



## Statement on the relevance of degradation products of Azadirachtin in aquatic systems

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### 1 Introduction

Insecticides and acaricides based on Azadirachtin are used against free feeding and sucking insect pests as well as mites in various crops. It is derived from neem seed kernels and as a plant extract consists of a mixture of several different compounds (e.g. limonoids). In the frame of the inclusion of the existing active substance Azadirachtin in Annex I of Council Directive 91/414/EEC questions were raised by EFSA and MSs regarding the metabolism of Azadirachtin in aquatic systems.

These questions were induced by the fact that it is not possible to synthesize <sup>14</sup>C-labelled Azadirachtin A (or any other of the limonoids contained in Azadirachtin) because of the complexity of the chemical structure (Strang, 2009). Although most recently the synthesis of Azadirachtin A has been accomplished, the synthetic procedure consisted of over 70 steps with an overall yield of 0.00015 % (Jauch, 2009). Radiolabelled synthesis is even more complicated and, thus, practically impossible.

Hence, a water/sediment study could not be carried out. Following SETAC (1995)<sup>1</sup> aquatic degradation studies are to be performed with radio-labelled active substances. The extremely complex structure of Azadirachtins also hampers the elucidation of metabolic and degradative pathways by exclusively analytical methods like HPLC, DC, GC or spectroscopic methods. For these reasons it was not possible to clarify the metabolic pathway or identify relevant metabolites.

Since the active substance Azadirachtin does not exist as a single compound, Azadirachtin A as a major biologically active single substance in Azadirachtin is taken as the analytical leading compound which is used as an analytical reference for quantification purposes.

In this statement the relevance of possible degradation products of Azadirachtin in aquatic systems will be discussed based on the available information on its fate in aquatic systems and on effects observed in aged water residue studies performed with Rainbow trout (*Oncorhynchus mykiss*) or *Daphnia magna*, resp.

### 2 Fate and behaviour of Azadirachtin in aquatic systems

In the hydrolysis study by Troß (1996) conducted at 30 and 40 °C using sterile buffer solutions of pH 4, 7 and 8 Azadirachtin A showed a hydrolytic half-life strongly dependent on the pH value. The results of this study extrapolated to 20 °C gave half-lives of 49.9, 19.5 and 4.4 days at pH 4, 7 and 8, respectively.

A second hydrolysis study by Molinari (2002) generally confirmed the results for Azadirachtin A outlined above. At 25 °C and using sterile buffer solutions of pH 4, 7 and 10 the hydrolytic half-lives 18.1, 9.6 and < 1 day(s), respectively, were found for Azadirachtin A. The respective half-lives for Azadirachtin B also tested in this study were calculated to be 24.0, 12.3 and < 1 day(s).

In the same study, a rapid disappearance of Azadirachtin A in river water samples was found. After temperature correction to 20 °C the respective DT<sub>50</sub> value for Azadirachtin A in river water amounted to 13.7 days.

As already mentioned under point 1 the identity of hydrolysis products could not be determined in these studies due to the structural complexity of ingredients in the technical material and the inability to synthesise (radio-labelled) Azadirachtin.

The rapid degradation of Azadirachtin A in natural waters was also confirmed by published data showing half-lives of Azadirachtin A in water being mostly far below 13.7 days (Szeto & Wan, 1996; Sundaram et al., 1995).

The photolytic half-life in surface layers of aqueous systems for the Azadirachtin variant NeemAzal was calculated to be 1.8 months and 5.5 days for solar radiation intensities in January or July, respectively, assuming standard conditions as defined in ABIWAS (Werle, 1995).

Based on the quantum yield and absorption properties found in an aquatic photodegradation study by Hennecke (2008) for Azadirachtin A direct photolysis is assessed to be a process of minor importance for the fate of Azadirachtin A in surface water. Moreover no stable metabolite was generated by aquatic photolysis during these irradiation experiments.

<sup>1</sup> SETAC (1995): Procedures for assessing the environmental fate and ecotoxicity of pesticides, SETAC-Europe 1995



Only little information is available on the fate of Azadirachtin A in water/sediment systems. The persistence of Azadirachtin A in stream water and sediment of a forest environment was investigated by Sundaram et al. (1997). A natural lentic system was simulated by placing glass aquaria in the forest floor. Sediment samples (pH 6.21, organic matter content: 8.4%) in petri dishes were placed at the bottom and aquaria were filled with water (pH 6.32), both collected from a nearby stream. The aquaria were fortified with the formulation Neem-EC (21 g Azadirachtin A/kg) dissolved in stream water resulting in two different initial concentrations of 0.219 and 0.407 mg Azadirachtin A/L. Water and sediment samples were taken regularly up to 384 hours after application and analysed for Azadirachtin A by HPLC. The persistence of Azadirachtin A ranged from 8 to 13 days in water and from 2 to 3 days in sediment. The  $DT_{50}$  value for stream water was about 35 hours regardless of the dosage rate applied. The Azadirachtin A residues in sediment increased with time, reached maximum concentrations of 0.007 mg/L and 0.018 mg/L at 27 hours after treatment at the two dosages, respectively, and declined gradually afterwards. These results indicate a faster degradation in the sediment compared to the water phase and clearly demonstrate that aquatic sediments in a forest environment could seldom act as efficient sinks for Azadirachtin A. Since samples were only analysed for Azadirachtin A no information on possible degradation products are available from this published outdoor study.

### 3 Evidence from aged water residue studies with *Oncorhynchus mykiss* and *Daphnia magna*

As it is presented above, Azadirachtin A in water is mainly subject to base-catalysed hydrolysis. Also indirect photolysis and microbial degradation are expected to contribute significantly to the degradation of Azadirachtin A in natural water bodies under conditions of use. Due to reasons stated under point 1 no identification of degradation products and no chemical or physical characterisation of these could be performed.

In order to characterise the degradation products of Azadirachtin with regard to their ecotoxicological potential, fish and aquatic invertebrates were exposed to aged Azadirachtin (NeemAzal) residues in water (Teigeler, 2009, Simon, 2009).

#### 3.1 Study design

NeemAzal was applied into the water phases of two water-sediment systems of different trophic conditions to give a final concentration of 45 mg NeemAzal/L, corresponding to 13.8 mg Azadirachtin A/L and 3.55 mg Azadirachtin B/L, a concentration at which effects on fish and aquatic invertebrates could be expected. The water-sediment systems were maintained under simulated sunlight with an irradiation intensity of at least 70 W/m<sup>2</sup> and a temperature of 20 ± 2 °C at water pH 7.7 – 9.3 in order to enable degradation of NeemAzal components by chemical and biological processes. Water samples were taken 1 hour, and on day 3, day 7, day 14 and day 21 after treatment for chemical analysis (Geschke, 2009) and the exposure of test animals. Before sampling, the water column and sediment layer were gently mixed to enhance an even distribution of water soluble compounds originating from the water and from sediment.

In consecutive bioassays fish (*Oncorhynchus mykiss*) and water fleas (*Daphnia magna*) were exposed to these (undiluted or diluted) water samples to determine the effects of NeemAzal water residues.

##### *Oncorhynchus mykiss*

In the first bioassay, fish were exposed to water samples taken 1 hour after treatment and to dilutions of 1:2, 1:4, 1:8 and 1:16 thereof to determine a concentration dependency, if possible. In the following bioassays fish were only exposed to undiluted water samples. A control group in fish holding-water was run in parallel to each bioassay.

##### *Daphnia magna*

In the first bioassay, daphnids were exposed to water samples taken 1 hour after treatment and to dilutions of 1:2, 1:4, 1:8 and 1:16 thereof to determine a concentration dependency. In the bioassays which started 3, 7 and 14 days after treatment, daphnids were exposed to undiluted water samples and to dilutions of 1:2, 1:4 and 1:8 thereof. In the bioassay started 21 days after treatment daphnids were exposed to undiluted water samples only. A control group in daphnia holding water was run in parallel to each bioassay.



### 3.2 Content of Azadirachtin A and B in the test solutions

In both water-sediment systems Azadirachtin A declined rapidly from the water phase, see **Table 1**. Initially 11.97 and 14.45 mg Azadirachtin A/L were determined in the water originating from the clayey silt and sandy sediment systems, respectively, and a decline to 0.05 mg Azadirachtin A/L (0.4 % of initial) and 0.11 mg Azadirachtin A/L (0.8 % of initial) within 21 days was observed. This corresponds to a reduction of 99.6 % and 99.2 % for the clayey silt and sandy sediment systems, respectively.

Azadirachtin B declined rather slowly from the water phase. Initially 2.81 and 3.13 mg Azadirachtin B/L were determined in the water originating from the clayey silt and sandy sediment systems, respectively, and a decline to 1.68 mg Azadirachtin B/L (59.7 % of initial) and 2.32 mg Azadirachtin B/L (74.2 % of initial) within 21 days was observed. This corresponds to a reduction of 40.3 % and 25.8 % in the clayey silt and sandy sediment systems, respectively.

Under the assumption that the decline of Azadirachtin A and Azadirachtin B is representative for all other components of Azadirachtin, we expect in the water samples from the clayey silt sediment a fraction of 40.3 to 99.6 % and from the sandy sediment systems a fraction of 25.8 to 99.2 % of the Azadirachtin components to be altered and thus to be degradation products of Azadirachtin.

NeemAzal is not classified as ready biodegradable in activated sewage sludge, which would require a mineralisation of 60% within a 28-days period under conditions optimised for sewage sludge activity. Therefore it is justified to assume that the majority of components are not mineralised under the conditions in the two water-sediment systems and thus still present in the water samples used in the bioassays.

NeemAzal is derived from a watery extraction of the neem seed kernels. Thus its components are rather hydrophilic and thus are likely to be distributed in the water column rather than bound to the sediments. Furthermore, the relatively low K<sub>oc</sub> of Azadirachtin A in the range of 20.6-875.1 mL/g, suggests a weak to moderate binding to the sediment.

Thus it is concluded, that fish and water fleas which were exposed to aged water residues were actually exposed to degradation products of NeemAzal.

**Table 1: Concentrations of Azadirachtin A und Azadirachtin B in water at start of the bioassays**

Time after soil treatment	Measured concentrations (mg/L)		Percentage of initial concentrations (%)	
	Azadirachtin A	Azadirachtin B	Azadirachtin A	Azadirachtin B
Clayey silt sediment – water system				
1 hour	11.97	2.81	100	100
3 days	10.28	2.88	85.9	102
7 days	0.11	2.75	0.92	97.9
14 days	0.05	1.94	0.42	68.9
21 days	0.05	1.68	0.42	59.7
Sandy sediment – water system				
1 hour	14.45	3.13	100	100
3 days	11.33	3.37	78.9	108
7 days	2.43	3.42	16.8	109
14 days	0.06	2.70	0.41	86.3
21 days	0.11	2.32	0.76	74.2

### 3.3 Effects on *Oncorhynchus mykiss* and *Daphnia magna*

Neither in the first bioassay with *Oncorhynchus mykiss* performed one hour after application of NeemAzal to the water-sediment system, nor in consecutive bioassays with water samples containing degradation products of NeemAzal, effects on fish were observed. Thus, the EC<sub>50</sub> is clearly exceeding 45 mg NeemAzal/L. Furthermore it is concluded that the entirety of degradation products present in the test solutions, which are in the range of 25.8 to 99.2 % of the applied NeemAzal, is not more toxic than freshly applied NeemAzal.



In the first two bioassays with *Daphnia magna* performed with water samples taken 1 hour or 3 days after application, resp., a clear dilution-response relation was observed for both water sources. The EC<sub>50</sub> were determined to be 11.3 and 11.4 mg NeemAzal/L for 1-hour water samples of the clayey silt sediment and sandy sediment, respectively (see **Table 2**). For 3-days water samples the EC<sub>50</sub> were determined to be 28.7 and 31.7 mg initial NeemAzal/L for the clayey silt and sandy sediment-water systems, respectively. In the following bioassays the EC<sub>50</sub> values were > 45 mg NeemAzal/L for aged water samples from both sediment-water systems.

**Table 2: Endpoints for acute effects on *Daphnia magna* in the bioassays**  
(all values based on nominal concentrations and given in mg NeemAzal/L)

Time after soil treatment	NOEC	EC <sub>10</sub>	EC <sub>50</sub>
Clayey silt sediment – water system			
1 hour	5.6	8.3	11.4
3 days	22.5	19.9	28.7
7 days	≥ 45.0	41.6	49.0
14 days	≥ 45.0	≥ 45.0	≥ 45.0
21 days	≥ 45.0	≥ 45.0	≥ 45.0
Sandy sediment – water system			
1 hour	5.6	9.6	11.3
3 days	22.5	29.5	31.7
7 days	≥ 45.0	17.6	≥ 45.0
14 days	≥ 45.0	≥ 45.0	≥ 45.0
21 days	≥ 45.0	≥ 45.0	≥ 45.0

The decline of effects was correlated with the decline of Azadirachtin A in the water samples, which suggests that Azadirachtin A is the driving factor for effects on aquatic invertebrates. Further on, the entirety of degradation products present in the test solutions, which are in the range of 25.8 to 99.6 % of the applied NeemAzal, is less toxic than freshly applied NeemAzal.

#### 4 Conclusions

An identification of degradation products of the individual components of Azadirachtin is impossible due to the lack of corresponding radiolabelled material. Likewise a prediction of environmental concentrations of all degradation products is impossible due to missing identification and quantification and in consequence the lack of knowledge on basic physical-chemical properties of degradation products.

For studies which involve aquatic systems, an analytical quantification of degradation products of the individual components of Azadirachtin is impossible due to the lack of analytical methods. However, a rough estimation of the quantity of the entirety of degradation products of Azadirachtin is possible based on the decline of measurable components, e.g. Azadirachtin A and Azadirachtin B.

In two series of studies with water samples in which up to 99.6 % of Azadirachtin A and up to 40.3 % of Azadirachtin B were degraded the effects on daphnids were clearly less severe compared to water samples containing unaltered components of NeemAzal.

It is concluded, that under conditions of applications of Azadirachtin in agricultural uses, degradation products in water are less toxic than the unaltered neem seed kernel extract.



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