APPENDIX 7

FORMAT FOR THE COMPILATION OF *TIER II* SUMMARIES - MICROBIAL PEST CONTROL AGENT

PART 1

Section 1 Identity, biology, and characterisation of the microbial pest control agent; Biological properties of the microbial pest control agent; Further information on the microbial pest control agent; Proposals including justification of the proposals for the classification and labelling of the microbial pest control agent; manufacturers methods for the MPCA; quality control information for the MPCA

The example of a summary and assessment of data which follows is intended to illustrate the approach recommended for the preparation of *Tier II* summaries and assessments. The material included has not been critically assessed for its technical content. The data included in the following summary and evaluation are not based on a real submission.

Applicant should be aware that these guidelines are intended to provide a degree of flexibility. Where in particular cases, it is more appropriate to present the data and information in another format, applicants may do so. In such cases it is recommended that the applicant discuss the format proposed with the Regulatory Authority of the Country to which application is to be made.

1. Identity of the Microbial Pest Control Agent

IIM 1.1 Applicant

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IIM 1.2 Producer

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IIM 1.3 Scientific information

IIM 1.3.1 Name and species description *(Example Bacillus spec. 1)*

Taxonomic name and strain

Species:	*Bacillus spec. 1*
First description: *author*	*1970*
Strain:	*ABCD*
Genus:	*Bacillus*
Family:	Bacillaceae
Division:	Bacteria

Alternatives / synonyms / common names of the micro-organism

There is no common name for the organism

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IIM 1.3.2 **Collection and culture reference number**

The strain is deposited in German Collection of Micro-organisms and Cell Cultures (DSMZ), Mascheroder Weg 1b, D-38124 Braunschweig, Germany
Reference number: DSMZ *ABCD*.

Strain origin

This strain originates from a natural, indigenous wild type and is not genetically modified.

IIM 1.3.3 **Test procedures and criteria used for identification**

The organism has been identified in the German Collection of Micro-organisms and Cell Cultures (DSMZ), in Germany. The identification based on microscopic examination and biochemical parameters which characterise different *Bacillus* species and provide useful identification tools (author, 1999).
Besides the basically relevant positive Catalase reaction inherent to all *Bacillus* species, further biochemical key parameters identifying strain *ABCD* of *Bacillus spec.1* are e.g.: positive Voges-Proskauer reaction, growth in 7% NaCl, and Casein decomposition. The strain *ABCD* of *Bacillus spec.1* is further characterised by the method of random amplified polymorphic DNA (RAPD) analysis.
(Data and criteria for identification see author 1999, Doc. K-IIB, Sec. 1, P. 1.3/01, and author 1999, Document J, Annex IIB, Sec. 1, P. 1.4/03, p. 8-10).

IIM 1.4 **Composition of Technical Grade of MPCA**

IIM 1.4.1 **Content of micro-organism (and metabolite, if appropriate) in terms of g/kg, % w/w, cfu's/ml or appropriate potency units *(Example Bacillus spec. 1)***

Microbiological purity of the micro-organism

The content of pure micro-organism in *ABCD* Technical is *20.6* % by weight on average, ranging from 16.22 to 24.98, and in terms of colony forming units 6×10^{10} cfu/g (100 g/ kg) are stated. The technical product was proved to contain in minimum 5×10^{7} cfu/g of the active ingredient, *B. spec.1*, thus meeting the required minimum concentration for end use specification; inert ingredients consist of *B. spec.1* fermentation solids and/ or solubles and residual moisture (author 1999, Doc. J-IIB, Sec. 1, P. 1.4/01).

IIM 1.4.2 **Composition of the microbial material used for manufacture of end use products in terms of g/kg (% w/w) for each ingredient *(Example Bacillus spec. 1)***

IIM 1.4.2.3 **Identity and content of impurities, additives and extraneous micro-organisms**

The completed fermentation material (broth) of each fermentation run (batch) is tested by counts of colony forming units (cfu) of *B. spec.1*, microscopic examination, optical density and is tested for contaminants by plating analysis, esp. with regard to human pathogens. Content of cfu and contaminants may additionally be determined for the Technical Powder.
The test results showed no detectable levels of human pathogens (*Escherichia coli, Staphylococcus aureus, Salmonella spp., Vibrio cholerae*) or other contaminants (yeasts, molds, coliforms) (author 1999, Doc. J-IIB, Sec. 1, P. 1.4/01, pp. 15-16).

The end-product *ABCD* Technical was determined to be 100 % pure for *B. spec.1* and did not contain any other micro-organism (author 1999, Doc. J-IIB, Sec. 1, P. 1.4/03).

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Author (1999) gives a number of employed agents for pH adjustment and lists fermentation media components in detail (page 7 of Doc. J-IIB, Sec. 1, P. 1.4/01).

The overall content of *B. spec.1* fermentation solids and/ or solubles in *ABCD* Technical achieves on average 900 g/kg (90 % by weight), it retains moisture at an average of 5 % (at 20 to 25°C) (author 1999, Doc. J-IIB, Sec. 1, P. 1.4/02).

or

Justification of the non-submission of data

Section 1 **Data Point IIM 1.4.2.3**	**Composition of the microbial material used for manufacture of end use products in terms of g/kg or g/L (for US and Canada also in % w/w) for each ingredient**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [X] Scientifically unjustified [ ]** **Other justification [ ]**	
Detailed justification: This information is not required because the Technical Grade of MPCA is a hypothetical stage in a continuous production process of the end use product.		
Undertaking of intended data submission [ ]	**-**	

IIM 1.4.3 **Quality criteria for the production and storage of the MPCA *(Example Bacillus spec. 1)***

IIM 1.4.3.1 **Method of production and quality control**

Including:
criteria for consistency and integrity of the master and working seed stock, typically, measures of biological activity and phenotypic or genotypic properties:
1. acceptable range for content of MPCA, in appropriate terms;
2. absence of human/ mammalian pathogens;
3. absence or maximum accepted level of known mammalian toxins, if their presence is suspected at any stage in process, or if MPCA is closely related to a toxigenic human pathogen
4. maximum accepted level for microbial impurities, using suitable indicators of an unhygienic process

ABCD Technical shall be produced by pure culture fermentation procedures with adequate control measures during production to detect any changes from the characteristics of the parent strain or contamination by other micro-organisms.

IIM 1.4.3.2 **Acceptable range for content of MPCA**

The end-product *ABCD* Technical showed no detectable levels of any other micro-organisms as *B. spec.1* (author 1999, Doc. J-IIB, Sec. 1, P. 1.4/03).
The content of pure micro-organism in *ABCD* Technical is *20.0* % by weight on average, ranging from *18 % to 22 %*, and in terms of colony forming units 6×10^{10} cfu/g are stated (author 1999, Doc. J-IIB, Sec. 1, P. 1.4/02).

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IIM 1.4.3.3 Presence of human/mammalian pathogens

Each seed transfer is checked for purity both microscopically and by streak plating. The completed fermentation material (broth) of each fermentation run (batch) is tested by counts of colony forming units (cfu) of *B. spec.1*, microscopic examination, optical density and is tested for contaminants by plating analysis, esp. with regard to human pathogens. Content of cfu`s and contaminants may additionally be determined for the Technical Powder.
The test results showed no detectable levels of human pathogens (*Escherichia coli, Staphylococcus aureus, Salmonella spp., Vibrio cholerae*) or other contaminants (yeasts, molds, coliforms) (author 1999, Doc. J-IIB, Sec. 1, P. 1.4/01, pp.).

IIM 1.4.4 Quality control data *(Example Bacillus spec. 1)*

Analytical profile of batches

Five lots of *ABCD* Technical were analysed for following parameters (author 1999, Doc. J-IIB, Sec. 1, P. 1.4/03):

- Purity for the active ingredient, strain *ABCD* of *B. spec.1*
- Determination of any other micro-organism (bacteria or fungi) by [*name, method*]
- Titers for all detected microbes
- Quantitation of moisture content

For the relevant information see Document J.

IIM 1.4.5 Theoretical discussion regarding (a) the formation and/or presence of unintentional ingredients *(Example Pseudomonas spec. 1)*

IIM 1.4.5.1 Presence of unintentional ingredients

P. spec.1 has no known production of exotoxins that can be demonstrated *in vitro*. As most tested Gram-negative bacteria, though, it contains endotoxins acting on other bacteria.

Author *et al* stated that *P. spec.1* produced phenazine –1-carboxylic acid. However, *P. spec.1* strain *ABCD* has been tested for phenazine production using a number of different techniques and no phenazine production could be found (author 1999, Doc. K-IIB, Sec.).

Centrifuged supernatant from *P. spec.1* strain *ABCD* was fractionated with column chromatography. Biological activity from five different column fractions was compared in three different bioassays (author 1999, Doc. K-IIB, Sec). Two of the fractions showed biological activity in all three bioassays. Further fractionation and isolation identified the biochemical product DEF.

Figure 1. Structure of metabolite 1.

Metabolite 1

Evidence shows that the bacteria are not likely to be associated with the phyllosphere (author 1999, Doc. K-IIB, Sec). Therefore metabolite 1 is unlikely to be present. Also the degradation of metabolite 1 should prevent any residue contaminating the aerial parts of the crop (author 1999, Doc. K-IIB, Sec). The analysis of shoots and of grain at harvest confirms that the residues are below the limits of determination.

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IIM 1.4.6 **Physical and chemical properties, if MPCA is produced as a manufacturing product that is stored prior to formulation of end use products *(Example Bacillus spec. 1):***

see example under point 2.1-2.5 – FORMULATED PRODUCT

Data point	Comments	Guideline and Method	Test material purity and specification	GL Y/N	findings	Reference
Physical state						Author 1999
Density						
Viscosity or surface tension						
Explosivity, corrosive character, oxidising properties						
Technical characteristics as appropriate						

or **Justification of the non-submission of data**

Section 1 Data Point IIM 1.4.6	**Physical and chemical properties, if MPCA is produced as a manufacturing product that is stored prior to formulation of end use products**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	Official use only
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [X] Scientifically unjustified [ ]** **Other justification [ ]**	
Detailed justification: Data are not necessary because the MPCA is **not** stored prior to formulation of the end use product.		
Undertaking of	**-**	

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intended data submission [ ]		

International regulatory status of micro-organism

There is no registration of other strains of this micro-organism registered in Germany.

1. Biological properties of the micro-organism

IIM 2.1 Origin of the isolate *(Example Bacillus spec. 1)*

This strain originates from a natural, indigenous wild type, isolated from a field soil in *ABCD* (USA) in 1999 (author 1999).

Method of isolation

Isolation of *Bacillus* species includes heat treatment of the suspended soil sample and subsequent streak plating using enrichment media (author 1999).

History of the organism and its uses

A number of soil microbes were isolated from soil samples and screened for their antagonistic properties on biological control of seed-borne pathogens of cereal 1989-1991 by the *name*. *Bacillus spec.1* strain *ABCD* was further developed as biofungicide by *name* (author 1999).

History of use of closely related strains or species

B. spec.1 is relevant as a model-organism for cell-biological research and has a potential as a commercial producer of the products of genetic engineering (author 1999).
The capability of *B. spec.1* (and other bacteria of this Genus) to produce exo-enzymes acting as proteases or cellulases is commercially used e.g. for tannery and for the production of additives for detergents (author 1999) and in food industry (author 1999).
Other scientists investigate the potential use of antifungal compounds as biological control agents against plant pathogens in agriculture (author 1999).

IIM 2.2 Natural occurrence and geographical distribution *(Example Bacillus spec. 1)*

B. spec.1 is an ubiquitous -not geographically restricted- inhabitant of the soil, from which it is spread to associated environments, including plants and plant materials, foods (cereals), animals and their faeces (by ingestion of spores) and is also naturally found in aquatic environments (fresh water, estuarine and coastal waters) (author 1999).

Level of natural occurrence

The level of natural occurrence of *B. spec.1* in the soil is not definitely stated in the supplied literature. Indications for their general prevalence can be derived from high levels of presumably soil-born *Bacillus* spp. spores in straw approaching 10^5cfu/g (author 1999), and from the high numbers of *Bacillus* spp. found in coastal waters (where they constitute up to 20% of total bacterial population) and from the major contribution of their endospores in estuarine and coastal sediments (achieving up to 80% of the heterotrophic flora) (author 1999).

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IIM 2.3 Information on target organism(s)

IIM 2.3.1 Description of the target organism(s) *(Example Bacillus spec. 1)*

Fungal plant pathogens: *Botrytis, Verticillium, Alternaria* and *Cladosporium.*

Description of the target organism(s) *(Example Virus spec. 1)*

Product name SC is recommended for use as a spring treatment in apple orchards to control the codling moth *Cydia pomonell*a. Virus particles must be ingested by larvae to be effective. Applications should be timed so that early-instar larvae on the surface of the leaf or fruit come in contact with the virus before entering the fruit.

IIM 2.3.1 Mode of action *(Example Bacillus spec. 1)*

Control of fungi: Competition for living space and nutrients (author 1999)
Enzymatic lysis of cell walls of fungal pathogens (author 1999)
Hyperparasitism (author 1999)

Mode of action *(Example Virus spec. 1)*

Product name SC is a biological insecticide containing the Granulovirus *Virus spec. 1*. The larval stage of the insect life cycle is the most susceptible to infection with baculovirus polyhedra or granules. Polyhedra are ingested when the insect larva feeds on contaminated food, and dissolve in the alkaline environment of the insect mid gut to release the virus particles. After penetrating the peritrophic membrane, the virus lipoprotein envelope fuses with the plasma membrane of a gut wall cell and releases its nucleocapsid into the cytoplasm. The nucelocapsids transport the virus DNA to the nucleus of the cell. Once in the nucleus, the virus produces two distinct structural forms in a biphasic replication cycle. First in the infected gut cells, nucleocapsids are formed without being occluded within a polyhedrin (or granulin) structure. These nucleocapsids bud through the nuclear membrane of the host cell and then through the plasma membrane while acquiring the virus-encoded gp67 kDa protein which allows for attachment to other susceptible cells within the host, such as fat cells, hemocytes and epidermal cells. These extracellular viruses (ECV) are released into the hemolymph and infect other cells or sometimes only a specific cell-type. These second waves of infection produce two forms of the virus: ECVs and the occluded virus particles within a polyhedral matrix in the nucleus. The accumulation of polyhedra within each cell, and within the insects as the cells rupture proceeds until the host dies. Virus particles are released from the decaying host into the environment where they can infect another larva.

Infective dose and transmissibility (in case of pathogenic effect) *(Example Virus spec. 1)*

Bio-assays in the laboratory have shown that V*irus spec.1* is very effective against larvae of codling moth *Cydia pomonell*a. The LD50 is dependent on the larval instar: the youngest larvae are the most susceptible (author 1999).

Tab. 1: LC50 (occlusion bodies (OBs/ml)) and LD50 (no. OBs) values for *virus spec. 1* against *Cydia pomonell*a larval instars.

Larval instar	**LC50 (OBs/ml)**	**LD50 (OBs)/animal**
1	1.9×10^5	4
2	9.1×10^3	3

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3	2.8×10^4	20

The natural route of infection of baculoviruses is per os, where larvae ingest the virus by feeding on infected leaf material. The virus can be spread through a population by contamination of leaves through decomposing infected caterpillars.
Additionally some vertical transmission is also possible. Females which were infected as caterpillars, but survived, do produce a small portion of virus contaminated eggs. The caterpillars which hatched from infected eggs will die quickly. Also predators and parasites can transmit the virus (author, 1999).

IIM 2.4 **Host specificity range and effects on other species than the target organism(s) *(Example Bacillus spec. 1)***

Bacillus spec.1 is not characterised by a distinct host specificity since growth of *B. spec.1* is not dependant upon a host but upon supply with decomposable organic matter (author 1999).
B. spec.1 strain *ABCD* is active against a number of plant pathogenic fungi including *Botrytis, Verticillium, Alternaria* and *Cladosporium*. Using *B. spec.1* strain *ABCD* does not cause any permanent changes in the natural microflora. There might be a slight short-term decrease in the number of soil fungi after treatment. However, relative numbers of different fungi return back to original level within 1-2 months and there are no long-term effects on non-target soil microflora (author 1999).

The potential suppression of mycorrhizal species was tested by applying the antimycotic molecule *name*, an antibiotic produced by strains of *B. spec.1* (author 1999):
In vitro the saprophytic growth of the fungus *Glomus spec.3* was inhibited by *name*, while *in vivo*, in the presence of the tomato host plant, the antibiotic did not restrict or impede the mycorrhizal symbiosis at any stage– simultaneously under field conditions *name* inhibited the pathogen *Botrytis spec.2* and effectively reduced the infection (author 1999).

Host specificity range and effects on other species than the target organism(s) *(Example Virus spec. 1)*

Cross-infectivity studies have shown that most baculoviruses have a narrow host range, never exceeding the order and usually not the family of the host from which the virus was originally isolated. Commonly, the host range is restricted to the genus of the competent host. Viruses from the genus *Granulovirus* have only been isolated from arthropods and the host range of GVs is generally narrower than viruses from the genus *Nucleopolyhedrovirus* (NPV).

The environmental effects studies submitted indicated that the active ingredient in *Product name* SC, *virus spec. 1*, is specific to the original codling moth host. Host-range testing on 14 terrestrial arthropod species showed that *virus spec. 1* was pathogenic and infective only to the intended target, i.e., codling moth, and was not able to infect larvae of six other insect species belonging to the order Lepidoptera, including two other species of the family Tortricidae (obliquebanded leafroller and spruce budworm). Although increased mortality was noted in the cabbage looper, no evidence was found of infectivity caused by *virus spec. 1*. Increased mortality, but no infectivity, was also noted in treated housefly larvae and in the hatching of adult parasitoid wasps from virus-infected codling moth larvae. Housefly mortality was not attributed to *virus spec. 1* but to other properties of the crude virus preparation or to an activated latent (occult) virus. Lower hatching success of parasitoid wasps from virus-infected codling moth larvae was a consequence of the virus killing the codling moth larvae before pupation of the parasitoid. No mortality or infectivity was noted in six other nontarget arthropod species tested, including the beneficial honeybee and Asian ladybeetle. Studies on an aquatic arthropod (freshwater daphnia) and non-arthropod invertebrate (earthworm) showed no apparent treatment-related effects and evidence in the

published literature indicate that GV baculoviruses are not likely to produce adverse effects (mortality or infectivity) in either of these two groups of non-target organisms. Published studies on other baculoviruses have also shown a very low potential for adverse effects in wild mammals and birds. As well, many years of experimental and commercial applications of other baculoviruses on agricultural crops and forests have demonstrated that plants are not adversely affected by these viruses.

Although no data were provided for effects on beneficial predators or parasites specific to apple orchards in eastern Canada, it is anticipated that use of *Product name* SC would have minimal impact on the natural predators and parasites in apples orchards which contribute to biological control of arthropod pests of apples. However, one drawback of the host specificity of *virus spec. 1* is that additional control options (e.g., insecticides) might be required for control of apple pests (e.g., leafroller, apple maggot) which might also have been controlled with insecticide applications timed for control of codling moth.

IIM 2.5 **Life cycle of the micro-organism *(Example Bacillus spec. 1)***

All spore-formers, including members of the Genus *Bacillus*, undergo a cycle consisting of several discernible phases: germination, outgrowth, multiplication, and sporulation. The germinating spore develops towards a metabolising cell capable of outgrowth. Vegetative growth occurs by cell division. The primary cell formed at the end of outgrowth can, under some conditions, such as insufficient nutrients, divide asymmetrically and proceed directly to sporulation (author 1999). A specific review about spore germination is given by author (1999).
The endospore plays a dominant role in the biology and the life-cycle of *B. spec.1* and relatives (author 1999). It is a dormant structure which enables the micro-organism to survive when environmental conditions turn unfavourable for vegetative growth and is a vehicle for dispersal by dust and air streams, as it is easily blown up (author 1999). The global distribution of *Bacillus* spp. may largely be derived from the endospore-forming capability.
Basically the endospore is the most heat tolerant bacterial life-form, enduring temperatures >80°C or even >100°C (author 1999).
The endospore does not present an obligate stage in the life-cycle, vegetative growth by cell-division may maintain predominant - or even the norm, unless e.g. lack of nutrients occurs (author 1999).
In a dry state endospores can remain viable for several years: after 50 years lasting storage of dry soil 10% of the spores remain their capability to germinate (author 1999).
Competition for nutrients (esp. carbon) occurs among the saprophytic members of the micro-flora within the natural habitat, the soil, and in the rhizosphere. Successfully competing bacteria inhibit fungal spore germination (fungistasis) and therefore competition is believed to present one potential mode of action in the suppression of fungal plant diseases like *Fusarium* wilts (author 1999).

IIM 2.7 **Information regarding closely related species**

p.m.

IIM 2.7 **Relationships to known plant or animal or human pathogens *(Example Bacillus spec. 1)***

B. spec.1 and close relatives are regarded as non-pathogenic micro-organisms being granted the status “organism GRAS” (generally regarded as safe) by the U.S. Food and Drug Administration, while other species of the Genus *Bacillus* are known as toxin forming pathogens (author 1999):

B. anthracis	causes anthrax in humans and animals (author 1999)
B. cereus	causes gastro-enteritis (via food) and opportunistic infections (author 1999)
B. thuringiensis	acts as an insect pathogen (author 1999)

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IIM 2.8 **Relationships to known plant or animal or human pathogens *(Example Bacillus spec.* Physiological properties (especially effects of environmental parameters on growth, infectivity, dispersal and colonisation ability) *(Example Bacillus spec. 1)***

Infectivity

Animals/ Humans:
Data about *B. spec.1* acting as a pathogen are rare. Author (1999) reported insect- and mammalian-pathogenicity of some of the *B. spec.1* strains isolated from diseased mosquito larvae, causing mortality due to invasive infection (with subsequent decomposition of larvae) after ingestion by larvae or after intraperitoneal injection in mice respectively.
Evidence for *B. spec.1* -as one of several possible causative agents- causing mastitis has been found in some cases (author 1999).

Author (1999) considers risks from use of *B. spec.1* as low, but states immuno-compromised individuals inoculated by high numbers of the micro-organism may be susceptible to an infection. Reviewing clinical records covering a 6-year period author (1999) ascribe bacterial infections with participating *Bacillus* species mainly to contamination of operative wounds, and rarely found life-threatening invasive infections, always going along with other serious infections or diseases, like leukemia and pneumonia. Finally, author (1999) states that the only health problem in fermentation facilities may be sensitisation of workers to the *B. spec.1*, since this name compound is capable of causing allergic reactions in individuals who are repeatedly exposed to it (author 1999).
ABCD strain of *B. spec.1* did not exert pathogenic or toxic impacts on mammals, as proved in relevant toxicological studies submitted (author 1999).
In addition, no hazardous effects of strain *ABCD* of *B. spec.1* were observed in testing non-target organisms (invertebrates, arthropods, fish and birds) that might be exposed to it under conditions of use (author 1999).
In conclusion any adverse impacts and risks of field application of strain *ABCD* of *B. spec.1* for exposed animals can be evaluated as being negligible.

Plants:
A few studies relate incidence of soft-rot disease on certain crops or crop products to *B. spec.1* (author 1999). Author (1999) report about plant toxicity symptoms caused by application of isolated, pure antibiotics produced by *B. spec.1* strains, in most cases the tested antibiotics caused slight to mediate symptoms in exposed corn seedlings.

Dispersal routes

Endospores of *B. spec.1* may easily be distributed with soil or dust particles and via aerosols. Under conditions of use drift and spacious transport may occur with surface water and with the wind swirling up these particles or aerosols (author 1999). During aerolization vegetative cells of *B. spec.1* are exposed to severe environmental stress factors (desiccation, UV-radiation, temperature), therefore survival of vegetative cells is limited (author 1999).

Environmental requirements

Generally *B. spec.1* reproduces under aerobic conditions, although in the presence of glucose and nitrate anaerobic growth occurs (author 1999).
B. spec.1 is reported to occur predominantly in the resting stage (endospore), unless fresh organic matter has been supplied to the soil (author 1999). In any case, application of organic matter, e.g. manure, will support growth of existing *B. spec.1* populations.

The influence of pH on growth of *B. spec.1* was tested by author (1999), the pH-range for growth was found to be pH 6,5 to 7,5. The *name* report (1999; Doc. K-IIB, Sec. 1, P.1.3/01) states the optimum pH range for vegetative growth of strain *ABCD* of *B. spec.1* is pH 6.5.
In the efficacy comparison tests carried out at different pH values of growth substrate, *B. spec.1* strain *ABCD* proved to be effective at pH values of growth substrates normally used in horticultural production (pH range from 5.0 to 7.5). The best disease control efficacy was achieved at soil pH values around pH 7 (neutral).

Author (1999) defined the temperature range for growth of various *B. spec.1* strains: maximum growth was achieved at 45 – 55 °C; the minimum temperature allowing growth was 10 to 20 °C. The *name* report (1999, Doc. K-IIB, Sec. 1, P.1.3/01) confirmed 55 °C as the maximal temperature permitting growth of strain *ABCD* of *B. spec.1*, the lowest temperature tested was 20 °C, allowing growth. In liquid cultivation of *B. spec.1* optimal growth occurred at 37 to 42 °C and production of an antibiotic was also shown to be temperature dependent (author 1999).

_ The results from several references (authors) indicate that *B. spec.1* will survive under a broad spectrum of environmental conditions. No negative impacts on *name* efficacy in controlling crop diseases are anticipated under normal European growing season conditions (~ 10 to 30°C) (author 1999).

Colonisation ability

The efficacy of strain *ABCD* of *B. spec.1* against phytopathogens is largely based upon colonisation of the leaf surface (author 1999).
Tests on the survival of strain *ABCD* of *B. spec.1* on tomato leaves were conducted under field conditions and demonstrated that cells grew up to day 5 followed by a sharp decline in colony forming units (cfu). The results indicated that the employed strain was potentially be able to survive for a couple of days under field conditions (author 1999, Doc. K-IIB, Sec. 4, P. 6.1/01).

When *B. spec.1 ABCD* is sprayed to the foliar part of the plants, the organism can be still detected one month after the *B. spec.1 ABCD* gradually decreases. E.g. on cucumber leaves, 45 %, 10 %, 0.7 % and 0.3 % of *B. spec.1* was viable after 1, 2, 3 and 4 weeks, respectively.
A general aspect of concern is the fact that the leaf surface of temperate plants is described as a stressed environment with low water and nutrient levels forcing microbes into slightly protected environments such as under spines and in epistomatal cavities (author 1999). Usually much less than 1% of the leaf surface is covered by micro-organisms and competition for space is most likely to occur in the limited micro-habitats rendering protection against ultra-violet radiation and drying (author 1999), explaining the slight occurrence of growing saprophytic bacteria on the leaf surface.
These unfavourable conditions do not impede the efficacy of *B. spec.1* since the preparation will be added several times in a spraying sequence and *B. spec.1* cells will reproduce as long as the plant pathogen will be present, furthermore a long-term survival is not necessitated for the induction of systemic resistance in the plant which presents one mode of action.

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IIM 2.9 **Description of any plasmids or other extra chromosomal genetic elements if applicable *(Example Bacillus thuringiensis var. 1)***

Bacillus thuringiensis var. 1 produces parasporal, proteinaceous, crystal inclusion bodies during sporulation. The genes for these bodies are located on a 100kb plasmid (*name*). The ingestion of these crystal inclusion bodies are insecticidal to larvae of the order Lepidotera and to both larvae and adults of a few Coleoptera (author, 1999).

or

Justification of the non-submission of data

Section 1 **Data Point**	**Description of any plasmids or other extra chromosomal genetic elements if applicable**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ] Scientifically unjustified [ ]** **Other justification [X]**	
Detailed justification: **Such elements are not involved in pesticidal activity, pathogenicity, toxicity, etc. ...**		
Undertaking of intended data submission [ ]	**-**	

IIM 2.10 **Genetic stability and factors affecting it *(Example Bacillus spec. 1)***

Genetic stability
Information on the genetic stability of natural traits related to the biocontrol activity of *B. spec.1* are rare, focus is attracted on genetic engineering and instability problems of inserted DNA (author 1999). Research on basic genetic mechanisms has extensively made use of *B. spec.1* as a model system, e.g. to study plasmid stability characteristics. A number of studies investigated the fate of transferred recombinant or naturally occurring plasmids, and stability was shown to be influenced by environmental factors, the stage of the host cell and the plasmid size (author 1999).

Gene transfer
A *natural* way of DNA transfer is through bacteriophages, that may package some percent of the chromosomal DNA and have been employed for analysis of the genetic structure.
(author 1999).

Under natural conditions genetic exchange of chromosomal DNA (here: linked markers for antibiotic resistance) was shown to occur between strains of *B. spec.1* growing together in soil, presumably by transformation (author 1999): within one week the mixed soil culture developed towards dominance of one phenotype based on one specific combination of transferred genes out of more than 100 initially present phenotypes - due to adaptive changes caused by selection.

Interspecies gene transfer has been proved by author (1999), who suggested conjugation between *B. spec.2* and *B. spec.1* as the transfer mechanism of a plasmid carrying tetracycline resistance. The plasmid transfer from *B. spec.2* to *B. spec.1* was detectable in sterile, nutrient amended soil at a frequency of 1 x 10^6, but it turned negligible in non-sterile soil. The plasmid transfer also declined with temperature and moisture content (author 1999).

The cited references indicate that interspecies gene transfer between *B. spec.1* and other micro-organisms, e.g. human pathogens, is not anticipated under field conditions. With regard to environmental concern of the deliberate release of genetically engineered micro-organisms author (1999) state that several factors limit the transfer of genes between bacteria, including physiological and environmental (stress) conditions.

Detailed discussion of relationship of micro-organism to any known human dermatophyte *(Example Bacillus spec. 1)*

Bacillus spec. 1 strain ABCD is not closely related to any known human dermatophyte. Dermal toxicity/irritation studies did not reveal signs of infectivity in any of test animals.

or

Justification of the non-submission of data

Section 1 **Data Point**	**Detailed discussion of relationship of micro-organism to any known human dermatophyte**	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA	**Official use only**
Other existing data [ ] **Limited exposure [ ]**	**Technically not feasible [ ]** **Scientifically unjustified [ ]** **Other justification [X]**	
Detailed justification: *B. spec.1* and close relatives are regarded as non-pathogenic micro-organisms being granted the status "organism GRAS" (generally regarded as safe) by the U.S. Food and Drug Administration. After literature research in *[names of the data banks]* there are no indications of relationship of *B. spec.1* to any known human dermatophyte		
Undertaking of intended data submission [ ]	**-**	

Information on toxic metabolites of MPCA *(Example Bacillus spec. 1)*

B. spec.1 produces different exo-enzymes contributing to the decay of organic matter. The extracellular enzyme *name* is known to elicit allergic or hypersensitive reactions in individuals repeatedly exposed to it and its toxigenic properties are assessed to be very low. *B. spec.1* does not produce significant quantities of extracellular enzymes or toxins and is generally considered to have a low degree of virulence to humans (author 1999). The results of the submitted toxicological studies on rodents demonstrate that *ABCD* strain of *B. spec.1* does not produce toxins (author 1999, Doc. K-IIB, Section 3, Point 5.5).

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Antibiotics

Different antibiotic molecules were identified as products from different strains of *B. spec.1*.
Author (1999) have listed and described several antibiotics produced by certain strains of *B. spec.1*, *names*. These antibiotics, consisting of a cyclic *name* with a lipophilic _-amino acid side-chain of variable length, reported to interact with the cytoplasmic membrane and to be important for the displayed antifungal activities (author 1999).
Other antibiotics were defined as *name*, isolated from fermentation broth of certain strains of *B. spec.1*; these antibiotics were shown to act antibacterial and to act against human pathogens (author 1999).
However, no antibiotics used in human or animal medicine are known to be produced by *B. spec.1* (author 1999).

IIM 2.12 **Resistance / sensitivity to antibiotics *(Example Bacillus spec. 1)***

In the natural habitat spontaneous mutants of *B. spec.1* are reported to occur, exhibiting resistance towards antibiotics, e.g. towards streptomycin in strains isolated from composts (author 1999).
Author (1992) located resistance genes on different plasmids isolated from compost bacteria by transferring each plasmid to competent and specifically prepared bacteria cells of *B. spec.1*. Plasmids carried resistance towards erythromycin and tetracycline.
Bacillus species, including *B. spec.1*, were isolated from infected patients (mainly from their wound drainage) and found to be uniformly resistant to a variety of antibiotics (penicillin, ampicillin, oxacillin, methicillin and colistin (author 1999).

The cases of *B. spec.1* acting as a causal agent for disease or even causing mortality are rare and always associated with individuals showing immuno-suppression, while less severe infections by contamination of wounds are more likely to occur (author 1999).
In this context the apparent lack of naturally induced interspecies gene transfer and consequently the improbability of a potential shifting of resistance genes into human pathogens supports the safety of *B. spec.1*.

An antibiotic susceptibility testing for *B. spec.1* strain *ABCD* was performed with certain commonly used antibiotics such as kanamycin (0.03 mg/mL), ampicillin (0.1 mg/mL), bacitracin (10 U/mL), cefaclor (0.03 mg/mL), chloramphenicol (0.03 mg/mL), erythromycin (0.01 mg/mL), tetracycline (0.03 mg/mL), streptomycin (0.01 mg/mL) and trimethroprim / sulfamethoxazole (1.25 g/Ml, 23,75 g/ml) (author 1999). Strain *1* strain *ABCD* was shown to be sensitive to all nine antibiotics at the tested concentrations. Spontaneous mutations within the seed vials used for fermentation are excluded since the culture is consistently stored at –80°C (author 1999, Doc. J-IIB, Section 1, P. 1.4/01).

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3 Further Information on the Microbial Pest Control Agent (Function, Mode of Action, Handling)

IIM 3.1 **Function**

Bacillus. spp 2, strain EFGH, is to be used to control lepidopterous pests.

IIM 3.3 **Fields of use**

Agriculture

IIM 3.4.1 **Details of existing and intended uses** (crops, groups of crops, plants or plant products treated or protected)

Bacillus. spp 2, strain EFGH, is for use on vegetables and cole crops

IIM 3.4.2 **Details of harmful organisms against which protection is afforded**

The following lepidopterous pests are the target species for these uses of Bacillus. spp 2, strain EFGH: Armyworm, Beet armyworm, Cabbage budworm, Cabbage looper, Corn earworm, Diamondback moth, Southern armyworm, and Tomato hornworm.

IIM 3.4.3 **Effects achieved**

When used as specified in the label instructions, Bacillus. spp 2, strain EFGH, will protect these crops against the listed lepidopterous pests.

IIM 3.5 **Information on mode of action and metabolites**

IIM 3.5.1 and **Statement of the mode of action of the Microbial Active Substance in terms of biochemical physiological mechanism(s) and biochemical pathway(s) involved**

Following ingestion, the crystalline inclusion bodies are dissolved and then converted to active -endotoxins (Cry1Aa, Cry1Ac, and Cry1C - analyzed as being present in Bacillus. spp 2, strain EFGH according to the methods of Crickmore et al. 1998) by insect proteases. The active toxins bind to specific receptor sites and produce pores in the insect gut which is lethal to the insect. In addition, Bacillus. spp 1, strain ABCD expresses the VIP-3 protein which increases the activity of the -endotoxins.

IIM 3.5.2 **Details of active metabolites (especially toxins) and degradation products, cross referenced to the toxicological and residues data provided.**

There are no other active metabolites and degradation products that are known to contribute to the toxicity of Bacillus. spp 2, strain EFGH. Some related strains are known to produce various toxins other than the -endotoxins, however the presence of these are monitored and controlled during the manufacturing process.

IIM 3.5.3 degradation **Information relative to the formation of active metabolites (especially toxins) and products.**

There are no active metabolites and degradation products of toxicological significance.

IIM 3.6 **Information on the possible occurrence of the development of resistance or cross-resistance.**

No development of resistance in susceptible insects has been seen with similar products used in similar ways. Generally, the crystalline -endotoxin does not persist in the environment long enough for insects to develop resistance at the doses utilized. It is readily degraded by UV light.

IIM 3.7 A material safety data sheet for the Microbial Active Substance

A standard MSD for *Bacillus* species is provided.

IIM 3.8 Detailed instructions for safe disposal

The standard means of safe disposal is by controlled incineration at an approved chemical waste facility. This is a standard process and no further detailed instructions are required. This process will be sufficient to inactivate the microbial spores in this product.

IIM 3.9 Procedures for the decontamination of water in case of an accident

There is no readily available method for decontamination of water for this microbial product. Antimicrobial disinfectants would be marginally effective, even at full strength, and the water would dilute them. Based on the submitted ecological effects studies, and studies of the -endotoxins produced by this strain, minimal adverse ecological effects would be anticipated from contamination of water of Bacillus. spp 2, strain EFGH. Furthermore, the -endotoxins are quickly degraded in water.

IIM 3.10 Other/special studies

No additional issues have been identified that might require special studies.

IIM 3.11 Crops or products to be protected or treated

Bacillus spp 2, strain EFGH may be used on all vegetables and cole crops.

IIM 3.12 Measures to render microorganism harmless, in case of an accident

Bacillus spp 2, strain EFGH is very species specific and is harmless to non-target species and humans, thus there is no need to render the microorganism harmless, in case of an accident. If you wish to inactivate the spores, you may use heat (see IIM 3.8.1 and IIM 32.8.2) or antimicrobial disinfectants approved for Bacillus spore inactivation.

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PART 2

Section 2 Analytical Methods

The example of a summary and assessment of data which follows is intended to illustrate the approach recommended for the preparation of *Tier II* summaries and assessments. The material included has not been critically assessed for its technical content. The data included in the following summary and evaluation are not based on a real submission.

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4. Methods for Analysis, Manufacturing, Quality Control and Post-registration monitoring of the MPCA

IIM 4.1 **Methods to preserve and maintain the master seed stock (Methods to prevent loss of virulence of seed stock of the micro-organism *(Example Bacillus spec. 1)***

The seed bank (initial stock) of strain *ABCD* of *B. spec.1* is stored in liquid nitrogen to avoid any changes in the strain. A general supply of ampoules for production has been prepared. The original ampule for this supply is always taken from liquid nitrogen seed bank mentioned above. Storage temperature for this general supply is deep freeze –80 ^{o}C. Before approving the ampoules to be used for production, purity and culturing properties are tested by immediate microscopic morphology on a glass slide and by colony morphology appearing after streak plating., preventing any mutation to occur (author 1999).
The strain is also deposited at *name* and could be retrieved from there.

To ensure efficacy every lot of final product, *name*, is assayed for activity against plant pathogens.

IIM 4.2 **Method of Production *(Example Bacillus spec. 1)***

Description of the production process:

ABCD Technical is the spray-dried end-product of a liquid, fed batch fermentation process using *B. spec.1* strain *ABCD* cultures which are maintained as frozen vials stored in 10 % name in a –80 °C freezer. The fermentation process takes 12-hours in minimum.
A detailed description and flow-diagram of the production process is given in author (1999, Doc. J-IIB, Sec. 1, P. 1.4/01). All of the following statements and data are cited from this document.

Process steps or sections include:	see pp. in cited Doc.
☌ culture maintenance of *B. spec.1* (frozen cells in liquid media)	5
☌ inoculation of seed fermenter	5
☌ cultivation of *B. spec.1* under *name*	6
☌ at depletion of *name* source initiation of fed batch phase	6

Methods for establishing purity of seed stock

Master seed vials are maintained frozen at –80°C in a sterile glycerol solution. Inoculation of production seed vials are performed under sterile conditions and techniques in a Class II A/B biological hood.

Techniques used to ensure a uniform product/ assay methods for standardisation:

Basically a uniform product is achieved by employing a standard fermentation process, carefully performing all process steps, and by applying quality control.

During the fermentation process, at each seed transfer point, the identity of the fermented micro-organism is checked by the following procedure:
A drop of the seed (liquid media with suspended cells) is placed on a nutrient broth agar and streaked out. A drop is also placed on a glass slide for optical control of the immediate microscopic appearance of the liquid culture. The plate is incubated and developing colonies are checked for appropriate microscopic and macroscopic morphology.

Quality control analysis is performed on every Lot of *ABCD* Technical to ensure a consistent product. This includes:

- counts of colony forming units (cfu) of *B. spec.1* (content of active ingredient)
- determination of moisture content of technical powder (or dry weight in the broth)
- test for human pathogens and other contaminants by *name*.

IIM 4.3 **Quality control and post-registration monitoring methods *(Example Bacillus spec. 1)*:**

1. to detect, isolate, and enumerate the micro-organism
2. to differentiate a mutant or genetically-modified micro-organism from the parent strain.
3. to detect spontaneous change in major characteristics of micro-organism.
4. to define content of micro-organism in appropriate terms incl. standardisation, sensitivity, reproducibility, statistical validity, and representative data to validate the bioassay.
5. to show control to a specified and acceptable level, of microbial impurities and of any other impurities of toxicological concern, including toxic metabolites, which are known or suspected to be present at any stage of the manufacturing process.
6. to show absence of any human and mammalian pathogens.

IIM 4.3.1 **Methods for the analysis of the micro-organism**

In principle *B. spec.1* is identified and analysed using biological methods, i.e. plating on organic growth media. The central criterion for identification of *Bacillus* species is the endospore-morphology. For identification of different strains additional criteria are microscopic appearance, colony morphology and physiological characteristics. During fermentation and production processes the immediate microscopic appearance of a drop of liquid culture is continually checked for purity control.
Agar plates are generally used for microscopical and macroscopical identification of micro-organisms.

Definite identification of strain *ABCD* is achieved by applying following parameters:

- Presence of single, gram-positive rods with peritrichous flagella
- Form of spore (if present):
- Colour of the culture: light cream (brownish) to cream
- Morphology of colony (shape, elevations, margins):

Further information on these characterising parameters of *B. spec.1*, distinguishing this strain from others, is given in the *name* report 1997 (see Doc. K-II, Sec. 1, P. 1.3/01)

The original isolate of strain *ABCD* of *B. spec.1* has been identified by additional biochemical and physiological key criteria, including namely (*name* report 1999, Doc. K-IIB, Sec. 1, P. 1.3/01):

- ☿ Anaerobic growth
- ☿ pH of Voges-Proskauer reaction
- ☿ Maximum temperature growth
- ☿ Growth in.....

Descriptions of corresponding standard methods of identification are given by name (1999).

To confirm, whether the *B. spec.1* type bacterium originate from the strain *ABCD* or from some other source, a molecular identification by RAPD/PCR technique is possible.

Methods for providing information on possible variability of seed stock/ active organism

The use of pure seed stock for inoculation of production seed vials excludes the problem of variable content of active organism.
Purity control of the initial seed vials is performed by randomly selecting a number of vials to check their appropriate microscopic and macroscopic morphology (conferring to colony forming after plating on nutrient broth agar) and by regular quality checks determining content of active ingredient in the broth or Technical Powder (by counts of colony forming units).

IIM 4.3.2 Methods to differentiate a mutant micro-organism from the parent wild strain

Justification:
Each fermentation run is started with initial seed stock culture, which is maintained as frozen vials. Thus mutations in the original parent strain *ABCD* isolated from *name* are excluded.

IIM 4.3.3 Quality control measures applied to the fermentation process

- ☿ checking each “seed” transfer for purity (both microscopically and by *method*), and
- ☿ continually monitoring the growth of *B. spec.1* both microscopically and by optical density of the *B. spec.1* liquid culture in the production fermenter.

The completed fermentation material (broth) of each fermentation run (batch) is tested by:

- ☿ counts of colony forming units (cfu) of *B. spec.1*,
- ☿ microscopic and macroscopic examination of colony morphology,
- ☿ determination of contaminants by *method*, esp. with regard to human pathogens.

During the fermentation process environmental conditions (temperature, pH, media) are adjusted to regulate growth of *B. spec.1* and to reduce growth of potential contaminants. The seed medium is steam sterilised at 121,5°C, 15 psi for 30 minutes prior to inoculation. All process steps up to the inoculation of the fermenter are performed in a Class II A/B biological hood under aseptic conditions and techniques. If there is any abnormal behaviour of fermentation, product batch is discharged by sterilisation.

Quality control measures applied to *ABCD* Technical

The minimum Quality control sample will be.....
Quality control comprises of:

- ☿ Determination of moisture content or dry weight
- ☿ Determination of content of active ingredient
- ☿ Tests for microbial contaminants
- ☿ Detection of human pathogens

Determination of moisture content

Using an oven or infrared moisture analyser three replicates of 1 to 10 g samples will be analysed with the average reported. Details are given in the cited Document on p. 14 (*author* 1999, Doc. J-IIB, Sec. 1, P. 1.4/01).

Determination of content of active ingredient
Quantitative determination of viable *B. spec.1* is based on counts of colony forming units evolving on nutrient broth agar after streak plating of serial dilutions of *ABCD* samples (in five replicates) and incubation for 1 to 5 days. For details see p. 14 of cited Document (author 1999, Doc. J). Further information on determining aerobic colony forming units (cfu) are given by author 1999 (see Doc. K-IIB, Sec.2, P. 4.1/03).

The detection limit of plating analysis applied to fermentation broth or Technical Powder is ... cfu/g (see p. 16 of cited Document, author 1999, Doc. J).

IIM 4.3.6 **Test for microbial contaminants and detection of human pathogens**

The final product is tested for contamination by culturing samples of each production batch simultaneously with the viability test. Samples of production batches are also sent to *name*, where the microbiological purity is ensured using the following standard methods:

ISO 4833-91 (mesophiles)
NMKL 44-1990 (coliforms)
ISO 7899-84 (faecal streptococci)
ISO 7954-87 (moulds)
ISO 6888-83 (staphylococci)
ISO 6579-93 (salmonella)

_ NMKL = Nordic committee of food analysis

Quality control methods *(Example Virus spec. 1)*:

The criteria for determining the quality of the product, specifically bacterial contamination, include (I) a maximum of one contaminant bacterium per 1000 viruses (i.e., a virus to bacteria ratio of 1000:1 or less) and (ii) the use of microbe-specific selection media to identify any primary human pathogenic bacteria. The presence of any primary human pathogens, regardless of the virus to bacteria ratio, will result in destruction of the batch. In addition to these tests, an intraperitoneal (IP) injection test (in mice) will be performed on each production batch. The IP test is to assure that no primary human pathogens are present in the ground insect material that may be present in the final formulation. Because numerous literature reports indicate that the possibility of human pathogens being present in these insects is very small, this test should not result in the loss of much, if any of the product. However, the microbe-specific media testing, along with the IP tests, should eliminate the possibility of primary human pathogens being present in the final *Product name* SC formulation.

IIM 4.4 **Methods to determine storage stability of the micro-organism *(Example Bacillus spec. 1)***

Storage stability of *ABCD* Technical has been determined covering a two-year period (author 1999; Doc. K-IIB, Sec. 2, P. 4.1/05). The employed test method is to determine the microbiological titer (cfu/g) of five different lots of test substance at different time points up to two years following storage under warehouse conditions. Stability is derived from consistent titer values. Experimental design and test procedure are described in detail on pp. 7 to 8 of the cited document.

IIM 4.5 **Post-registration monitoring methods to determine and quantify residues of viable or non-viable micro-organism and metabolites *(Example Bacillus spec. 1)***

The following methodology is used to detect viable residues of *B. spec.1* of strain *ABCD*

(author 1999):

- The samples are suspended in water and cultivated by spread plate method to detect the possible presence of bacteria in the sample.
- *B. spec.1* is identified on the basis of *method*
- To confirm, whether the *B. spec.1* type bacterium originate from the strain *ABCD* or from some other source, a molecular identification by RAPD/PCR technique is possible.

Non-viable residues:
Not relevant. There are no observations of such metabolites etc. that could cause non-viable residues originating from the bacterium *B. spec. 1* of strain *ABCD*.

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PART 3

Section 3 Toxicological Studies and Exposure Data and Information

The example of a summary and assessment of data which follows is intended to illustrate the approach recommended for the preparation of *Tier II* summaries and assessments. The material included has not been critically assessed for its technical content. The data included in the following summary and evaluation are not based on a real submission.

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IIM 5.1 **Summary: potential of microbial pest control agent to be hazardous to humans with consideration of its pathogenic potential, its ability to infect and pattern of clearance, and its toxicological effects**

All toxicological studies prepared with the active substances *abcd* revealed no effects to human or animal health. The fungus does not affect any organism except the host *Sclerotinia sp*. and a toxin is not produced. Toxic metabolism or degradation products do not occur.

IIM 5.2 **Occupational health surveillance report on workers during production and testing of MCPA**

Clinical cases and poisoning incidents did not occur in the laboratories of the applicant. There are no indications for a toxic potential regarding to the information of the medical record of the employees involved in the manufacture of ABCD.

IIM 5.2.4 **Published reports of adverse effects**

In the publications no incidents are mentioned. Clinical signs and poisoning symptoms are unknown, therefore, first aid measures and therapeutic regimes for the non-toxic active substance *ABCD* can not be recommended.

IIM 5.3 **Basic studies**

IIM 5.3.2 **Acute oral infectivity, toxicity and pathogenicity**

Pure active substance *ABCD*, 25 mg dissolved in 100 ml sterile physiological saline to 1.9 x 109 CFU/ml. 10^8 CFU was administered to male and female Sprague-Dawly rats by oral gavage.
Rats were observed for 14 days. Clinical signs and body weight were monitored at days 4, 7, 10 and 14. Histopathological research of the spleen, liver and kidneys was performed at days 4, 7 and 14.

No rats died during the study. No significant differences in body weight were observed compared to the control animals. Histopathological research of the organs revealed no toxicity or infectivity of either organ by the microbe. Clearance of the faeces was shown by linear regression from day 4-14 days to occur at day 23. It was concluded that *ABCD* was not toxic or infectious in the rat under the circumstances of this study (no labelling required).

IIM 5.3.3 **Acute intratracheal/inhalation infectivity, toxicity and pathogenicity**

According to the results about the acute toxicity and because of the temperature conditions which have an optimum between 20 to 25°C (*ABCD* can not exist in warm-blooded organisms), further studies concerning the short-term toxicity were not prepared.

IIM 5.3.4 Acute intravenous/intra peritoneal infectivity

Pure active substance of *ABCD*, 0.25 mg dissolved in 100 ml sterile physiological saline to 1 x 10^7 CFU/ml. 1.0 ml was injected intraperitoneally into Sprague-Dawly rats.
Test animal were observed for 28 days. Clinical signs and body weight were monitored at days 4, 7, 10 and 14. No histopathological research was performed of organs. Clearance of the microbe from the blood was tested at days 2, 8, 15, 22 and 28.
No significant differences in body weight were observed compared to the controls. No abnormal findings were noted at necroscopy. No infectivity/colonies were found from blood samples at day 2, 8, 15, 22, 29. It was concluded that the micro-organism is non-toxic and non-pathogenic in rats (no labelling required).

IIM 5.3.5 Genotoxic potential

According to the results about the acute toxicity and because of the temperature conditions which have an optimum between 20 to 25°C (*ABCD* can not exist in warm-blooded organisms), further studies concerning the genotoxicity potential were not prepared.

IIM 5.3.6 Cell culture study, for viruses and viroids

The genotoxicity of different NPVs has been determined on the basis of *in vitro* tests, *in vivo* tests and cell culture studies. Negative results were seen consistently in a mouse cell line, in a muntjak cell line, in bone marrow cells of Chinese hamster and NMRI mice, and in a human cell line. There is no evidence of virus replication in mammalian cell cultures, and no changes in sister chromatid exchanges or chromosome aberrations compared to control animals were observed. Based on the available data it can be concluded that ABCD will not induce genotoxic effects in mammals.

IIM 5.4 Toxicity studies on metabolites

The micro-organism *X. xxx* produces at growth, a relevant metabolite ABC, that is toxic (EC 50 for YYY is xyz g/kg). ABC will not be present in concentrations exceeding X g/l in the formulated product at the point of formulation. Measurements of the ABC content in the bacterial broth before the formulation will be included in the regular quality control of the production. The presence of residues of ABC on the treated material will not exceed the stated amount of xyz g/kg.

The micro-organism *X. xxx* is not present on food or feed above the stated background level for the organism (ref).

IIM 5.5 Other/special studies

According to the results about the acute toxicity and because of the temperature conditions which have an optimum between 20 to 25°C (*ABCD* can not exist in warm-blooded organisms), further studies concerning the (1) short-term toxicity, (2) genotoxicity potential, (3) long-term toxicity and carcinogenicity, (4) reproductive toxicology, (5) teratogenity potential and (6) neurotoxicity potential were not prepared.

IIM 5.6 Summary of mammalian toxicity and overall evaluation

See: Tier III summary

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PART 4

Section 4 Residues in or on Treated Products, Food and Feed

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IIM 6.1 Rationale for waiver of residue data based on information showing that MPCA is not hazardous to mammal

IIIM 8 Rationale to waive residue studies on MPCP

Regarding to the biological properties of the active substance of ABCD, *abcd*, an indiginous soil sclerotia-parasite, no studies were prepared because (1) the fungus *abcd* is an indiginous soil compartment and was detected in several soils, (2) fungus *abcd* is a high specialised mycoparasite and is not able to exist in strange organisms, (3) the concentration of the mycoparasite *abcd* depends on the concentration of the host *Sclerotinia* spp. and (4) the product ABCD, including the active substance *abcd* as a spore suspension, must be incorporated in the soil directly after application. The product is used for *Sclerotinia* spp. soil decontamination before sowing or planting. A direct treatment of plants is not possible and the way of application (before sowing or planting and incorporation in soil after application) therefore bring about that no residues in or on products, feed and feeding stuffs can occur.

The micro-organism *X. xxx* produces at growth, a relevant metabolite ABC, that is toxic (EC 50 for YYY is xyz g/kg). ABC will not be present in concentrations exceeding X g/l in the formulated product at the point of formulation. Measurements of the ABC content in the bacterial broth before the formulation will be included in the regular quality control of the production. The presence of residues of ABC on the treated material will not exceed the stated amount of xyz g/kg. The residual amount on cereal for consumers will be below the detection limit for ABC. This will be confirmed by measurements of ABC at regular intervals.

The micro-organism *X. xxx* is not present on food or feed above the stated background level for the organism (ref).

Analytical techniques have been developed to determine ABC residues in the manufactured product, on the treated material and on the harvest (ref).

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PART 5

Section 5 Fate and behaviour in the environment

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IIM 7 Fate and Behaviour studies on the Microbial Pest Control Agent in the environment

IIM 7.1 **Sufficient information on the origin, properties, survival and residual metabolites of the microorganism to assess its fate and behaviour in the environment. Information provided in parts 2 - 6 may suffice.**

Report: IIM 7.1.1 - Jones, B. Hare, E. (1996), Soil Microcosm Fate and Persistence, Vatrobe, Inc. Unpublished Report CR35-A21

Guideline: US EPA 885.5200. Expression in a Terrestrial Environment.

GLP: Fully GLP compliant in accordance with US 40 CFR 160.

Materials and Methods: The study was conducted during the period January 2, 1995 to June 2, 1996, by Cincinnati Research, Inc. Cincinnati, Ill. USA. The test organism used was *Beauvaria wendiensis strain XYZ1*. Laboratory studies were conducted to examine Physical Stability under Temperature Extremes using pure cultures on nutrient agar in petri dishes and Sunlight Effects, in which conidia in sterile water under glass cover slips were exposed to direct natural sunlight. Viability and germination were assessed by plating the treated cultures onto nutrient agar. A greenhouse microcosm study was conducted in sterile soil pots containing Crested Wheatgrass and Alfalfa exposed to natural sunlight. An aerosol condidia preparation was sprayed onto each plant and soil. The population levels of *B. wendiensis* was sampled daily and plated onto pertri dishes containing nutrient agar. The daily sampling was repeated for 30 days.

Findings: Temperature Effects: *Beauvaria wendiensis strain XYZ1* conidia maintained original viability for 56 days at 5⁻C and 25⁻C. At 50⁻C, the conidia steadily lost viability with germination of 6.8% at 14 days and 0.2% at 28 days. The germination half-life at 50⁻C was 4.6 days and the quarter-life was 10.0 days at that temperature.

Physical Stability: The viability of *Beauvaria wendiensis strain XYZ1* conidia decreased very rapidly after two hours of direct exposure to direct natural sunlight. The estimated half-life of *B. wendiensis* conidia was 2.58 hours and the quarter-life was 3.11 hours based on the results of this study.

Persistence on Crested Wheatgrass and Alfalfa: The conidia persisted for four days in the upper canopy and about 16 days in the middle canopy of the test crops. Persistence was greater in the middle canopy of the broadleaf crop, alfalfa, than the grass crop, rested wheatgrass.

Conclusions: Temperature Effects: Since exposure at 5⁻C and 25⁻C resulted in virtually no loss of viability from 56 days exposure and these temperatures are more representative of field conditions than 50⁻C, the study indicates that conidia of *Beauvaria wendiensis strain XYZ1* will likely be unaffected by ambient field temperatures for at least 56 days and remain infections to susceptible insects.

Physical Stability: These data predict that conidia *Beauvaria wendiensis strain XYZ1* are rapidly inactivated by natural sunlight under the conditions of the study. However, the exposure scenario (directly exposed glass cover slips) is not analogous to typical field, foliage and soil surface habitats of the insect pests for which the product is intended to infect and control. The natural habitat can provide ample niches of shade and humidity which would favor *Beauvaria wendiensis strain XYZ1* conidia survival and infectivity for target pest species as well as susceptible nontarget insects.

Persistence on Crested Wheatgrass and Alfalfa: The conidia persisted for four days in the upper canopy and about 16 days in the middle canopy of the test crops. Persistence was greater in the middle canopy of the broadleaf crop, alfalfa, than the grass crop, rested wheatgrass. The major environmental factor reducing persistence of conidia was thought to be ultraviolet light which is consistent with the published findings of others and the previous study. These data do not preclude effects on nontarget insects directly after application and within a few days of application, particularly to plants with foliage that provides shade

Report: IIM 7.1 - Xxxxxx, X. and X. Xxxxxxxxxxx, X. (1999), Isolation of *Beauvaria wendiensis* from various biotopes and plant species in Sweden and from soil and plant samples taken at various locations in Switzerland, Vatrobe, Inc. Unpublished Report 801

Guideline: Guidelines were not available at the time the test was performed.

GLP: No. University facility, not completely GLP compliant.

Materials and Methods: The study was conducted during the period July 1, 1999 - September 25, 1999, by Plant Biocontrol Laboratories, University of Sweden, Uppsala, Sweden. The test organism used for comparison to environmental isolates was *Beauvaria wendiensis strain XYZ1*. Soil and plant samples were taken from locations in Sweden and Switzerland. The Sweden samples were gather from the vicinity of Uppsala (three different locations representing five different biotopes) and from farm fields (16 localities) in southern and eastern Sweden. The Swiss material was sampled from farm fields at 22 different locations. The samples were analysed using selective media. Colonial morphology aided the selection of colonies for comparison with *Beauvaria wendiensis strain XYZ1* using RAPD-PCR fingerprinting. (Cravanzola F, Piatti P, Bridge P, Ozino O - 1997. Detection of genetic polymorphism by RAPD-PCR in strains of the entomopathogenic fungus *Beauveria brongniartii* isolated from the European cockchafer (*Melolontha* spp.). Letters in Applied Microbiology, 25, 289-294.)

Findings: Seventy-one (71) isolates showed resemblance to the isolate *B. wendiensis strain XYZ1* with respect to colony character. Isolates resembling *B. wendiensis strain XYZ1* were found in most samples but not on all plant material from the Uppsala area. Several tested isolates also showed the same RAPD-PCR fingerprint as *B. wendiensis strain XYZ1*.

Conclusions: Fungi resembling the strain XZY1 are a group of soil fungi that occur in all the biotopes sampled and are common in cultivated soils

Beauvaria wendiensis has the same routes of dispersal as other strains of soil fungi. It can be dispersed via the atmosphere, with dust particles and aerosols, but it also adheres to and is transported by animals of different kinds. The organism can probably survive and have a certain

degree of self-reproduction in the soil ecosystems it encounters when used. However, since it is already common and has a low competitive ability, it is regarded to have no possibility to re-establish. *B. wendiensis* cells are motile, but as for most motile fungi, the self-mobility in the ecosystem is probably very restricted.

IIM 7.2 Other/special studies

Additional Fate and Behaviour studies are not needed because sufficient information is available from the two section 7 studies, above, to support a risk assessment for these uses of *Beauvaria wendiensis strain XYZ1*.

Appendix 7 **Format for the compilation of *Tier II* summaries - Microbial Pest Control Agent**

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PART 6

Section 6 Ecotoxicological studies and risk assessment

The example of a summary and assessment of data which follows is intended to illustrate the approach recommended for the preparation of *Tier II* summaries and assessments. The material included has not been critically assessed for its technical content. The data included in the following summary and evaluation are not based on a real submission.

Applicant should be aware that these guidelines are intended to provide a degree of flexibility. Where in particular cases, it is more appropriate to present the data and information in another format, applicants may do so. In such cases it is recommended that the applicant discuss the format proposed with the Regulatory Authority of the Country to which application is to be made.

IIM 8 Effects on non-target organisms

IIM 8.1 Effects on Birds

Report: IIM 8.1 - Faire, A. (1996), Avian Oral Study: Mallard Duck. Report, pp 5. Vatrobe, Inc. Unpublished Report No. 712, July 05, 1996

Guideline: US EPA 885.4050

GLP: Yes (laboratory certified by the National Certification Authority of Sweden)

Materials and Methods: The study was conducted during the period January 10, 1995 - January 25, 1996, by Norcen, Inc. Institute of Ecology Testing, Motola, Sweden, Report NC345123. The test organism used was *Beauvaria wendiensis strain XYZ1*, obtained from Vatrope, Inc.. The test species was Mallard Duck, 17 to 19 days old at the start of the study. Body weights were recorded weekly. Standard XXXXX- brand duck diet containing no antibiotics was used throughout the study (analyses submitted separately). They were housed, 5 birds per cage, in 5000 cm^2 x 32 cm high mesh cages. They were kept at 21 deg C and relative humidity levels from 45 to 60%. The lighting was fluorescent and was timed for 8 hours of light and 16 hours of dark. The total feed consumption was recorded at weekly intervals. The TGAI was administered in a maximum hazard dose of 2,667 mg/kg per bird per day for five days in capsular form. The concentration of TGAI in the diet ranged from 5.0 x 10^8 to 1.05 x 10^{12} cfu/ml. The control groups consisted of 10 birds each and the maximum hazard dose group consisted of 30 birds. In addition to the non-dosed negative control group, a control group was treated with an equivalent amount of heat-killed TGAI, and another control group was treated with 2000 mg/kg/ bird weight of milipore-filtered supernatant from the TGAI growth culture by gavage. Visual observations for signs of intoxication, abnormal behavior, regurgitation, or sickness were made for 120 minutes after dosing and at least 4 times daily for the 30 day duration of the study. Gross necropsies were performed on 10 randomly chosen birds from the maximum hazard dose group. Samples taken from the digestive tract, lungs, and blood of the necropsied birds were pour planted onto selective media and incubated.

Findings: There were no differences seen among the groups with regards to body weight increases or food consumption (insert tables). No mortalities were seen in control or treated groups. Nor was there any evidence of toxicity or pathogenicity caused by the TGAI. The necropsies revealed no abnormalities or lesions. No colonies representative of *Beauvaria wendiensis* were detected in any of the tissues sampled.

Conclusions: *Beauvaria wendiensis XYZ1* belongs to a group of commonly occurring soil-fungi and is thus part of the natural environment which birds are exposed to. Since *XYZ1* is

sensitive to low pH it is unlikely that it will survive and reproduce in the birds interior. In addition, the maximum growth temperature *in vivo* of this fungus is 32 deg C which would prevent it from growing at the higher body temperature of birds, thus making it unlikely this could be an avian pathogen. The mallard duck study further supports these conclusions since there was no evidence of toxicity or pathogenicity caused by the test organism.

IIM 8.2 Effects on Fish

Report: IIM 8.2 - Gentner, F.; and Couch, J. (1975), Freshwater Fish testing in Bluegill sunfish and Rainbow trout, Vatrobe, Inc. Unpublished Report No. 706, July 01, 1995

Guideline: US EPA 885.4200

GLP: Yes, in accordance with US 40 CFR 160.

Materials and Methods: The study was conducted during the period April 3, 1974 - July 27, 1974, by Gulf Breeze Aquatic Testing Labs, Gulf Breeze, Fla. USA, Report GB25. The test organism used was *Beauvaria wendiensis strain XYZ1*, obtained from Vatrope, Inc.. The test species was Bluegill Sunfish, *Lepomis macrochirus*, 17 to 19 days old at the start of the study. There were two deviations from the guidelines: 40 fish were used in a Maximum Hazard Dose rather than 30 and the tests were carried out for 35 days rather than 30.
Include complete materials and methods summary.

Findings: Record the findings

Conclusions: Summarize the conclusions

IIM 8.3 Effects on Aquatic invertebrates

A study was not conducted because the use pattern would not result in significant exposure to aquatic invertebrates.

IIM 8.4 Effects on Algal Growth and Growth Rate.

Studies were not conducted because the use pattern would not result in significant exposure to algae.

IIM 8.6 Effects on ~~Aquatic or~~ Terrestrial Plants

A study was not conducted because the use pattern would not result in significant exposure to terrestrial plants and the microorganism is not related to any known plant pathogen.

IIM 8.7 Effects on ~~Honey~~ Bees

Report: IIM 8.7 - Honey Bee testing Vatrobe, Inc. Unpublished Report No.708, July 21, 1997

Guideline: US EPA 885.4380

GLP: Yes, in accordance with US 40 CFR 160.

Materials and Methods: The study was conducted during the period May 9, 1997 - June 9, 1997, by Eco Animals Lab, Braunschweig, Germany, Report 2357DE. The test organism used was *Beauvaria wendiensis strain XYZ1*, obtained from Vatrope, Inc.

Appendix 7 **Format for the compilation of *Tier II* summaries - Microbial Pest Control Agent**

Company name **Month and year** **Microbial Pest Control Agent (Name)** **page of**

Include complete materials and methods summary.

Findings: Record the findings

Conclusions: Summarize the conclusions

IIM 8.8 Effects on ~~Non-target~~ Terrestrial Arthropods other than bees

Report: IIM 8.8 - Non-target terrestrial arthropods - parasitic wasps (Parasitoid) Vatrobe, Inc. Unpublished Report No.709a, September 21, 1997

Guideline: US EPA 885.4340

GLP: Yes, in accordance with US 40 CFR 160.

Materials and Methods: The study was conducted during the period June 9, 1997 - August 9, 1997, by Eco Animals Lab, Braunschweig, Germany, Report 2358DE. The test organism used was *Beauvaria wendiensis strain XYZ1*, obtained from Vatrope, Inc.
Include complete materials and methods summary.

Findings: Record the findings

Conclusions: Summarize the conclusions

Report: IIM 8.8 - Non-target terrestrial arthropods - ladybird beetles (Foliage Dwelling predators) Vatrobe, Inc. Unpublished Report No.709b, September 21, 1997

Guideline: US EPA 885.4340

GLP: Yes, in accordance with US 40 CFR 160.

Materials and Methods: The study was conducted during the period June 9, 1997 - August 9, 1997, by Eco Animals Lab, Braunschweig, Germany, Report 2358DE. The test organism used was *Beauvaria wendiensis strain XYZ1*, obtained from Vatrope, Inc.
Include complete materials and methods summary.

Findings: Record the findings

Conclusions: Summarize the conclusions

IIM 8.9 Effects on Other Terrestrial Invertebrates

Studies were not conducted on other terrestrial invertebrates. *Beauvaria wendiensis strain XYZ1* is a naturally-occurring soil borne fungus that is common in the terrestrial environment. No epizootics have been attributed to *Beauvaria* species and they do not appear to be capable of maintaining sufficient levels in the environment to cause an epizootic. Thus, even if another invertebrates is susceptible to this fungus, it will not be impacted significantly except in the area of treatment with artificially high levels of this fungus.

IIM 8.9.1 Effects on earthworms

An earthworm study was not conducted because *Beauvaria wendiensis strain XYZ1* is a naturally-occurring soil borne fungus that has not been modified in any aspect. It is likely that the fungus and

its relatives constitutes food for earthworms and at least does not cause them harm at the expected exposures for these use patterns. Tests for toxicity of *XYZ1* to earthworms have, for these reasons, not been regarded appropriate.

IIM 8.10 Effects on ~~non-target~~ soil micro-organisms

A soil micro-organism study effects on non-target soil micro-organisms. Because it is a naturally occurring soil known fungus, it is not likely to cause harm at the expected exposures at these use patterns.