ARTICLE IN PRESS

Available online at www.sciencedirect.com

ScienceDirect

Ecotoxicology and Environmental Safety ▮ (▮▮▮▮) ▮▮▮–▮▮▮

Ecotoxicology and Environmental Safety

www.elsevier.com/locate/ecoenv

Highlighted Article

Occurrence of the transgenic corn *cry1Ab* gene in freshwater mussels (*Elliptio complanata*) near corn fields: Evidence of exposure by bacterial ingestion ☆

M. Douville, F. Gagné*, C. André, C. Blaise

Fluvial Ecosystem Research, Environment Canada, 105 McGill Street, Montréal, Québec, Canada H2Y 2E7

Received 17 October 2007; received in revised form 30 January 2008; accepted 2 February 2008

Abstract

The purpose of this study was to examine the contamination of *cry1* and *cry1Ab* genes from *Bacillus thuringiensis* and transgenic corn in feral freshwater mussels collected from sites located in proximity of corn fields. In addition, mussels were transplanted for 2 months to a site in the Huron River, upstream to the Richelieu River, which is subject to intensive corn farming. Mussels were significantly contaminated by both genes in their gills, digestive glands, and gonads, as determined by qPCR methodology. Gene sequence analysis confirmed the presence of transgenic corn *cry1Ab* gene in mussel tissues. In an attempt to explain the presence of the transgene in mussel tissues, heterotrophic bacteria were grown from surface water and sediment samples on agar plates in the Richelieu River in May and August. The transgene was found at two out of six surface water samples and in one sediment sample. The study revealed that exposure to transgenic corn *cry1Ab* gene in mussels seems to proceed by ingestion of microorganisms during feeding.

Keywords: Mussels; Transgenic corn; *cry1Ab* gene; *Bacillus thuringiensis*; Gene detection

1. Introduction

Bacillus thuringiensis (Bt) encodes for δ-endotoxins, *cry1Ab*, among others, that are highly toxic to coleopteran, dipteran, and lepidopteran insects. Its toxic properties have been recognized for more than 40 years and Bt is still in extensive use for the control of pest infestations in crops and to protect the human population from biting insects such as mosquitoes (Soberon et al., 2007). Bt is a Gram-positive bacterium that produces a crystalline protein during sporulation that is highly toxic to insects after ingestion and exposure to the alkaline environment and various proteases of the insect gut (Karim and Dean, 2000). The crystals are then dissolved and digested to yield a truncated and more soluble state leading to epithelium pore formation, inflammation, starvation, and death. More recently, corn was genetically modified to express the toxic form of *cry1Ab* from Bt-*kurstaki* in all its tissues (Zimmermann et al., 1998). Moreover, the transgene was designed for sustained expression by including the 35S promoter from the cytomegalovirus (CMV) promoter and flanked by intron 1 of the corn-specific heat shock protein 70 at the upstream (5′) region. Transgenic corn has been marketed for effective pest management in agriculture and is currently in use in North America (Brodman et al., 2002).

The increasing commercial use of transgenic crops and the spraying of Bt suspensions has raised concerns about the release of recombinant DNA (rDNA) and feral DNA from biotechnology into aquatic ecosystems. Through the turnover of Bt and the degradation of plant biomass, large quantities of DNA are released to receiving environments (Dale et al., 2002). Under certain conditions, extracellular DNA can persist in the aquatic environment (Douville et al., 2007), thereby increasing the probability of horizontal gene transfer (HGT) events from plants to

☆ Freshwater mussels were collected by permission of the Ministère du Développement Durable, de l'Environnement et des Parcs [Québec Ministry of Sustainable Development, Environment and Parks]. They were handled in such a way to minimize stress and discomfort.

*Corresponding author. Fax: +1 514 496 7398.
E-mail address: francois.gagne@ec.gc.ca (F. Gagné).

0147-6513/$ - see front matter
doi:10.1016/j.ecoenv.2008.02.006

Please cite this article as: Douville, M., et al., Occurrence of the transgenic corn *cry1Ab* gene in freshwater mussels (*Elliptio complanata*) near corn fields: Evidence of exposure by bacterial ingestion. Ecotoxicol. Environ. Saf. (2008), doi:10.1016/j.ecoenv.2008.02.006

microorganisms and from extracellular DNA to microorganisms. The capacity of microorganisms to acquire novel DNA traits represents a means to adapt more rapidly to changes in the environment by acquiring and selecting new genes for survival. HGT is considered a rare event in eucaryotes but it is more common in prokaryotes, as mentioned above. However, transgenic plant DNA containing a prokaryotic sequence associated with the bacterial promoter (CaMV 35S) or antibiotic-resistant genes could permit the downward transfer from plant to bacteria (Bertolla et al., 2000). In view of the (deliberate) release of genetically modified organisms into aquatic and terrestrial ecosystems, an understanding of the processes implicated in inter-species gene transfers is crucial to maintaining our genetic biodiversity (Wolska, 2003). In addition, the cumulative effects of anthropogenic pollution and other stressors such as global warming on the frequency of intra- and inter-species genetic exchanges in aquatic ecosystems are virtually unknown at present.

The purpose of this study was therefore to examine the contamination of *cry1Ab* genes from Bt corn in the freshwater mussel *Elliptio complanata* in areas influenced by agricultural activity. The levels of cry1 gene from endemic source of Bt were measured to determine whether the cry1Ab levels followed a similar pattern of exposure. Bivalves accumulate large quantities of microorganisms during feeding and respiration (Watanabe et al., 1997) and have been used as sentinel species for bioavailability studies of gene products derived from biotechnology.

2. Materials and methods

2.1. Site characteristics and sampling

Freshwater mussels (*E. complanata*) were collected from the Richelieu River at Île aux Noix, Quebec, and from Balsam Lake, Ontario, on June 2004 (Fig. 1). These sites are in close proximity to corn field where both transgenic and non-transgenic corn were grown. In the Richelieu River area (Île aux Noix and Huron River), about 30% of corn has a transgenic origin (conservative estimation of 300 ha of land reserved for corn). Mussels were also collected from a remote area (Lake Opasatica, town of Rouyn-Noranda, North-West of the province of Québec) as a reference site devoid of any agriculture contamination. Mussels from Île aux Noix were maintained in 300-L aquarium under constant aeration. They were fed twice a week with suspensions of *Pseudokirchneriella subcapitata* (10–30 million algae /L). Twenty Île aux Noix mussels were transplanted to the Huron River, 35–40 km downstream of the Richelieu River, in an area of intensive corn farming, for 2 months (September–October 2004) to verify if changes of gene contamination occurred. The Huron River site is also affected by intensive farming of both wild and transgenic Bt corn. Balsam Lake is located in an Ontario provincial park 144 km northeast of Toronto. Mussels were collected from the northern part of the lake. The water flows from south to north; corn is grown near the southern portion of the lake (hence, mussels were located downstream of the contamination source). After sampling, mussels were kept on ice until their same-day arrival at the laboratory and allowed to depurate for 24 h at 15 °C before freezing at −80 °C. Surface water (4 L) and sediments (0.5 kg) were collected from the Richelieu River area in May and August 2005, for the determination of heterotrophic bacteria counts (Bodycote, Sainte-Foy, Quebec).

2.2. Mussel tissue preparation and morphologic assessments

Mussels were first measured (shell length in mm) and weighed (g wet weight of mussel, soft tissues, gonads, gills, digestive glands) for the

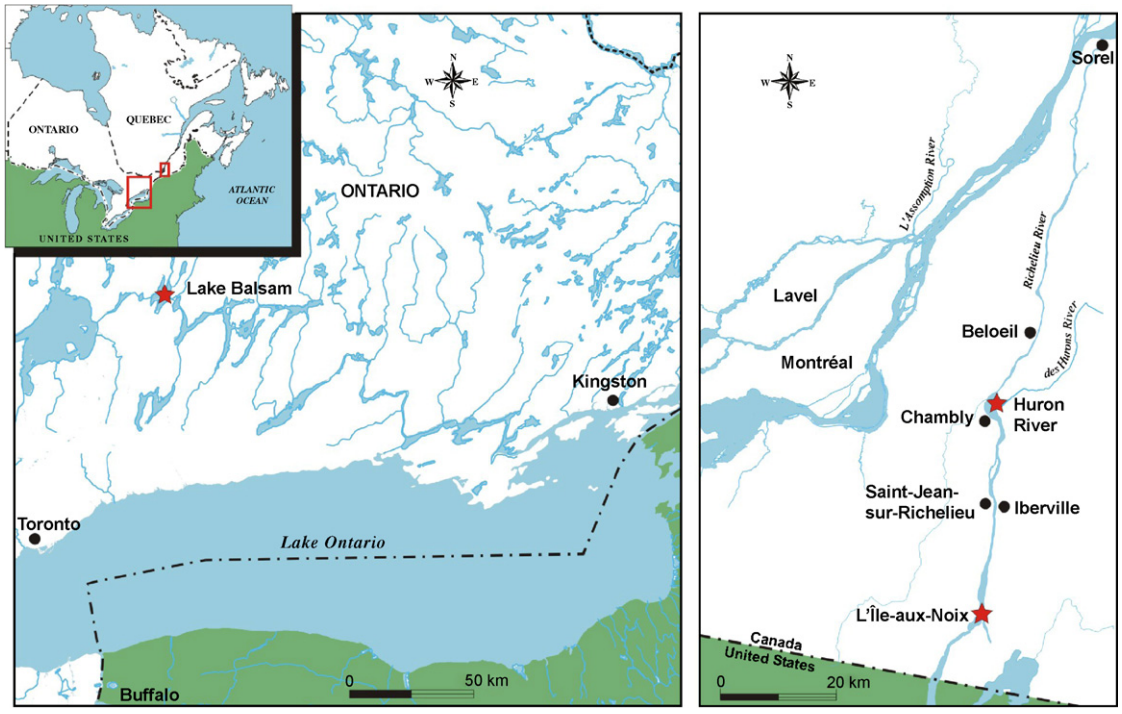

Fig. 1. Site locations for the mussels contamination study. Mussels were collected in Balsam Lake (Ontario) and in the Richelieu River at Île aux Noix (Québec, Canada). Mussels from the Richelieu River were transplanted for 2 months in netted cages and placed at the des Hurons River near the confluence of the Richelieu River.

Please cite this article as: Douville, M., et al., Occurrence of the transgenic corn *cry1Ab* gene in freshwater mussels (*Elliptio complanata*) near corn fields: Evidence of exposure by bacterial ingestion. Ecotoxicol. Environ. Saf. (2008), doi:10.1016/j.ecoenv.2008.02.006

determination of condition factor (total weight/shell length), soft tissue weight ratio (g soft tissues/g total weight) and gonado-somatic index (mg of gonad tissue/g of soft tissue, wet weight). Visceral masses of gonad, digestive gland and gill were dissected out on ice and tissues were homogenized with a Polytron tissue homogenizer in ice-cold 10 mM Tris–acetate buffer, pH 8, containing 1 mM EDTA and 1 mM dithiothreitol at a 1:5 weight:volume ratio. Aliquots of each homogenate were taken for DNA extraction and total protein determination (Bradford, 1976).

2.3. DNA extraction and preparation

DNA was extracted from mussel tissues by following Minton and Mayer's (1994) method, with some modifications. Extractions of biological samples were done in separate sequences (first, DNA was extracted in tissues from mussels, followed by bacterial DNA extraction the following year after extensive cleaning of the working areas) and took place in a different laboratory from the one housing the real-time PCR instrument, in order to limit cross-contamination events. Tissue homogenates were lysed in 100 mM Tris–HCl lysis buffer (1:2 volume ratio), pH 8, containing 5 mM EDTA, 0.2% sodium-dodecyl-sulfate and 200 mM NaCl, with proteinase K at a final concentration of 100 μg/mL, at 55 °C in a water bath until dissolution. The processed tissues were then centrifuged at 15,000*g* at 4 °C for 5 min and the resulting supernatant mixed with one volume of phenol–chloroform–isoamyl alcohol (25:24:1, v/v/v) and centrifuged again at 15,000*g* for 5 min at 4 °C. The aqueous upper layer was mixed with 5 M NaCl (1:20 volume ratio) and two volumes of ice-cold isopropanol. Samples were set aside at −80 °C for 30 min and then centrifuged as described above. Pellets were mixed with ice-cold ethanol at 70% and centrifuged again. Pellets were dried at 37 °C for about 10 min and dissolved in Tris–EDTA (TE) buffer (10 mM Tris–HCl at pH 8.0 and 1 mM EDTA) at 4 °C. Any bacteria that had grown on the agar plates (in heterotrophic conditions) were collected with a sterile inoculating loop under a UV-sterilized laminar flow hood and mixed with protease-based lysis buffer (i.e. 20 mM Tris–HCL, pH 8.0, 2 mM sodium EDTA, 1.2% Triton® X-100 and 20 mg/mL lysozyme) at 37 °C for 30 min. Bacterial (plasmid) DNA was extracted using the Plasmid extraction kit by QIAGEN Inc. (Valencia, CA, USA). DNA purity was evaluated by absorbance at 260 and 280 nm (in a quartz cuvette, Beckman DU70 spectrophotometer). In case of impurity (i.e. A260/A280 < 1.8), extracts were re-extracted by phenol–chloroform–isoamyl alcohol as described above. Total amounts of DNA extracted in each samples were quantified by absorbance at 260 nm.

2.4. Analysis of cry1 genes by real-time polymerase chain reaction

DNA extracted from tissues of mussels was tested, along with bacteria, for the presence of *cry1* and *cry1Ab* genes from Bt and Bt corn, respectively. A quantitative fluorescence real-time polymerase chain reaction (qPCR) procedure was conducted using the iCycler iQ™ real-time detection system (BIO-RAD Laboratories, Hercules, CA, USA) with SYBR Green I as the fluorescent dye. The reaction took place in a volume of 50 μL, containing 25 μL of iQ™ SYBR Green Supermix (Bio-Rad Laboratories), 0.5 μM of forward and reverse primers, and 5 μL of test sample (between 25 and 100 ng DNA). For bacterial *cry1*, universal primers were used as described in Ben-Dov et al. (1997). For Bt corn, primers for genetically modified corn were used as recommended by Kuribara et al. (2002). For Bt cry1 we used the following primers: forward: 5′-ATG ATT CAT GCG GCA GAT AAA C-3′ and reverse: 5′-TTG TGA CAC TTC TGC TTC CCA TT-3′; for *cry1Ab* from Bt corn: forward (hsp70): 5′-GAT GCC TTC TCC CTA GTG TTG A-3′ and reverse (*cry1Ab*): 5′-GGA TGC ACT CGT TGA TGT TTG-3′. The following qPCR conditions were used: 3 min initial denaturation at 95 °C, 35 (for bacterial *cry1*) or 40 cycles (for Bt corn *cry1Ab*) consisting of a 0.5 min denaturation step at 95 °C, a 1 min annealing step at 59 °C, and a 1 min extension step at 74 °C. Fluorescence measurements were taken at 80 °C for 10 s. A melt-curve analysis was performed immediately afterward using 70 steps of 0.5 °C increments for 10 s, starting at 59 °C and ending at 94 °C. Agarose gel electrophoresis was performed at a 2% agarose concentration. Staining was done using the fluorescent probe SYBR®Green at 1 × for 40 min (Molecular Probes, Eugene, OR, USA). Sequence analysis of amplified DNA (amplicon) obtained by PCR reaction with the Bt corn *cry1Ab* gene was performed in bacterial DNA samples and gonads, gills and digest glands of mussels from the different sites. The reported detection limit of the qPCR method was 200 pg DNA, which corresponds to 40 genomic copies (Douville et al., 2007). The sequencing was completed at the McGill University and Genome Quebec Innovation Centre (Montréal, Quebec, Canada). PCR products were purified (Multiscreen; Millipore, Bedford, MA, USA), and sequencing was performed using BigDye Terminator (version 3.1) and analyzed on ABI 3730XL sequencers (Applied BioSystems, Foster City, CA, USA). Genomic DNA from mussels collected from a pristine site (Abiti-Temiscamingue, Québec) devoid of agriculture or urban areas were used as negative controls.

2.5. Data analysis

The following numbers of mussels were used for each site: 8 for Île aux Noix, 16 for the Huron River site, and 8 for Balsam Lake. For detection of *cry1* and *cry1Ab* genes, any data below the method's detection limit were reported as 0 at the statistical analysis. Data distribution was analyzed using the Shapiro–Wilk W test for the normality of distribution and Levene's test for the homogeneity of variances. In all cases the distribution was not normal and the variances were not homogeneous. Non-parametric tests were then selected for analysis. Site differences were determined using a Kruskall–Wallis ANOVA followed by a rank sum test (Mann–Whitney) for inter-site or intra-site comparisons. The sites were compared between Île aux Noix with des Hurons and Balsam lake sites. A Spearman R rank correlation analysis was performed to seek out trends between the various endpoint measurements in mussels across the three study sites ($N = 32$ individuals). All statistical tests were performed using the Statistica software package (version 7.0).

3. Results

3.1. General health status of mussels

The health status of freshwater mussels was estimated by tracking various morphologenic, reproductive, and stress biomarkers. The condition factor and tissue weight ratio changed significantly across the study sites in some cases (Fig. 2). The condition factor of mussels from Balsam Lake was significantly lower than that of the mussels at the Île aux Noix site in the upper Richelieu River. The soft tissue weight ratio was significantly lower in mussels transplanted to the Huron River site, but significantly higher in mussels from Balsam Lake. The condition factor and tissue weight ratio were significantly correlated ($r = -0.53$; $p < 0.01$), suggesting that shell growth was affected more than soft tissue biomass.

3.2. Occurrence of Bt cry1 and transgenic cry1Ab genes in mussels

Mussels collected at all study sites contained detectable amounts of *cry1* gene from Bt (Fig. 3). The Bt *cry1* gene was found in all tissues examined, in descending order of concentrations: gills, digestive glands, and gonads. The levels of Bt *cry1* gene in gonadal tissues were not different among sites (rank ANOVA: $p > 0.1$). However, *cry1* levels

Please cite this article as: Douville, M., et al., Occurrence of the transgenic corn *cry1Ab* gene in freshwater mussels (*Elliptio complanata*) near corn fields: Evidence of exposure by bacterial ingestion. Ecotoxicol. Environ. Saf. (2008), doi:10.1016/j.ecoenv.2008.02.006

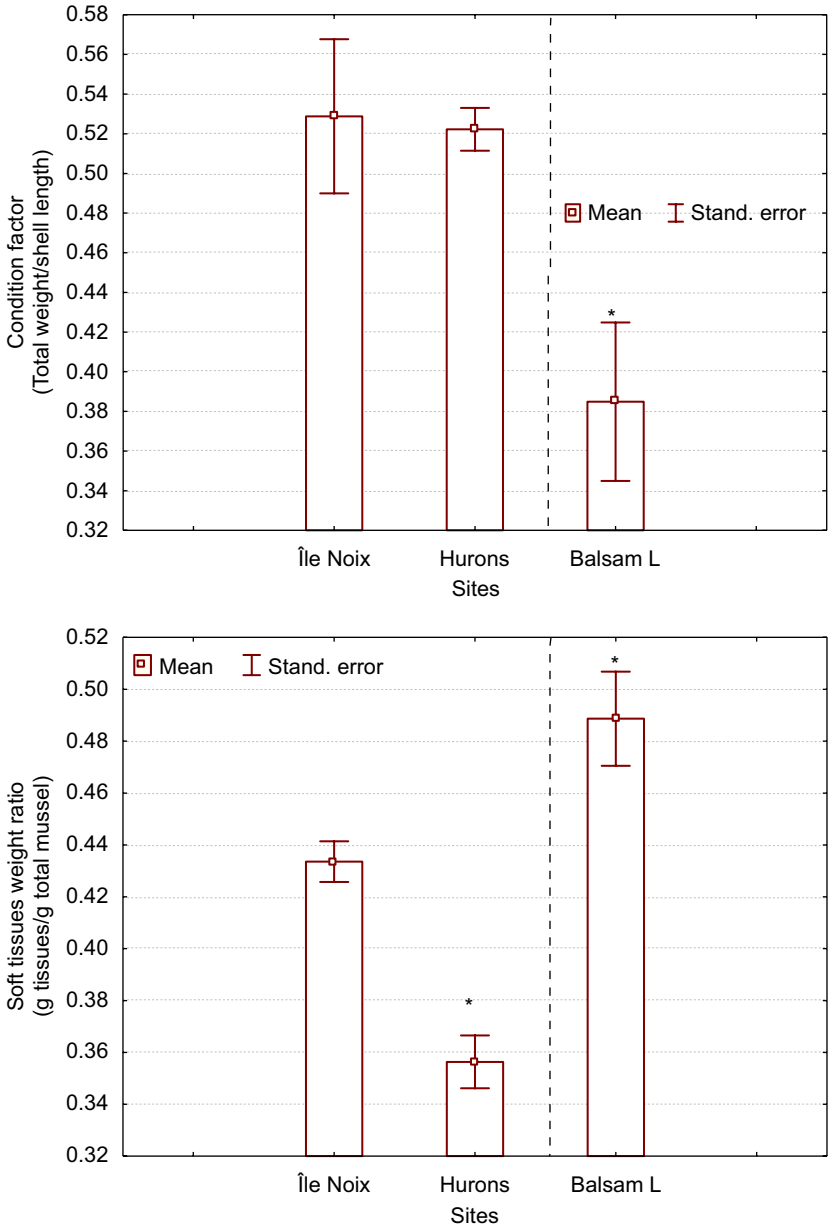

Fig. 2. Morphological characteristics of freshwater mussels exposed to an agricultural area. Freshwater mussels were analyzed for condition factor and soft tissue weight ratio. The data represent the mean with standard error. Asterisks (*) indicate a significant difference from the upstream Île aux Noix site in the Richelieu River. The dashed line separates the field sites from the Richelieu River and Balsam lake areas.

in digestive glands and gills were significantly higher at Balsam Lake compared to Ile Noix and Hurons sites ($p<0.05$). The sum of the Bt gene from the three organs was also significantly higher at Balsam Lake compared to the other sites ($p<0.001$). A correlation analysis revealed that the Bt *cry1* gene detected in digestive gland was significantly correlated with the levels found in gonad ($r=0.58$; $p<0.01$). Gill Bt *cry1* gene was correlated with soft tissue weight ratio ($r=0.5$; $p<0.01$) and negatively so with condition factor ($r=-0.71$; $p<0.001$). The sum of Bt *cry1* gene in tissues was significantly correlated with condition factor ($r=-0.73$; $p<0.001$) and soft tissue weight ratio ($r=0.56$; $p=0.001$).

The occurrence of *cry1Ab* from transgenic corn was also examined (Fig. 4). The Bt corn *cry1Ab* gene was also detected in mussel tissues, in descending order: gills, digestive glands, and gonads. Mussels collected at the pristine site (Opasatica Lake) were devoid of transgenic *cry1ab* gene. The gene from transgenic corn did change significantly in the digestive gland (rank ANOVA: $p=0.02$). The transgene was significantly higher in the gills of mussels from Balsam Lake relative to the two other sites, as was the sum of transgenic *cry1Ab* genes (rank sum test: $p<0.01$). No significant difference was found between sites for the transgene present in gonadal tissues. The sum of transgenic *cry1Ab* in tissues of mussels from Balsam

Please cite this article as: Douville, M., et al., Occurrence of the transgenic corn *cry1Ab* gene in freshwater mussels (*Elliptio complanata*) near corn fields: Evidence of exposure by bacterial ingestion. Ecotoxicol. Environ. Saf. (2008), doi:10.1016/j.ecoenv.2008.02.006

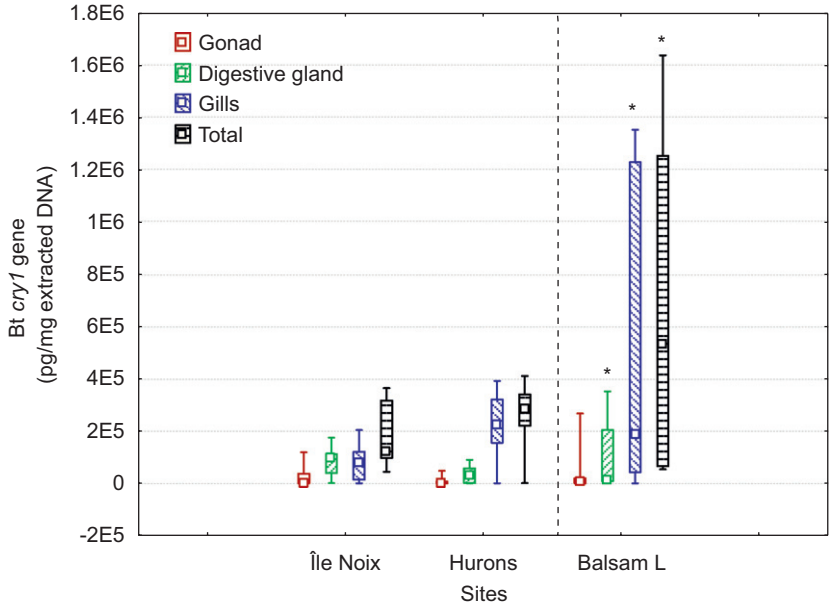

Fig. 3. Levels of Bt cry1 gene in organs of *Elliptio complanata* mussels. Mussel organs were analyzed for the presence of cry1 gene. The data are expressed as the median with 25–75% percentiles (box) and 1–99% percentiles (bars). Asterisks (*) represent a significant difference from the Île aux Noix site in the Richelieu River. The dashed line separates the field sites from the Richelieu River and Balsam lake areas.

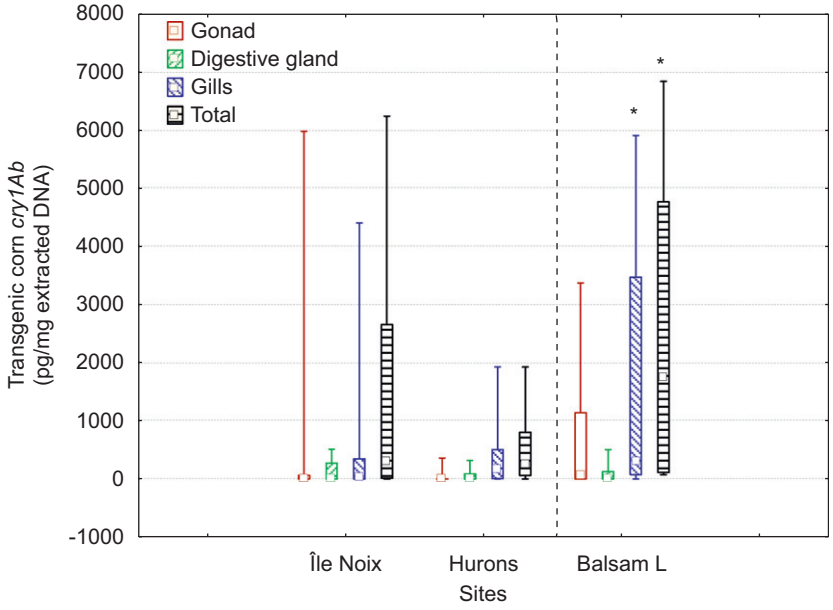

Fig. 4. Levels of *cry1Ab* gene from transgenic corn in *Elliptio complanata* mussel organs. Mussel organs were analyzed for the presence of *cry1Ab* gene derived from transgenic corn. The data are expressed as the median with 25–75% percentiles (box) and 1–99% percentiles (bars). Asterisks (*) represent a significant difference from the Île aux Noix site upstream in the Richelieu River. The dashed line separates the field sites from the Richelieu River and Balsam lake areas.

Lake was significantly higher than that in mussels from sites in the Richelieu River (Anova on ranks: $p<0.05$ and rank sum test: $p=0.05$). A correlation analysis revealed that Bt corn *cry1Ab* in digestive gland was correlated with *cry1* from Bt in gonad ($r=0.60$; $p<0.001$) and digestive gland ($r=0.62$; $p<0.001$). Gill *cry1Ab* gene from Bt corn was significantly correlated with *cry1* from Bt found in the gills ($r=0.73$; $p<0.01$), condition factor ($r=-0.42$; $p<0.05$) and soft tissue weight ratio ($r=0.43$; $p<0.05$). Gonad *cry1Ab* levels was significantly correlated with *cry1* from Bt in the gonad ($r=0.60$; $p<0.001$) and in the digestive gland ($r=0.62$; $p<0.001$). The sum of *cry1Ab* in tissues was significantly correlated with condition factor ($r=-0.49$; $p<0.01$), soft tissue weight ratio ($r=0.50$; $p<0.01$), and sum of Bt *cry1* in tissues ($r=0.60$; $p<0.001$).

Please cite this article as: Douville, M., et al., Occurrence of the transgenic corn *cry1Ab* gene in freshwater mussels (*Elliptio complanata*) near corn fields: Evidence of exposure by bacterial ingestion. Ecotoxicol. Environ. Saf. (2008), doi:10.1016/j.ecoenv.2008.02.006

Given that mussels were contaminated by *cry1Ab* genes from transgenic corn which could be the result of the presence of transformed bacteria in mussel's tissues, the occurrence and sequence of the transgene in heterotrophic bacteria from surface waters and sediments were also examined. In surface waters, heterotrophic bacterial cultures revealed that two of six water samples collected in late spring (May 2006) had positive qPCR results, giving a 113 bp-amplicon (Fig. 5). Furthermore, sequencing of this amplicon was 100% identical to the sequence reported for Bt corn (Kuribara et al., 2002) as seen in Table 1. However, no detection was observed in surface water samples collected in August 2006. In sediments, only one sample (2.5 km downstream of the Huron River site) was positive based on qPCR and again, after DNA sequencing, the transgene's sequence was confirmed to be *cry1Ab* from transgenic corn (Kuribara et al., 2002). Most mussels contained *cry1Ab* gene from Bt corn, with the mussels from Balsam Lake containing the highest levels of the transgene and all being contaminated (Table 2). It is noteworthy that

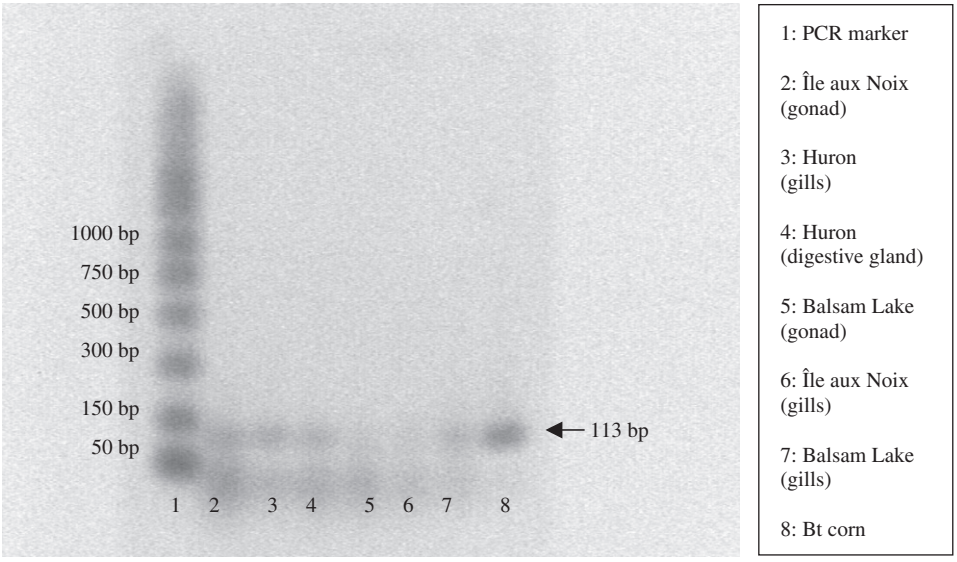

Fig. 5. Agarose gel electrophoresis of Bt corn *cry1Ab* amplicons of mussel tissues. Amplicons from *cry1Ab* from transgenic corn were isolated on 2% agarose gel electrophoresis using a SYBR®Green fluorescent probe.

Table 1
Detection of Bt corn *cry1Ab* gene in heterotrophic bacteria collected in surface water and sediments near a Bt cornfield

Samples	Detection of Bt corn *cry1Ab* gene (ng/mg bacterial DNA)		Sequence
	Surface water	Sediments	
May 2006			
16.5 km upstream of des Hurons River	ND[a]	ND	–
15 km upstream of des Hurons River	330 ± 70	133 ± 27	GATGCCTTCTCCCTAGTGTTGACCAGTGTT ACTCACATAGTCTTTGCTCATTTCATTG TAATGCAGATACCAAGCGGCCATGGACA ACAACCCAAACATCAACGAGTGCATCC
8 km upstream of des Hurons River	62 ± 12	ND	GATGCCTTCTCCCTAGTGTTGACCAGTGTT ACTCACATAGTCTTTGCTCATTCATTGA ATGCAGATACCAAGCGGCCATGGACAAC AACCCAAACATCAACGAGTGCATCC
Confluence of des Hurons and Richelieu rivers	ND	ND	
30 km downstream of Richelieu River	ND	ND	
82 km downstream of (confluence with St. Lawrence River)	ND	ND	
August 2006			
Bt corn sequence from Kuribara et al. (2002)	ND (for all sites)	ND (for all sites)	GATGCCTTCTCCCTAGTGTTGACCAGTGTT ACTCACATAGTCTTTGCTCATTTCATTG TAATGCAGATACCAAGCGGCCATGGACA ACAACCCAAACATCAACGAGTGCATCC

[a]ND: not detected.

Please cite this article as: Douville, M., et al., Occurrence of the transgenic corn *cry1Ab* gene in freshwater mussels (*Elliptio complanata*) near corn fields: Evidence of exposure by bacterial ingestion. Ecotoxicol. Environ. Saf. (2008), doi:10.1016/j.ecoenv.2008.02.006

Table 2
Detection of Bt corn *cry1Ab* gene in *Elliptio complanata* from sites in Quebec and Ontario

Sample	Number of positive mussels	Sequence
Île aux Noix Richelieu River	5/8	tgcagataccaagcggccatggacaa caacccaaacatcaacgagtgcatcc
Transplanted mussels ines Hurons River	12/16	tgcagataccaagcggccatggacaa caacccaaacatcaacgagtgcatcc
Balsam Lake	8/8	tgcagataccaagcggccatggacaa caacccaaacatcaacgagtgcatcc
DNA sequence of Kuribara et al. (2002)		tgcagataccaagcggccatggacaa caacccaaacatcaacgagtgcatcc

75% of mussels caged for 2 months in the Huron River were contaminated by the transgene, which is apparently higher, albeit not significantly so, than the initial rate of contamination (62%) in mussels at Île aux Noix (proportion comparison test: $p = 0.26$). Thus, *cry1Ab* was found in surface waters/sediments in some heterotrophic bacterial preparations and in mussel's tissues where the gills and digestive gland contained most of the transgene.

4. Discussion

4.1. Occurrence and activity of DNA in aquatic ecosystems

The production of transgenic material over a large surface area (i.e. crops) releases large amounts of rDNA into the environment. The release of rDNA into the environment increases the likelihood of HGT events. In a previous study, surface waters and sediments were contaminated by Bt corn *cry1Ab* and Bt *cry1* when collected in areas of intensive agricultural activity (Douville et al., 2007), suggesting that HGT could occur at sites affected by intensive agricultural activities in the Richelieu River. Extracellular DNA also persists in some conditions in aquatic environments, pervading the bacterial community and increasing the likelihood of HGT. The present data support this possibility—in heterotrophic bacterial populations, at least—where some bacteria found in surface waters and sediments contain the plant transgene. No occurrence of *cry1Ab* gene, or below than detection limit at least, was observed in samples of bacteria collected in August. This was corroborated by the observation that in mussels caged for 2 months in the Huron River, they were less contaminated by transgenic DNA but not with *Cry1* from Bt.

The mechanism by which transgenic DNA might be transferred horizontally could proceed, in theory, by three means. The first means consist of reintegration of DNA containing the antibiotic resistance and bacterial promoter of the co-transfection plasmid pV-ZMGT10 genes by the microflora in the rhizosphere. However, this is not likely since the kanamycin resistance gene (nptII) did not remain in the plant but the actual limit of detection was not mentioned in the report (De Schrijver and Moens, 2003). Would it be plausible that very low amounts of the plasmid persisted in the plant's cells? The second pathway, although unlikely as mentioned above, could proceed through the transfer of nopaline synthetase (nos) gene of the plasmid to bacteria in the rhizosphere. The plasmid used for maize transformation contained the nopaline synthetase gene of *Agrobacterium tumefaciens* (De Schrijver and Moens, 2003). This bacterium is able to horizontally transfer DNA between itself and plants, which cause tumors in plants in some cases. Moreover, this bacterium could also pervade (opportunistic pathogen) higher organisms whose immune system are weakened and maintained its ability to transfer its DNA in eukaryotes in the laboratory. It is possible that the transgene might be reintroduced in naturally occurring *A. tumefaciens* in the rhizosphere or that the plasmid was not entirely removed in the host (albeit unlikely according to the above). The third pathway could be the transfer by the enhanced cauliflower mosaic virus promoter which was also found in the plasmid and remains in the plant. Mutations could render the viral promoter less specific to the cauliflowers and transforms bacteria in the rhizosphere. However, further testing is warranted to confirm these hypotheses in addition whether the cry1Ab was readily inserted in the mussel's genome. Although we found transgenic *cry1Ab* in mussel tissues, we do not know if it was the result of internalized bacteria or DNA transfer to the mussel genome took place. Our data only supports that a transfer from plant to (heterotrophic) bacteria took place and finds their way in mussels since they feed on benthic microflora. Gene expression of foreign, prokaryote, DNA to higher eukaryotes has been shown in mammals injected intramuscularly with plasmid containing the δ-endotoxin from Bt (Pang, 1994). Indeed, antibodies were raised against the δ-endotoxin from Bt in mice and rabbits by injecting isolated plasmid from Bt into their muscles. Hence, the possibility exists that foreign DNA could be expressed in mammalian tissues. However, further research will be required to assess whether transgenic *cry1Ab* is actually expressed in mussel's tissues.

The transformative potential of *Pseudomonas stutzeri* by plasmid DNA containing neomycin phosphotransferase gene from a transgenic Bt corn line was detected for up to 48 h in both groundwater and river water (Zhu, 2006), indicating the relatively limited persistence of rDNA in controlled settings. Conversely, immunoreactive *cry1Ab* proteins were found above detection limits in bulk soil 7 months after harvesting, with no apparent effects on bacterial soil community structure (Baumgarte and Tebbe, 2005). This suggests that proteins, and perhaps DNA, persist for many months after corn churning and composting at the end of the farming period (Zwahlen et al., 2003a). The long-term exposure of the transgene would increase the probability of HGTs to the bacterial community and

Please cite this article as: Douville, M., et al., Occurrence of the transgenic corn *cry1Ab* gene in freshwater mussels (*Elliptio complanata*) near corn fields: Evidence of exposure by bacterial ingestion. Ecotoxicol. Environ. Saf. (2008), doi:10.1016/j.ecoenv.2008.02.006

eventually taken up by mussels during feeding. This is more likely in northern areas where the corn stem churning and composting occur at the beginning of winter (late November and early December in the area under study). Indeed, *cry1Ab* gene product was detected more frequently in spring before the seeding of Bt corn the following year (Douville et al., 2007). Only composting at high temperatures (>48 °C) was effective in rapidly degrading rDNA in corn (Guan et al., 2005). The *cry1Ab* protein from Bt corn appeared to influence the composition of the soil microbial community within the first 72 h, leading to increased soil respiration (Mulder et al., 2006). However, these effects returned to normal 3 weeks after the initial addition of transgenic corn, suggesting no lasting effects on the microbes. In another study, the cultivation of Bt maize significantly increased the saturated-to-unsaturated lipid ratios in soils, which appeared to negatively affect microbial activity in the soil (Dinel et al., 2003) and indicated that Bt maize can influence soil community structure. Bt was found to influence somewhat the function of aquatic microbial communities (Kreutzweiser et al., 1996). Indeed, microbial respiration was affected in the artificial concentration in microcosms corresponding to 100-fold the expected environmental concentration of 20 IU/mL of Bt application. However, the reduced decomposition activity, the net mass loss of leaf material between Bt treatment and the control was not significant.

4.2. Bioavailability and potential for environmental effects of cry1Ab and cry1 genes

Bivalves are well known to accumulate large amounts of microorganisms and particles during feeding. In the present study, mussels contained significant amounts of *cry1* from Bt in gills and digestive glands, suggesting a dietary intake of Bt in mussels, which thrive at the water/sediment interface in aquatic ecosystems. A similar pattern of *cry1Ab* from transgenic corn was also found, albeit at concentrations of about two orders of magnitude less. This was supported by the significant correlations of Bt *cry1* and Bt corn *cry1Ab* in gills ($r = 0.73$; $p < 0.001$) and digestive glands ($r = 0.62$; $p < 0.001$). To the best of our knowledge, this is the first account of *cry1Ab* transgene contamination in freshwater mussels and this contamination appeared to arise from mussels feeding on bacteria, as evidenced by the occurrence, albeit at a low frequency, of the transgene in heterotrophic bacteria from surface waters and sediments near cornfields. However, whether the transgene was actually associated to the mussel genome, cannot be answered in the present study. Indeed, the study only reveals that *cry1Ab* from transgenic corn was found in mussels and this could be the result of ingested of transformed bacteria. Current data about the potential toxic consequence of Bt gene and transgenic *cry1Ab* gene on non-target aquatic species and information on freshwater mussels are lacking at present. The effects or implications of the Bt genes taken up into mussel tissues are not known, but the correlation analysis indicated that condition factors were negatively correlated with the presence of Bt gene. In a study of salmon fed genetically modified maize, superoxide dismutase activity in the liver and distal intestine were significantly higher (Sagstad et al., 2007). Intestinal catalase and heat shock protein 70 in the liver were significantly induced in fish fed transgenic maize compared to wild maize or to a fish reference diet. Transgenic corn feed seemed to bring significant changes in plasma granulocytes and monocyte proportion, suggesting inflammation (oxidative stress). In the snail *Helix aspersa* infected by a nematode and co-exposed to cadmium and Bt maize, infected snails grew more slowly when fed Bt corn than the wild corn feeding group (Kramarz et al., 2007). In earthworms, significant weight loss (18%) resulted when the worms were fed Bt corn compared to a moderate weight gain of 4% in worms from the wild corn feeding group (Zwahlen et al., 2003b).

5. Conclusions

Freshwater mussels collected in areas under the influence of both Bt corn and wild corn cultivation contained, in their tissues, *cry1* and *cry1Ab* genes from Bt and Bt corn, respectively. The genes accumulated more heavily in the gill and digestive gland, but some traces of the genes were found in the gonad. This contamination seems to proceed by feeding on bacteria or particles that are maintaining or stabilizing the genes in the aquatic environment suggesting the HGT (plant to bacteria) was occurring. The adverse effects of these biotechnology products in mussels are not clear in such altered, agriculture-dominated environments, but a trend analysis did reveal that condition factor and oxidative status (results not shown) were significantly related to their presence. However, concurrent contamination by chemical pollutants and their potential adverse effects in these agricultural watersheds cannot be excluded. The potential for adverse effects of the Bt genes on mussel health should be determined by toxicity assays in which Bt wild type and recombinant genes are fed to cultured, healthy mussels. Future research is warranted to elucidate the ecotoxicological properties of rDNA in non-target aquatic organisms. Whether the genes were expressed at the mRNA level in mussels remains also an unanswered question and merits further investigation. Our study supports the contention that freshwater mussels can be effective, relevant bioindicators of contamination by genetically modified organisms in aquatic ecosystems.

Acknowledgments

We thank Chad Boyko (Environment Canada, Burlington, Ontario) for providing the mussels from Balsam Lake; Sophie Trépanier for assistance in the field, and Tana McDaniel (CCIW, Ontario) for providing information on agricultural activities near Balsam Lake. The manuscript was edited by Patricia Potvin and the maps of the study

Please cite this article as: Douville, M., et al., Occurrence of the transgenic corn *cry1Ab* gene in freshwater mussels (*Elliptio complanata*) near corn fields: Evidence of exposure by bacterial ingestion. Ecotoxicol. Environ. Saf. (2008), doi:10.1016/j.ecoenv.2008.02.006

areas were prepared by François Boudreault, both from Environment Canada. This project was funded by the Canadian Research Strategy of Biotechnology Canada.

This work was funded by the Environmental Management of Biotechnology for Regulation and Research of Environment Canada.

References

Baumgarte, S., Tebbe, C.C., 2005. Field studies on the environmental fate of the *cry1Ab* Bt-toxin produced by transgenic maize (MON810) and its effect on bacterial communities in the maize rhizosphere. Mol. Ecol. 14, 2539–2551.

Ben-Dov, E., Zaritsky, A., Dahan, E., Barak, Z., Sinai, R., Manasherob, R., Khamraev, A., Troiskaya, E., Dubitsky, E.A., Berezina, N., Margalith, Y., 1997. Extended screening by PCR for seven cry-group genes from field-collected strains of *Bacillus thuringiensis*. Appl. Environ. Microbiol. 63, 4883–4890.

Bertolla, F., Kay, E., Simonet, P., 2000. Potential dissemination of antibiotic resistance genes from transgenic plants to microorganisms. Infect. Control Hosp. Epidemiol. 21, 390–393.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Brodman, P.D., Ilg, E.C., Bertoud, H., Hermann, A., 2002. Real-time quantitative polymerase chain reaction methods for four genetically-modified maize varieties and maize DNA content in food. J. AOAC Int. 85, 646–653.

Dale, P.J., Clarke, B., Fontes, E.M., 2002. Potential for the environmental impact of transgenic crops. Nat. Biotechnol. 20, 567–574.

Dinel, H., Schnitzer, M., Saharinen, M., Meloche, F., Pare, T., Dumontet, S., Lemee, L., Ambles, A., 2003. Extractable soil lipids and microbial activity as affected by Bt and non Bt maize grown on a silty clay loam soil. J. Environ. Sci. Health B 38, 211–219.

Douville, M., Gagne, F., Blaise, C., Andre, C., 2007. Occurrence and persistence of *Bacillus thuringiensis* (Bt) and transgenic Bt corn *cry1Ab* gene from an aquatic environment. Ecotoxicol. Environ. Saf. 66 (2), 195–203.

Guan, J., Spencer, J.L., Ma, B.L., 2005. The fate of the recombinant DNA in corn during composting. J. Environ. Sci. Health B 40, 463–473.

Karim, S., Dean, D.H., 2000. Pesticidal and receptor binding properties of *Bacillus thuringiensis cry1Ab* and *cry1Ac* delta-endotoxin mutants to *Pectinophora gossypiella* and *Helicoverpa zea*. Curr. Microbiol. 41, 430–440.

Kramarz, P.E., de Vaufleury, A., Zygmunt, P.M., Verdun, C., 2007. Increased response to cadmium and *Bacillus thuringiensis* maize toxicity in the snail *Helix aspersa* infected by the nematode *Phasmarhabditis hermaphrodita*. Environ. Toxicol. Chem. 26, 73–79.

Kreutzweiser, D.P., Gringorten, J.L., Thomas, D.R., Butcher, J.T., 1996. Functional effects of the bacterial insecticide *Bacillus thuringiensis* var. *kurstaki* on aquatic microbial communities. Ecotoxicol. Environ. Saf. 33, 271–280.

Kuribara, H., Shindo, Y., Matsuoka, T., Takubo, K., Futo, S., Aoki, N., Hirao, T., Akiyama, H., Goda, Y., Toyoda, M., Hino, A., 2002. Novel reference molecules for quantitation of genetically modified maize and soybean. J. AOAC Int. 85, 1077–1089.

Minton, R.L., Mayer, G.C., 1994. A method for the isolation of total genomic DNA from the Zebra mussel, *Dreissena polymorpha*. In: Proceedings of the Fourth International Zebra Mussel Conference, Madison, WI, USA, March. 〈http://sgnis.org/publicat/proceed/1994/543_551.pdf〉.

De Schrijver, A., Moens, W., 2003. Report on the molecular characterization of the genetic map of event Mon810. Service of Biosafety and Biotechnology, Scientific Institute of Public Health, IPH/15/20/SBB/03-0409, Belgium.

Mulder, C., Wouterse, M., Raubuch, M., Roelofs, W., Rutgers, M., 2006. Can transgenic maize affect soil microbial communities? PloS. Comput. Biol. 2, e128.

Pang, A.S.D., 1994. Production of antibodies against *Bacillus thuringiensis* delta-endotoxin by injecting its plasmids. Biochem. Biophys. Res. Commun. 202, 1227–1234.

Sagstad, A., Sanden, M., Haugland, O., Hansen, A.C., Olsvik, P.A., Hemre, G.I., 2007. Evaluation of stress- and immune-response biomarkers in Atlantic salmon, *Salmo salar L.*, fed different levels of genetically modified maize (Bt maize), compared with its near-isogenic parental line and a commercial Suprex maize. J. Fish Dis. 30, 201–212.

Soberon, M., Fernandez, L.E., Perez, C., Gill, S.S., Bravo, A., 2007. Mode of action of mosquitocidal *Bacillus thuringiensis* toxins. Toxicon 49, 597–600.

Watanabe, M.F., Park, H.D., Kondo, F., Harada, K., Hayashi, H., Okino, T., 1997. Identification and estimation of microcystins in freshwater mussels. Nat. Toxins 5, 31–35.

Wolska, K.I., 2003. Horizontal DNA transfer between bacteria in the environment. Acta Microbiol. Pol. 52, 233–243.

Zhu, B., 2006. Degradation of plasmid and plant DNA in water microcosms monitored by natural transformation and real-time polymerase chain reaction (PCR). Water Res. 40, 3231–3238.

Zimmermann, A., Hemmer, W., Liniger, M., Luthy, J., Pauli, U., 1998. A sensitive detection method for genetically modified MaizeGuard™ corn using nested PCR system. Lebensm. Wissn. Technol. 31, 664–667.

Zwahlen, C., Hilbeck, A., Gugerli, P., Nentwig, W., 2003a. Degradation of the *cry1Ab* protein within transgenic *Bacillus thuringiensis* corn tissue in the field. Mol. Ecol. 12, 765–775.

Zwahlen, C., Hilbeck, A., Howald, R., Nentwig, W., 2003b. Effects of transgenic Bt corn litter on the earthworm *Lumbricus terrestris*. Mol. Ecol. 12, 1077–1086.

Please cite this article as: Douville, M., et al., Occurrence of the transgenic corn *cry1Ab* gene in freshwater mussels (*Elliptio complanata*) near corn fields: Evidence of exposure by bacterial ingestion. Ecotoxicol. Environ. Saf. (2008), doi:10.1016/j.ecoenv.2008.02.006