Government of Canada Gouvernement du Canada

Final Screening Assessment of Bacillus thuringiensis strain ATCC 13367

Environment and Climate Change Canada

Health Canada

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Synopsis

Pursuant to paragraph 74(b) of the Canadian Environmental Protection Act, 1999 (CEPA), the Minister of the Environment and the Minister of Health have conducted a screening assessment of Bacillus thuringiensis strain ATCC[1] 13367 (B. thuringiensis strain ATCC 13367).

B. thuringiensis strain ATCC 13367 is a facultative anaerobic Gram-positive bacterium. As a species, Bacillus thuringiensis (B. thuringiensis) is generally considered ubiquitous and commonly found in terrestrial and aquatic habitats. B. thuringiensis is able to form spores which can withstand harsh environmental conditions and survive under conditions of nutrient depletion. Various characteristics of B. thuringiensis make it suitable for use as an active ingredient in commercial and consumer products, including degreasers, detergents, and additives in bioremediation and biodegradation and in various industrial processes.

B. thuringiensis is known particularly for the production of crystal proteins (Cry toxins) which are toxic for various Orders of insects (mainly lepidopterans, dipterans and coleopterans). In particular, B. thuringiensis strain ATCC 13367 is known to produce a Cry 1B (Cry1Ba) toxin, which is known to be selectively toxic towards insect species of the Order Lepidoptera, and towards a few species of the Orders Diptera and Coleoptera. Despite the ubiquity and abundant use of various B. thuringiensis subspecies, , there are no known adverse population–level effects on target species in the ecosystems where it is used, and no adverse effects on non-target terrestrial or aquatic plants, vertebrates or invertebrates

B. thuringiensis is not considered a human pathogen and to date no mammalian pathogenicity and toxicity study has demonstrated that commercial spore preparations of any B. thuringiensis subspecies cause adverse effects by any route of exposure. B. thuringiensis has been isolated from a few gastrointestinal, ocular and wound infections. Some B. thuringiensis strains, including ATCC 13367, have been reported to produce enterotoxins and membrane damaging toxins. These toxins are known as important factors for pathogenicity of a close relative, Bacillus cereus, in humans. However, the significance of the presence of these virulence factors in B. thuringiensis in relation to human infections is not clear. The scientific literature reports very few cases of infection linked to

[1] American Type Culture Collection

B. thuringiensis. B. thuringiensis is resistant to several clinical antibiotics, but effective treatments against infection are available.

This assessment considers the aforementioned characteristics of B. thuringiensis strain ATCC 13367 with respect to environmental and human health effects associated with consumer and commercial product use and industrial processes subject to CEPA, including releases to the environment through waste streams and incidental human exposure through environmental media. To update information about current uses, the Government launched a mandatory information-gathering survey under section 71 of CEPA, as published in the Canada Gazette, Part I, on October 3, 2009 (section 71 Notice).

Based on the information available, it is concluded that B. thuringiensis strain ATCC 13367 does not meet the criteria under paragraph 64(a) or (b) of CEPA as it is not entering the environment in a quantity or concentration or under conditions that have or may have an immediate or long-term harmful effect on the environment or its biological diversity or that constitute or may constitute a danger to the environment on which life depends. It is also concluded that B. thuringiensis strain ATCC 13367 does not meet the criteria under paragraph 64(c) of CEPA as it is not entering the environment in a quantity or concentration or under conditions that constitute or may constitute a danger in Canada to human life or health.

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1. Introduction

Pursuant to paragraph 74(b) of the Canadian Environmental Protection Act, 1999 (CEPA), the Minister of the Environment and the Minister of Health are required to conduct screening assessments of living organisms added to the Domestic Substance List (DSL) by virtue of section 105 of the Act to determine whether they present or may present a risk to the environment or human health (according to criteria set out in section 64 of CEPA[2]). Bacillus thuringiensis strain ATCC 13367 was added to the DSL under subsection 25(1) of CEPA 1988 and under subsection 105(1) of CEPA because it was manufactured in or imported into Canada between January 1, 1984 and December 31, 1986.

This screening assessment considers hazard information obtained from the public domain and from unpublished research data, as well as comments from scientific peer reviewers. Exposure information was obtained from the public domain and from a mandatory CEPA section 71 Notice published in the Canada Gazette, Part I, on October 3, 2009. Further details on the risk assessment methodology used are available in the Risk Assessment Framework document "Framework for Science-Based Risk Assessment of Micro-Organisms Regulated under the Canadian Environmental Protection Act, 1999" (Environment and Climate and Health Canada 2011).

In this report, data that are specific to the DSL-listed strain B. thuringiensis strain ATCC 13367 are identified as such. Strain-specific data were obtained from several sources: the Nominator, the American Type Culture Collection (ATCC), unpublished data generated by Health Canada[3] and Environment and Climate Change Canada[4] research scientists and peer-reviewed scientific literature. Where strain-specific data were not available, surrogate information from B. thuringiensis literature searches was used. When applicable, literature searches conducted on the organism included its synonyms, and common and superseded names. Surrogate organisms are identified in each case to the taxonomic level provided by the source. Literature searches were conducted using scientific literature databases (SCOPUS, CAB Abstracts, Google Scholar and NCBI PubMed), web searches, and key search terms for the identification of human

[2] A determination of whether one or more criteria of section 64 of CEPA are met is based on an assessment of potential risks to the environment and/or to human health associated with exposure in the general environment. For humans, this includes, but is not limited to, exposure from air, water and the use of products containing the substances. A conclusion under CEPA may not be relevant to, nor does it preclude, an assessment against the criteria specified in the Hazardous Products Regulations, which is part of the regulatory framework for the Workplace Hazardous Materials Information System (WHMIS) for products intended for workplace use.

[3] Testing conducted by Health Canada's Environmental Health Science and Research Bureau

[4] Testing conducted by Environment and Climate Change Canada's Ecotoxicology and Wildlife Health Division

health and environmental hazards. Information identified up to July 2016 was considered for inclusion in this report.

2. Decisions from Domestic and International Jurisdictions

2.1 Domestic

The Public Health Agency of Canada (PHAC) classified B. thuringiensis to be in Risk Group 1 (as a species) and furthermore, classified B. thuringiensis strain ATCC 13367 also to be in Risk Group 1 for human and terrestrial animals excluding terrestrial invertebrates (personal communication PHAC 2016).

The Canadian Food Inspection Agency (CFIA) does not require a plant protection permit to import B. thuringiensis under the CFIA's Plant Protection Act (CFIA 2015). Also, B. thuringiensis is assigned to be in Risk Group 2 for aquatic animals and is considered to require containment level 2 because of its activity as an aquatic animal pathogen (personal communication CFIA 2013).

Health Canada's Pest Management Regulatory Agency (PMRA), granted full registration for the sale and use of B. thuringiensis ssp. kurstaki (first registered in 1962), B. thuringiensis ssp. israelensis (first registered in1982), B. thuringiensis ssp. tenebrionis (first registered in 1990) and B. thuringiensis ssp. aizawai (registered in 2015) as active ingredients for use as microbial pest control agents with domestic, commercial and restricted classifications under the Pest Control Products Act (PMRA-HC 2006). There is currently a total of 38 registered pesticide products containing living B. thuringiensis as the active ingredient for use against lepidopteran, coleopteran and dipteran insects (PMRA-HC 2016a).

Health Canada's Health Products and Food Branch has a legislated responsibility for pre-market assessment of novel foods and novel food ingredients as detailed in Division 28 of Part B of the Food and Drug Regulations (Novel Foods). Health Canada has issued letters of no objection to several lines of cotton, corn, tomato and potato all containing Cry toxins from B. thuringiensis and deemed that foods derived from these are acceptable, safe and do not raise concerns for human health (Health Canada 2016). In addition, viable spores of B. thuringiensis Berliner as an agricultural chemical or their components or derivatives, that are present in or on the food is exempt from paragraph 4(1) (d) of the Food and Drugs Act under Division 15, Section B.15.002 of the Food and Drugs Regulations.

Approvals of B. thuringiensis on crops and their use in livestock feed demonstrate environmental and animal feed safety as determined by the CFIA and are summarized in numerous decision documents (CFIA 2016).

2.2 International

Between 1961 and 1995, the United States Environmental Protection Agency (US EPA) registered 177 products containing viable B. thuringiensis for use against lepidopteran, coleopteran and dipteran insects (US EPA 1998).

Criteria for specific tolerances and exemption of viable spores of B. thuringiensis Berliner for pesticidal chemical residues in food have been established in Section 180.1011 of Subpart D of Code of Federal Regulations (US EPA 2002).

A number of member countries of the Organization for Economic Cooperation and Development (OECD) have registered B. thuringiensis products for managing agricultural invertebrate pests (AAFC 2005).

The European Food Safety Authority (EFSA) completed risk assessments to support authorization by the European Commission of nine B. thuringiensis pesticide active substances as laid down in Annex I Council Directive 91/414/EEC of the Regulation (EC) No 2229/2004 (European Commission 2015).

The International Programme on Chemical Safety part of the World Health Organisation (WHO) has produced an environmental and human health risk assessment of microbial pest control agents based on B. thuringiensis (WHO 1999).

No other regulatory decisions were found regarding B. thuringiensis[5]

[5] Government agencies and organizations searched include: the United States Environmental Protection Agency; United States Food and Drug Administration; United States Animal and Plant Health Inspection Services; United States Department of Agriculture; American Biological Safety Association; World Health Organization; United States Centers for Disease Control; Biosecurity New Zealand; Australian Department of Health; European Food Safety Authority; European Centre for Disease Prevention and Control; and the Invasive Species Specialist Group.

3. Hazard Assessment

3.1 Characterization of Bacillus thuringiensis strain ATCC 13367

3.1.1 Taxonomic identification and strain history

Binomial name: Bacillus thuringiensis

Taxonomic designation:

Kingdom:	bacteria
Phylum:	firmicutes
Class:	bacilli
Order:	bacillales
Family:	Bacillaceae
Genus:	Bacillus
Species:	Bacillus thuringiensis
Subspecies:	Bacillus thuringiensis ssp. thuringiensis (Type strain: ATCC 10792^{T})
DSL strain:	ATCC 13367

On the DSL, listed as Bacillus thuringiensis strain ATCC 13367

Other strain numbers for B. thuringiensis strain ATCC 13367: BCRC 11029, CCRC 11029, CCUG 22499, CBDD 27, R. Davis USDA NRS 1328, IAM 11064, IFO 13866, JCM 20337, NBRC 13866, NIH B-13, Steinhaus B13, HD-737 (Dawyndt et al. 2005; US DA 1988)

Synonyms, common and superseded names:

B. cereus ssp. thuringiensis Smith et al.
B. thuringiensis Berliner 1915
B. thuringiensis var thuringiensis
B. thuringiensis Berliner
B. thuringiensis ssp. thuringiensis

Nomenclature:

The species B. thuringiensis was first described by Berliner and hence the designation Bacillus thuringiensis Berliner which refers to the author who first described the species. ATCC 13367 is a strain of Bacillus thuringiensis ssp. thuringiensis (Bacillus thuringiensis Berliner or Bacillus thuringiensis Berliner 1915). Regardless of the name used by the author of the scientific articles used in the report, for the purpose of this report, the name B. thuringiensis will be used when discussing the species at large and B. thuringiensis ssp. thuringiensis will be used when referring to the subspecies that includes ATCC 13367. Although the term serovar has been used synonymously with subspecies in the published scientific literature (e.g., serovar kurstaki, serovar israelensis, serovar aizawai, etc.), for the purpose of this report the term serovar will be used only for flagellar H-antigen specificity (serovar H-1, serovar H-14, etc.) and the term subspecies (ssp.) will be used otherwise (ssp. thuringiensis, ssp. kurstaki, ssp. israelensis, etc.). Reports of adverse effects attributed to B. thuringiensis ssp. thuringiensis were considered in this screening assessment as close surrogate information for B. thuringiensis strain ATCC 13367.

Strain history:

Publications in the scientific literature by various culture collections indicate that strain B13 isolated from a moth larva was deposited at ATCC as B. cereus ssp. thuringiensis ATCC 13367. It was later validated and identified by ATCC as B. thuringiensis Berliner based on biochemical characteristics (ATCC 2014; Steinhaus and Jerrel 1954).

> Chain of custody: ATCC 13367 <<--R Davis 1328, USDA<<--E. A Steinhaus B-13 (ATCC 2014)

3.1.2 Phenotypic and molecular characteristics

B. thuringiensis is a member of the B. cereus group, which consists of seven very closely related species: B. cereus, B. thuringiensis, B. anthracis, B. weihenstephanensis, B. cytotoxicus, B. pseudomycoides and B. mycoides (Helgason et al. 2004; Guinebretière et al. 2013). Differentiation of B. thuringiensis and B. cereus is not possible on the basis of cellular morphology, utilization of organic compounds, or by comparison of ribosomal sequences and spacer regions (Baumann et al. 1984; Priest et al. 2004), characterization of cell content of fatty acids (Vaisanen et al. 1991) or sugar utilisation (Wunschel et al. 1995).

Fatty acid methyl-ester (FAME) analyses by Health Canada scientists showed a high similarity with B. cereus, which is expected given the genetic similarity among B. cereus group members.

B. thuringiensis differs most obviously from other B. cereus group members in the production of insecticidal crystal (Cry) proteins, which can be visualized by light microscopy or detected by protein screening (Bernhard et al. 1997; Carlson and Kolsto 1993). Many variations in amino acid sequences of Cry toxins exist which often have insecticidal specificity to particular species.

3.1.2.1 Morphological characteristics

The species B. thuringiensis comprises many subspecies that are differentiated by their insecticidal target range. These include subspecies thuringiensis, kurstaki, israelensis, tenebrionis and aizawai.

The morphology of Cry protein crystals is a function of the Cry toxin produced, which in turn confers a specific insecticidal target species range within the orders Lepidoptera, Coleoptera, Diptera and Orthoptera (Hansen et al. 1998; Tyrell et al. 1981). The crystals produced by various B. thuringiensis subspecies have various forms: bipyramidal (Cry1), cuboidal (Cry2), fat rectangular (Cry3A), irregular (Cry3B), spherical (Cry4A and Cry4B), and rhomboidal (Cry11A) (Schnepf et al. 1998). The subspecies thuringiensis, kurstaki, kenyae, alesti, and tolworthi are known to produce crystals typical of Cry1 proteins that are bipyramidal (Bravo et al. 1998; Obeidat et al. 2004; Schnepf et al. 1998; Tyrell et al. 1981) and typical of Cry2 proteins that are cuboidal (Schnepf et al. 1998). The subspecies israelensis is known to produce an amorphous composite of three crystal types of which two are round or spherical (Hansen et al. 1998; Obeidat et al. 2004) and the third is a bar-shaped inclusion (Ibarra and Federici 1986). The subspecies tenebrionis produce rhomboid square crystals (Herrnstadt et al. 1987).

B. thuringiensis strain ATCC 13367 vegetative cells, spores and colonies are morphologically typical of the B. cereus group members (Table 1-1). Discrepancies in the table between data from the nominator, Health Canada, the ATCC and Bergey's manual are within the range of acceptability of B. thuringiensis and may be due to different culture conditions.

Table 1-1:Cell and Colony Morphologies of B. thuringiensis strain ATCC 13367, ATCC 10792 and B. thuringiensis Berliner 1915

Characteristic	ATCC 13367	B. thuringiensis ssp. thuringiensis (ATCC 10792)[d]	Berliner 1915[e]
Gram stain	Gram negative in young culture and prevailingly Gram positive in older cultures[a] Gram positive [b, c, d]	Gram positive	Gram positive
Cell morphology	Rods; cells may be curved, bent or frequently swollen [a] Medium rods [c] Stout rods [d]	Short stout rods, ends only slightly rounded	Rods

Motility	Non motile [a] Motile [b, c] Slightly motile to non-motile; Perichitrous flagellation [d]	Slightly motile to non-motile; Perichitrous flagellation	Motile
Crystal	N/A [a, **b,** c] Small, diamond-shaped parasporal body [d]	Oval, not swollen; diamond-shaped to cuboidal parasporal crystal	Cuboid, bipyramidal, spherical to ovoid, flat to rectangular or diamond shaped parasporal crystal
Spore	N/A [a, **b,** c] Cylindrical with round ends, average size 1.0 by 1.6 µm; usually formed in 48 hours; spores lie obliquely in the sporangium [d]	Cylindrical with round ends, average size 1.0 by 1.5 µm; sub terminal; usually formed in 24 to 48 hours; spores lie obliquely in the sporangium	Ellipsoidal, sub terminal spores ~ 1.3 µm in length and ~0.8µm in diameter [f]
Colony size	5-10mm [b]	N/A	N/A
Cell Size	0.5 to 0.7 by 1.0 to 3.0 µm occurring in an angular arrangement coccoid cells 0.6 to 0.8 µm in diameter; rudimentary branching may occur in liquid media [a]	1.7 by 4.0 µm	1.0 to1.2 by 3.0 to 5.0 µm
Colony Form	Irregular [a, b, c]	Irregular	Circular to irregular
Colony Elevation	Flat [b, c]	Flat	N/A
Colony Margin	Undulate [a, b, c]	Slightly lobed	Entire or undulate crenate or fimbriate edges
Colony texture (surface)	Dry [b,c]	Dry	Matte to granular
Colony opacity	Opaque [b] Darker center [c]	Transmitted light: cream white Opaque Reflected light: brownish white	N/A
Pigmentation	Off-white [b] Cream [c]	N/A	Whitish to cream

N/A data not available (not reported by the source)
[a] appearance on nutrient agar at 30°C as reported by the Nominator
[b] appearance on TSB agar after 7 days of growth at room temperature in testing by Health Canada scientists
[c] appearance on blood agar after 24-36 hours at 37°C as reported by ATCC
[d] based on information from Heimpel and Angus 1958 on strain NRS 1328 (Heimpel and Angus 1958)
[e] based on information summarising phenotype of several strains from Bergey's manual (Logan and De Vos 2009)
[f] based on information from (Chung et al. 2010)

3.1.2.2 Serotyping

The flagellar H-antigen has been used to classify B. thuringiensis into 69 serotypes with 13 sub-antigenic groups, giving a total of 82 serovars (Lecadet et al. 1999). Although there is some correlation between flagellar antigen serovar and insecticidal toxicity reviewed in (US EPA 1998), a particular crystal type may be produced by more than one H-serovar. B. thuringiensis ssp. thuringiensis, including ATCC 10792 belongs to serotype H-1 (Heimpel 1967; Norris and Burges 1965) and presumably ATCC 13367 does as well, however, no specific data were found or generated for ATCC 13367.

3.1.2.3 Genomic characteristics

Genotypic differentiation of B. thuringiensis and B. cereus is not possible by:

- DNA homology analysis (Kaneko, Nozaki, Aizawa 1978),
- ribotyping (Priest et al. 1994),
- 16S rRNA gene fingerprinting, 23S and 5S rRNA gene restriction fragment length polymorphism (RFLP) (Joung and Côté 2001a; Joung and Côté 2001b)
- analysis of the 16S-23S internal transcribed sequence (Bourque et al. 1995; Lechner et al. 1998; Wunschel et al. 1995),
- PCR analysis of genes encoding B. cereus-like toxic products (Asano et al. 1997; Damgaard et al. 1996), or
- pulsed field gel electrophoresis and multilocus enzyme electrophoresis (MLEE) (Carlson and Kolsto 1993; Carlson et al. 1994).

Likewise, B. cereus group species are indistinguishable through 16S rDNA sequence analysis (Ash et al. 1991; Chang et al. 2003; Chen and Tsen 2002; La Duc et al. 2004; Lechner et al. 1998). This was confirmed using 16S rRNA gene sequences of B. thuringiensis strain ATCC 13367, analyzed by Health Canada research scientists, which have 98.37% homology with those of B. thuringiensis ATCC 10792 in the proprietary MicroSeq ® ID library and more than 98% homology with those of other members of the B. cereus group included on the database (B. thuringiensis ATCC 33679, B. cereus ATCC 14579, B. anthracis Ames and B. mycoides ATCC 6462). Similarly, using the Ribosomal Database Project, (Release 11 https://rdp.cme.msu.edu/), ATCC 13367 has a close relationship with B. cereus, and B. weihenstephanensis.

The genetic relatedness between members of the B. cereus group is so close that from a strictly phylogenetic point of view they can be seen as a single species; however, more in depth genetic methods have been extensively used to demonstrate phylogenetic relationships and to understand the few genomic variations among the B. cereus group. These include:

- full genomic sequencing (Ivanova et al. 2003),
- amplified fragment length polymorphism (AFLP) (Hill et al. 2004; Hoffmaster et al. 2006; Ticknor et al. 2001a; Ticknor et al. 2001b),
- rep-PCR fingerprinting (Cherif et al. 2003a),

- 16S rRNA and 23S rRNA gene sequence analysis (Ash et al. 1991),
- multi locus enzyme electrolysis (MLEE) (Helgason et al. 2000; Priest et al. 2004)
- multi-locus sequence typing (MLST) (Helgason et al. 2004; Priest et al. 2004; Tourasse et al. 2006a; Tourasse et al. 2006b) and
- suppression subtractive hybridization (SSH) (Radnedge et al. 2003).

The B. cereus group members are usually divided into three main phylogenetic clades based on MLST studies (Sorokin et al. 2006). Clade I contains B. anthracis, some B. cereus strains and some B. thuringiensis strains, mostly from clinical sources. Clade II comprises mostly strains of B. thuringiensis. Some clinical isolates of B. cereus and an environmental isolate including DSL strain B. cereus ATCC 14579 also occur in Clade II. Finally Clade III consists of B. mycoides and B. weihenstephanensis which are considered non-pathogenic (Carlson et al. 1994; Didelot et al. 2009; Helgason et al. 2004; Priest et al. 2004; Sorokin et al. 2006; Vassileva et al. 2006). A phylogenetic tree based on alignment of 16S rRNA in the B. cereus group using bootstrapped neighbor-joining method showed that B. thuringiensis ssp. thuringiensis and other subspecies such as tenebrionis, morrisoni, kurstaki, sotto, israelensis belong to Clade II and are divergent from Clade I which includes B. thuringiensis ssp. konkukian and B. thuringiensis Al-Hakam, both alleged human pathogens (Ibrahim et al. 2010). However the B. cereus group species can be differentiated by PCR analysis for cry genes.

3.1.3 Biological and ecological properties

3.1.3.1 Natural occurrence

B. thuringiensis is considered to be ubiquitous (de Been et al. 2006; Martin and Travers 1989), with worldwide distribution and it has been isolated from all continents, including Antarctica (Forsyth and Logan 2000).

B. thuringiensis ssp. thuringiensis is mostly found in terrestrial habitats, as illustrated below, but is also found in aquatic environments (Iriarte et al. 2000; Martinez and Caballero 2002)

Specifically, B. thuringiensis has been isolated from the following environments:

Soil:

- cultivated agricultural soils, non-cultivated soils, deserts, tropical and tundra rain forests (Bernhard et al. 1997; De Lucca II, Simonson, Larson 1981; Landen et al. 1994; Martin and Travers 1989; Obeidat et al. 2004; Stotzky 2000).

Phylloplane and Rhizosphere:

- leaves, the phylloplane of arboreous and herbaceous plants, including deciduous and coniferous trees, grass and cabbage as well as from mushroom compost

(Bernhard et al. 1997; Damgaard et al. 1997; Kim 2000; Mizuki et al. 1999; Smith and Couche 1991)
- o fresh fruits and vegetables either as natural contaminants or as residues of B. thuringiensis based insecticides (Frederiksen et al. 2006)
- o insect cadavers (Bernhard et al. 1997; Carozzi et al. 1991), also, it has been recovered from soils from sericulture (Xavier et al. 2007)
- o stored grain products, mills and maize grinders (Bernhard et al. 1997; De Lucca et al. 1982; Ejiofor and Johnson 2002; Kim 2000; Kim et al. 1998; Meadows et al. 1992; Obeidat et al. 2004) and various food items such as pasta, pita bread and milk (Damgaard et al. 1996)

Aquatic:

- o stagnant or dried ponds, water, marine sediments, brackish sediments in mangrove swamps (Ichimatsu et al. 2000; Iriarte et al. 2000; Maeda et al. 2000; Maeda et al. 2001)

3.1.3.2 Growth parameters

B. thuringiensis ssp. thuringiensis can convert nitrates to nitrites, and utilises citrate slowly as a carbon source. It grows anaerobically in the presence of glucose (Heimpel and Angus 1958); however, under anaerobic conditions, growth of B. thuringiensis is slow and sporulation may be inhibited. B. thuringiensis can grow facultatively anaerobically but under anaerobic conditions, growth of B. thuringiensis is slow and sporulation may be inhibited; therefore, oxygen supply is essential requirement for longer term survival and persistence (Argôlo-Filho et al. 2013; Khetan 2000). Germination and growth have been proposed to be inhibited by other indigenous microorganisms and poor nutrient availability (West et al. 1985).

B. thuringiensis germination and growth have a restricted pH range with an optimal growth at a pH of 7.5 ± 1.0 for B. thuringiensis ssp. kurstaki and B. thuringiensis ssp. israelensis (Seligy et al. 1997). Similarly, B. thuringiensis ssp. thuringiensis grown on nutrient agar shows optimum growth at pH 6.7 and 6.4, a 10-fold reduction in growth at 6.0 and 5.6, a further 10,000-fold reduction at pH 5.1 and no growth at pH 4.4 (Saleh et al. 1970a).

B. thuringiensis grows over a wide temperature range. The minimum temperature for growth is 10-15°C and the maximum is 40-45°C (De Vos et al. 2009). It was reported to grow well between 28 and 35°C (Heimpel and Angus 1958).

Data generated by Health Canada research scientists showed that B. thuringiensis strain ATCC 13367 grew well in trypticase soy broth (TSB) and in fetal bovine serum at 27°C, 32°C, 37°C and 42°C, but growth was limited in sheep serum-containing mammalian culture media and Dulbecco's modified eagle medium (DMEM) at those temperatures (Table B-1). In addition, B. thuringiensis strain ATCC 13367 grew well in various solid media at 28°C and 37°C (Table B-2).

3.1.3.3 Life Cycle (Sporulation)

The life cycle of B. thuringiensis can be divided into 4 phases (Khetan 2001):

- Phase I—Vegetative Growth: A population of vegetative cells will grow exponentially under favourable conditions of moisture content, temperature, pH oxygen and nutrient availability. When a population of vegetative cells passes out of the exponential phase of growth, usually as a result of nutrient depletion, sporulation is induced.
- Phase II—Transition to Sporulation: The tricarboxylic cycle is depressed and the organic acids, acetate and pyruvate which have been accumulated during vegetative growth are metabolized, supplying the cell with energy and carbon for spore and crystal synthesis.
- Phase III—Sporulation and the Biosynthesis of Cry toxin: Sporulation is divided into seven different stages, from chromosome replication and separation to the formation of a separate structure inside the cell. During sporulation B. thuringiensis produces the Cry toxins. Cry toxins are contained within the parasporal crystal inclusions that are synthesized adjacent to the endospore (Bourque et al. 1993; Carozzi et al. 1991; Ceron et al. 1994; Schnepf et al. 1998).
- Phase IV—Spore Maturation and Cell lysis: The spore acquires full resistance properties (for example, against desiccation and high temperature), and outer spore coat layers are developed during late sporulation stages. Lastly, the mother cell lyses, releasing the mature spore into the environment. The vegetative growth cycle resumes when the spore encounters a nutrient rich environment (El-Khoury et al. 2016; Hilbert and Piggot 2004). Germination of spores is another important part of the life cycle as it is linked to pathogenicity. B. thuringiensis has been known to germinate and persist in the gastrointestinal tract and digestive system of mammals (Wilcks et al. 2006a; Zhang et al. 2012).

3.1.3.4 Survival, persistence and dispersal in the environment

3.1.3.4.1 Persistence and survival of B. thuringiensis vegetative cells and spores

A population of vegetative cells will grow exponentially under favourable conditions of moisture content, temperature, pH, oxygen and nutrient availability. For B. thuringiensis, these conditions are mostly found within the host or target insect. Little data is available in the scientific literature on the survival and replication of B. thuringiensis in aquatic environments. Moreover, information summarised in regulatory decisions from domestic and international jurisdictions suggest that B. thuringiensis is not known to proliferate in a vegetative state in aquatic environments (PMRA-HC 2006; US EPA 1998; WHO 1999). Data on vegetative cell populations of B. thuringiensis spp. thuringiensis inoculated in non-sterile soil showed that the population declines within 2 days and forms spores (Akiba 1986).

Much more data on persistence of B. thuringiensis spores in terrestrial environments are available, but they are highly variable, especially in soil. There is a wide range of

persistence reported from a few days to several years, as illustrated in the following summary of findings:

- Spores of B. thuringiensis survived up to 10 days in lake and sewage waters, without a change in their number (Furlaneto et al. 2000);
- Spores of B. thuringiensis ssp. israelensis persisted for at least 22 days in stagnant water and mud (Ohana et al. 1987);
- B. thuringiensis ssp. kurstaki viable spores persisted for about 40 days in fresh water and for more than 70 days in sea water (Menon and De Mestral 1985);
- B. thuringiensis ssp. kurstaki was recovered up to 12 days after aerial spray in a near-by river (Menon and De Mestral 1985);
- B. thuringiensis spores remained viable for about three months in the soil (Saleh et al. 1970b);
- B. thuringiensis spores incubated in natural soil in both laboratory and nature declined in number for the first 2 weeks; thereafter, the number of viable spore remained constant for at least 8 months (Petras and Casida Jr. 1985);
- B. thuringiensis persisted in the form of spores in the top few centimetres of soil for 7 years (Hendriksen and Hansen 2002);
- B. thuringiensis cells introduced into soil in sprays persisted for 88 months (Vettori et al. 2003);
- B. thuringiensis ssp. thuringiensis spores in soil (mainly in forest ecosystems) survived for several years after spray applications (reviewed in (Addison 1993));
- Spores survived with a half-live in the range of 120 days in the topsoil layer of an agricultural field (Pedersen et al. 1995);
- The half-life of spores of B. thuringiensis ssp. thuringiensis on soybean leaves was > 24h; since there was a 90 % decline in spore viability during the first day (Ignoffo et al. 1974); and
- B. thuringiensis ssp. kurstaki spores applied extensively in North America could persist in urban environments for at least 4 years (Van Cuyk et al. 2011).

B. thuringiensis spores are highly resistant to heat (to 80°C), desiccation and drought, enabling the bacterium to survive periods of stress under adverse environmental conditions (Petras and Casida Jr. 1985; West et al. 1985) (reviewed in (Lambert and Peferoen 1992)). However, solar or ultra violet radiation, temperature, humidity, wind and rain can limit persistence of B. thuringiensis spores (Brar et al. 2006). Spores can be rapidly inactivated by UV radiation and sunlight. Survival can drop more than 90% within 20 minutes of exposure to sunlight (Brar et al. 2006; Griego and Spence 1978).

Similar to data from the literature on other B. thuringiensis strains, B. thuringiensis strain ATCC 13367 spores applied at an initial density of ~$1x10^6$ CFU/g soil persisted in microcosm soil at a level of ~$1x10^5$ CFU/g soil for a full 105-day study period (Providenti et al. 2009).

3.1.3.4.2 Persistence of insecticidal toxins

Some published reports on the persistence of Cry toxins in soil show short half-lives, whereas others show low-level residues lasting for many months (reviewed in (Clark et al. 2005)), as illustrated by the following summary of reports:

- B. thuringiensis Cry toxins introduced into soil in sprays can persist for 28 months (Vettori et al. 2003);
- The half-life of Cry toxins of B. thuringiensis ssp. thuringiensis on soybean leaves was > 24 h since there was a reduction of 65% insecticidal activity during the 1st day. Some insecticidal activity could be detected at 7 days post application (Ignoffo et al. 1974); and
- Studies monitoring Cry toxins produced in transgenic B. thuringiensis crops show rapid break down of Cry toxins and low persistence in the soil (Icoz and Stotzky 2008; Li YunHe et al. 2007; Palm et al. 1996).

Factors leading to the deactivation of Cry1Ac toxin from transgenic B. thuringiensis crops include microbial degradation, higher temperature and sequestration in a solid matrix. The deactivation in non-sterile medium at 24ºC was fastest in soil with a half-life of 1.5 days, followed by sediment with a half-life of 3.9 days and the slowest in water with a half-life of 12.8 days, which indicates that this toxin may persist for a longer period in aquatic systems (Li YanLiang et al. 2013). The free Cry toxins of B. thuringiensis ssp. kurstaki and B. thuringiensis ssp. tenebrionis could be removed by degradation by indigenous micro-organisms (Koskella and Stotzky 1997). The Cry toxin of B. thuringiensis spp. aizawai is degraded by soil microorganisms at an exponential rate with a half-life of about 3-6 days (West et al. 1984).

Persistence of Cry toxins is enhanced when toxins are bound to surface active particles in the environment (Koskella and Stotzky 1997; Lee et al. 2003; Stotzky 2004). Purified Cry toxins can be rapidly adsorbed by clay minerals, humic acids and organo-mineral complexes and could persist in both cultivated and forest soil (Crecchio and Stotzky 1998; Crecchio and Stotzky 2001; Tapp et al. 1994). Low molecular weight organic acid ligands increase the adsorption of B. thuringiensis toxin by soil minerals (Fu et al. 2007). Toxins adsorbed and bound on clay and humic acids retain insecticidal activity; however, humic acids reduce biodegradability (Crecchio and Stotzky 1998; Koskella and Stotzky 1997; Tapp and Stotzky 1995a; Tapp and Stotzky 1995b). The insecticidal activity retained in the soil varies with the type of soil (composition) and the pH of the soil (Tapp and Stotzky 1998).

3.1.3.4.3 Dispersal

There is little to no multiplication of B. thuringiensis outside the insect host, and B. thuringiensis rarely spreads from the point of inoculation on land (Snarski 1990). B. thuringiensis spores are dispersed by non-anthropogenic transport such as water, wind and migrating animals (Bernhard et al. 1997) or rain (Pedersen et al. 1995). B. thuringiensis spores resist downward leaching in soil, and remain in the top few

centimeters of the soil profile (Hendriksen and Hansen 2002). In soils receiving 45 cm of simulated rainfall, B. thuringiensis was detected to a depth of 3-6 cm (Akiba 1991).

After the application of B. thuringiensis on land, it may be dispersed by birds and mammals feeding on infected target insects (Meadows 1993). Dispersal of B. thuringiensis spores by carabid beetles and other surface active insects has been detected up to a distance of 135m from the point of application (Pedersen et al. 1995). Many animals have been shown to excrete B. thuringiensis in their feces. These include voles (Swiecicka and De Vos 2003), Japanese deer (Ohba and Lee 2003), 14 species of wild mammals in Korea (Lee et al. 2003) and 11% of rodents and 17% of insectivore mammals examined for a Polish National Park (Swiecicka et al. 2002).

3.1.3.5 Horizontal Gene Transfer

Many toxin-encoding genes in B. thuringiensis are carried on plasmids (Berry et al. 2002; Levinson et al. 1990; Zhong et al. 2000), which can be transferred from cell to cell by conjugation, transformation and transduction (Gonzalez Jr et al. 1982; Lecadet et al. 1980; Reddy et al. 1987; Ruhfel et al. 1984; Santos et al. 2010; Thorne 1978; Wilcks et al. 1998). B. thuringiensis strains can exhibit complex plasmid profiles, with up to 17 plasmids, in sizes ranging from 2 to 600 kb, which can be found in one strain (Gonzalez Jr. and Carlton 1980; Kronstad et al. 1983; Lereclus et al. 1982).

Health Canada scientists, through a plasmid extraction protocol (Reyes-Ramírez and Ibarra 2008) followed by gel electrophoresis, did not detect plasmids in the genome of B. thuringiensis strain ATCC 13367. Without evidence for plasmid content, B. thuringiensis strain ATCC 13367 cannot be implicated in the conjugal transfer of DNA including virulence factors to other bacteria in the environment. While it is possible that B. thuringiensis strain ATCC 13367 could acquire virulence plasmids from pathogenic relatives, the probability of such an occurrence is no higher than for other naturally occurring strains of B. thuringiensis.

3.1.3.6 Resistance to Antibiotics, Metals and Chemical Agents

B. thuringiensis has shown resistance to heavy metals. It can biosorb the heavy metals most frequently present in polluted aquatic and soil environments such as cadmium, copper, chromium, nickel, zinc, cobalt and mercury (El-Helow et al. 2000; Hassen et al. 1998; Mendil et al. 2008; Oves et al. 2013; Öztürk 2007). B. thuringiensis produces a broad spectrum β-lactamase and is thus resistant to penicillin, oracillin, ampicillin, and cephalosporins. It is also resistant to trimethoprim (De Vos et al. 2009; Luna et al. 2007). However, B. thuringiensis is generally sensitive to gentamicin, levofloxacin, moxifloxacin, rifampicin, amikacin, ciprofloxacin, vancomycin, chloramphenicol, erythromycin, tetracycline, clindamycin, gatifloxacin, and quinupristin/dalfopristin (Callegan et al. 2006; Hernandez et al. 1998; Luna et al. 2007; Rosenquist et al. 2005; Turnbull et al. 2004).

Table 1-2 presents the antibiotic susceptibility profile (MICs µg/mL) of B. thuringiensis strain ATCC 13367.

Table 1-2: Antibiotic susceptibility profile for B. thuringiensis strain ATCC 13367

Antibiotic	Susceptible [a]	Intermediate [a]	Resistant [a]	MIC µg/mL [b]
Amoxicillin	≤ 0.12	-	≥ 0.25	> 24 (R)
Aztreonam	N/A	N/A	N/A	> 24
Cefotaxime	≤ 8	16-32	≥ 64	> 24 (R)
Doxycycline	N/A	N/A	N/A	0.37
Gentamicin	≤ 4	8	≥ 16	3.9 ± 2 (S)
Nalidixic Acid	N/A	N/A	N/A	8.4 ± 3.3
Trimethoprim	N/A	N/A	N/A	>24
Vancomycin	≤4	N/D [c]	N/D [c]	1.4 ± 0.3 (S)

[a] Interpretive criteria (Patel et al. 2010)
[b] The work was conducted using TSB-MTT liquid assay method (Seligy et al. 1997). The reported values are based on a minimum of seven independent experiments. Values correspond to the minimal inhibitory concentration (µg/ml) for B. thuringiensis strain ATCC 13367 (20,000 CFU/well) grown in the presence of antibiotic for 24 h at 37°C. R: resistant, S: susceptible
[C] The rare occurrence of resistant strains precludes defining any results categories other than "susceptible"
N/A Not Available. Recommendations not made because limited data exist for Bacillus sp.
N/D Not Determined.

3.1.3.7 Pathogenic and toxigenic characteristics

3.1.3.7.1 Pathogenesis

B. thuringiensis is known for its entomopathogenic properties due to the production of crystal proteins (Cry toxins) and other virulence factors, which are thought to facilitate its development within the insect host.

Studies conducted to support pesticide registrations of various other B. thuringiensis strains and transgenic Bt plants show an absence of pathogenic effects in non-target species.

For summaries of these pathogenicity and toxicity studies, refer to:

- OECD - Consensus Document on Safety Information on Transgenic Plants Expressing Bacillus thuringiensis - Derived Insect Control Protein (OECD 2007)
- U.S.EPA Reregistration Eligibility Decision (RED) Bacillus thuringiensis (US EPA 1998)
- WHO - Environmental Health Criteria 217 Microbial Pest Control Agent Bacillus thuringiensis (WHO 1999)
- PMRA – Proposed Acceptability for Continuing Registration – Re-evaluation of Bacillus thuringiensis - https://www.canada.ca/en/health-canada/services/consumer-product-safety/reports-publications/pesticides-pest-management/decisions-updates.html#rvd-drv (PMRA 2006).

3.1.3.7.2 Toxins

B. thuringiensis species can produce a variety of toxins such as Crystal protein (Cry) toxins, Cyt toxins, β-exotoxin and Vip, which are primarily responsible for its pathogenesis in insects and which exhibit various activities and host ranges. Some B. thuringiensis toxins, such as β-exotoxin, can be toxic to mammals at high doses Some strains of B. thuringiensis are known to produce other toxin types including hemolysins and enterotoxins that have diarrheal properties, and are similar to those produced by B. cereus (Hansen and Hendriksen 2001).

See Appendix D: Host range of Cry toxins for a summary table the host range of the Cry and Cyt toxins.

Crystal protein (Cry) toxins

The mode of action of the Cry toxin is still being elucidated. According to the classical model (Figure 1-1), the crystal proteins are first ingested as protoxins, which are solubilized and proteolytically converted to smaller protease-stable polypeptides in the insect midgut. The activated toxins then bind to specific receptors at the surface of midgut epithelial cells, allowing them to insert into the membrane and form poorly selective pores which are permeable to small molecules such as inorganic ions, amino acids and sugars. The presence of the pores in the plasma membrane interferes with cell physiology by abolishing transmembrane ionic gradients and leads to osmotic lysis of the cells due to the massive influx of solutes from the midgut lumen. In turn, destruction of the cells results in extensive damage to the midgut epithelial tissue and death of the intoxicated larvae (as reviewed in Vachon et al. 2012). The events leading to pore formation remain relatively poorly understood, and are explained by competing models: the sequential binding model (Figure 1-2) and the signaling pathway model (Figure 1-3) (Vachon et al. 2012; Bravo, et al. 2007; Bravo et al. 2011; Dorsch et al. 2002; Knowles and Dow 1993; Knight et al. 1994; Vadlamudi et al. 1993; Vadlamudi et al. 1995).

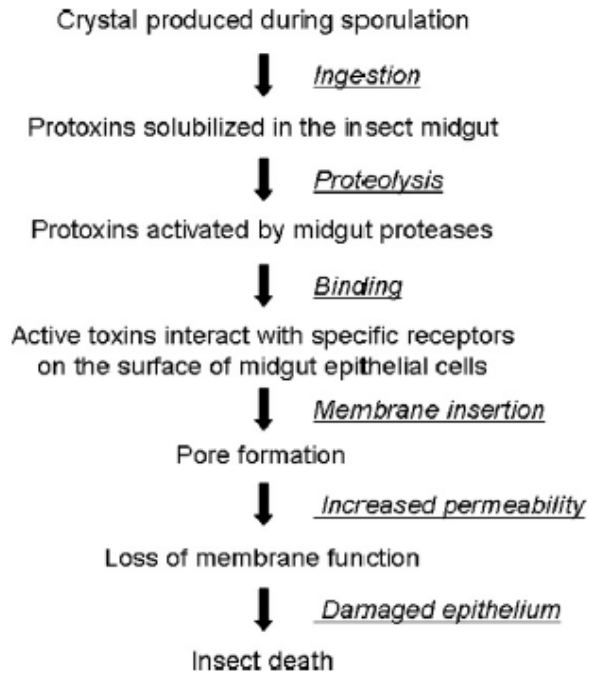

Figure 1-1: Schematic representation of the steps leading to pore formation and insect death according to the classical model of B. thuringiensis mode of action (Vachon et al. 2012)

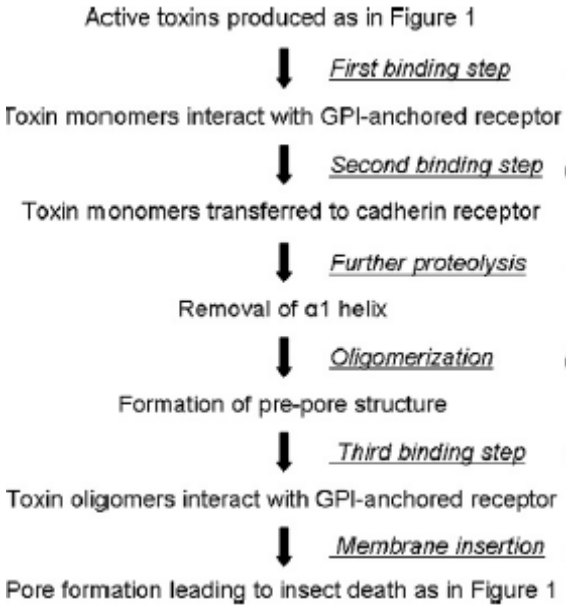

Figure 1-2: Schematic representation of the steps leading to pore formation and insect death according to the sequential binding model (Vachon et al. 2012)

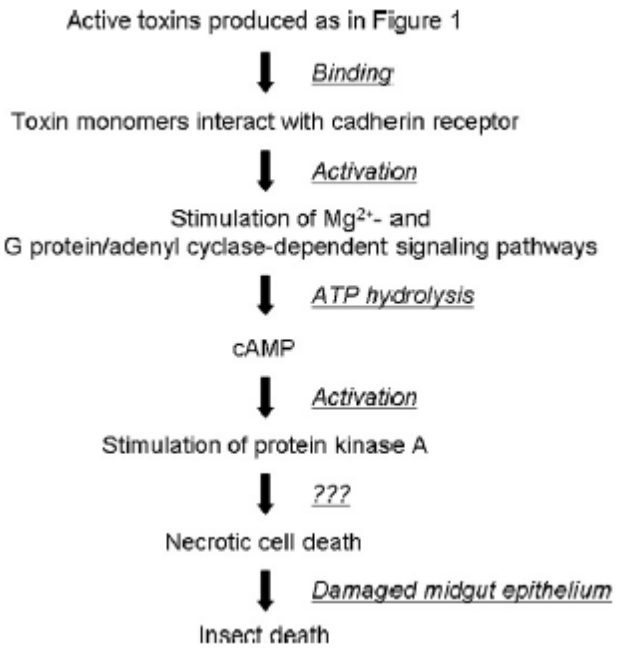

Figure 1-3: Schematic representation of the steps leading to insect death according to the signaling pathway model (Vachon et al. 2012)

Most strains of B. thuringiensis carry and express more than one cry gene. The spectrum of insecticidal activity of each strain depends upon the combination of individual Cry toxins present in their parasporal crystals (Carlson and Kolsto 1993; Gonzalez et al. 1982) and their level of expression (Masson et al. 1998). Different Cry toxins are specifically active against different insect orders, especially Lepidoptera, Coleoptera, Diptera but also against other invertebrates such as nematodes (Adang et al. 1985; Bravo et al. 2007; de Barjac and Frachon 1990) and as reviewed by (de Barjac 1978; Heimpel 1967; Herrnstadt et al. 1987; Hofte and Whiteley 1989; Schnepf et al. 1998). Historically the cry1 genes were understood to encode proteins toxic to lepidopterans; the cry2 genes to encode proteins toxic to both lepidopterans and dipterans; the cry3 genes to encode proteins toxic to coleopterans; and the cry4 genes to encode proteins toxic to dipterans alone (Crickmore et al. 1998). The current nomenclature, based solely on amino acid identity, allows closely related toxins to be ranked together (Crickmore et al. 2014).

Genomic DNA from DSL B. thuringiensis strain ATCC 13367 was subjected to whole genome sequencing and DNA contigs were annotated by PROKKA software and BLASTn queries. Only the cry1Ba4 gene was identified among the annotated sequences in B. thuringiensis strain ATCC 13367. Targeted PCR amplification was also used to confirm the absence of other cry genes (see Appendix C: Virulence factors of). This is consistent with the strong association of the Cry1B toxin with B. thuringiensis ssp. thuringiensis (Martínez et al. 2005). Cry1 toxins are mostly lepidopteran specific and insoluble at acidic or neutral pH but soluble at alkaline pH, with pH values of 10 or

above required for effective solubility (Hofte and Whiteley 1989; Huber et al. 1981; Lecadet et al. 1999).

A relational B. thuringiensis toxin specificity database giving known insecticidal activities of Cry1B and Cry1Ba toxins identified in ATCC 13367 showed that these are principally active against Lepidoptera; however, they also affect several Coleoptera and some Diptera species (COGEM 2014; van Frankenhuyzen 2009; van Frankenhuyzen 2013):

Lepidoptera:

- Actebia fennica,
- Agrotis ipsilon,
- Artogeia rapae,
- Bombyx mori,
- Cacyreus marshalli,
- Chilo suppressalis,
- Choristoneura fumiferana,
- Conopomorpha cramerella,
- Crocidolomia binotalis,
- Cydia pomonella,
- Diacrisia obliqua,
- Diatraea grandiosella, Diatraea saccharalis,
- Epinotia aporema
- Epiphyas postvittana,
- Helicoverpa zea, Helicoverpa armigera, Helicoverpa punctigera
- Heliothis virescens,
- Hellula undalis,
- Hyphantria cunea,
- Lambdina fiscellaria,
- Lymantria dispar
- Malacosoma disstria,
- Mamestra brassicae
- Orgyia leucostigma,
- Ostrinia nubilalis,
- Pectinophora gossypiella,
- Perileucoptera coffeella,
- Phthorimaea operculella,
- Pieris brassicae,
- Plutella xylostella,
- Pseudoplusia includens,
- Spodoptera exigua, Spodoptera littoralis,Spodoptera frugiperda,
- Thaumetopoea pityocampa,
- Trichoplusia ni
- Wiseana cervinata, Wiseana copularis, Wiseana jocosa,

They are also active against

Diptera:

- Lucilia cuprina,
- Musca domistica, and

Coleoptera:

- Anoplophora glabripennis
- Anthonomus grandis
- Chrysomela scripta,
- Hypothenemus hampei
- Leptinotarsa decemlineata
- Phaedon cochleariae

Dipteran toxicity of Cry1Ba is of less interest from a non target safety perspective because those toxicities occur at high dose levels compared to Diptera-active proteins (COGEM 2014).

Cyt Toxins

Not all B. thuringiensis species are known to produce Cyt toxins. Cyt toxins have been reported in dipteran-specific B. thuringiensis strains such as subspecies israelensis (Chang et al. 1993; Crickmore et al. 1995; Crickmore et al. 1998) and reviewed by (De Maagd et al. 2003; Gill et al. 1992; Palma et al. 2014). B. thuringiensis ssp. israelensis is known to be mosquitocidal and produces a mixture of Cyt toxins which have cytolytic and hemolytic activity in vitro and are toxic towards Aedes, Culex and Anopheles species (Crickmore et al. 1995; Wu et al. 1994). Some Cyt toxins have been reported to also be possibly active against cancer cells (van Frankenhuyzen 2009). Certain B. thuringiensis strains produce Cyt toxins, which possess cytolytic activity against a variety of eukaryotic cells and erythrocytes (Knowles et al. 1989).

The Cyt toxins of mosquitocidal B. thuringiensis display a mechanism of cell-membrane interaction different from that of the Cry toxins. Cyt toxins do not bind to protein receptors but directly form pores in the membrane (Bravo et al. 2007; Gill et al. 1987; Knowles et al. 1989; Thomas and Ellar 1983) or destroy the membrane in a detergent-like interaction (Butko 2003).

Health Canada scientists subjected genomic DNA from B. thuringiensis strain ATCC 13367 to whole genome sequencing, and cyt genes were not detected in B. thuringiensis strain ATCC 13367 following in silico screening.

Vegetative Insecticidal Protein

Vegetative insecticidal proteins (Vip 1-4) are secretable proteins of B. thuringiensis, some of which are toxic to species with little susceptibility to Cry toxins (Estruch et al. 1996; Palma et al. 2012) (and reviewed in (Palma et al. 2014)). Vips are secreted by

approximately 15% of B. thuringiensis strains starting at mid-log phase of vegetative growth and continuing during sporulation. They have potent broad spectrum insecticidal activity (Estruch et al. 1996; Palma et al. 2012). Vip1 and Vip2 function as a binary toxin. Vip1/Vip2 showed toxic activity against coleopteran larvae (e.g., Diabrotica spp.) and aphid pests (Palma et al. 2014). The Vip3 toxins have a wide spectrum of activity against lepidopteran insects (Estruch et al. 1996; Milne et al. 2008; Palma et al. 2012), including Black Cutworm (Agrotis ipsilon), Fall Armyworm (Spodoptera frugiperda), Beet Armyworm (Spodoptera exigua), Tobacco Budworm (Heliothis virescens), and Corn Earworm (Helicoverpa zea) (Estruch et al. 1996). Three major subfamilies of Vip3 exist, designated Vip3A, Vip3B and Vip3C. There is evidence that Vip3 proteins act by oligomerization and form pores. Vip3A toxins bind to and lyse insect gut epithelial cells (Lee et al. 2003). The target and mode action of Vip4 toxins are not known.

Health Canada scientists subjected genomic DNA from B. thuringiensis strain ATCC 13367 to whole genome sequencing and vip genes were not detected in B. thuringiensis strain ATCC 13367 following in silico screening.

β-exotoxin

β-exotoxin is a thermostable nucleotide analogue, formerly known as thuringiensin, exhibiting insecticidal properties and produced during vegetative growth in certain strains of B. thuringiensis, including some strains of B. thuringiensis spp. thuringiensis. It exhibits non-specific activity, killing a wide range of pest invertebrates including lepidopterans, dipterans, hymenopterans, hemipterans, isopterans, orthopterans, nematodes and mites (reviewed by (Glare and O'Callaghan 2000)). β-exotoxin causes lesions in the liver, kidney and adrenal glands of vertebrates (Boucias and Pendland 1998) and chromosomal aberrations in human blood cultures through the inhibition of the DNA-dependant RNA polymerase (Meretoja et al. 1977). Analysis of several soil isolates of B. thuringiensis found approximately 58% of strains produced active β-exotoxin (Perani et al. 1998). Because β-exotoxin is toxic towards vertebrates, most commercial preparations of B. thuringiensis are prepared from isolates that lack the ability to produce β-exotoxin (Hernández et al. 2003) and as reviewed in (McClintock et al. 1995). The WHO has banned the use of β-exotoxin producing strains from public use to avoid the potential for adverse effects in non-target organisms (Hernández et al. 2001; Ohba et al. 1981). Synthesis of the β-exotoxin requires the presence of a cluster of 11 genes usually located on a plasmid (Liu et al. 2014).

Health Canada scientists subjected genomic DNA from DSL B. thuringiensis strain ATCC 13367 to whole genome sequencing and β-exotoxin gene was not detected following in silico screening.

Bacillus cereus-like toxins

Some strains of B. thuringiensis , including B. thuringiensis ssp. thuringiensis, produce the diarrheal-type enterotoxins that characterize B. cereus (Abdel-Hameed and Landen 1994; Damgaard 1996; Hansen and Hendriksen 2001; Hyldebrink Damgaard 1995;

Jackson et al. 1995; Jensen et al. 2002; Rosenquist et al. 2005); albeit at lower levels than those associated with B. cereus (Hyldebrink Damgaard 1995). Hemolysin BL (HBL), non-hemolytic enterotoxin (NHE) and cytotoxin K (CytK) are enterotoxins that have been linked to food poisoning outbreaks involving B. cereus (Fagerlund et al. 2010; Lund and Granum 1997; Lund et al. 2000; Schoeni and Wong 2005; Stenfors Arnesen et al. Granum 2008) and that may form pores in the membrane of mammalian intestinal epithelial cells, causing osmotic lysis (Beecher and Wong 1997; Hardy et al. 2001; Haug et al. 2010). B. cereus produces other virulence factors known to play a role in its pathogenicity and ability to cause gastrointestinal and other types of infection. These include hemolysins (hemolysin I (aka cereolysin O), hemolysin II [HlyII] and III [HlyIII]), Enterotoxin FM (EntFM now known as CwpFM a potential cell wall peptidase implicated in adhesion, biofilm formation and virulence), phospholipase C (PLC) of which three variants are recognized: phosphatidylinositol hydrolase (PI-PLC), phosphatidylcholine hydrolase (PC-PLC) and sphingomyelinase (SMase). The transcription factor PlcR is considered a virulence factor as it is involved in the expression of most known virulence factors in B. thuringiensis, including phospholipase C, proteases, cell surface proteins, hemolysins and enterotoxins during vegetative growth (Agaisse et al. 1999; Bouillaut et al. 2005; Gominet et al. 2001; Lereclus et al. 1996; Salamitou et al 2000; Tran et al 2010).

Health Canada scientists confirmed the presence of hbl, nhe, cytK, hemolysin I, hemolysin II and III and entFM (cwpFM) genes in B. thuringiensis strain ATCC 13367 by in silico screening of the whole genome sequence and PCR analysis as well as protein expression of HBL and Nhe by immunochromatography.

Other virulence factors

B. thuringiensis produces other virulence factors, which are thought to facilitate its development within the insect host and to contribute to its pathogenicity and toxicity. These include degradative enzymes such as phospholipases, and a number of extracellular compounds including the S-layer proteins (SLP), that contribute to virulence (Gohar et al. 2005; Mignot et al. 2001; Pena et al. 2006).

B. thuringiensis also produces metalloproteases, a serine alkaline protease and a cysteine protease, which play a role in gene expression and cell lysis for sporulation. These proteases also play a role in entomotoxicity by ensuring proper maturation of spores and of the insecticidal crystalline protein, cleaving antibacterial proteins of insect hosts, and converting inactive protoxins to active toxins (Brar et al. 2007). The immune inhibitor A (InhA) metalloprotease has shown that it specifically cleaves antibacterial peptides produced by insect hosts, suggesting that it may contribute to the virulence of B. thuringiensis. InhA and InhB interfere with the humoral defense system in pupae of Hyalophora cecropia (Edlund et al 1976; Grandvalet et al. 2001).

The secreted insecticidal protein (Sip) is a secreted protein that shows activity against coleopteran larvae but its mode of action remains unknown (Donovan et al. 2006).

B. thuringiensis produces dense biofilms under various conditions, possibly including those at the host intestinal epithelium (Fagerlund et al. 2014). Biofilms may confer resistance to antimicrobial agents and could contribute to enhanced persistence (Auger et al. 2009). Some serovars of B. thuringiensis (H4 and H13) produce chitinase at low levels. Chitinase can enhance the insecticidal activity that (Liu et al. 2002). Parasporins are proteins associated with parasporal inclusions of B. thuringiensis which are non-hemolytic, but can kill human leukemic T cells (MOLT-4) and human cervical cancer cells (HeLa), although not normal T cells. This cytocidal activity occurs only when parasporins are degraded by proteases (trypsin and proteinase K) (Katayama et al. 2005; Mizuki et al. 2000; Ohba et al. 2009).

Health Canada scientists confirmed the presence of InhA and chitinase genes in B. thuringiensis strain ATCC 13367 by in silico screening. See Table A-4 for Virulence factors and toxins associated with B. thuringiensis present in B. thuringiensis strain ATCC 13367.

3.1.4 Effects

3.1.4.1 Environment

Terrestrial and aquatic plants

Literature reviews indicate no known adverse effects on terrestrial and aquatic plants from B. thuringiensis in spite of a long history of exposure to a wide range of naturally occurring strains of B. thuringiensis and widespread use of pesticidal strains in agriculture, forests and aquatic environments. In pathogenicity and toxicity testing[6] performed by Environment and Climate Change Canada scientists, strain ATCC 13367 did not cause any pathogenic or toxic effect in the model plant used. There was no difference in shoot or root length; shoot or root mass in Trifolium pretense (Red Clover) grown in artificial or field-collected soils exposed to six repeated treatments of 5.2×10^6 vegetative cells of ATCC 13367 per gram of soil (Princz 2005).

Based on the various modes of action of B. thuringiensis toxins, no adverse effects in plants are expected; moreover, genetically modified plants expressing Cry toxins are not affected by it.

Furthermore, some B. thuringiensis strains have even been reported to possess biocontrol potential against some plant pathogens such as Erwinia carotovora (Dong et al. 2004) or Fusarium roseum var sambucinum (Sadfi et al. 2001).

[6] Tests were conducted at the Biological Assessment and Standardization Section, Soil Biotechnology Lab according to “Guidance Document for Testing the Pathogenicity and Toxicity of New Microbial Substances to Aquatic and Terrestrial Organisms (EPS 1/RM/44, March 2004).

Terrestrial and aquatic vertebrates

To date no mammalian or avian toxicity study has demonstrated any adverse health effect from any B. thuringiensis subspecies spores or from commercialised strains (reviewed by (McClintock et al. 1995; PMRA-HC 2006; US EPA 1998)).

Infectivity and pathogenicity studies submitted to the US EPA to support registration of various B. thuringiensis subspecies as insecticides consistently show clearance of B. thuringiensis organisms from rodents after oral, pulmonary, or intravenous administration and significant adverse effects on body weight gain, clinical observations or necropsy findings are absent (as reviewed (McClintock et al. 1995)).

In addition, B. thuringiensis subspecies currently registered as pesticides, including subspecies kurstaki, tenebrionis, israelensis and aizawai have been tested in mallard ducks and bobwhite quails for non-target effects as part of studies required to support registration. None of B. thuringiensis subspecies showed toxicity to these avian species after acute and subacute testing (US EPA 1998).

Oral toxicity testing of a B. thuringiensis ssp. thuringiensis commercial spore preparation performed in rats showed no fatalities or signs of toxicity at a dose of $2x10^{12}$ spores (Fisher and Rosner 1959). In rats fed with spores, B. thuringiensis vegetative cells were detected in fecal and intestinal samples of all animals. B. thuringiensis spores were capable of germination in the gastrointestinal tract; however, vegetative cells survived poorly in the gut and no cytotoxic effect was detected (Wilcks et al. 2006a). Moreover, there has been no report of adverse effects in insectivorous mammals after ingestion of moribund insects killed by B. thuringiensis commercial pesticides(Bellocq et al. 1992) (and as reviewed in (Glare and O'Callaghan 2000)). No adverse effect on the indigenous gut bacteria was observed when 10^8 B. thuringiensis spores were fed to rats (Wilcks et al. 2006b). Vegetative cells of B. thuringiensis ATCC 10792^T decreased by 90% in 4h in cattle rumens while the spores did not decrease even after 24h (Adams and Hartman 1965)

B. thuringiensis ssp. kurstaki, B. thuringiensis ssp. israelensis and B. thuringiensis ssp. tenebrionis known to produce B. cereus type enterotoxins showed no negative effects when given to rats at oral doses of $1x10^{12}$ spores (+crystals), over three weeks, or a single subcutaneous dose of $1x10^6$ spores (+crystals) (Bishop, Johnson, Perani 1999). In chickens fed with vegetative cells of B. thuringiensis ssp. kurstaki, bacteria were detected up to day 4 in the digestive system and feces. In comparison, chickens fed with spores of B. thuringiensis ssp. kurstaki, bacteria were detected for a period of 13 days in in the digestive system and feces. No signs of adverse effects were observed in any of the chickens (Zhang et al. 2012).

There is no information available on effects of B. thuringiensis ssp. thuringiensis on aquatic vertebrates, possibly due to the fact that only B. thuringiensis ssp. israelensis and B. thuringiensis ssp. kurstaki have been used as biocides against aquatic pests in the past.

B. thuringiensis subspecies currently registered as pesticides, including subspecies kurstaki, tenebrionis, israelensis and aizawai have been tested in Trout and Bluegill as part of studies required to support registration. No toxicity or pathogenicity was noted from exposure to any B. thuringiensis subspecies (US EPA 1998).

No known equivalent to the Cry toxin receptor is found in mammalian species and therefore these toxins are considered harmless to mammals (Betz et al. 2000; Broderick et al. 2006; Hofte and Whiteley 1989; US EPA 1998) and based on the mode of action of Cry toxins, no adverse effects are expected in terrestrial and aquatic vertebrates.

Invertebrates

A variety of B. thuringiensis strains are used as insecticides. They are generally active against the larval stage of the target insects and have limited toxicity in adult insects. Different B. thuringiensis strains have different and specific target insect ranges based on the various Cry toxins that they produce. Historically terrestrial larvae of lepidopterans were the only known susceptible target for B. thuringiensis Berliner. Later on, new strains of B. thuringiensis were discovered bearing other forms of Cry toxin, thus changing the range of susceptible targets and expanding the application of B. thuringiensis as a biocide to include, for example, aquatic larvae of Mosquitos and Black Flies (B. thuringiensis spp. israelensis), Gypsy Moths, Spruce Budworm and Forest Tent Caterpillars (B. thuringiensis spp. kurstaki) and Colorado Potato Beetle (B. thuringiensis spp. tenebrionis). Most other invertebrates are not susceptible to Cry toxins (as reviewed in (English and Slatin 1992). Because of the distinct host specificities of different B. thuringiensis strains, the assessment of effects in target species in this report will focus on describing the effects of B. thuringiensis ssp. thuringiensis, B. thuringiensis strain ATCC 13367 and the insecticidal toxin known to be present in B. thuringiensis strain ATCC 13367, Cry1Ba.

Effects on target species

Numerous B. thuringiensis subspecies have been isolated from dead and dying insect larvae, and in most cases, the isolate has toxic activity against the insect from which it was isolated. Based on Heimpel and Angus (Heimpel and Angus 1960), B. thuringiensis ssp. thuringiensis is primarily toxic to lepidopterans and commercial preparations based on B. thuringiensis ssp. thuringiensis are active against the Lepidoptera (Arthur and Angus 1965).

Laboratory experiments using B. thuringiensis ssp. thuringiensis against larvae of Thymelicus lineola (lepidopteran, European Skipper) showed death after ingestion. Effects included sluggishness, inhibition of feeding, death within 24-48 h at 22°C and the presence of vegetative cells in the host. In field trials, B. thuringiensis ssp. thuringiensis was effective against Colias eurytheme (lepidopteran, Alfalfa Caterpillar) (Arthur and Angus 1965). The ED_{50} (median effective dose, including paralysis in 6 h) of parasporal inclusion free of spores for B. thuringiensis ssp. thuringiensis is reported as

26 µg/g for Bombyx mori (lepidopteran, Silkworm) larvae and LD_{50} (median lethal dose) in 48 hours is 5 µg/g (Angus 1967).

The purified Cry1Ba toxin, which is present in ATCC 13367, caused 40% mortality at a dose of 8,000 ng/cm^2 in first instar larvae of Asymmathetes vulcanorum (coleopteran, Colombian Potato Weevil) (Gómez et al. 2012). The LC_{50} (median lethal concentration) of Cry1B toxin in first instar larvae of Spodoptera exigua (lepidopteran, Beet Armyworm) is 0.86 µg/cm^2 (Qiong et al. 2012).

Cry1B is toxic to Hypothenemus hampei (coleopteran, Coffee Berry Borer) by feeding assays (López-Pazos et al. 2009). Cry1B has variable toxicity to Spodoptera frugiperda (lepidopteran, Fall Armyworm) and its toxicity varies with the origin of the target insect populations (Monnerat et al. 2006). Cry1B is highly active against first instar Thaumetopoea pityocampa (lepidopteran, Pine Processionary) larvae with a LC_{50} of 1830 pg/µL (Rausell et al. 1999).

Effects on non-target species

Testing of B. thuringiensis strain ATCC 13367 spores by Environment and Climate Change Canada scientists found no significant effects on survival or reproduction in Folsomia candida (Collembollan, Springtail) or Eisenia andrei (Haplotaxida Tiger Worm) exposed to concentrations of 5.67x10^6 or 25.5x10^6 of vegetative cells per gram of soil[7] (Princz 2005).

The purified Cry1Ba toxin, which was detected in ATCC 13367, was tested in Honey Bees, and no significant effect was observed on survival rate of adults bees fed with the toxin (Malone et al. 2001; Malone et al. 1999). As there is little information on the effects of B. thuringiensis ssp. thuringiensis or Cry1B and Cry1Ba toxin in non-target terrestrial invertebrate species, the effects of better-studied subspecies on non-target terrestrial invertebrates will be also considered here.

Commercial formulations of B. thuringiensis ssp. kurstaki do not measurably affect abundance, distribution, diversity or feeding behaviour of non-target soil micro arthropods (Addison et al. 2006), nor did they have significant effect on survival of adult honey bees (Malone et al. 1999).

The purified Cry1Ac toxin was tested against 14 species of insects and no significant effect was observed in Myzus persicae (hempitera), Blatella germanica (blattodea), Aedes aegypti (diptera), Leptinotarsa decemlineata, Diabrotica undecimpuncatata, Anthonomus grandis (coleoptera), benefical Apis millifera (hymenoptera, Honey Bee),

[7] Tests were conducted at the Biological Assessment and Standardization Section, Soil Biotechnology Lab according to “Guidance Document for Testing the Pathogenicity and Toxicity of New Microbial Substances to Aquatic and Terrestrial Organisms (EPS 1/RM/44, March 2004).

Nasonia vitripennis (hymenoptera, Parasitic Wasp), Chrysopa carnea (neuroptera, Green Lacewing), or Hippodamia convergens (coleoptera, Ladybug) (Sims 1995). Bioassays of purified B. thuringiensis toxins indicate that Cry9C and Cry1F toxins are relatively non-toxic to Monarch first instar larvae. Monarchs were sensitive to Cry1Ab and Cry1Ac toxins, but sensitivity decreases in older larvae (Hellmich et al. 2001).

Many studies are available on the effect of transgenic crops bearing various Cry toxins (especially Cry1Ab, Cry3Bb1) on soil macro-organisms such as Woodlice, Collembolans, Mites, Earthworms, Snails and Nematodes in the field. Generally no significant effects were observed when comparing transgenic crops to non-transgenic crops (reviewed in (Icoz and Stotzky 2008; Yu et al. Wu 2011)). Meta-analysis of studies regrouping 26 arthropod taxa demonstrated no effect of transgenic B. thuringiensis corn on common herbivores, or predatory and parasitoid arthropods in fields in southern Europe (Comas et al. 2014). Meta-analysis of 42 field experiments involving transgenic crops bearing various Cry toxins indicates that non-target invertebrates are generally more abundant in transgenic cotton and transgenic maize fields than in non transgenic fields managed with insecticides; however, in insecticide free control fields, some non-target taxa are also less abundant (Marvier et al. 2007).

Reports of effects of pollen from transgenic B. thuringiensis Cry toxin-expressing plants on butterflies are contradictory, with some studies reporting effects on monarch larvae (Jesse and Obrycki 2000; Losey et al. 1999), and others reporting no adverse effects on butterflies (Hellmich et al. 2001; Wraight et al. 2000). Such a difference in effect between larvae and adults is expected. The effects observed seem to be limited to pollen from one particular hybrid of transgenic B. thuringiensis-corn out of the three tested (Hellmich et al. 2001; StanleyHorn et al. 2001; Zangerl et al. 2001).

A report drafted to study the impact of genetically modified (GM) crops expressing multiple B. thuringiensis proteins and whether interactions between B. thuringiensis proteins can be predicted showed that B. thuringiensis pesticidal proteins can be classified as having high toxicity to non-target invertebrates when active in the 0.01 - 0.10 µg/mL range (below 25% percentile; dipteran active proteins), medium toxicity when active in the 0.10 -10 µg/mL range (lepidopteran, dipteran and coleopteran-active proteins), and low toxicity when LC_{50} are in the 10-100 µg/mL range (above 75% percentile; coleopteran and nematodan – active proteins) (COGEM 2014).

Aquatic invertebrates

Historically, terrestrial larvae of lepidopterans were the only known susceptible target for B. thuringiensis Berliner. Similarly information received from other jurisdictions and the historical record of ATCC 13367 indicates that the target range of ATCC 13367 most likely would have been terrestrial invertebrates belonging to the insect order lepidoptera and as such all aquatic invertebrates are considered non-target species of the DSL strain. As there is little information on the effects of B. thuringiensis ssp. thuringiensis on aquatic species, the effects of better-studied subspecies used in the aquatic environment and tested on non-target aquatic invertebrates will be considered here.

B. thuringiensis subspecies currently registered as pesticides, including subspecies kurstaki, tenebrionis, israelensis and aizawai have been tested on Daphnia, Grass Shrimp and Copepods as part of studies required to support pesticide registration. Subspecies kurstaki and israelensis were moderately toxic and aizawai was highly toxic to Daphnia, but none was toxic to Marine Grass Shrimp or Copepods (US EPA 1998).

Pesticidal strains of B. thuringiensis ssp. israelensis have been deliberately applied to aquatic environments for mosquito and blackfly control. Effects on non-target species in this context are summarized below:

- B. thuringiensis ssp. israelensis tested against chironomid larvae in experimental ponds and in golf course ponds showed reductions in populations of chironomid midges (Diptera) (Ali 1981);
- B. thuringiensis ssp. israelensis used in golf course ponds had no adverse effects on non-target insects such as rotifers, Cyclops spp., Daphnia spp., Baetis spp., ostracods, corixids, notonectids or coleopterans (Ali 1981); and
- B. thuringiensis spp. israelensis spore preparations used to control blackfly in aquatic environments showed no negative effects on non-target aquatic macro invertebrates (Heptahenia group, Hexagenia, Anthopotamus, Dicrotendipes) with the exception of Petrophila and Polypedilum where a small decrease in number was detected (Jackson et al. 1994).

Exposure of Acropora millepora and Acropora tenuis (Coral Larvae and Juvenile Corals) to B. thuringiensis ssp. israelensis insecticide, at concentrations 10 to 100 fold higher than the effective concentration on target Mosquitoes, showed no negative effect on different stages of development. Lanthella basta (Adult Corals and Sponges) showed no evidence of Coral or Sponge disease (Negri et al. 2009).

Population level effects from widespread application of B. thuringiensis: Ecological Effects

A field study following aerial applications of B. thuringiensis used against Jack Pine Budworm in Ontario under natural conditions showed no detectable impact on the abundance of small mammals (Rodents and Shrews) in the treated area (Innes and Bendell 1989). An integrated 6-year study of the effect of B. thuringiensis ssp. israelensis on zooplankton, insects and breeding birds in wetlands showed insect densities were reduced; however, no negative effect was observed in zooplankton or breeding birds (Hershey et al. 1998; Niemi et al. 1999). A two year study of B. thuringiensis ssp. israelensis as a larvicide on benthic macroinvertebrate communities in wetlands showed effects in insect populations but minimal effects on non-insect macroinvertebrates and it was generally considered safe for non-target species (Hershey et al. 1998). A field study performed in a stream channel showed no effect of B. thuringiensis ssp. kurstaki applied at concentration 100-times higher than the expected environmental concentration of formulated insecticides, on a variety of aquatic

insects collected from the natural environment (Kreutzweiser et al. 1992). Tests of the effects of B. thuringiensis ssp. kurstaki on the microbial community in a natural aquatic environment, when applied at concentrations 100 and 1000 times higher than the expected environmental concentration of formulated insecticides, concluded that B. thuringiensis ssp. kurstaki had no adverse effect on the microbial community and posed little risk to the aquatic environment (Kreutzweiser et al. 1996).

Some studies report the effects of Cry toxins. The addition of purified Cry toxin to soil in a glass house experiment caused no significant or measurable effect on the microbial community (Griffiths et al. 2007). In addition, the amount of Cry1Ab toxin released in root exudates and biomass of transgenic B. thuringiensis-corn had no effect on a species of earthworm or on the total numbers of nematodes, protozoa, bacteria, or fungi extracted from the experimental microcosms or soil (Muchaonyerwa et al. 2002; O'Callaghan et al. 2005; Saxena and Stotzky 2001).

The insecticidal property of B. thuringiensis is mostly due to the presence of Cry toxins, the toxic effects of which are limited to larval stages of the target host and are ineffective against adults. There is no report in the literature showing effects of B. thuringiensis used as insecticide on adult populations of target hosts. Also, there is no report in the literature of any adverse ecological effects on biodiversity or food chain supply following the use of commercial preparation of B. thuringiensis against pests found in aquatic or terrestrial environments.

3.1.4.2 Human health

In general, B. thuringiensis is considered non-pathogenic to humans. Despite its widespread occurrence in nature, B. thuringiensis has been infrequently isolated from clinical samples, and infections in humans are rare. The incidence of infections in immunocompetent individuals is extremely low.

One study on human volunteers was found in the literature, in which volunteers ingested 1 g of a commercial preparation of B. thuringiensis spores ($9x10^9$ cells) in capsules daily for 5 days and 5 out of 18 subjects also inhaled 100 mg of the powder ($9x10^8$ cells) daily for 5 days. No observable adverse effects were recorded for the duration of the study (Fisher and Rosner 1959).

Surveillance studies in areas where B. thuringiensis is used as a pesticide provide data on a large population exposed to spores. One surveillance study was performed on two populations, one of 80,000 and one of 40,000, within the spray area of a commercial preparation of B. thuringiensis spores. The four largest clinical laboratories were enrolled and all clinical samples positive for Bacillus sp. collected during the spray and one month after were tested for B. thuringiensis. B. thuringiensis was isolated from 55 patients. Of these isolates, 52 were deemed to be contaminants and not the cause of any clinical illness. For the other three, B. thuringiensis isolates could not be established with certainty as the cause of the infection as these patients had pre-existing conditions (Green et al. 1990). Microbiological and epidemiological surveillance of another area

sprayed with a commercial preparation of B. thuringiensis spores did not reveal any related cases of diarrhea or infection linked to B. thuringiensis, in the general population or in ground spray-workers, even though B. thuringiensis was isolated from patient clinical samples during the spray period (Noble et al. 1992).

A reporting mechanism is in effect in Canada for all pesticides. There is a total of 58 incident reports filed for pesticides indicating B. thuringiensis as the active ingredient. Most reports were classified as minor, and symptoms reported included skin rash, coughing, irritated throat, headache, insomnia, runny nose, anxiety, bronchitis, nose bleed, edema, congestion, itchy skin, sneezing, difficulty breathing, diarrhea, hives and asthma attack. One incident was reported as major and six as moderate. Of these, few could have resulted from exposure to B. thuringiensis. A total of 11 reports were from people with pre-existing asthma (PMRA-HC 2016b).

A few commercial and non commercial B. thuringiensis strains possessing the three enterotoxin genes hbl, nhe and cytK have been isolated from fruits and vegetables such as tomatoes, cucumbers and peppers, however no cases of infection were linked to these strains (Frederiksen et al. 2006).

To date no mammalian toxicity study has demonstrated that commercial spore preparations of any B. thuringiensis subspecies cause adverse health effects by any route of exposure (reviewed in (McClintock et al. 1995; PMRA-HC 2006; US EPA 1998)). No known toxins or metabolites of B. thuringiensis have been identified to act as endocrine disrupters or immunotoxicants (US EPA 1998).

No known equivalent to the Cry toxin receptor is found in mammalian species; and therefore, these toxins are considered harmless to humans and other mammals (Betz et al. 2000; Broderick et al. 2006; Hofte and Whiteley 1989; US EPA 1998).

Experimental studies

In vitro and in vivo tests were conducted by Health Canada scientists to evaluate the potential of B. thuringiensis strain ATCC 13367 to cause cytotoxicity and adverse immune effects. No change was observed in human colonic epithelial cells (HT29) exposed to B. thuringiensis ATCC13367 spores up to 24h after exposure. B. thuringiensis strain ATCC 13367 was hemolytic to red blood cells from various sources at both 28°C and 37°C (see Table B-2: Growth characteristics of B. thuringiensis strain ATCC 13367 on solid media at various temperatures). BALB/c mice were endotracheally exposed to 10^6 or 10^5 spores or vegetative cells of B. thuringiensis strain ATCC 13367. Overall spore exposure showed no adverse effects and B. thuringiensis spores were cleared within 4 days post exposure. However, endotracheal exposure to vegetative cells (10^5 – 10^6) produced shock-like symptoms in mice, (including lethargy, ruffled fur, hunched appearance and respiratory distress) within 2h after exposure, and granulocyte infiltration in the lung 4h after exposure (Tayabali et al. 2011).

BALB/c mice intratracheally instilled with 3.4 x 10^6 to 3.5 x10^5 CFU B. thuringiensis ssp. kurstaki and B. thuringiensis ssp. israelensis spores (commercial B. thuringiensis based biopesticides) showed an acute inflammatory response. The response was dominated by neutrophils after 24 hours and followed by normalization of neutrophil numbers and inflammation dominated by lymphocytes and eosinophils on day 4 and few inflammatory cells present in the lung lumen after 70 days which may lead to sub chronic lung inflammation most likely due to the prolonged presence of B. thuringiensis spores triggering and maintaining the inflammatory response (Barfod et al. 2010).

BALB/c mice repeatedly exposed to low dose aerosol inhalation of 2.52L/hour per mouse, theoretically calculated to be 1.9 x 10^4 CFU B. thuringiensis ssp. israelensis and 2.3 x 10^3 CFU B. thuringiensis ssp. kurstaki spores per exposure one hour per day for 5 days a week or for two weeks. 70 days after the end of the aerosol exposure, 3 out of 17 mice had interstitial lung inflammation. Plethysmography showed that inhalation of aerosol did not induce airway irritation (Barfod et al 2010).

Case Reports of infection or toxicity

Even though B. thuringiensis is not considered a human pathogen, some cases have been reported implicating it as a causative agent of infection.

B. thuringiensis has been associated with ocular infections and described to have potential ocular toxicity. Antibiotic susceptibility studies show efficacy of ciprofloxacin and vancomycin to treat ocular infections (Callegan et al. 2006).

- One farm worker developed an ocular infection and corneal ulcer in one eye accidently splashed with a commercial B. thuringiensis ssp. kurstaki product. The corneal ulcer resolved after 14 days of treatment with subconjunctival injections of gentamycin and cefazolin sodium (Samples and Buettner 1983).
- Combined toxin production as a group (enterotoxins, phospholipases, hemolysins and proteases) and motility have been associated with ocular virulence of B. thuringiensis in a rabbit eye infection model (Callegan et al. 2005).
- One case of periorbital cellulitis caused by B. thuringiensis was reported in a 7-year-old female (Peker et al. 2010).

B. thuringiensis has also been associated with surface wounds.

- B. thuringiensis was isolated from burn wounds (Damgaard et al. 1997).
- B. thuringiensis ssp. konkukian serotype H34 was isolated from open wounds (later reported as strain 97-27) (Hernandez et al. 1998). Experimental evidence showed that this strain can cause infection in immunosuppressed mice after cutaneous inoculation (Hernandez et al. 1999) and that it clusters in Clade I, as does B. anthracis (Hill et al. 2004). Subsequent comparison of the complete genome of B. thuringiensis 97-27 spp. konkukian revealed differences in virulence, metabolic competence, structural components and regulatory mechanisms and the phylogenetic tree suggests it is distinct from other B.

thuringiensis and is more like a pathogenic B. cereus strains or B. anthracis (Han et al. 2006).

- A research worker developed a soft tissue infection following an accidental hypodermic needle injury when handling a growth medium containing spores and Cry toxin crystals of B. thuringiensis ssp. israelensis and Acinetobacter bacteria. Intoxication resulting from a synergetic effect of the Acinetobacter and the Cry toxin crystals seemed to be the source of the pathology observed (Warren et al. 1984).

B. thuringiensis has been associated with bacteremia in a neutropenic patient and may have been responsible for severe pulmonary disease (Ghelardi et al. 2007).

Some authors believe that cases of illness caused by B. thuringiensis may have been misdiagnosed as B. cereus, as the former may not produce its characteristic insecticidal toxin crystals when incubated at 37°C, owing to the loss of the plasmids carrying the toxin genes (Granum and Lund 1997; Granum 2007). Therefore, the number of cases of B. thuringiensis food-borne illness may be under reported. The scientific literature reports that B. thuringiensis misidentified as B. cereus was found in patients suffering from gastroenteritis (Jackson et al. 1995). Bacillus isolated from food specimens initially identified as B. cereus through phenotypic methods and later identified as B. thuringiensis through PCR has been implicated in food poisoning from strawberries (McIntyre et al. 2008). Also, B. thuringiensis has been implicated in 4 outbreaks of food poisoning, with symptoms including nausea, diarrhea, abdominal cramps, vomiting, fever and headache (McIntyre et al. 2008). However, these B. thuringiensis isolates were from food specimens associated with the outbreaks and not from any clinical specimens from the outbreak, and therefore, it cannot be confirmed that B. thuringiensis was responsible. In comparison, B. cereus has been reported as the causative agent of food-related outbreaks in more than 100 events reported in Canada only.

Allergenicity

In guinea pigs, hypersensitivity tests were done by repeatedly administering a commercial spore preparation of B. thuringiensis by subcutaneous injection and topical application to abraded and intact skin over a 3-week period to prime the immune system caused slight erythema and edema, indicative of local irritation. No reaction was observed from application to intact skin. Challenge was performed two weeks after the last application. There was no evidence of allergic response (Fisher and Rosner 1959).

No severe allergic reaction to commercial B. thuringiensis preparation was reported among occupationally exposed ground spray workers. Where reactions were observed, reported symptoms included headache, nose, throat and eye irritation, dry skin and chapped lips (Noble et al. 1992).

There are no reports of sensitization to commercial B. thuringiensis formulations, which in turn supports the lack of allergic concerns with Cry toxins (as reviewed in (McClintock et al. 1995)). Only one study on farm workers showed that some tested individuals

showed induction of IgE and IgG antibodies and tested positive for skin allergy tests after spaying commercial B. thuringiensis pesticides, but there was no evidence of occupationally related respiratory symptoms (Bernstein et al. 1999).

Digestive fate studies conducted with Cry toxins produced in transgenic B. thuringiensis-plants and bioinformatic studies assessing the potential for allergic cross-reactivity of various Cry toxins revealed that none of the Cry toxins are of significant concern for allergenicity (Betz et al. 2000; Randhawa et al. 2011).

3.2 Hazard severity

3.2.1 Environment

The environmental hazard potential of B. thuringiensis strain ATCC 13367 for terrestrial and aquatic plants, terrestrial and aquatic vertebrates, and most terrestrial and aquatic invertebrates is estimated to be low. However, it is estimated to be high for larvae of some species of the order Lepidoptera and few species of the orders Coleoptera and Diptera.

1) B. thuringiensis can be differentiated from the other members of the B. cereus group by the production of Cry toxins, or presence of cry genes and associated bipyramidal crystals.
2) Historical as well as strain history data point to B. thuringiensis strain ATCC 13367 belonging to the subspecies thuringiensis.
3) The insecticidal effect of B. thuringiensis comes mostly from activity of the specific Cry toxins expressed, each of which has a narrow host range.
4) Analysis of the whole genome of B. thuringiensis strain ATCC 13367 revealed that it contains only the cry1Ba gene, indicating that adverse effects of B. thuringiensis strain ATCC 13367 are limited to larvae of the species that are susceptible to Cry1Ba: Lepidoptera:
    - Chrysomela scripta, Leptinotarsa decemlineata, Lucilia cuprina, Musca domistica, Hyphantria cunea, Diacrisia obliqua, Bombyx mori, Pectinophora gossypiella, Phthorimaea operculella, Lambdina fiscellaria, Conopomorpha cramerella, Wiseana cervinata, Wiseana copularis, Wiseana jocose, Malacosoma disstria, Cacyreus marshalli, Orgyia leucostigma, Perileucoptera coffeella, Agrotis ipsilon, Actebia fennica, Helicoverpa zea, Helicoverpa armigera, Heliothis virescens, Mamestra brassicae Pseudoplusia includes, Spodoptera exigua, Spodoptera frugiperda Spodoptera littoralis, Pieris brassicae, Artogeia rapae, Plutella xylostella, Chilo suppressalis, Ostrinia nubilalis, Crocidolomia binotalis, Diatraea saccharalis, Diatraea grandiosella, Hellula undalis, Thaumetopoea pityocampa, Choristoneura fumiferana, Cydia pomonella, Epiphyas postvittana, Epinotia aporema;

    Diptera:

    Lucilia cuprina, Musca domistica; and

    Coleoptera:

Anaplohora glabripennis, Anthonomus grandis, Chrysomela scripta, Leptinotarsa decemlineata, Phaedon cochleariae, Hypothenemus hampei.

5) Some fermentation by-products or exotoxins of B. thuringiensis may cause toxicity/pathogenicity towards daphnia, Honey Bees and other non-target insects; however, no toxicity has been attributed to Cry1Ba, except in the susceptible species identified above.
6) Unpublished Environment and Climate Change Canada data show that B. thuringiensis strain ATCC 13367 does not cause any adverse effects in toxicity testing on terrestrial invertebrates Folsomia candida (Collembolan), and Eisenia andrei (Tiger Worm) and the terrestrial plant Trifolium pretense (Red Clover).
7) B. thuringiensis pathogenicity and toxicity studies demonstrate that it has no adverse effects on non-target species including terrestrial and aquatic plants and vertebrates. Therefore, under normal circumstances it is not considered a hazard to healthy livestock or other organisms in the environment.
8) Despite its occurrence in nature and frequent and repeated releases into the environment as an insecticide, there is no evidence in the literature that any strains or subspecies of B. thuringiensis have adversely affected the targeted species at a population level in the environment causing epizootics or any consequential adverse effect on the receptor ecosystem.
9) A search in the species at risk public registry shows that none of the arthropods that the Cry1Ba is toxic against are listed as extirpated, endangered, threatened and of special concern (SARA 2016).

3.2.2 Human Health

The human hazard potential of B. thuringiensis strain ATCC 13367 is assessed to be low-medium.

1) B. thuringiensis can be differentiated from the other members of the B. cereus group by the production of Cry toxins, or presence of cry genes and associated bipyramidal crystals.
2) B. thuringiensis grows well at 37°C and 42°C.
3) Analysis of the genome of B. thuringiensis strain ATCC 13367 reveals that it contains genes for the following toxins: Cry1B, Cry1Ba, Nhe, HBL, CytK, hemolysin II and III, EntFM (now known as CwpFM), cereolysin O and phospholipases C.
4) B. cereus-like enterotoxins, Nhe, HBL and Cytk implicated in outbreaks of food-borne illness have historically been associated with B. cereus, and no commercialised strain of B. thuringiensis has been conclusively linked to any food poisoning outbreak; however, because B. cereus group members are difficult to differentiate, B. thuringiensis may be underreported as a causative agent of food-borne disease.
5) Cry toxins produced by B. thuringiensis are not toxic to mammals, because the mammalian gut lacks the unique receptor needed for its mode of action and the alkaline environment which permits activation of the toxin.

6) Various pathogenicity and toxicity studies have been conducted and to date no mammalian study has demonstrated that commercial spore preparations of any B. thuringiensis subspecies has led to any adverse health effects in mammals or shown that Cry toxins are toxic to mammals.
7) Very few infections are linked to B. thuringiensis. Those reported include ocular and wound infections and gastrointestinal illness. Although similar in nature to infections causedby B. cereus, the prevalence of these infections is much lower or could possibly be under reported.
8) In case of B. thuringiensis infection, effective antibiotics are available for treatment.
9) Public surveillance data (from countries including Canada), toxicity and pathogenicity data available on several strains of B. thuringiensis used broadly as insecticides (OECD 2007; PMRA-HC 2006; US EPA 1998; WHO 1999) show a general lack of adverse effects in humans.

Hazards related to micro-organisms used in the workplace should be classified under the Workplace Hazardous Materials Information System (WHMIS)[8].

4. Exposure Assessment

4.1 Sources of exposure

This assessment considers exposure to B. thuringiensis strain ATCC 13367 resulting from its addition to consumer or commercial products and its use in industrial processes in Canada.

B. thuringiensis was nominated to the DSL in 1993 for use in consumer and commercial products and added to the list in 1997.

Responses to a voluntary questionnaire sent in 2007 to a subset of key biotechnology companies, combined with information obtained from other federal government regulatory and non-regulatory programs, indicate that between 10,000 and 100,000 kg of products potentially containing B. thuringiensis strain ATCC 13367 were imported into Canada in 2006-2007 for consumer and commercial use.

Although the 2007 voluntary survey indicated that products potentially containing B. thuringiensis strain ATCC 13367 were imported for consumer and commercial use, no

[8] A determination of whether one or more criteria of section 64 of CEPA are met is based on an assessment of potential risks to the environment and/or to human health associated with exposure in the general environment. For humans, this includes, but is not limited to, exposure from air, water and the use of products containing the substances. A conclusion under CEPA may not be relevant to, nor does it preclude, an assessment against the criteria specified in the Hazardous Products Regulations, which is part of the regulatory framework for the Workplace Hazardous Materials Information System (WHMIS) for products intended for workplace use.

such uses were indicated during the section 71 of CEPA mandatory information gathering.

The Government conducted a mandatory information-gathering survey under section 71 of CEPA, as published in the Canada Gazette, Part I, on October 3, 2009 (section 71 Notice). The section 71 Notice applied to any persons who, during the 2008 calendar year, manufactured or imported B. thuringiensis strain ATCC 13367, whether alone, in a mixture or in a product. Responses to the section 71 Notice indicates products containing B. thuringiensis strain ATCC 13367 were imported in Canada during the 2008 reporting year, for pest control use, and in very small quantities for research and development (R&D). Although the section 71 Notice was intended to gather information about the specific strains on the DSL, the reported pest control uses seem unlikely to pertain specifically to B. thuringiensis strain ATCC 13367. Respondents that reported pest control uses market products containing other subspecies and strains of B. thuringiensis that are currently registered under the Pest Control Products Act (PCPA). As no products containing strain ATCC 13367 were registered in the past or are currently registered under the PCPA, it is believed that respondents have erroneously identified activities with other strains as pertaining to strain ATCC 13367. In addition to this, as per PMRA, B. thuringiensis strain ATCC 13367 has not been registered or used as a pesticidal strain in Canada. The identified R&D uses are not expected to result in widespread release to the environment. Although no other uses were reported for B. thuringiensis strain ATCC 13367 during the mandatory survey, it is available for purchase from the ATCC.

As B. thuringiensis strain ATCC 13367 is on the DSL, and so can be used in Canada without prior notification, it could be an attractive choice for commercialization. A search performed from the Canadian Patent database, indicates that:

- B. thuringiensis strain ATCC 13367 can be used as a microbial pesticide (Bok et al. 1993); however, such use must be in compliance with the PCPA for use in Canada;
- B. thuringiensis can be used in a insecticidal composition admixed with pyrethrum (Westall 1980) and in a process for improved control of aquatic insects (Branton and Kase 1987) but must be in compliance with the PCPA in such use in Canada; and
- Isolated and purified protein derived from B. thuringiensis ssp. thuringiensis displays cytotoxic effects against tumor cells and is also a method of treating a neoplastic cell (Aggarwal and Padilla 1996).

A search in the public domain on the patent database for the DSL-listed B. thuringiensis revealed the presence of strain ATCC 13367 as a antagonistic organism (Bok et al. 1993) and in treatment of plants in a microbial pesticide which may be in the form of cell, spores or suspension (Branly and Atkins 2001).

A search of the public domain (MSDS, literature and patents) revealed the following consumer, commercial and industrial applications of B. thuringiensis in general. These

represent possible uses of the DSL strain, as strain ATCC 13367 is likely to share the characteristics (modes of action) with other commercialized B. thuringiensis strains:

- Degradation of polar organic solvents (Middleditch and Lee 1994) ;
- Remediation of heavy metals or aromatic compounds (Ibeanusi 1998; Kafilzadeh and Mokhtari 2013; Mishra and Malik 2013);
- Bioremediation of halogen-contaminated soil (Orolin et al. 1998);
- Bioremediation of waste water (Cline 2001; Keeton Jr. 2007);
- Improving efficiency of phytoremediation (Babu et al. 2013; Kumar and Chandra 2004);
- Biosorption of heavy metals found frequently in polluted environments (Hassen et al. 1998);
- Plant growth promotion (Raddadi et al. 2007);
- Use as a probiotic for animal health (Kweon et al. 2012; Reneshwary et al. 2011); and
- B. thuringiensis strains can also be used to produce enzymes, biochemicals and biopolymers:

    - B. thuringiensis bacteriocins, autolysins, lactonases and other proteins could potentially be used as biopreservatives in the food industry, as biocontrol agents in agriculture and aquaculture (Bibiana and Nithyanand 2014), and in human health care to control pathogens, or as anticancer or antifungal agents (Cherif et al. 2003b; Choi et al. 2007; Raddadi et al. 2004; Raddadi et al. 2007; Yamashita et al. 2000);
    - B. thuringiensis chitinases, esterases could be used in the detergent industry; and
    - B. thuringiensis chitinases could be used for the production of biopolymers (Schallmey et al. 2004).

4.2 Exposure characterization

4.2.1 Environment

Environmental exposure to B. thuringiensis strain ATCC 13367 is estimated to be low based on responses to the voluntary survey and the section 71 Notice, and considering that reported pest control uses are unlikely to pertain to the DSL strain. It is recognized that B. thuringiensis strain ATCC 13367 is available for purchase from the ATCC, and could be used for several other consumer, commercial, industrial and agricultural activities identified as potential uses of B. thuringiensis strain ATCC 13367 as described in Section 2.1 Sources of Exposure. The following environmental exposure scenarios are therefore considered, based on potential future uses of B. thuringiensis strain ATCC 13367, and on the characteristics listed above.

In the event that potential future uses are realized in Canada, terrestrial species, including vertebrates, invertebrates and plants could be exposed to B. thuringiensis

strain ATCC 13367 through application of plant growth promotants to agricultural fields and crops; biodegradation, bioremediation and application to plants to improve the efficiency of phytoremediation. However, the exposure to terrestrial species including vertebrates, invertebrates and plants through the application of insecticides to forests and agricultural fields to control lepidopteran pests is not expected as this strain is not registered as an insecticide and more effective strains are currently in use. Aquatic applications could also expose terrestrial species through irrigation systems. Should potential future uses be realized in Canada, aquatic species, including vertebrates, invertebrates and plants, could be exposed to B. thuringiensis strain ATCC 13367 through applications such as water and wastewater treatment. Aquatic species could also be exposed as a result of runoff from terrestrial applications to soil of products containing B. thuringiensis strain ATCC 13367 or of land-applied treated sewage sludge, or waste effluent from commercial or industrial activities.

Organisms at sites of application are likely to be the most directly exposed. In particular, species feeding or drinking near treated or contaminated soils could ingest B. thuringiensis strain ATCC 13367 or inhale air-borne viable cells or spores. Terrestrial and aquatic exposure to B. thuringiensis strain ATCC 13367 as a result of its release from facilities manufacturing enzymes or biochemicals is expected to be limited by the application of good manufacturing practices (for example, conformity with municipal and provincial waste water regulations and manufacture in a contained facility).

The magnitude of exposure to B. thuringiensis strain ATCC 13367 of environmental species and the Canadian ecosystem will depend on the mass or volume released in the environment, on its persistence and survival in the receiving environment, the nature of the use and on the proximity of environmental species to the sites of application or disposal.

B. thuringiensis is an ubiquitous organism frequently isolated from soil. Studies in the scientific literature that contain data on population levels of B. thuringiensis in the natural environment are limited. Vegetative cells could survive and multiply in host and dead insect cadavers under favourable conditions, but are not expected to persist in the environment. Persistence data obtained by Environment and Climate Change Canada on B. thuringiensis strain ATCC 13367 and information in the literature on B. thuringiensis indicate that B. thuringiensis strain ATCC 13367 spores are likely to persist in terrestrial environment from a few weeks to several months or even years under favourable conditions.

Should potential uses be realized in Canada, insecticidal proteins could also be released. The Cry toxins are known to degrade rapidly once solubilized and have a relatively short persistence in the environment. Free insecticidal proteins bind tightly to soil particles and so are unlikely to enter aquatic systems through runoff. Any free insecticidal proteins that do enter the aquatic ecosystem through run off will likely remain bound to the sediment and therefore less bioavailable.

Due to the expanding commercialization of microbial-based products, some potentially containing B. thuringiensis strain ATCC 13367, there is a likelihood of an increase in the use and release of this micro-organism in the environment. While large inputs of B. thuringiensis strain ATCC 13367 into the environment could result in concentrations greater than background levels of B. thuringiensis, high numbers of vegetative cells are unlikely to be maintained in water and in soil due to microbial competition (Leung et al. 1995; van Veen et al. 1997). Also, those uses are not expected to lead to exposure greater than that resulting from use of other B. thuringiensis strains and subspecies as pesticides.

4.2.2 Human

Human exposure to B. thuringiensis strain ATCC 13367 is estimated to be low based on responses to the voluntary survey and the section 71 Notice and considering that reported pest control uses are unlikely to pertain to the DSL strain. B. thuringiensis strain ATCC 13367 is available for purchase from the ATCC and given that consumer, commercial, industrial and agricultural activities were identified as potential uses in Section 2.1 Sources of Exposure, exposure scenarios arising from potential future uses of the B. thuringiensis strain ATCC 13367 have been considered.

Direct human exposure to B. thuringiensis strain ATCC 13367 would be expected to be the greatest through its use in consumer products containing spores or viable cells. Handling and application of such products would be expected to result in direct exposure of the skin and eyes and inhalation of aerosolized droplets or air-borne spores.

Inadvertent ingestion following use on or near food preparation surfaces is possible. The use of such products in food preparation areas could result in the contamination of surfaces and foods at the time of product application possibly resulting in ingestion.

The general population could be exposed as bystanders during commercial application of products for biodegradation, bioremediation and water and wastewater treatment, pest control agents and agricultural applications. The extent of bystander exposure would depend on the mode of application, the volume applied and the proximity of bystanders to the site of application. In general, exposure for bystanders and general population is expected to be low for potential uses of B. thuringiensis strain ATCC 13367.

Human exposure to bodies of water and soils treated with B. thuringiensis strain ATCC 13367 could result in exposure of the skin and eyes as well as inadvertent ingestion. Human activity on soils recently treated with B. thuringiensis strain ATCC 13367 could result in air-borne spores, which could then be inhaled and could expose the skin and eyes. These exposures are expected to be low relative to its direct use in consumer products.

Release of the DSL B. thuringiensis strain ATCC 13367 from facilities manufacturing enzymes or biochemicals could occur, but is expected to be limited by the application of good manufacturing practices, in which measures should be taken to minimise releases of production micro-organisms.

Any health risk associated with drinking water is expected to be negligible as B. thuringiensis does not proliferate in aquatic habitats or other drinking water sources. In the event that the organism enters municipal drinking water treatment systems through release from intended and potential uses, the water treatment process, which includes coagulation, flocculation, ozonation, filtration and chlorination, is expected to effectively eliminate this micro-organism and so limit ingestion through drinking water.

In the event that consumer, commercial or industrial activities increase or change, the human exposure to B. thuringiensis strain ATCC 13367 could change based on the exposure scenarios described above.

5. Risk Characterisation

In this assessment, risk is characterized according to a paradigm whereby a hazard and exposure to that hazard are both required for there to be a risk. The risk assessment conclusion is based on the hazard, and on what is known about exposure from current uses.

Based on responses to the section 71 survey, and considering that reported pest control uses are unlikely to pertain to the DSL strain, exposure to B. thuringiensis strain ATCC 13367 from ongoing consumer, commercial and industrial uses is considered to be low, and, given its hazard, the risk is therefore low.

The determination of risk from current uses is followed by consideration of the estimated hazard in relation to foreseeable future exposures (from new uses).

B. thuringiensis strain ATCC 13367 has not been registered or used as a pesticidal strain in Canada, and more effective insecticidal strains are currently commercialized. In the unlikely event that B. thuringiensis strain ATCC 13367 is developed as a pesticidal strain, there is potential for widespread environmental release. In Canada, microbial pest control agents and end-use pesticide products containing B. thuringiensis strain ATCC 13367 would be subject to registration under the PCPA. These uses of the DSL strain would therefore undergo a complete risk assessment, and any necessary risk mitigation measures would be applied by Health Canada’s PMRA.

Other potential uses could also result in environmental release but releases are expected to be in lesser quantities than used for pesticide applications.

Risk to environment from potential future uses

Hazard has been estimated for DSL B. thuringiensis strain ATCC 13367 to be low for terrestrial and aquatic vertebrates, terrestrial and aquatic plants, and most terrestrial and aquatic invertebrates; however, it is estimated to be high for larvae of certain insect species. Health Canada scientists have shown that B. thuringiensis strain ATCC 13367 contains the Cry1Ba toxin gene. Cry1Ba is known to be selectively toxic towards insect species of the Order Lepidoptera, as well as certain species of the Orders Diptera and Coleoptera. Nevertheless, other B. thuringiensis subspecies and strains have been used extensively as biological pest control agents in agricultural and forestry applications and surveillance data does not link the use of B. thuringiensis to any long-term adverse effect at the ecosystem level or at the population level of target or non-target species.

Considering all lines of evidence available, it is estimated that larval stages of susceptible insects would be adversely impacted by releases of B. thuringiensis strain ATCC 13367 at the site of release, during the application period; however, there is no evidence to suggest that overall populations of these susceptible species will be adversely affected. The risk from potential future uses therefore remains low.

Risk to humans from potential future uses

Hazard has been estimated for DSL B. thuringiensis strain ATCC 13367 to be low-medium for human health, with the hazard elevated from low because of the potential for B. cereus-like enterotoxin genes to be expressed and to cause food poisoning.

Exposure through ingestion is of primary concern since B. thuringiensis strain ATCC 13367 contains hbl, nhe and cyt k genes and produces HBL and Nhe toxins, which are implicated in gastrointestinal disease. The use of products containing B. thuringiensis strain ATCC 13367 in food preparation areas could result in the contamination of foods, and subsequent lapses in proper food handling practices could allow bacteria to proliferate. Cycles of reheating and inadequate refrigeration are particularly problematic for spore-forming bacteria like B. thuringiensis, because spores are known to survive high temperatures. Spores may also enter the vegetative cycle under favourable conditions. In this way, the number of viable cells in food increases in exponential fashion, eventually reaching a level that can lead to human gastrointestinal infection.

Nevertheless, other B. thuringiensis subspecies and strains have been used extensively as biological pest control agents in agricultural and forestry applications leading to human exposure and available surveillance data does not link the use of B. thuringiensis to any increase of infection in by-standers of sprayed areas.

6. Conclusion

Based on the information presented in this screening assessment, it is concluded that B. thuringiensis strain ATCC 13367 is not entering the environment in a quantity or concentration or under conditions that:

- have or may have an immediate or long-term harmful effect in the environment or its biological diversity;
- constitute or may constitute a danger to the environment on which life depends; or
- constitute or may constitute a danger in Canada to human life or health.

Therefore, it is concluded that B. thuringiensis strain ATCC 13367 does not meet the criteria set out in section 64 of the CEPA.

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Appendices

Appendix A: Fatty Acid Methyl Ester Analysis

Table 0-1: Fatty acid Methyl ester (FAME) analysis of B. thuringiensis strain ATCC 13367

Data generated by Health Canada scientists. Data presented show the best match between the sample and two MIDI databases (clinical and environmental). The table reports the number of matches (fraction of total number of tests) and the fatty acid profile similarity index (in parentheses; average of all matches). MIDI is a commercial identification system that is based on the gas chromatographic analysis of cellular fatty acid methyl esters.

Environmental **Matched strain-8/15 (0.625)**	**Clinical** **Frequency – Best Match (Similarity Index)**
B. thuringiensis – entomocidus 8/15 (0.625)	B. cereus-GC subgroup 5/9 (0.776)
B. cereus-GC subgroup 4/15 (0.311)	B. thuringiensis-GC subgroup B 4/9 (0.535)
B. mycoides-GC subgroup B (Bacillus cereus group) 2/15 (0.125)	Only two matches
B. thuringiensis-sotto 1/15 (0.397)	Only two matches

Appendix B: Growth of B. thuringiensis strain ATCC 13367 in various media

Table B-1: Growth of B. thuringiensis strain ATCC 13367 in liquid media at various temperatures

Medium	27°C	32°C	37°C	42°C
Trypticase Soy Broth	+	+	+	+
Sheep Serum	~	~	~	~
Fetal Bovine Serum	+	+	+	+
Dulbecco's Modified Eagles Medium	~	~	~	-

– no growth, + growth, ~ low level growth or delayed growth (after 15h)

Data generated by Health Canada scientists. Growth of B. thuringiensis strain ATCC 13367 in broth culture, as measured by increase in absorbance at 500 nm, in four different growth media and over a range of temperatures: Concentration of bacteria at time zero was 1×10^6 CFU/mL. Measurements were taken every 15 minutes over a 24-hour period with a multi-well spectrophotometer.

Table B-2: Growth characteristics of B. thuringiensis strain ATCC 13367 on solid media at various temperatures

Medium	28°C	37°C
Citrate Agar[a]	+	+
Nutrient Agar	+	+
Mannitol Salt Agar[b]	-	-
Growth on Starch agar [c]	+	+
Growth on Urea agar	+	+
Catalase activity[d]	+	+
Growth on Sheep blood agar	+	+
Hemolysis of Sheep blood[e]	+	+

Testing conducted by Health Canada's Environmental Health Science and Research Bureau
(+) Positive for growth or test
(-) Negative for growth or test
[a] The ability to use citrate as the sole carbon source
[b] Isolation and differentiation of Staphylococci
[c] Differential medium that tests the ability of an organism to produce extracellular enzymes that hydrolyze starch
[d] Catalase enzyme assay measures by enzymatic detoxification of hydrogen peroxide
[e] Hemolysis of sheep blood

Appendix C: Virulence factors of B. thuringiensis

Table C-1: Virulence factors and toxins associated with B. thuringiensis present in B. thuringiensis strain ATCC 13367

Virulence Factor / Toxins (genes)	Immuno-chromatography [a]	PCR amplification[b]	BLASTn query of ATCC 13367 whole genome contigs
Cry toxin [c,d,e]	N/A	N	+ (Cry1Ba4)[e,f]
Thuringiensin biosynthesis gene cluster	N/A	N/A	N
Vegetative Insecticidal Proteins (vip3A, vip3B, vip3C)[d,e]	N/A	N	N
Cyt 1[e]	N/A	N/A	N
Cyt 2[e]	N/A	N/A	N
Cytotoxin K (cytk)	N/A	+	+
Sphingomyelinase (sph)	N/A	?	+
Cerolysin O (thuringiolysin O, tlo)	N/A	+	+
Hemolysin II	N/A	+	+
Hemolysin III	N/A	+	+
Enterotoxin FM (CwpFM)	N/A	+	+
Hemolysin BL (HBL)	+	+	+
Non hemolytic enterotoxin (NHE)	+	+	+
Phosphatidylcholine-specific phospholipase C (PC-plc)	N/A	+	+

Virulence Factor / Toxins (genes)	Immuno-chromatography [a]	PCR amplification[b]	BLASTn query of ATCC 13367 whole genome contigs
Transcription factor PlcR	N/A	N/A	+
FhlA (flhA)	N/A	N/A	+
Immune inhibitor A metalloprotease (inhA)	N/A	N/A	+
Neutral peptidase B metalloprotease (nprB)	N/A	N/A	+
Metalloprotease (nprP2)	N/A	N/A	+
Protease (sfp)	N/A	N/A	+
Chitinase	N/A	N/A	+
Parasporins (Cry31, Cry46, Cry41, Cry45)[e]	N/A	N/A	N

Testing conducted by Health Canada's Environmental Health Science and Research Bureau
[a] Protein detected in culture of B. thuringiensis strain ATCC 13367 using immunochromatography. 200µl of an overnight culture of B. thuringiensis strain ATCC 13367 grown in Brain Hearth Infusion broth was applied to Duopath® Cereus Enterotoxins test device https://www.emdmillipore.com/CA/en/product/Duopath-Cereus-Enterotoxins,MDA_CHEM-104146#overview
[b] Amplicon presence and size consistent when compared with B. cereus ATCC 14579 or B. thuringiensis ssp kurstaki (Foray 48B) (+ =present/consistent; ? = inconsistent amplicon size; N=not detected; N/A = not tested)
[c] Primer sequences (Rosas-Garcia et al. 2008)
[d] Primer sequences (Jain et al. 2012)
[e] Cry, Cyt, Vip protein status was also determined from genome contig files using BtToxin_scanner (Ye et al. 2012)
[f] Refer to http://www.btnomenclature.info/

Appendix D: Host range of Cry toxins

Table D-1: Host spectrum of Cry and Cyt toxins associated with B. thuringiensis

Host	Cry or Cyt toxin
Lepidoptera	Cry1A-K, Cry2A, Cry7B, Cry8D, Cry9A-C,E, Cry15A, Cry22A, Cry51A
Diptera	Cry1A-C, Cry2A, Cry4A-B, Cry10, Cry11A-B, Cry16A, Cry19A-B, Cry20A, Cry24C, Cry27A, Cry32B-D, Cry39A, Cry44A, Cry47A, Cry48A, Cry49A, Cyt1A-B, Cyt2A-B
Coleoptera	Cry1B, Cry3A-C, Cry7A, Cry8a-G, Cry9D, Cry14A, Cry18A, Cry22A-B, Cry22A, Cry34A-B, Cry35A-B, Cry36A, Cry37A, Cry43A-B, Cry55A, Cyt1A, Cyt2C
Rhadbitida	Cry5A, Cry6A-B, Cry12A, Cry13A, Cry14A, Cry21A, Cry55A
Hemiptera	Cry2A, Cry3A, Cry11A
Hymenoptera	Cry3A, Cry5A, Cry22A
Gastropoda	Cry1Ab
Human-cancer cell	Cry31A, Cry41A, Cry42A, Cry45A, Cry46A (parasporins)
Bacteria	Cry1A, Cry3A, CryD-like, Cry4Ba, Cry11Aa, Cyt1Aa