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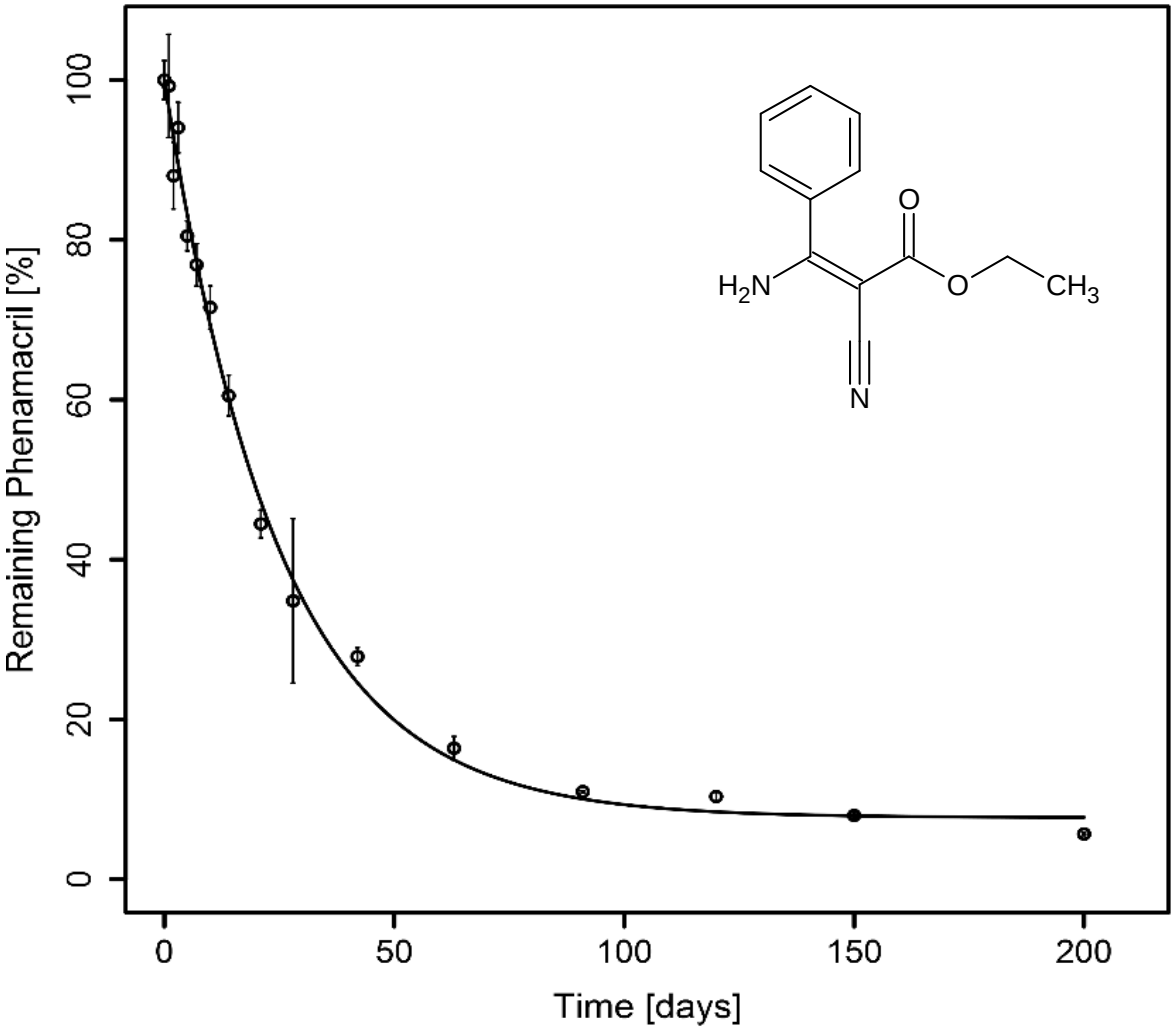
Remaining Phenamacril [%]
0
20
40
60
80
100
0
50
100
150
200
Time [days]
O
H_2N
O
CH_3
N

Aerobic dissipation of the novel cyanoacrylate fungicide phenamacril in soil and sludge incubations

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Abstract: The cyanoacrylate, ethyl (2*Z*)-3-amino-2-cyano-3-phenylacrylate (phenamacril), has been introduced as an effective agent against several fungi species belonging to the *Fusarium* genus. However, in current literature, knowledge about the environmental behavior of this fungicide is limited and there are no data on the degradation in the environment. By performing tests on inherent degradability as well as degradation studies in soils this study provides the only published information regarding the environmental stability and degradation kinetics of this compound. Tests for inherent/ready biodegradation revealed the phenamacril is inherently degradable with zero order kinetics, even though the degradation is comparatively slow. Degradation of phenamacril in soil was found to occur following first order kinetics with a final plateau with a half live of 17.1 days (i.e. more rapidly than Tebuconazole but less rapidly than Octylisothiazolinone).

Keywords: fungicide, phenamacril, biodegradation, soil, agriculture

Declaration of interest: none

Introduction:

Phenamacril *(*ethyl (2*Z*)-3-amino-2-cyano-3-phenylacrylate, structural formula in figure 1) has been suggested as a potential fungicide against *Fusarium*. *Fusarium* is a filamentous ascomycete fungi widely known for its ability to produce a multitude bioactive secondary metabolites (Hansen et al., 2015; Sørensen et al., 2009; Summerell and Leslie, 2011). This is including mycotoxins such as zearalenone (ZEA), fumonisins, nivalenol (NIV), deoxynivalenol (DON), T-2 toxin and Fusarenone X (FUS) (Bottalico, 1998; Geng et al., 2014; Nelson et al., 1994; Summerell and Leslie, 2011; Yu and Keller, 2005). As these are either directly harmful to humans or endangering food production, there is a high motivation to control *Fusarium*, e.g., by using pesticides. However, there are not a lot of active ingredients available that are effective on *Fusarium*. Phenamacril is *Fusarium* specific (Li et al., 2008; Zhang et al., 2015) and capable of reducing the crop infection Fusarium Head Blight (FHB). Additionally it is able to control wheat scab (Li et al., 2008; Zhang et al., 2010). With an EC_{50} of 0.126 µg mL^{-1} (approx. 0.583 µM) on the target organism (Li et al., 2008) phenamacril proves to be a potent and selective fungicide against specific *Fusarium*.

However, all pesticide use needs to undergo a risk assessment and there are concerns on potential persistence of phenamacril in soil and thus, potential contamination of the groundwater (EC, 2009; Younes and Galal-Gorchev, 2000; Pimentel and Levitan, 1986). Pesticide concentrations in soil are controlled by usage rate, biodegradation rate constants, sorption to soil, partitioning of the compound between soil and water and the amount of water percolating through the soil due to, e.g., rainfall. Some of these processes are controlled by the physicochemical properties of the compound (e.g. ionizability, water solubility, lipophilicity, molecular weight, etc.), while others by properties of the soil and environmental conditions (Arias-Estévez et al., 2008; Gevao et al., 2000; Pimentel and Levitan, 1986). Sorption and mobility of phenamacril have been studied with three Chinese soils (Wu et al., 2016). Phenamacril exhibited low to medium mobility in the tested soils (Wu et al., 2016). Opposite to sorption, there are no degradation data of phenamacril available, though the compound has been shown to be chemically stable (Donau et

al.,2017). This study was conducted to gain biodegradation kinetics of phenamacril in soil to test whether this compound might be a suitable candidate for the European market. Two key factors for the risk assessment were addressed in this study: i) is the compound inherently degradable and ii) is its degradability in soil high enough to make this compound indeed a candidate for a European registration. Degradation experiments in sludge were performed in parallel to test for ready or inherent biodegradability (OECD, 2009, 1992, 1981).

Materials and Methods

MATERIALS

Soil. Soil samples for the incubation experiments were collected at a depth of 0-20 cm from an agricultural field in autumn 2016 (Field 101 26; University of Copenhagen experimental farm, Tåstrup, Denmark) used for growing barley. The field soil was fertilized exclusively with inorganic fertilizers (NPKS). The soil received two different fungicide application in the past: 1) propiconazole (IUPAC name: 1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]-1,2,4-triazole) (2006) and 2) tebuconazole (IUPAC name: 1-(4-chlorophenyl)-4,4-dimethyl-3-(1,2,4-triazol-1-ylmethyl)pentan-3-ol) (single summer applications 2008-2013). Phenamacril has never been used on this field. The soil was loamy sand texture, slightly acidic (pH 6.4), contained about 12.7% total moisture and an organic carbon content of 1.6%. The soil material was stored at 4℃ until usage. Prior to the experiment, the soil was sieved to reach particle sizes below 2 mm (Bollmann et al., 2017).

Sludge. Sludge was collected at Bjergmarken wastewater treatment plant (WWTP) in Roskilde, Denmark. Bjergmarken WWTP is a conventional treatment plant using activated sludge treatment and has a capacity of 125.000 PE. Bjergmarken is designed for biological removal of organic matter, nitrification, denitrification and biological phosphorus removal. The treated wastewater consists of 80% household and 20% industrial wastewater. The pH of the wastewater of this plant is usually around 8.0.

Analytical standards. Phenamacril was prepared by dissolving ethyl benzimidate hydrochloride ((2.5 g, 12.8 mmol, 97%, Sigma Aldrich) in dry ethanol (10 mL, 99.9% (Vol.), CCS Healthcare AB)). Ethyl cyanoacetate (1.5 mL, 13.8 mmol, >98%, Aldrich Chemistry) was added. The solution was cooled to 0℃ and freshly distillated triethyl amin e (4.5 mL, 32.1 mmol, >99%, Sigma Aldrich) was slowly added (9 mL h^{-1}). The solution was kept at 0℃ for 15 minutes and subsequently heated to 75℃ under N_2 atmosphere for 15 hours. Water (10 mL) was added to the reaction and the mixture was cooled to 0℃ for 1 hour. The solvents were red uced *in vacuo* (gradually lowering the pressure to 10 mbar at 40℃) to a volume of approximately 10 mL. The aqueous solution was cooled to 0℃ and the formed precipitate was filtered and washed with 3 x 10 mL cold (0℃) water. The resulting white crystals were resolubilized in a minimum of hot acetone and recrystallized in cold (0℃) water. The compound was confirmed by NMR spectra recorded in $CDCl_3$ as previously described (Donau et al., 2017). The yield of this reaction was 87% of the theoretical value. The purity of the compound was determined to be >98% by ^{1}H-NMR. The ^{1}H-NMR spectrum can be found in Fig SI 4).

A selectively ^{2}D-labelled (1,1,1,2,2-pentadeutero-ethyl)-3-amino-2-cyano-3-phenyl acrylate (further referred to as phenamacril-D_5, structural formula Fig SI 1) was prepared as internal standard for quantification in MS according the following protocol: Methyl 3-amino-2-cyano-3-phenyl acrylate was prepared by the protocol described above, but exchanging the solvent to methanol (Sigma Aldrich), using methyl cyanoacetate (99%, Sigma Aldrich) and adjusting the temperature to 65℃. Potassium hydroxide (0.21 g, 3.7 mmol) and methyl 3-amino-2-cyano-3-phenyl acrylate (0.20 g, 1.0 mmol) were dispersed in ethanol-d_6 (1 g, 19.1 mmol) and heated to 65℃ for 6 hours. H eating was turned off and the reaction cooled to room temperature. Water (25 mL) was added and the product extracted with dichloromethane (3 x 15 mL). The combined organic phase was washed with brine (2 x 25 mL). The solvents were reduced *in vacuo* (10 mbar at 40℃) and the resulting product purified by column chromatography on a silica column using dichloromethane as eluent. The yield of this reaction was 90% of the theoretical. The purity of the compound was determined to be >95% by ^{1}H-NMR. The ^{1}H, ^{13}C and ^{2}H-NMR spectra can be found in Figures SI 5-7).

NMR. All NMR spectra were recorded on a BRUKER AVIII-600 MHz NMR spectrometer equipped with a cryogenic probe. ^{1}H and ^{13}C spectra were referenced to internal TMS, ^{2}H spectra were referenced indirectly using Ξ =0.1535060886.

INCUBATIONS

Soil incubations. It was aimed for conducting the incubations at a realistic level. The recommend usage for phenamacril is 375 g ha^{-1} (Zhang et al., 2010). Assuming a homogenous distribution in a 20 cm deep layer and considering a soil density of 1.7 g cm^{-3} results in a target concentration of 188 ng g_{soil}^{-1} phenamacril. The incubations were conducted as parallel incubations of 48 individual incubations in 10 g samples in 60 mL brown glass jars (microcosm). Each of the soil samples was spiked with phenamacril by performing a primary spike to sand to avoid a change in the microbiological community by the solvent of the spike. The spiking was performed by adding 20 µL of a methanolic solution of 0.1 mg L^{-1} (i.e. a total amount of 2 ng) of phenamacril to 150 mg sand. The spiked sand was left overnight for the solvent to evaporate, before being added to the soil microcosm. Each pre-spiked portion (150 mg) of sand was added to a 10 g soil subsample and mixed heavily, to a resulting fungicide concentration of 200 ng g_{soil}^{-1} in each microcosm. The 48 glass jars with the 10 g of spiked soil were covered with aluminum foil to avoid light penetration and then closed with a plastic lid to avoid an otherwise quick loss of water. The samples were incubated in darkness at 22℃ in a dark temperature stabilized cupboard for 0, 1, 2, 3, 5, 7, 10, 14, 21, 28, 42, 63, 91, 120, 150, and 200 days, respectively. Once a week, the jars were opened and weight controlled; thus, ensuring that the incubations were conducted aerobically. If necessary, the water content of the soil was restored by using tap water. High frequency sampling was conducted in the beginning and low frequency sampling at the end of the incubation, as first or second order kinetics are expected. Thus, logarithmic (in time) sampling was conducted. For each incubation period, three microcosms were transferred to a freezer at -18℃ (without further stabilization) until isochronus extraction and analysis.

Incubation in activated sludge (i.e. sludge from a wastewater treatment plant (WWTP) (OECD, 2009)). The incubations were conducted in 250 mL Erlenmeyer flasks with sludge to which a combination of methanolic solutions of phenamacril and Ibuprofen were added. Ibuprofen was used as a positive control. In detail: into different empty Erlenmeyers phenamacryl and ibuprofen were spiked to reach 1) 1 mg L^{-1} phenamacril, 2) 1 mg L^{-1} phenamacril and 1 mg L^{-1} Ibuprofen, and 3) 1 mg L^{-1} Ibuprofen (positive control). The concentration was chosen, as tests for inherent degradation are usually conducted at high (i.e., mg L^{-1}) concentrations to enable catabolism (OECD1981,1992, 2009). The spiked flasks were left overnight for the methanol to evaporate to avoid the methanol being used as additional carbon source. 100 mL of a mixture of activated sludge and effluent wastewater (1:2 ratio (V/V) of sludge:wastewater both from WWTP Bjergmarken, Roskilde) was added to each reactor (Erlenmeyer flask). The reactors were vigorously stirred, but not artificially aerated. After predefined time periods, 1.5 mL samples were taken from each reactor, centrifuged at 6000 rpm for 10 minutes and the supernatant collected. The samples were stored at -18℃ until analyzed in isochronous measurements.

EXTRACTION AND ANALYSIS

Sludge Analysis. To 490 µL of each water sample, 10 µL of the internal standard (1 µg mL^{-1} methanolic solution of phenamacril-D_5) was added. The samples were analyzed using HPLC-MS/MS.

Soil Extraction and Analysis. A subsample (1 g soil) of each incubation was mixed with 1.5 g hydromatrix (*Varian*, 181 Palo Alto, CA, USA) and extracted using accelerated solvent extraction (ASE 350, *Dionex*, Sunnyvale, CA). Void volume of the 11 mL cells was filled with Ottawa sand. The cells were extracted at 110℃ and 1000 psi with acetonitrile in a single extraction cycle. Extraction settings: A static time of 5 minutes, preheating time of 1 minute, flush 60% and purge 60 seconds were used. Method validation and phenamacril recovery are available in the

supplementary information (Table SI 1). A subsample of 990 µL was taken from the primary extract and 10 µL internal standard (1 µg mL^{-1} methanolic solution of phenamacril-D_5) was added. Afterwards, the samples were analyzed using high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). Limit of quantification (10 ng/g), recovery rate (83%) and reproducibility (SD=5%) was obtained from extracting the same soil spiked to target concentrations as used for the incubations the full documentation is contained in Table SI 1.

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HPLC-MS/MS target analysis. The concentration of phenamacril in the samples was analyzed by high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) on an Ultimate 3000 dual gradient low pressure mixing HPLC-system (*Dionex,* Sunnyvale, CA, USA) coupled to an API 4000 triple-quadrupole-MS (*AB Sciex*, Framingham, MA, USA). The separation was performed at 20℃ using a Synergy Polar-RP colu mn (L = 150 mm, ID = 2 mm, particles = 4 µm, *Phenomenex*, Torrance, CA, USA) with a constant flow of 300 µL min^{-1}. An acidic gradient was used with 0.2% formic acid in water (A) and 0.2% formic acid in methanol (B): 0-2 min 0% B, 2-7 min 0% → 100% B, 7-9 min 100% B, 9-9.5 min 100% → 0% B, 9.5-12 min 0% B. For both compounds two MRM (multireaction monitoring) transitions where analyzed: Phenamacril (Transition 1: 217→104 Da, declustering potential (DP): 41V, collision energy (CE): 31V, exit potential (CXP): 9V; Transition 2: 217→171 Da, DP: 32V, CE: 15V, CXP: 10V), phenamacril-D5 (Transition 1: 222→104 Da, DP: 34V, CE: 34V, CXP: 8V; Transition 2: 222→171 Da, DP: 40V, CE: 17V, CXP: 10V). Ionisation voltge was 5500 V at 500 ^{o}C.

Results and Discussion

Degradation in soil

As phenamacril is a fungicide intended for use in agriculture, the biodegradation of the compound was tested in soil. For this study, 200 days incubation time was chosen to gain information how much residues would be left in the soil before a potential application in the next year/season.

The degradation of phenamacril is summarized in Figure 2. During the timeframe of the incubation of 200 days, the concentration of phenamacril decreased from 200 ng g_{soil}^{-1} to 10.4 ± 0.20 ng g_{soil}^{-1}. This corresponds to a decrease by 96.4% in this time period in the chosen soil. Three different models were fitted to the data;

i) first-order degradation (eq. 1) resulting in a poor fit for concentrations after 28 days of incubation.

(eq. 1) $C=C_o*exp(-k*t)$

ii) first-order degradation with a final plateau (eq. 2) exhibited a very close fit

(eq. 2) $C=P+(C_o-P)*exp(-k*t)$

iii) first-order with double exponential decay (eq. 3) resulting in a good fit. However, compared to the first-order with plateau, the extra variable in the double exponential decay mostly improved the fit to the values at 200 hours.

(eq. 3) $C=(C_o*R)*exp(-k_1*t)+(C_o*(1-R))*exp(-k_2*t)$

(plots of *i*) and *iii*) are shown in the supplementary material fig SI 3 and 4).

With C = concentration at a given time point t; C_0 = starting concentration; k = reaction rate constant; P = Plateau and R = the ratio of compound degraded by the two degradation mechanisms, k_1 and k_2 the rate constants.

As applying extra variables increases the risk of overfitting, the simpler model, single-first-order with plateau, was finally taken to present the data (Figure 2). A first-order degradation with a final plateau is not uncommon in biological systems. The plateau or second phase could be a result of several factors such as:

a) A compound becoming non-available as carbon source at the lower concentrations as there is a small fraction of the compound that is not available to the degrading organisms as it forms fractions strongly bound to the soil.

b) Depletion of an additional compound needed for co-degradation, a lot of enzymes need reactant and co-reactant to perform reactions, if one is not (or no longer) present the reaction can no longer be performed. For a lot of enzymes this can be energy delivering systems such as ATP/ADP or NADH.

c) Considering equilibrium reactions rising concentrations of the reaction product can at some point block the reaction in a way that the educt concentrations do no longer change. Thus a metabolite reaching a concentration that blocks further degradation (Casas et al., 2015; Torresi et al., 2017).

d) If a biodegradation is based on a very selective enzyme, the organism is investing energy and carbon into making the enzyme, which can be outbalanced if the substrate concentrations are high – typically mg/L (as the carbon from the to be degraded compound can be harvested) if the concentrations, however drop below certain values (usually around µg/L) the investment in the enzyme cannot be returned and the organism will not be able to maintain the degradation enzymes thus the reaction shows a plateau.

However, exploring the cause for the kinetic behavior in this case requires further studies. From the resulting degradation model, the half-life of phenamacril in soil was determined to be 17.1 days, the plateau was determined to be at 0.014 ±0.003 µg g^{-1} and the initial rate constant 0.040 ± 0.002 d^{-1}. The quantitation with extraction and HPLC-MS/MS measurements was conducted with a standard deviation of < 10% (see SI). However, with 48 data points and the fitting of the model derives a residual standard error of 5.7%. The half-life of phenamacril is lower than that determined for Terbutryn (231 d), Isoproturon (100 d) and Mecoprop (44 d), but higher than Iodocarb (1.05 d) or Octylisothiazolinone (9.3 d), which were tested earlier in the same soil with a similar experimental setup (Bollmann et al., 2017). This indicates that phenamacryl is not among those compounds that are hardly processed by the soil between applications (e.g. two times a year). In that way it would be a less problematic compound and the risk of accumulation of the total applied load in soil would be low. However, phenamacril is not reaching a concentration near zero (or the limit of determination) (Figure 2) before a potential new application can occur (6 months), making the compound still a pseudo-persistent one with residues present at least several months after

application (Bollmann et al., 2017). The plateau from the degradation indicates towards long term presence of the compound once sprayed and thus implies higher risks of leaching of this compound into groundwater, than the relative short half-life might indicate. In contrast to the consequences of the plateau, the reasonably short half-life time of phenamacril may contribute to decrease the risks of higher concentrations ending in ground- or surface waters.

The only data that is published on sorbtion of phenamacril in soil is referring to three Chinese soils. In Jiangxi red soil moderate mobility (based on K_D=5.2) was observed, while in Taihu paddy soil and Northeast China black soil very little mobility was observed (based on K_D=29.4 and 46.5) (Wu et al., 2016). This would be relatively high in the Danish context in which propiconazole with K_D=1-40 (pesticidvarsling, 2003) is considered as relative high. – A transfer of this knowledge, however, to the loamy and sandy soils in Denmark is probably not easy and should not be tried theoretically as long as the binding mechanisms are not 100% resolved. This would indicate that if a use on Danish soil was considered, a full sorption study eventually using field and lysimeter studies would be recommended. A full risk assessment, however is beyond the topic of this study.

Degradation in sludge (test on inherent and readily degradability)

On top of the degradation or dissipation of phenamacril in soil, its degradation in activated sludge was tested to assess whether this compound is readily, inherently or not degradable as it is foreseen for the registration, evaluation and assessment of chemicals in Europe (REACH). For this test, an incubation period of 300 h was chosen even though this is massively exceeding residence times in classical sludge plants (typically 20 h) as suggested in the background documents of (OECD, 2009). The bioactivity of the sludge was ensured by co-addition of ibuprofen to selected reactors (see SI). The data are summarized in Figure 3. The data was fitted to a zero-order equation (eq. 4) as it does not fit to first order. Over the timeframe of the experiment (300 hours > 10 d), the concentration of phenamacril decreases slowly but significantly (i.e. > 20%), while the removal of ibuprofen occurs as expected rapidly (half-life of about 2 h, see SI Fig. SI2). The removal of phenamacril is relative slow (rate constant: -0.5 ± 0.1 mg L^{-1} h^{-1}). Phenamacril is thus

classified as inherently but not readily biodegradable. Readily and inherently degradability are different criteria in the OECD guidelines. Inherently aims for checking whether the compound degrades at all - phenamacril does. Readily aims for whether the compound is degraded under realistic conditions in a way that it does not show up in the environment-phenamacril does not fulfil this criterion.

(eq. 4) $C=C_o+(-k*t)$

Concerning removal in a wastewater treatment plant, there is little indications for substantial removal of phenamacril by degradation, especially considering typical residence times of 20 h. Removal by sorption to sludge might still occur, but there is no data on this.

Conclusions

In summary, this contribution has added knowledge on the degradation kinetics of phenamacril in several environmental settings. Phenamacril is inherently, but not readily, degradable.

Phenamacril degrades in soil with a first-order kinetics ending in a plateau of 0.014 µg g^{-1} with a half-life of 17.1 days, which makes it more rapidly degradable than most current pesticides. However, the determined probable plateau is causing reasons for concern. The study found that phenamacril did only degrade slowly in activated sludge treatment, which could cause a concern if the compound leaches to surface- or groundwater or production residues were introduced into the wastewater. Whether or not this compound is or is not posing a risk to the groundwater is requiring detailed modelling to which this study is only contributing the basis for the biodegradation part.

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363 **Figures:**

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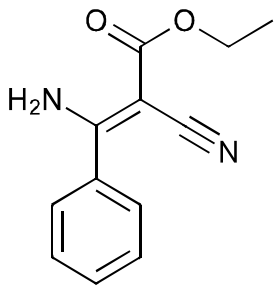

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367 **Figure 1:** Chemical structure of ethyl (2*Z*)-3-amino-2-cyano-3-phenylacrylate (phenamacril)

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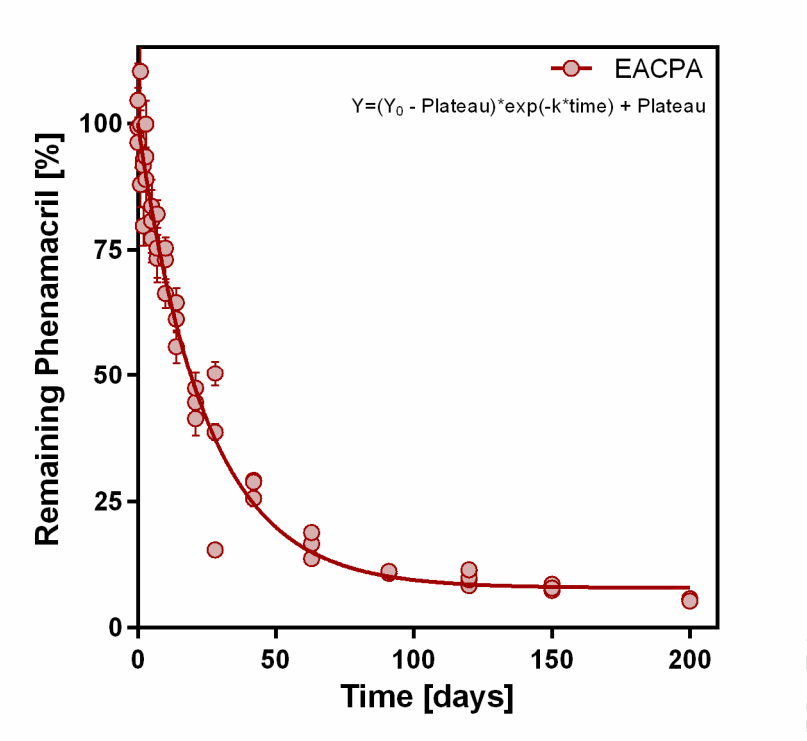

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371 **Figure 2:** Remaining phenamacril (EACPA) as a function of time during soil incubation. The black
372 line represents the resulting model when fitting the degradation to a first-order kinetic with a
373 plateau. Each dot represents a single incubation experiment, terminated at the given time point the
374 values have been normalized to the starting concentration. In total 48 independent incubations
375 were conducted to gain this dataset.

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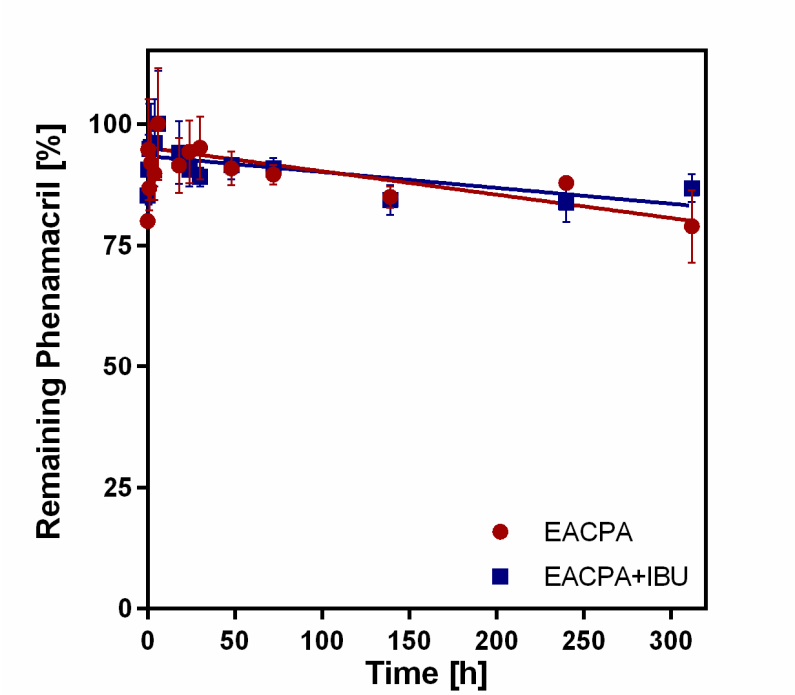

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379 **Figure 3:** Degradation of phenamacril (ECPA) in the active sludge reactors. The represented data
380 are average values of two reactors (red circles): 1 mg L^{-1} phenamacril and (blue squares): 1 mg L^{-1}
381 phenamacril + Ibuprofen (IBU)), which have been normalized with respect to the highest obtained
382 average value. The line represents a zero-order modelling of the removal. This modelling is based
383 solely on data from 6 hours to 300 hours, thus excluding the initial mixing/resolvation phase from
384 the modelling.

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- Phenamacril degrades slowly in soil incubations
- Phenamacril degrades even slower and with first order kinetics in sludge