# The mobility and persistence of the insecticidal Cry1Aa toxin, Bt (*Bacillus thuringiensis*) in soils

Nordine Helassa[A], Sylvie Noinville[B], Philippe Déjardin[C], Hervé Quiquampoix[A], Siobhán Staunton[A]

[A]UMR 1222 Eco&Sols, INRA, place Viala, 34060 Montpellier, France Email staunton@montpellier.inra.fr
[B]UMR 7075 - LADIR, CNRS, 2 rue Henri Dunant, 94320 Thiais, France
[C]UMR 5635 IEM, CNRS, 2 Place Eugène Bataillon, 34095 Montpellier, France

**Abstract**
The rapid worldwide expansion of genetically modified crops containing the Bt trait gives rise for concern as to the possible impact on non target species. These plants release insecticidal proteins, known as cry into soil during growth and decomposition of crop residues. The fate of the toxin is strongly influenced by its interactions with soil, in particular adsorption which modifies its biological properties, its mobility and its persistence. We describe three series of studies to investigate (i) the adsorption properties (ii) the mobility of the adsorbed protein and (iii) the decline of toxin in soil as a function of time and microbial activity. We conclude that the toxin will be strongly immobilised in soil and that microbial degradation does not determine the observed decline of toxin over periods of weeks and months.

**Key Words**
Adsorption, protein, genetically modified plants, microbial activity, soil sterilisation

## Introduction

Soil dwelling bacteria, *Bacillus thuringiensis* (Bt), synthesize crystalline proteins (Cry) during sporulation that after ingestion by larvae are solubilised and truncated to produce highly target specific, insecticidal proteins. Target specificity results both from the chemical conditions in the insect mid-gut and the presence of receptors in cell membranes allowing pore formation and rapid insect death. Genes for some of these Cry proteins have been inserted into various plants, and since 1996 such genetically modified (GM) plants have been commercialised. These Bt crops often give better yields and improved crop quality in comparison with non Bt crops. There is considerable public concern about the wide-spread cultivation of these crops. Given the very rapid increase in the area of agricultural land world wild used to grow these crops, understanding of potential negative side effects are necessarily limited. Although the bacteria exist spontaneously in soil and various preparations of spores and crystals are widely used for pest control, including in organic farming, there are important differences between bacterially produced toxin and that of GM crops. Firstly, GM plants synthesise directly the toxin, thus bypassing two of the steps that confer target specificity (solubilisation in the alkaline pH of insect mid-gut and enzymatic activation of the protein). Secondly, GM plants synthesize the protein throughout the plant and continuously during plant growth, whereas the activity of commercial preparations of spores and crystals is rapidly lost.

There has been considerable research on the possible impact of Bt crops on non target organisms. To date there has been no strong evidence of direct negative effects. An important factor in determining the exposition of non target organisms to Bt toxin is the interaction of the proteins with soils, and the resulting mobility and persistence of the toxin in the soil environment.

We have investigated the adsorption, the mobility and the persistence of one Cry protein, Cry1Aa, in contact with various soils and soil minerals.

## Materials and Methods

The protein was produced by culture of a genetically modified strain of the bacterium (*B. thuringiensis* subsp. *kurstaki* HD-1 provided by CIRAD, Montpellier) cultivated in a fermentor, then purified and activated as previously described (Vié *et al*. 2001). Concentrated solutions of protein were maintained at high salt concentration and high pH to avoid polymerisation. The monomeric state of the protein was verified using dynamic light scattering (Zetasizer HS3000).

Reference clay minerals, montmorillonite and kaolinite, were size fractionated (<2 µm) by sedimentation, made homoionic with sodium and washed until salt free. Mica was freshly cleaved. Glass was acid-washed and, when required, made hydrophobic by silanisation. Four soils with contrasting texture and organic

matter content were selected for incubation experiments. When necessary the protein was labelled with a fluorescent probe (fluorescein isothiocyanate, FITC). Adsorption isotherms as a function of pH were measured in clay suspension with protein analysis by uv-spectroscopy (Helassa *et al*. 2009). Mobility of FITC-labelled protein adsorbed on montmorillonite was measured using FRAP (Fluorescence Recovery After Photobleaching) which has not hitherto been reported for proteins adsorbed on mineral surfaces. Soils were incubated with trace amounts of Cry1Aa under controlled conditions of temperature and moisture content. Various chemical and physical treatments varied the microbiological activity. Soils were sterilised by γ-irradiation, by autoclaving and by addition of $HgCl_2$, or maintained at 4°C to inhibit microbial activity. Microbial activity was enhanced by addition of trigger solutions of amino acids and/or glucose. Toxin was extracted and analysed using an ELISA test.

**Results and Discussion**

Adsorption of Cry1Aa on montmorillonite and kaolinite was found to be low affinity and data could be fitted to Langmuir isotherms, as since in Figure 1. Adsorption was measured as a function of pH, but the lowest pH that could be investigated was 6.5, roughly the isoelectric point, since protein oligomerized at acid pH. For both minerals the maximum of adsorption decreased markedly as pH was increased above the isoelectric point. Adsorption was about 40 times greater on montmorillonite than on kaolinite, in line with the difference in their specific surface area. Adsorption capacity with respect to surface area on both minerals followed he same trend as a function of pH, which is surprising given the strong pH dependence of surface charge on kaolinite (Helassa *et al*. 2009). Desorption in water or alkaline buffer was small, thus adsorption was largely irreversible, despite the low affinity isotherms. Desorption was very efficient when non-ionic or zwetterionic detergents were added which is important since current detection techniques of the protein require that it be in solution (Helassa *et al*. 2009).

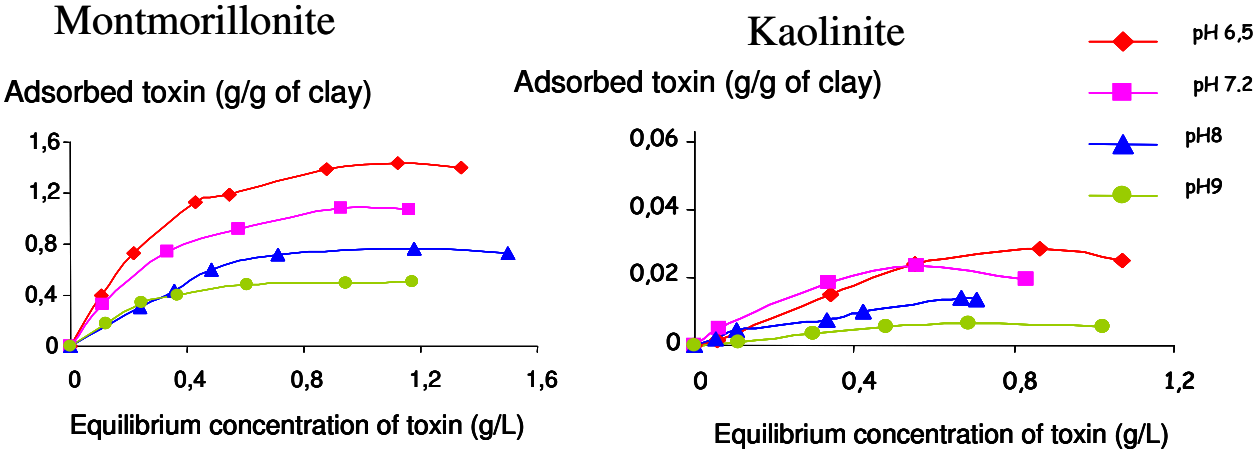


**Figure 1. Adsorption isotherms of monomeric Cry1Aa on montmorillonite and kaolinite as a function of pH. The amount of protein adsorbed was calculated by depletion from the initial solutions and proteins quantitatively measured by uv-visible spectroscopy at 280 nm.**

It is often assumed that proteins are immobilised by adsorption, however there is little experimental proof of this. The mobility of an adsorbed fluorescent molecule can be deduced by the rate at which fluorescence is recovered after photobleaching. This technique, FRAP, has never previously been applied to environmentally relevant mineral surfaces and proteins. FITC-labelled protein was adsorbed onto montmorillonite in suspension, centrifuged and washed with water to remove excess protein in solution, centrifuged and the pellet smeared onto a glass microscope slide. Protein concentration in remaining solution was negligible. Fluorescence intensity was scanned with a confocal scanning laser microscope and a disc of 12 µm diameter photobleached with and argon laser and the bleached zone scanned for at least 30 minutes. Figure 2a shows a typical image of the bleached zone, and Figure 2b the repeated scans across a section of the bleached zone. There is some variation in the base line of fluorescence intensity, due to small variations in the intensity of the excitation beam. However the shape of the intensity curve did not vary, and this is considered to be a better indicator of the absence of lateral diffusion. We found no evidence of mobility of the adsorbed protein at any pH value between 6.5 and 9 and no mobility at two levels of protein loading on the clay mineral surface. We thus conclude that the protein is unlikely to diffuse far from its point of introduction in the soil and while this limits dispersion, it could lead to the build-up of hot spots.

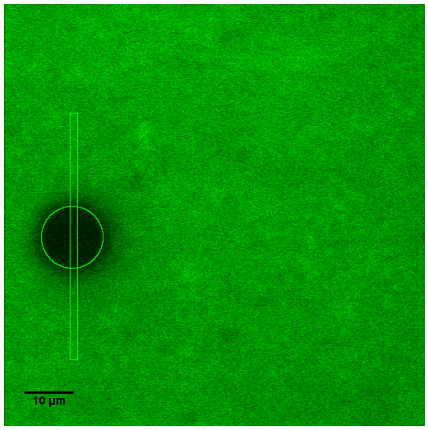


**Figure 2a. Typical fluorescence image after photobleaching of a uniform disc of 12 µm of FITC-labelled Cry1Aa adsorbed on montmorillonite.**

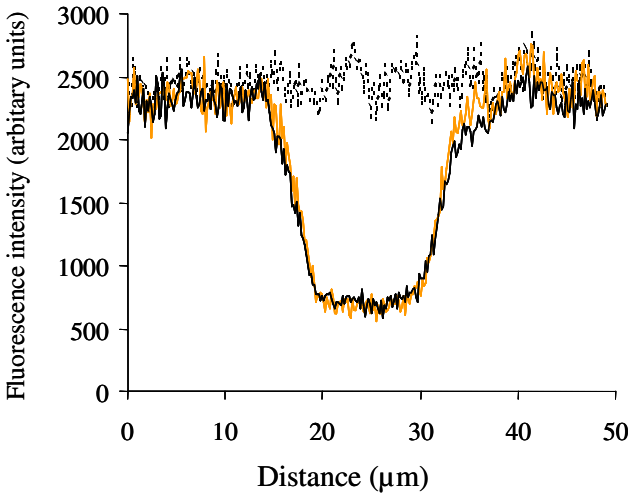


**Figure 2b. Fluorescence intensity profiles of FITC-labelled Cry1Aa adsorbed on montmorillonite before (top, flat curve), immediately after and 500 sec after photobleaching.**

It is often stated that adsorption of proteins on soil mineral surfaces protects them against microbial breakdown and thus prolongs their persistence in soil. We monitored the detection of Cry1Aa incubated in four contrasting soils and varied the level of microbial activity by sterilising or boosting activity. Two of the soils had clayey texture but differed in their organic matter content, whereas the other two were sandy textured with contrasting organic matter content. After different incubation periods, soils were destructively sampled, the protein extracted in an alkaline solution containing surfactant and another protein (bovine serum albumin). This extractant was found to give similar, high extraction yields for all the soils. The protein extracted was quantified using an Elisa test.

Figure 3 shows the trends in proteins extracted and detected for each of the four soils. There are only small differences in the persistence of the toxin in the contrasting soils. The clayey texture does not appear to provide additional protection against degradation, nor enhance irreversible fixation.

If the decline of detectable Bt toxin with time was due to microbial breakdown, then enhancement of microbial activity would increase the rate of decline and conversely sterilisation or inhibition of microbial activity would maintain a higher level of detectable protein. Figure 4 shows the absence of any effect of sterilisation by either γ-irradiation or autoclaving for one soil. Similar results were obtained for all soils. Concurring results were obtained when microbial activity was boosted with amino acids and/or glucose and when activity was inhibited by $HgCl_2$. In marked contrast, at 4°C, when hydrophobic interactions may be favoured, adsorption was smaller and the rate of decline over a 3-week period much slower. We conclude that the decline in extractable, detectable toxin was not determined by microbial activity. However we have not yet been able to distinguish between two possible reasons: firstly that protein becomes more irreversibly bound to soil surfaces with time thus decreasing extractability; secondly that changes in secondary structure of the protein that were maintained after extraction made the protein less well recognised by the Elisa test.

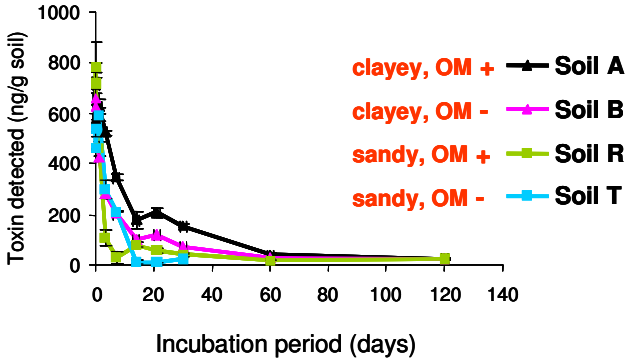


**Figure 3. Cry1Aa extracted and detected as a function of incubation period with each soil.**

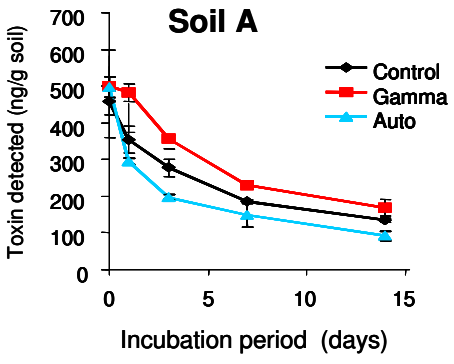


**Figure 4. Cry1Aa extracted from Soil A (clayey top soil) and detected as a function of incubation time and sterilisation.**

**Acknowledgements**
This work was financed by the French Agence Nationale de la Recherche (ANR) and the doctoral programmes of the Région Languedoc-Roussillon and the INRA.

**References**

Helassa N, Quiquampoix H, Noinville S, Szponarski W, Staunton S (2009) Adsorption and desorption of monomeric Bt (*Bacillus thuringiensis*) Cry1Aa toxin on montmorillonite and kaolinite. *Soil Biology & Biochemistry* **41**, 498-504.

Icoz I, Stotzky G (2008) Fate and effects of insect-resistant Bt crops in soil ecosystems. *Soil Biology and Biochemistry* **40**, 559-586.

James C (2007) Global Status of Commercialized Biotech/GM Crops: 2007. (ISAAA) Brief No 37, Ithaca, NY.

Vié V, Van Mau N, Pomarède P, Dance C, Schwartz JL, Laprade R, Frutos R, Rang C, Masson L, Heitz F, Le Grimellec C (2001) Lipid-induced pore formation of the *Bacillus thuringiensis* Cry1Aa insecticidal toxin. *Journal of Membrane Biology* **180**, 195-203.