 ± 51 | 21 | 99 ± 3 | 156 ± 51 | 33 |
| **NW** | | | | 79 ± 17 | 242 ± 76 | 31 |

***Tests in DG and NW soils were run simultaneously, therefore the same Lufa 2.2 control was used. Survival and reproduction are mean values of five replicates (± SD) of the Lufa 2.2 control, and of 10 replicates (± SD) in the two pooled controls, except for reproduction of the pooled controls of LF soil which had one outlier removed (n = 9). CV = coefficient of variance, repr. = reproduction. Soil abbreviations: CO = Coimbra soil, LF = Lufa 2.2, DG = Dutch grassland, NW = North Wales soil. For their characteristics, see Table 4.***

The controls and solvent controls of the four soils showed no significant differences (Student's t-test; $P > 0.05$), and the two controls for each soil were therefore pooled. One significant outlier ($P > 0.05$) in reproduction data of control in LF soil was removed.

After 28 days of exposure, the adult survival of the two pooled controls for CO, LF and DG soils (Table 16) met the survival validity criteria (survival above 80 %), although for the NW soil the control survival was 79 %, so slightly below the criteria.

The average number of juveniles in CO, LF, DG and NW soils was in all cases above 100, but the coefficient of variance (CV) was slightly higher in three soils than the recommended 30 %.

It is known that *F. candida* prefers soils with a high amount of organic matter (Wiles and Krogh, 1998). In our experiment however, the number of juveniles was highest in the CO soil, which had the lowest OM content (2.37 %) while the second highest number of juveniles was counted in the NW soil with the highest OM content (14.7 %). This could be attributed to the general physical condition of the animals used in the toxicity experiment in CO soil, as the reproduction in the Lufa 2.2 controls run simultaneously also was the highest (Table 16). The reproduction in the DG soil, having 10.6 % OM, was lower than in LF and CO soils which had substantially lower OM content. But other soil properties, like pH and particle size distribution, may also have affected the performance of the animals.

According to Fountain and Hopkin (2005) *F. candida* has a slight preference for a soil with pH 5.6, where the level of reproduction appeared to be the highest. In our toxicity tests, LF soil with pH 5.67 was the closest to that value, but had the second lowest reproduction. The highest reproduction was found in CO soil with a pH of 5.85, which also is close to the preferred value. NW had the lowest pH (5.04), but the low pH did not seem to have any significant impact on the reproduction.

It seems like that both the OM content and pH did not influence the springtail reproduction in the controls and that the results were likely to be due to the condition of the animals rather than physical properties of the soil. Nonetheless, since the animal performances were generally good and differences between different controls rather small, is not is not very likely that the variations in control performance had any influence on the outcome of the toxicity tests with the different soil types.

### *5.7.2.2 Influence of soil properties on the toxicity of chlorantraniliprole*

The pH measurements at the beginning and end of the test, summarized in Table 17, confirmed the values derived from an earlier study on the same soils (Table 4; Waalewijn-Kool, 2013). Soil pH showed a slight decrease during the test for CO, LF and NW soils and an increase in DG soil (Table 17). Often, a decrease in pH is due to repeatedly moistening the soil over time. The pH values were however, steady between the replicates of each treatment and did not show any significant differences over the CAP concentration range.

***Table 17: pH of the four different soils at the start and end of the 28-day toxicity tests with chlorantraniliprole and* Folsomia candida.**

| Soil | CO | LF | DG | NW |
|---|---|---|---|---|
| **pH start** | 5.97 ± 0.013 | 5.71 ± 0.007 | 6.74 ± 0.007 | 5.15 ± 0.01 |
| **pH end** | 5.81 ± 0.024 | 5.39 ± 0.008 | 6.98 ± 0.005 | 4.68 ± 0.007 |

***All values presented are mean values (± SEM) of all the treatments for each soil, including two replicates per treatment. Soil abbreviations: CO = Coimbra soil, LF = Lufa 2.2, DG = Dutch grassland, NW = North Wales soil. For their characteristics, see Table 4.***

Data on the survival and reproduction of *F. candida*, exposed to a range of CAP concentrations in all tested soil types are collected in Table 18. Survival was high for the CO and DG soils, even at the highest concentration tested (2.5 and 6.25 μg/g dw, respectively). On the other hand, in LF and NW soil the survival was gradually decreasing with increasing CAP concentration. Since LF and NW have very different OM contents as well as pH values, this effect could not be assigned to any of these parameters.

***Table 18: Survival (%) and reproduction of* Folsomia candida *exposed to chlorantraniliprole in four test soils.***

| soil | CO | | LF | | DG | | NW | |
|---|---|---|---|---|---|---|---|---|
| c (µg/g dw) | survival (%) | reproduction | survival (%) | reproduction | survival (%) | reproduction | survival (%) | reproduction |
| **0** | 91 ± 19 | 302 ± 36 | 99 ± 26 | 184 ± 49 | 99 ± 3 | 156 ± 51 | 79 ± 17 | 242 ± 76 |
| **0.026** | 100 ± 0 | 301 ± 115 | 100 ± 0 | 162 ± 36 | | | | |
| **0.064** | 98 ± 5 | 272 ± 68 | 98 ± 5 | 126 ± 31 | 98 ± 5 | 175 ± 45 | 88 ± 22 | 251 ± 87 |
| **0.16** | 96 ± 6 | 140 ± 31 | 98 ± 5 | 100 ± 23 | 100 ± 0 | 163 ± 80 | 86 ± 6 | 212 ± 81 |
| **0.40** | 84 ± 20 | 60 ± 30 | 86 ± 21 | 27 ± 26 | 98 ± 5 | 119 ± 16 | 70 ± 10 | 165 ± 47 |
| **1.00** | 98 ± 5 | 19 ± 7 | 68 ± 23 | 0 ± 0 | 98 ± 5 | 68 ± 30 | 68 ± 13 | 82 ± 23 |
| **2.50** | 92 ± 8 | 10 ± 6 | 56 ± 23 | 0 ± 0 | 92 ± 13 | 7 ± 4 | 46 ± 22 | 5 ± 6 |
| **6.25** | | | | | 90 ± 7 | 0 ± 0 | 40 ± 23 | 0 ± 0 |

***Survival and reproduction for each soil are mean values of five replicates (± SD) and of ten replicates (± SD) in the controls (c = 0 µg/g dw), except for reproduction of the pooled controls of LF soil which had one outlier removed (n = 9). Soil abbreviations: CO = Coimbra soil, LF = Lufa 2.2, DG = Dutch grassland, NW = North Wales soil. For their characteristics, see Table 4. Concentrations are expressed as nominal values.***

In all tested soils, CAP severely affected springtail reproduction. Even where the survival at the highest CAP concentrations was still high, the number of instars was very low. From the reproduction data, a clear concentration-response relationship could be plotted for each soil tested (Figure 48). The total number of juveniles for each treatment varied between the different soils, but the decrease in reproduction was similar for the two low organic soils (CO and LF soil) compared with the ones having higher OM contents (DG and NW soils). By calculating $EC_{50}$ and $EC_{10}$ values for each soil (Table 19), it became evident that the effect of CAP on reproduction significantly decreased ($P < 0.05$) with increasing OM content. CO soil, with the lowest OM content however, showed a lower toxicity than LF soil, and DG soil had a lower toxicity than NW soil with the highest OM content. The differences in $EC_{50}$ were however, not significant (Likelihood ratio test, $X^2 < 3.84$; $P > 0.05$). The highest toxicity ($EC_{50}$ = 0.14 µg CAP/g dw) was observed in the LF soil, having an OM content of 3.09 %, and the lowest in DG soil ($EC_{50}$ = 0.76 µg CAP/g dw), with an OM content of 10.6 %. Comparing the $EC_{50}$ values on the basis of an F-test, the null hypothesis was accepted, when the soils with low organic matter content (CO and LF soil) were compared to each other ($F_{CO, LF} = 0.027$) and analogous, when high organic soils (DG and NW soil) were compared to each other ($F_{DG, NW} = 0.248$). However, a significant difference ($P < 0.05$) was found when comparing the $EC_{50}$

values for the low organic (CO and LF) soils with those for the high organic soils (DG and NW soil) ($F_{CO, DG}$ = 23.380; $F_{CO, NW}$ = 19.79; $F_{LF, DG}$ = 29.60, $F_{LF, NW}$ = 25.61), confirming the effect of soil OM content on CAP toxicity to *F. candida*. All $EC_{50}$ and $EC_{10}$ calculations are based on nominal concentrations of CAP.

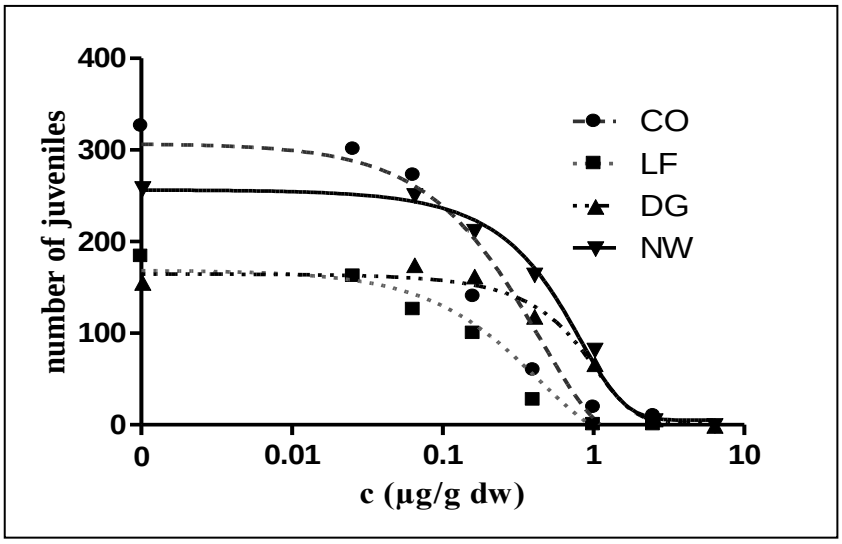



***Figure 48: Effect of chlorantraniliprole on the reproduction of* Folsomia candida *after 28 days of exposure in the four test soils***

***Presented are mean values derived from five replicates (10 replicates for controls and 9 replicates for LF control). Soil abbreviations: CO = Coimbra soil, LF = Lufa 2.2, DG = Dutch grassland soil, NW = North Wales soil. For their characteristics, see Table 4. Concentrations are expressed as nominal values.***

Unlike $EC_{50}$s, the 95% confidence intervals (CI) for the $EC_{10}$s are overlapping. Despite this, they still indicate a clear difference in toxicity and therefore are worth displaying. Because of the flat slope of the dose-response curve, $EC_{10}$s are prone to larger variation than the $EC_{50}$s.

The difference between the lowest and the highest $EC_{50}$ and $EC_{10}$ values, when OM content was increased more than two times, was a factor of 5.4 and 8.3, respectively.

***Table 19: $EC_{50}$ and $EC_{10}$ values for the reproduction toxicity of chlorantraniliprole to* Folsomia candida *after 28 days of exposure in four test soils.***

| Soil | OM (%) | pH | $EC_{50}$ (µg/g) | $EC_{10}$ (µg/g) |
|---|---|---|---|---|
| **CO** | 2.37 | 5.85 | 0.16 (0.085-0.209) | 0.04 (0.002-0.074) |
| **LF** | 3.09 | 5.67 | 0.14 (0.088-0.199) | 0.03 (0.004-0.056) |
| **DG** | 10.6 | 6.78 | 0.76 (0.433-1.09) | 0.25 (0.003-0.501) |
| **NW** | 14.7 | 5.04 | 0.62 (0.347-0.884) | 0.17 (0.002-0.346) |

***$EC_{50}$ and $EC_{10}$ values are presented as µg CAP/g dw with 95 % confidence intervals in parenthesis. $EC_{50}$ and $EC_{10}$ values are based on nominal CAP concentrations.***

An influence of OM content on toxicity has also been found in other studies. Consistent with our study, Martikainen and Krogh (1999), working on the sexually reproducing collembolan *Folsomia fimetaria*, showed a decrease in the effect on survival and reproduction of the insecticide dimethoate with increasing OM content of the soil. In early study, Martikainen (1996) obtained analogous results with dimethoate also in a reproduction test using *F. candida* as well as the earthworm *Aporrectodea caliginosa tuberculata*, for which the influence on animal survival and reduction of biomass was investigated.

There is no consistent opinion whether and how the pH itself influences the ecotoxicity for soil dwelling organisms. Crouau et al. (1999) showed that the increase of the soil pH itself (up to 6.9) negatively affected the reproduction of *F. candida*. The influence of pH on xenobiotic toxicity however, highly depends on the xenobiotic itself. It is known that for metals the solubility at lower pH is higher, which eventually increases their toxicity (Crouau and Pinelli, 2008). With higher solubility in water, the compound becomes more easily available to the organisms, as the main route of exposure is the pore water (van Gestel 1997, Smit and van Gestel 1998, Diddel and Römbke 2001). Since the solubility of CAP in different pH values is similar (pH 4: 0.972 mg/L, pH 7: 0.880 mg/L and pH 9: 0.971 mg/L; FAO, 2008), such small changes in the pH of the tested soils are not expected to affect the bioavailability of CAP. In general, the effect of the pH was found to be non-significant for the sorption/desorption behavior of organic chemicals (Delle Site, 2001). Also in our tests, no pH influence was observed on springtail toxicity. DG

soil, which had the highest pH (6.78) showed the lowest toxicity, while NW soil with the lowest pH (5.04) had the second lowest toxicity.

One can notice a small difference between the $EC_{50}$ value for the toxicity of CAP to *F. candida* in the first test (Chapter 5.7.1, $EC_{50}$ = 0.20 µg/g, test A), and the value for the LF soil obtained in this test ($EC_{50}$ = 0.14, test B). In both tests, Lufa 2.2 was used. Small differences in the $EC_{50}$ values between the two tests could be due to the different batch of the animals, different time of the toxicity test and different handling of the animals. Cation exchange capacity (CEC) and dissolved organic carbon (DOC) can additionally influence the toxicity of chemicals. Generally the CEC becomes important in the toxicity testing when dealing with ionic compounds. Since CAP has a very high dissociation constant $pK_a$ (around 11; EPA 2008, FAO 2008) and since the pH of the soil was in all cases lower than 7, no effect of CEC was to be expected.

### 5.7.3 Avoidance test of chlorantraniliprole to *Folsomia candida*

In all replicates for all treatments, the number of animals recovered at the end of the avoidance test with *F. candida* was above 85 %, except for 1 replicate at CAP concentration 10 µg/g dw, where the recovery was 75 %. Results of the avoidance test with CAP on *F. candida* are shown in Figure 49. Considering the mean of all replicates, neutral response (0 % avoidance) was found in the solvent control, meaning that an equal number of animals were found on both sides of the soil, while in the C/C test containers, on average 18 % more animals were recovered from one side than the other. But this is within the normal variation of such tests.

The statistical analysis showed no significant difference ($P > 0.05$) between the numbers of springtails found on the treated and untreated parts of the test containers for each CAP concentration. Yet, some interesting observations can be made. From Figure 49 we can see that the animals avoided the soil with the lowest CAP concentration (1 µg/g dw), with a mean avoidance of 23 % ($\sigma$ = 22 %). At all higher concentrations however, considerably more animals were found on the treated soil than in the control. The net mean avoidance was most negative at 10 µg/g dw (-38 %,

σ = 20 %), following by 33 µg/g dw (-34 %, σ = 21 %), and 100 µg/g dw (-29 %, σ = 21 %). No trend can therefore be observed along the CAP concentration range.

One reason why springtails were not repelled by the contaminated soil would be that they are attracted by CAP. Another explanation can be that the springtails were disabled to avoid the treated soil due to the effect of CAP. If the animals happened to contact the contaminated soil, we may hypothesize that a short term exposure of *F. candida* to CAP disabled the animals to move away and return to the clean soil. As CAP impairs the normal functioning of the muscles, this directly affects the locomotive capabilities of the affected target. As there was a tendency for springtails to avoid soil with the low CAP concentration, it leads us to suggestion that 1 µg CAP/g dw soil may already affect the movement capability of the animals, but less than at higher concentrations. The mean avoidance in the test containers with 3.3 µg CAP/g dw soil on one side, was already negative, but less negative than at 10 µg CAP/g dw and higher. This test may provide additional information - as avoidance did not change with increasing CAP concentrations above 10 µg/g dw, we may assume that there is a threshold concentration which affects the animals, and further increase of the concentration does not increase the effect. One explanation would be that, considering bioavailability according to the pore water hypothesis, this threshold concentration is reached when the pore water becomes saturated with CAP. According to this hypothesis, due to low solubility of CAP, higher amendments of CAP in the soil would have no effect on CAP concentration in the pore water. To confirm this, the actual CAP concentrations should be measured in the soil as well as in the pore water for each treatment.

When the animals from the test were collected and observed under the microscope, it was clearly seen that those exposed to higher concentrations of CAP had difficulties to move. An additional separate experiment confirmed this observation. When adult animals were placed on a compacted soil with the same concentration range as in the avoidance test, an effect on their locomotion activity was already observed after one day at the higher CAP concentrations. It could be noted that the avoidance test with *F. candida* was repeated with slightly different CAP concentrations. Similar results were obtained, which increased the confidence in our conclusions.

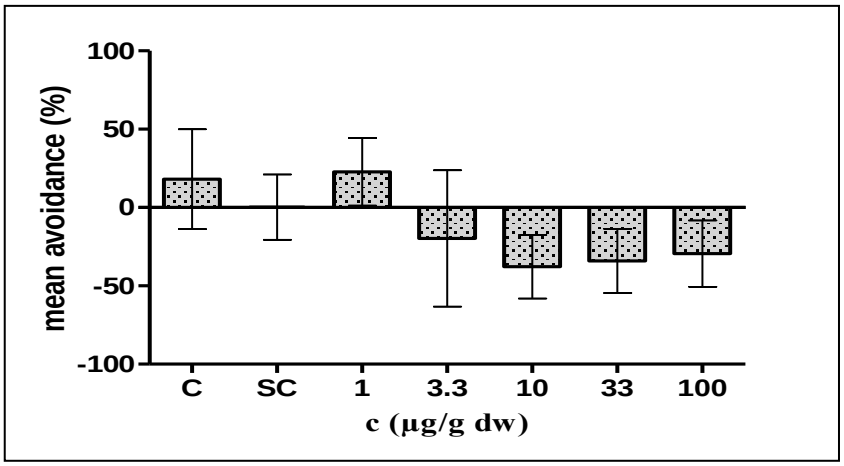



***Figure 49: Mean avoidance response (%) of* Folsomia candida *exposed to pure Lufa 2.2 soil (= control) or Lufa 2.2 soil spiked with chlorantraniliprole of different concentrations.***

***Concentrations are expressed as CAP nominal values.***

## 5.8 General discussion on toxicity of chlorantraniliprole to selected non-target organisms

Due to the considerable difference in amino acid sequence of mammalian and insect ryanodine receptors (RyRs), a different pharmacological action of anthranilamides was anticipated and proven for insects and mammals (Corodva et al., 2006). For same reasons, CAP is also not toxic to fishes and birds (EPA, 2008). This suggests that mode of action of CAP to a great extent depends on the type of RyR of the species. However, while CAP was toxic to non-target insects such as caddisfly, mayfly and non-biting midges at very low concentrations, it appeared to be less toxic to other non-target species also belonging to the insects, such as honey bees, lady bird beetles (EPA, 2008) and parasitoid wasps (Brugger et al., 2010).

Our toxicity test on *D. magna* and data from other sources (Barbee et al. 2010, EPA 2008) suggest that also crustaceans can be prone to CAP mode of action. Olivares et al. (1993) showed that microsomal sarcoplasmic reticulum (SR) fractions (where RyRs are situated) from lobster skeletal muscle were found to bind [3H]-ryanodine, a compound having the same mode of action as CAP (see Chaper 2.6). The data on CAP toxicity to crustaceans provided so far in our and other studies (*Daphnia magna* (present study), *Procambarus. clarkii* (Barbee et al., 2010)), G*ammarus*

*pseudolimnaeus* (EPA, 2008), could be explained by their close relation with the insects (Boore et al., 1998). It is possible that their ryanodine receptors show close homology to the one of insects. The same conclusion could be drawn for springtails. As springtails are suggested to be closely related to insect and crustaceans (Nardi et al., 2003) or even a sister group to Insecta (Delsuc et al., 2003), this could be one of the reasons explaining their high sensitivity to CAP. The effect of CAP was clearly expressed visually, as water fleas and springtails exposed to the highest tested CAP concentrations showed difficulties of moving in space. On the other hand, CAP in our study did not show any toxicity to the terrestrial crustacean, *P. scaber*, even at the very high concentrations (1000 µg/g dw, nominal). No behavioral changes, differences in locomotion ability, consumption rate and body mass change were noted between exposed animals and controls. Survival of the oribatid mites exposed to CAP was also comparable to the controls, and as with isopods, no differences in locomotion ability were observed after the animals were extracted. Since enchytraeids were fixated, the mobility of the animals could not be observed, however high survival and especially the absence of effects on the more sensitive reproduction endpoint suggest that enchytraeids were anyhow not affected by CAP. This does however, not exclude the occurrence of toxic effects of CAP in the natural environment. Effects may occur on the biomolecular level and may only become manifest upon long-term exposures, especially due persistence of CAP and its consequent ability to accumulate in the soil (EPA 2008, Health Canada 2013). It appears that the mode of action of CAP is selective to insects and related species, however it is not the only factor on which the toxicity of CAP could be predicted.

Differences in species sensitivity could also be due to the differences in exposure routes (Rundgren and van Gestel, 1998). The tested annelids, having a thin cuticle and therefore intense contact to the soil matrix, however, were still resistant to CAP action. To restore the water balance, springtails are known to actively take up pore water and with that chemicals dissolved in it (Rundgren and van Gestel, 1998), making them more susceptible to chemical effects. Based on results of several studies comparing the toxicity of compounds and different exposure routes (Rundgren and van Gestel, 1998), the exposure through food instead of soil would not be expected to cause increased toxicity of CAP to tested organisms.

The suggested reasons for CAP pharmacological activity could lie in the presence or absence of CAP-specific binding receptors, specie- and compound- dependent toxicodynamic processes of the organism and the sensitivity of the species.

While CAP affected the survival as well as reproduction of *F. candida*, only survival was affected of *D. magna*. One reason for this could be a fast degradation of CAP in water into less or non-toxic degradation products. In this case, the compound would acutely affect the survival but because of its fast degradation, long term effects on reproduction would not be expected. Since the media used for the test was slightly alkaline (with pH around 8, Table 11), CAP would be expected to degrade into compound **H**, which was shown not to be toxic to *D. magna*. However, the media was renewed every three days and the actual concentrations of CAP remained fairly constant. This proves that no considerable degradation of CAP occurred during the incubation period. Another reason could be related to the mode of action of CAP. Due that, CAP would affect the mechanism that is crucial for survival, but would in case of *D. magna* not interfere with reproduction at concentrations below the $LC_{50}$.
For the CAP chemical and photochemical transformation products EPA (2008) reports lower toxic potency than the parent compound. Our study agrees with their statement for compounds **B** and **H**, tested in acute and chronic toxicity test with *D. magna*.

# 6 CONCLUSIONS

The first goal of our study was to investigate the chemical and photochemical stability of the insecticide chlorantraniliprole (CAP) in water and to identify and characterize its transformation products. Our second goal was to determine the toxicity of CAP and some of its transformation products to selected non-target aquatic and terrestrial organisms.

The stability experiments showed that CAP in water can be degraded by both chemical and photochemical processes. We demonstrated that in tap water with naturally present bases (mostly hydrogen carbonate) and basic pH (8.0), CAP is slowly transformed to compound **H** by dehydration. Our photodegradation experiments showed that under UV-A light from simulated solar irradiation, CAP is transformed to the photostable compound **A** by dechlorination and subsequent intramolecular rearrangement to form an oxazine ring. Bases in water promote the transformation of **A** to an isomeric compound **B** by the opening of an oxazine ring, followed by intramolecular rearrangement and hydroxylation of the former oxazine group. Compound **B** is stable in the dark, however irradiation causes expulsion of its pyridine moiety to form product **C**. These two pathways (photolytic and transformation to compound **H**) seem to be the most important for the initial degradation of CAP in surface water with alkaline pH.

In pure deionized water with slightly acidic pH (6.1), a different transformation pathway was observed. Unlike in alkaline tap water, CAP in deionized water remained stable in the absence of sunlight. When irradiated with UV-A light, CAP degraded to compound **A** as before, but in this case the subsequent transformation products **B** and **C** were observed only in traces even when irradiation continued. Compound **A** is therefore the main transformation product of CAP in acidic water and compound **C** in basic water with naturally present electrolytes. The photodegradation half-life of CAP (starting concentration = 39 μM) irradiated under UV-A light was 5.2 days in deionized water and 4.2 days in tap water. Separate experiments demonstrated that humic acids and $NO_3^-$ have little influence on CAP photodegradation in water.

The toxicity tests showed that the two transformation products **B** and **H** exerted hardly any effect on the water flea *Daphnia magna*, while CAP was highly toxic with an acute $LC_{50}$ of 9.35 μg/L and a chronic $LC_{50}$ of 3.71 μg/L. The acute-to-chronic

ratio of 2.52 demonstrates that the toxicity of CAP increases with increasing exposure time, but CAP did not affect daphnid reproduction at concentrations below the $LC_{50}$

In a 28-day sediment toxicity test, CAP and its transformation product **H** did not affect survival and reproduction of the freshwater black worm *Lumbriculus variegatus* at concentrations up to 800 µg/g sediment dry weight (dw).

For terrestrial invertebrates, tested in Lufa 2.2 soil, CAP did not show any effect on survival and reproduction of the oribatid mite *Oppia nitens*, the enchytraeid *Enchytraeus crypticus* as well as survival, body mass change, consumption rate and behavior of the isopod *Porcellio scaber* at concentrations as high as 1000 µg/g dw. On the other hand, CAP severely affected the survival and reproduction of the springtail *Folsomia candida*, with a 28-day $LC_{50}$ of 5.14 µg/g and a reproduction $EC_{50}$ of 0.20 µg/g dw. When comparing different soil types, reproduction toxicity of CAP to *F. candida* was lower in soils with higher organic matter content, while differences in soil pH seemed not to affect CAP toxicity to *F. candida*. Avoidance tests with *F. candida* and behavioral observations suggest that CAP is affecting the locomotion ability of the sprigtails in a prompt way and already at low soil concentrations (the 2-day avoidance tests already showed effects at 10 µg/g dw; Lufa 2.2 soil).

Although the present work provided valuable and extensive information of the environmental fate of CAP and its toxicity to non-target organisms, at the same time it opened up new directions for future research. The investigation of chemical and photochemical stability of CAP in natural waters at lower concentrations and without the addition of organic solvents is highly encouraged. While our research focused mainly on the degradation pathway of CAP and the mechanisms of the formation of its transformation products, future studies should focus on determining degradation rates of CAP and its transformation products under natural environmental conditions. In the present toxicity studies of CAP on sediment worms and selected terrestrial invertebrates, the actual CAP exposure concentrations have not yet been assessed. This information would provide more confidence in the results, especially for the toxicity tests with *F. candida*, where effects of CAP were noted at very low concentrations. This would also provide insight into the stability of CAP in soils and sediments during these tests, possibly also explaining the observed effects. It would

also be helpful to know more about the bioavailability of CAP to terrestrial species. For this purpose a solid phase micro extraction method could be applied, which might need some adaption to cope with the slightly polar nature of this compound. *F. candida* could be used to examine the mode of action of CAP. For this, a toxicodynamic approach could be taken, in which behavioral and physical changes on animals placed on soil spiked at different concentrations should be followed in time. It would be also interesting to assess the effects of CAP and its degradation products at the biomolecular level, e.g. using cells or isolated ryanodine receptors and applying calcium imaging. Such studies in a combination with toxicodynamic as well as toxicokinetic studies could provide us with better explanations for the differences in the toxicity of CAP to different test species.

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