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Guidance document on Baculoviruses as plant protection products

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Foreword

The OECD's Working Group on Harmonization of Regulatory Oversight in Biotechnology decided in 1995 to focus its work on the development of *Consensus Documents* which are mutually acceptable among Member countries. These consensus documents contain information for use during the regulatory assessment of a particular product. In this series, the *Consensus Document on Information used in the Assessment of Environmental Applications involving Baculoviruses* was published by OECD [ENV/JM/MONO(2002)1] in 2002.

The OECD Expert Group on Biopesticides (EGBP) found that an update of the OECD Consensus Document [ENV/JM/MONO(2002)1] to cover the scientific progress on baculoviruses since 2002 would be highly appropriate. For this Guidance Document, literature on baculoviruses useful for industry and regulatory authorities was reviewed and used to address data points required for the assessment of baculoviruses as pesticide products.

The initial draft of this document was developed by Prof. Dr. Johannes Jehle and was overseen by the EGBP. The draft guidance was first sent for review to a scientific advisory group from Australia, Canada, Germany, the Netherlands and the US, as well as the International Biocontrol Manufacturers Association (IBMA) in January 2021. The draft document was sent to the EGBP for comments in December 2021 and January 2023.

The draft guidance was revised, based on comments received. The final document was approved by the Working Party on Pesticides in August 2023.

This document is being published under the responsibility of the Chemicals and Biotechnology Committee (CBC), which has agreed that it will be declassified and made available to the public.

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1 Glossary and Abbreviations

Glossary

Budded virus (BV)

A type of baculovirus that buds out of infected cells and spreads the infection within an insect and within cell culture. The BV derive their envelope from modified cell membranes.

Contaminant (microbial)

A (pathogenic/infective) micro-organism unintentionally present in the technical grade ingredient.

Genotype

In baculovirus literature, a genotype refers to cloned genetic variant of an isolate.

Infectivity/infectiveness

Infectivity is the ability of the organism to replicate and establish a focal point of infection. An infection may or may not result in overt disease.

Invasiveness

Invasiveness is the ability of the micro-organism to spread to adjacent or other tissues.

Metabolite

A metabolite is a general term to refer to any substance produced by a micro-organism. Viruses are unable to produce metabolites. For this document, metabolite refers only to secondary metabolites (i.e., metabolites which are not essential for basic life processes of the micro-organism).

Micro-organisms

Microbiological entities, cellular or non-cellular, capable of replication and/or of transferring genetic material. The definition applies to, but is not limited to, bacteria, fungi, protozoa, viruses and viroids; nematodes are not included.

Occlusion-derived virus (ODV)

Baculovirus phenotype that is derived from occlusion bodies. They obtain their envelope within the nucleus.

Peritrophic membrane

The peritrophic membrane (or peritrophic matrix) is a tube-like structure that separates food from the insect midgut epithelium. It is composed of chitin and protein.

Pathogenicity

The ability of a micro-organism to inflict injury and damage to a host upon infection; it depends on host resistance or susceptibility.

Pathogen

A pathogen is a micro-organism that is invasive, infective and exhibits pathogenicity.

Pathogenic potential

Pathogenic potential refers to the degree that a pathogen causes morbid symptoms.

Technical grade active ingredient (TGAI)

TGAI is the outcome of the manufacturing process of the micro-organism(s) intended to be used as active substance in plant protection products. It consists of the micro-organism(s) and may contain additives, metabolites, chemical impurities, contaminating micro-organisms and the spent medium/rest fraction resulting from the production process. For baculoviruses, the TGAI could also include insect parts, scales, setae, etc.

Toxin

A toxin is a (organic) substance that is produced in nature and is able to injure or cause damage in a living organism.

Virus isolate

A virus isolate refers to a virus that has been isolated from a host and propagated in culture (Fauquet et al., 2008). Note that this definition is different from isolate definition used in the Guidance Document for the Regulatory Framework for the Microorganism Group: Bacteriophages. ENV/CBC/MONO(2022)40.

Virus strain

A virus strain refers to viruses that belong to the same species and having stable and heritable biological, serological, and/or molecular differences (Fauquet et al., 2008).

Virus species

A species is the lowest taxonomic level in the hierarchy approved by the International Committee on Taxonomy of Viruses (ICTV, http://ictv.global). It constitutes a monophyletic group of viruses whose properties can be distinguished from those of other species by multiple criteria.

Abbreviations

AAVs	adeno-associated viruses
BEVS	baculovirus expression vector system
BIOHAZ	EFSA Panel on Biological Hazards
bp	base pair
BV	budded virus, budded viruses
CFU	colony forming unit
EC	European Commission
EFP	envelope fusion protein
EFSA	European Food Safety Authority
EGT	ecdysteroid-UDP-glucosyltransferase
ELISA	enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
et al.	et alii; and others
etc.	et cetera
EU	European Union
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FP	few polyhedra
GRAS	Generally Recognized As Safe
GV	granulovirus
HA	hemagglutinin
hcf	host cell factor
hrs	homologous repeated sequences
hrf	host range factor

ha	hectare
HTS	high-throughput sequencing
IAP	inhibitor of apoptosis
ICTV	International Committee on Taxonomy of Viruses
IgG	Immunoglobulin G
IPM	Integrated Pest Management
K2P	Kimura-two-parameter
kbp	kilo base pairs
L1 and L5	first instar and fifth instar
LC_{50}	median lethal concentration (50%)
LD_{50}	median lethal dose (50%)
lef	late expression factor
mg-	mixed genotype
MMP	matrix metalloprotease
MNPV	multiple nucleocapsid nucleopolyhedrovirus
MPCA	microbial pest control agent (active substance)
MPCP	microbial pest control product
MRL	maximum residue level
NA	neuraminidase
ni-	natural isolate
NOB	non-occluded baculoviruses
NPV	nucleopolyhedrovirus
OB	occlusion body, occlusion bodies
ODV	occlusion-derived virus
OECD	Organisation for Economic Co-operation and Development
ORF	open reading frame

PCR	polymerase chain reaction
per os	route of uptake is taken through the mouth (peroral)
PIB	polyhedral inclusion body (refers to the OB of NPVs)
pif	*per os* infectivity factor
PM	peritrophic membrane (also peritrophic matrix)
polh/gran	polyhedrin/granulin gene
PTM	potato tuber moth
QPS	Qualified Presumption of Safety
REBECA	Regulation of Biological Control Agents
REN	restriction endonuclease
RFLP	restriction fragment length polymorphism
sg-	single genotype
SNP	single nucleotide polymorphism
SNPV	single nucleocapsid nucleopolyhedrovirus
TE	transposable element
TGAI	technical grade active ingredient
vFGF	baculovirus-encoded fibroblast growth factor
VLPs	virus-like particles
VS	virogenic stroma
WHO	World Health Organization

For baculovirus names and their abbreviations, see Appendix Table A1.

2 Introduction

1. Baculoviruses have been explored for more than 75 years as microbial pest control agents (MPCA) and are commercially available as microbial pest control products (MPCP) for almost 50 years. Today baculoviruses are registered and used as MPCP in nearly all OECD countries. Since its publication in 2002, the OECD *Consensus Document on Information used in the Assessment of Environmental Applications involving Baculoviruses* [ENV/JM/MONO(2002)1] served as an international reference in the assessment of health and environmental risks posed by the use of baculoviruses in plant protection. It referred mainly to studies on baculoviruses related to human and environmental safety, which were carried out from the late 1960 to 1980 and reviewed the knowledge on baculovirus biology, morphology and genetics published until 1999. Since then, tremendous scientific progress has been achieved in understanding the molecular biology of baculoviruses, their ecology and evolution as well as in the concepts on species demarcation and classification; new methodologies were developed for identifying and studying baculoviruses. In addition, dozens of different baculoviruses have been meanwhile registered and safely used worldwide.

2. The OECD Expert Group on Biopesticides (EGBP) found that an update of the OECD Consensus Document [ENV/JM/MONO(2002)1] would be highly appropriate to cover the scientific progress on baculoviruses and to provide further guidance for industry and regulatory authorities in assessing the biological properties, potential hazards to human health, non-target organisms and the environment. For this, peer-reviewed literature on baculoviruses was reviewed using the SCOPUS database from 1999 to 2020. Where appropriate, the most recent relevant literature was added during the phase of reviewing the draft document in 2021.

3. The specific focus of this guidance document lies on the developments in identification (including taxonomy and classification) (Chapter 3), biological properties including mode of action, genetic stability, host resistance, population genetics and baculovirus ecology (Chapter 4), human health considerations (Chapter 5), residues (Chapter 6), environmental fate (Chapter 7), effects on non-target organisms (Chapter 8) and conclusions for safety assessment (Chapter 9).

4. This document is intended to provide general and specific information on baculoviruses and guidance to industry, regulatory authorities and others who have responsibility for assessments of baculoviruses used as biological control agents.

3 Identity

3.1 Taxonomy

3.1.1 Concepts of Classification

5. Baculoviruses (family *Baculoviridae*) are insect-specific viruses with an enveloped rod-shaped nucleocapsid and a circular double-stranded DNA genome. Further family-defining characteristics are their replication and nucleocapsid assembly in the nucleus, mRNA transcription from viral DNA by host- and virus-encoded RNA polymerases, and infection of larval stages of insects of the orders Lepidoptera, Hymenoptera, and Diptera (Harrison et al., 2018a). Prior to the Ninth Report of the International Committee on Taxonomy of Viruses (ICTV), the family *Baculoviridae* was divided into two genera, (i) the genus *Nucleopolyhedrovirus* and (ii) the genus *Granulovirus* (Volkman et al., 1995). This classification was mainly based on occlusion body (OB) morphology but lacked a phylogenetic basis as is nowadays accepted for many virus families. Using partial and complete genome sequences, a phylogeny-based classification was developed (Jehle et al., 2006a) and eventually accepted by the ICTV (Harrison et al., 2018a; Herniou et al., 2011).

6. “Baculo-” is derived from *baculum*, meaning “rod” in Latin and refers to the morphology of the nucleocapsid. “Nucleopolyhedro-” comes from “nuclear polyhedrosis”, referring to the multifaceted appearance of OB in the nuclei of infected cells, whereas “Granulo-“ derived from “granule” and “granulosis”, referring to the relatively small size of OB and their granular appearance in infected cells (Harrison et al., 2018a).

7. The given name of an individual baculovirus is currently composed of two to three parts, (i) the scientific name of host insect from which the virus isolate was initially isolated and (ii) a reference to the OB morphology as nucleopolyhedrovirus (NPV) or granulovirus (GV) and if necessary (iii) an additional identifier such as A, B etc.

8. According to the current taxonomy, the family *Baculoviridae* is classified into four genera, *Alphabaculovirus*, *Betabaculovirus*, *Gammabaculovirus* and *Deltabaculovirus*. Viruses of the genus *Alphabaculovirus* infect larvae of the insect order Lepidoptera and have polyhedral OB with multiple virions consisting of single or multiple enveloped nucleocapsids. The alphabaculoviruses can be further subdivided into two clades, group I and group II, which correlate with the type of envelope fusion protein used for infection. Members of the genus *Betabaculovirus* (previously *Granulovirus*) also infect larvae from the insect order Lepidoptera but have ovoid OB with a single virion consisting of a single enveloped nucleocapsid. Viruses of the genus *Gammabaculoviruses* and *Deltabaculoviruses* infect larvae of sawflies (order Hymenoptera) and mosquitoes (order Diptera), respectively. They have polyhedral OB embedding multiple virions with single enveloped nucleocapsids (Harrison et al., 2018a).

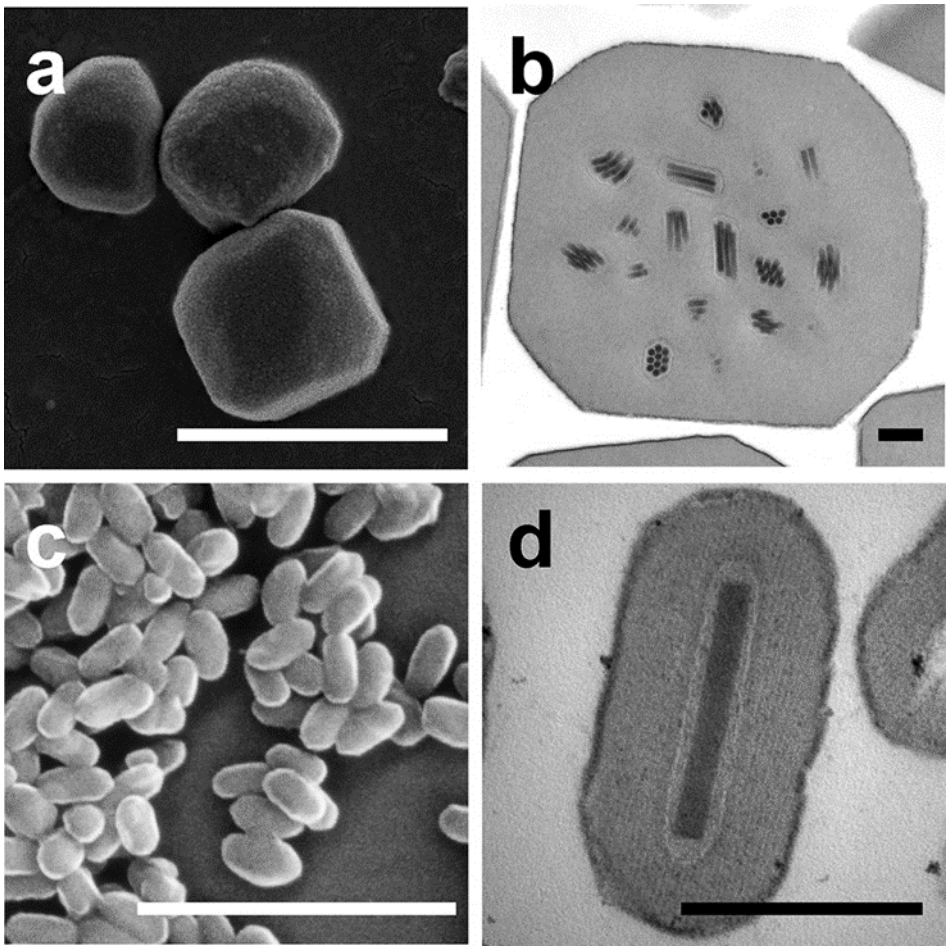

Fig. 1. Scanning and transmission electron micrographs of occlusion bodies of (a) Operophtera brumata nucleopolyhedrovirus MA, (b) Autographa californica multiple nucleopolyhedrovirus C6, (c) Spodoptera frugiperda granulovirus and (d) Mythimna unipuncta granulovirus #8. Scale bars, (a, c) 2 μ m, (b, d) 250 nm. (From: Harrison et al., 2018a; © The authors, under exclusive licence to Microbial Society (CCBY 4.0)).

9. Viruses previously classified as non-occluded baculoviruses (NOB) found in other arthropod orders, such as Lepidoptera, Orthoptera, Coleoptera, Crustacea and others, have been re-classified into separate virus families, such as *Nudiviridae*, *Hytrosaviridae* or *Nimaviridae,* because they are only distantly related to baculoviruses (Harrison et al., 2020; Kariithi et al., 2019; Wang et al., 2019a). Therefore, baculoviruses are not only arthropod-specific but rather insect-specific for the mentioned insect orders Lepidoptera, Hymenoptera and Diptera. With the formation of higher virus taxa, ICTV has recently introduced the order *Lefavirales* which harbors the three families *Baculoviridae, Nudiviridae* and *Hytrosaviridae*. Together with the family *Nimaviridae,* these four families form the class *Naldaviricetes*.

10. The species concept of viruses follows a phylogenetic and polythetic approach, constituting a virus species as a “monophyletic group of viruses whose properties can be distinguished from those of other species by multiple criteria." These developed criteria used for species distinction are mainly based on host range and specificity, cell and tissue tropism, pathogenicity, DNA restriction profiles, DNA and predicted protein sequences as well as sequence similarities (Adams et al., 2013).

3.1.2 Species Demarcation Criteria

11. In addition to proposing a new classification system for baculoviruses based on genome sequence, phylogenetic species demarcation criteria were developed for alpha- and betabaculoviruses, which are by far the largest groups of baculoviruses. Kimura-two-parameter (K2P) distances based on the concatenated nucleotide sequences of the highly conserved genes *polyhedrin/granulin* (*polh/gran), late expression factor 8 (lef-8)* and *lef-9* have been used to identify whether a given baculovirus is a variant of an already known baculovirus species or belongs to a new species: pairwise K2P distances of *polh/gran, lef-8 and lef-9* of <0.015 indicate that viruses belong to the same baculovirus species, whereas distances >0.050 indicate that viruses belong to different virus species. For K2P distances between 0.015 and 0.050 additional biological information such as host range, pathology etc. is required for species determination (Jehle et al., 2006b).

12. Comparison of genome sequences of Helicoverpa armigera nucleopolyhedrovirus G4 (HearNPV-G4), Helicoverpa armigera nucleopolyhedrovirus C1 (HearNPV-C1) and Heliothis zea single nucleopolyhedrovirus (HzSNPV) was in line with the K2P determination, concluding that these closely related NPVs were three variants of the same species *Helicoverpa armigera nucleopolyhedrovirus* (Chen et al., 2001; Jehle et al., 2006b; Zhang et al., 2005). Also, isolates of Rachiplusia ou multiple nucleopolyhedrovirus (RoMNPV), Anagrapha falcifera nucleopolyhedrovirus (AnfaNPV), Trichoplusia ni multiple nucleopolyhedrovirus (TnMNPV) and others appear to be members of the same species *Autographa californica multiple nucleopolyhedrovirus* (Harrison et al., 2012).

13. However, in another instance of species identification, the two viruses Mamestra configurata nucleopolyhedrovirus A and B (MacoNPV-A and MacoNPV-B), which derived from the same host insect *Mamestra configurata*, were classified to belong to two separate species as supported by genome comparison (Li et al., 2002a). Similarly, the two Agrotis segetum nucleopolyhedrovirus A and B (AgseNPV-A and AgseNPV-B) infect the same host insects but belong to two different species *Agrotis segetum nucleopolyhedrovirus A* and *Agrotis segetum nucleopolyhedrovirus B* as supported by genome sequencing and K2P determination (Wennmann et al., 2015).

14. As a consequence of the development of high-throughput sequencing (HTS) techniques, hundreds of baculovirus genomes have been sequenced and assembled into consensus genome sequences, facilitating a further approach of species identification based on 38 baculovirus core genes, which are a set of conserved genes found in all baculovirus genomes. This approach applies the same nucleotide distance-based criteria, but extended the K2P distance threshold from three marker genes *polh/gran, lef-8,* and *lef-9* to the 38 core genes data, with criteria of <0.021 for viruses belonging to the same species of a given baculovirus, while the threshold for different species has increased to >0.072, thus proposing a modified standard for phylogenetic lineage analysis and species identification (Wennmann et al., 2018). For members of the genus *Gammabaculovirus* and *Deltabaculovirus,* species are distinguished on the basis of differences in host range and specificity, genome sequence, gene content and gene order. Due to the very few recognized species in these genera it is unclear if the same K2P criteria developed for viruses from the genera *Alphabaculovirus* and *Betabaculovirus* can be applied.

15. With the Sixth Report of the ICTV, significant changes were introduced to baculovirus naming (Volkman et al., 1995). Since then, abbreviation of baculovirus names is based on a four-letter code for the host species, consisting of the first two letters of the genus name and species epithet (e.g. HearNPV), and on the elimination of the “M” and “S” indicator for NPVs with multiple or single enveloped nucleocapsids, respectively. However, some baculoviruses, well documented in the historic literature and already classified into species in the Fifth ICTV Report, retained their old names and abbreviations (e.g. AcMNPV, HzSNPV, CpGV and others), whereas for all isolates of prospective new species, the new guidelines were applied (e.g. HearNPV, AnfaNPV or AdorGV).

16. Nomenclature of viruses and virus species causes some confusion among non-taxonomists because according to ICTV rules, virus names are completely written in non-italics and can be abbreviated, whereas virus species have to be written in full italics and cannot be abbreviated. For example, the Mexican isolate of Cydia pomonella granulovirus, abbreviated CpGV-M, belongs to the species *Cydia pomonella granulovirus*. A new binomial nomenclature of virus species names has been developped (Siddell et al., 2020) and will be applied to baculoviruses starting 2023 (Appendix Table A1) (van Oers et al., 2023).

17. As of 2020, 84 baculovirus species have been recognized by ICTV (Harrison et al., 2018a; ICTV 2020) (Appendix Table A1).

3.1.3 Baculovirus Strain Definition

18. Micro-organisms are generally registered at strain level. However, the strain concept of bacteria and fungi referring to the origin from a single colony or spore cannot be applied to viruses. Viruses used as plant protection agents, such as baculoviruses or bacteriophages, are unique among micro-organisms in that they may comprise mixtures (=population) of different, often highly similar genotypes.

19. For most viruses, there is no formally accepted definition for any taxa below the species level, and no standardised approach has been established to deal with this issue. In scientific literature on viruses, the terms “strain” or “isolate” or even “variant” have been used uncritically and interchangeably (Fauquet et al., 2008). A “virus strain” definition has been proposed as “strains are best represented by viruses belonging to the same species and having stable and heritable biological, serological, and/or molecular differences” (Fauquet et al, 2008). This scientific concept implies that a viral strain may not consist of a single genotype but may consist of mixtures of genotypes belonging to the same species, it only requires an identifiable and stable inheritance of a character. The latter also appears the most important requirement from a regulatory view. To cope with the fact that baculoviruses generally exist in mixtures, in the EU, the term “isolate” has been used in regulatory documents (e.g. European Commission, 2008), however without further defining the nature of an “isolate”. There, the term “isolate” would not be different from the “strain” definition of Fauquet et al. (2008). It needs to be noted, that this concept of the terms “strain” or “isolate” are different from definitions applied for bacteriophages used in plant protection, where isolates have been defined as “a pure clone (genetically identical) derived from a wild population of a micro-organism” (OECD, 2022).

20. Because these ambiguities in scientific and regulatory documents in the use of the terms “strain” and “isolate” cannot be resolved until generally accepted descriptors of viruses below species level are defined, the terms “strain” will be used in this document as follows: *A “virus strain” refers to viruses that belong to the same species with stable and heritable biological, serological, and/or molecular properties that are distinguishable from those of other strains of the same species*. This definition comprises the term “isolate” used in EU as well as strains consisting of genetically purified clones (=genotypes). From a regulatory perspective, it is important that a registered baculovirus strain/isolate can be identified and distinguished from others by specific means. Following these considerations, three *senso stricto* types of baculovirus strains can be identified, which all would follow the strain definition introduced above:

1. Single genotype strain (sg-strain): Such strain consists of a pure genotype
2. Natural isolate strain (ni-strain): Such strain consists of a naturally stable mixture of genotypes derived from selection and propagation in a certain host.
3. Mixed genotype strain (mg-strain): Such strain could consist of a deliberately prepared mixture of defined genotypes.

21. Different strains of a given baculovirus can be distinguished on the molecular level by distinct DNA restriction endonuclease (REN) profiles or single nucleotide polymorphism (SNP) patterns based on genome sequencing data. The stable inheritance of the specific REN profiles or SNP patterns are a pre-requisite for strain identification (see Chapter 3.1.4).

3.1.4 Methods for Identification

22. During virus replication, polyhedral- or granule-shaped OB are produced in the nucleus or in a nucleo-cytoplasmic area. This histopathological feature distinguishes baculoviruses from other polyhedra-producing viruses, such as cypoviruses (*Reoviridae*) or entomopoxviruses (*Poxviridae*), for which OB are formed in the cytoplasm of infected cells.

23. To further identify baculoviruses at the species or strain level, biochemical, serological and molecular tools have been developed and were widely applied (OECD, 2002). Early studies used SDS-polyacrylamide gel electrophoresis of viral proteins, enzyme-linked immunosorbent assays (ELISA) and other immunological methods.

24. For many years, DNA restriction endonuclease (REN) analysis was used as standard to differentiate baculoviruses at the species, strain, isolate and genotype level, resulting in detailed restriction maps of baculovirus genomes (Erlandson, 2009; Escribano et al., 1999; Redman et al., 2010; Rezapanah et al., 2008; Rohrmann et al., 1978; Smith and Summers, 1978, 1979).

25. With the development of DNA sequencing techniques, whole genome sequences of baculoviruses became easily available (Ayres et al., 1994; Appendix Table A1). Using PCR methods, specific DNA fragments can be amplified and sequenced resulting in an identification and classification system based on a few highly conserved genes, such as *polh/gran, lef-8, lef-9, and per os infectivity factor 2 (pif-2=ac22)* (Herniou and Jehle, 2007; Herniou et al., 2004; Jehle et al., 2006b).

26. HTS techniques allow detailed insight to explore the genetic population structure of a baculovirus (Liu et al., 2011; Loiseau et al., 2020; Thézé et al., 2014). The HTS method has been shown to be highly useful to study the identity of baculoviruses (Gueli Alletti et al., 2017), explore the genetic variation and dynamics in a baculovirus population (Chateigner et al., 2015; Wennmann et al., 2020), to characterize baculovirus genomes (Krejmer et al., 2015), and to distinguish genotypes within baculovirus isolates and characterize genotype mixtures (Fan et al., 2020a, c). HTS is considered as the most advanced method for baculovirus genome characterization and identification.

Regulatory consideration and evaluation

27. Each baculoviral active substance should be identified and named at the strain level using the best available technology. Nowadays, full genome DNA sequencing is considered the most appropriate method, especially as genetic distances based on gene or genome sequences are used for species demarcation. Information should be provided whether the strains consist of single or multiple genotypes. Species identification should follow the ICTV criteria for baculovirus species demarcation. In the EU, approval of a baculovirus strain is extended to the baculovirus species level. A separate Guidance Document is available on how new strains of baculovirus species can be evaluated and added to the already approved strains/isolates (European Commission, 2008).

28. Taxonomy can change in time due to new methods and concepts used in virus systematics. The names of baculoviruses may change as well as the species affiliation. When a literature search is conducted, it is important to also take into account previous taxonomic names which may have been used in past publications. Phylogenetic analyses should be performed using different conserved baculovirus genes, preferably on basis of baculovirus core genes.

29. According to good scientific practice, each new baculovirus strain should be placed into an international collection.

3.2 Specification of Active Substance and Product

30. Purified baculovirus OB suspensions maintain their activity for decades when stored at -18 °C or lower, which is the appropriate temperature for long-term inoculum storage. Baculovirus biopesticide preparations contain OB harvested from infected insect cadavers reared at small or industrial scale and are formulated either as dry powders or as wet preparations (Hunter-Fujita et al., 1998). The most important morphological structure is the baculovirus OB itself, as it provides a unique degree of physical and biological stability to the infectious virion. Formulated products typically contain 10^{12} to 10^{13} OB/L (Ravensberg, 2011). Application rates vary from about 10^{10} OB/ha to 10^{13} OB/ha, which may be equivalent to the OB yield of about 200 infected larvae (Federici, 1999).

31. Due to their size of >500 nm and semicrystalline character, OB are specifically refractive in light microscopy. Thus, the concentration of purified OB of NPV and GV in a suspension is typically enumerated by direct counting in a hemocytometer using phase contrast and dark field microscopy, respectively (Eberle et al., 2012b; Hunter-Fujita et al., 1998). Rarely, some alternative methods based on electron microscopy counting (Hunter-Fujita et al., 1998), ELISA (Jones, 2000) or quantitative PCR (Tsurata et al., 2018) have been used to estimate OB numbers but might be difficult to be standardised. Concentration can also be indirectly estimated using the potency of baculovirus preparation in comparison to a standard with known concentration.

32. The activity of a production batch is typically determined by bioassays aiming to determine the median lethal dose (LD_{50}) or the median lethal concentration (LC_{50}) to which larvae of the targeted host insect were exposed (Eberle et al., 2012b; Hunter-Fujita et al., 1998). Such bioassays cannot be fully standardised but depend on the properties and requirements of host larvae to be inoculated. Different inoculation methods have been developed: (1) droplet feeding, (2) surface application, (3) diet plug assays, and (4) diet incorporation (Eberle et al., 2012b). As a rule, it is recommended that dose/concentration-response bioassays cover a mortality range between 10% to 90%. Therefore, the optimal range of dose/concentration and test duration for a certain host stage should be established in advance. This may require a large range of doses/concentrations to be applied to assess the rough position and the shape of the response curve. Then, five doses/concentrations should be arranged symmetrically around the assessed LD_{50} or LC_{50} point (Cory and Bishop, 1995). Usually, early larval instars are used because of their higher susceptibility and lower variation in weight. As a minimum number 30 - 35 larvae should be introduced for each virus dose/concentration and the untreated control. If technically feasible, only larvae surviving day 1 should be recorded for experimental evaluation, in order to exclude those larvae from the experiment that died from handling (Eberle et al., 2012b). Mortality observed in the treatments are corrected for mortality of the untreated control using the formula of Abbott (1925). At least three independent repetitions of the whole assays are necessary to randomize effects related to uncontrollable effects and laboratory conditions and thereby estimating the experimental error. To reduce variation of bioassay repetitions and between bioassays, insect rearing and bioassay conditions (temperature, day length, humidity, food, test length) need to be controlled and kept constant (Eberle et al., 2012b). The LD_{50} or LC_{50} values depend on the duration of the test. Usually, the longer the test duration, the higher the observed mortality and the lower the LD_{50} or LC_{50} values. Thus, such values cannot be cross-compared if not the identical experimental conditions are applied.

33. The shape of dose/concentration-mortality response curves is usually sigmoid. A log transformation of the doses/concentrations and a probit transformation of the mortality (Probit analysis) can be performed to facilitate comparison among different assays. Such Probit analysis results in a linear regression line. Heterogeneity (deviation from the Probit model) based on Chi-square estimation and fiducial limits are determined. The regression lines for different treatments can be compared for their slopes (hypothesis of parallelism). If the slopes of the probit-log dose/concentration lines are parallel, two virus batches can be compared based on their LD_{50} or LC_{50} position. If the probit lines are not parallel, the difference between batches will vary at different mortality levels. In this case, LD_{50} or LC_{50} and slope need

to be given when comparing bioassays (Jones, 2000). The same is applied when comparing batches based on their relative potencies which is the ratio between the LC_{50} of a test suspension and the LC_{50} of a standard.

Regulatory consideration and evaluation

34. The minimum content of the baculovirus OB (sometimes also called PIBs (polyhedral inclusion bodies) for alphabaculovirus OB or granules for betabaculovirus OB) should be reported. Appropriate terms relevant to baculoviruses, such as OB (or PIB/granule) per volume or weight should be applied. Because there are no concerns for human health or the environment due to exposure to baculoviruses and no metabolites are produced, information on the maximum content is not relevant.

35. Information on specificity, linearity, accuracy and precision of enumeration method should be provided.

36. Representative data on five batches of end-use products, consisting of both potency data (based on LD_{50} or LC_{50} values) and OB counts should be provided. For calculating the LD_{50} or LC_{50} values, appropriate statistical analysis methods need to be applied.

3.3 Identity and Content of Impurities, Additives, contaminating Micro-organisms

37. Most conceptual work and research on production, formulation and application of baculovirus biocontrol products were done in the 1960s to 1990s, culminating in a huge body of literature on practical aspects of baculovirus application (for review see Burges and Jones, 1998; Hunter-Fujita et al., 1998). However, public information on specific formulants in commercialized products is greatly restricted due to intellectual property interests by registration holders.

38. In general, baculovirus formulations aim to suppress microbial growth in the product, improve shelf-life of the product, facilitate spraying and application of the product, provide UV protection to the active ingredient, and stimulate uptake by host larvae. Since baculovirus products generally comply with organic and Integrated Pest Management (IPM) farming schemes, there might be further restrictions to meet the requirements of substances allowed to be used in organic farming.

Microbial Contaminants

39. Production of baculovirus active ingredients depends on a cellular environment supporting virus replication and OB formation. Although significant efforts and progress were made to scale-up production using cell culture fermentation for biotechnological and biomedical use of baculoviruses (van Oers et al., 2015), such *in vitro* systems are not available or cost-effective yet for most baculoviruses used as MPCA and can result in reduced *per os* infectivity of the produced OB, which is undesirable for any baculovirus being used in plant protection. Therefore, all commercial baculovirus MPCAs are produced *in vivo* in insect larvae which are kept in mass rearing facilities. Since the insect larvae used for production are unsterile and viral OB are harvested from infected larvae, it is not possible to avoid contamination of baculovirus preparations with the larval microbiome, especially the midgut bacterial flora (Alabouvette and Cordier, 2011; Hauschild, 2011). It is generally agreed that microbial contamination presents an inherent hazard associated with baculovirus-based MPCPs, thus microbial screening is essential to ensure that the products meet certain microbiological specifications. An Issue Paper on Microbial Contaminant Limits for Microbial Pest Control Products and proposed microbiological contamination screening requirements for baculovirus-based pest control products manufactured *in vivo* has been published (OECD, 2011).

40. Insect cadavers contain microbes at levels of up to 2×10^8 CFU per larva, mainly consisting of *Enterococcus* species with lower levels of yeasts and some Enterobacteriacae (Grzywacz et al., 1997; Lasa et al., 2008; OECD, 2011). Guillon (1997) reported that the natural larval midgut flora consists to a

large extent of *Bacillus cereus.* For this contaminant, a maximum level of 10^7 CFU/g or mL has been recommended, considering its ubiquity in the environment. The products need to be free (based on 25 g/mL product) from severe human pathogens, such as *Salmonella*, *Vibrio* and *Shigella.* Existing limits for food categories range between 10^3 to 10^5 CFU/g, and maximum food loads in worst-case scenarios will be far below these thresholds due to spray dilution, UV inactivation, rain wash-off and crop growth (OECD, 2011). As pointed out by Ravensberg (2011), microbial purity of baculovirus MPCPs is of crucial importance and needs to be carefully adhered to during the production process.

Unintentional ingredients

41. Baculoviruses are typically produced in susceptible host larvae reared on industrial scale. Though the manufacturing process should include steps (e.g. filtration, centrifugation etc.) to purify the OB, the final product may contain larval residues from production. There was a single case of allergenicity, characterized by slight eye irritation, reported for extremely high doses of the baculovirus product Gypchek, which contains LdMNPV as MPCA as well as larval remnants, such as setae (Lewis, 1981).

Shelf-life

42. Purification of the crude OB suspension is necessary to meet registration requirements and to keep the final product stable. Additives to suppress microbial growth during shelf storage include Sorbic acid, Tween 20, Tween 80, and potassium sorbate buffers for low pH, which both inhibits bacterial growth and stabilizes OB viability (Hunter-Fujita et al., 1998). As OB activity stays stable when refrigerated or frozen at -20 °C, commercial products retain their activity even longer under appropriate storage conditions. Activity of commercial CpGV products was unaffected for up to three years when stored at 2 °C or even 25 °C (Lacey et al., 2008). As noted in the same study, extended exposure to temperatures >30 °C cause considerable decline of activity. Test formulations of Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV) stayed stable for at least 18 months when stored at 4 °C and -20 °C but not at 25 °C (Lasa et al., 2008). In general, refrigeration or freezing is recommended for long-term storage of baculovirus MPCAs and MPCPs. Typical shelf-life tests would include larval bioassays and assessing the potency of the active substance. For shelf-life testing, the OECD Guidance ENV/JM/MONO(2016)54 should be taken into account (OECD, 2016).

Additives

43. Numerous additives have been investigated to improve spray distribution and virus uptake in the field (Hunter-Fujita et al., 1998). These additives can be categorized as surfactants, stickers, UV protectants and phagostimulants. Surfactants, such as wetters and spreaders, are used to improve mixing of the product into water, and to facilitate spray coverage on leaf surfaces, which are typically hydrophobic. Stickers, e.g. celluloses, molasses or vegetable gums, may improve adherence of OB to leaf surfaces. OB adherence depends on the size and surface charge of OB as well as on surface charge and hydrophobicity of leaf surfaces, and requires optimization for specific applications (Burges and Jones, 1998).

UV protection

44. Baculoviruses are quickly inactivated by UV radiation from the sun. The rapid decline of baculovirus viability necessitates frequent sprays to cover a season. For instance, CpGV products are sprayed up to 10-15 times per season in intervals of seven to ten days, depending on the sunshine duration and intensity. To delay UV inactivation, numerous additives have been tested during the last decades with varying levels of success. These additives can be classified into (1) reflectants, (2) general or selective absorbents, (3) chromophores, (4) free-radical scavengers, (5) host-derived materials and (6) other substances (Hunter-Fujita et al., 1998).

45. Considerable attention has been paid to optical brighteners belonging to the group of stilbene sulphonic acids, because they were found to increase the susceptibility of host larvae to virus infection. Laboratory and field experiments demonstrated a 2- to 3-log decrease of the LC_{50} in different virus-host

systems when optical brighteners were used, allowing a significant reduction in virus dosage and broadening the window of application to older instars. Such effects were demonstrated for Lymantria dispar multiple nucleopolyhedrovirus (LdMNPV) (Shapiro and Dougherty, 1994), Autographa californica multiple nucleopolyhedrovirus (AcMNPV) (Dougherty et al., 1996), AnfaNPV (Vail et al., 1996), HzSNPV/HearNPV (Ibargutxi et al., 2008; Shapiro and Vaughn, 1995), SeMNPV (Lasa et al., 2007; Murillo et al., 2003), and others. The effect of optical brighteners on the insect midgut was attributed to the degradation of the peritrophic membrane (PM) (Wang and Granados, 2000), inhibition of sloughing of infected midgut epithelial cells (Washburn et al., 1998) and inhibition of apoptosis of baculovirus-infected larval midgut cells (Dougherty et al., 2006). In general, exposure to optical brighteners may enhance host susceptibility to a homologous virus, whereas reports on host range extension are very rare (Shapiro and Dougherty, 1994; Shapiro and Vaughn, 1995) and are not considered as a risk.

Microencapsulation

46. To improve UV stability and to reduce the amount of UV protectants added to tank mixes, immediate binding of UV protectants to the OB have been explored and different techniques of microencapsulation were investigated (Hunter-Fujita et al., 1998). Important challenges of these techniques are that microencapsulated OB need to be stable in the environment but must retain OB viability as well as OB solubility in the larval midgut lumen to allow ODV release and infection of the midgut epithelial cells. Other critical issues are potential phytotoxic effects on leaves and fruits as well as a sufficient cost-benefit-ratio. Though many of these approaches show promising effects in laboratory experiments, only a fair level of success has been reported from field experimentation and none of them has been yet adopted commercially (Wilson et al., 2020).

Stimulation of uptake by host larvae

47. The only route to initiate midgut infection of host larvae is *per os* uptake of baculoviral OB during feeding. Substances stimulating feeding activity of larvae can increase the amount of ingested OB and thus increase the likelihood that a larva ingests a dose sufficient to initiate infection. Molasses (also used as a sticker), sucrose, and Coax have been frequently recommended to increase the potency of baculovirus application as feeding stimulants. However, experiences with such stimulants are variable and depend on the target pest species as well as the target crop (reviewed in Ballard et al., 2000; Burges and Jones, 1998).

Application practice

48. Baculovirus products can be applied with conventional spraying equipment that is also used for chemical pesticides, and they are highly compatible with other plant protection measures in organic and integrated agricultural production. They may be applied as tank mixtures together with other plant protection treatments (e.g. fungicide application) to reduce application costs. In these cases, it is important to determine any adverse effects such mixes may have on virus activity (Fritsch et al., 2008). Due to the particular nature of baculovirus OB, which is sensitive to alkaline as well as highly acidic milieus, pH in containers and tank mixtures needs to be between pH 5-8.

49. Currently, commercialised baculovirus biocontrol agents are targeted against lepidopteran and hymenopteran host larvae. With increasing larval stage and age, these larvae consume more and more plant material, causing a dramatic increase of damage during their development from early to late instars. There are numerous studies which indicate that food consumption by infected early instars is significantly reduced, whereas no or only limited damage reduction occurs when late instars are infected (Bianchi et al., 2000a; Farrar and Shapiro, 2003; Harper, 1973; Tanada and Reiner, 1962; Tatchell, 1981). In addition, early instar larvae are much more susceptible to baculovirus infection than late instars, and thus need significantly lower doses to become infected. Because most baculoviruses used as biological control agents kill their host larvae in the same stage as they become infected, targeting early instars has the highest effect on damage reduction. Therefore, field treatment is most effective against newly hatched

larvae or early instars, requiring a lower dosage for infection but earning highest benefits in preventing larval-caused damage.

Regulatory consideration and evaluation

50. It should be shown that the level and nature of contaminating micro-organisms are within the acceptable limits as stated in the OECD Issue Paper on Microbial Contaminant Limits for Microbial Pest Control Products (OECD, 2011). Batch analysis should be provided to show that the technical grade active ingredient (TGAI) complies with the before mentioned microbiological contamination screening requirements (OECD, 2011).

51. Additives and co-formulants must be listed, along with their content, identity (IUPAC name(s) and CAS number(s) of its constituent(s), trade name), and function. Unintentional ingredients should be specified.

52. Baculoviruses do not produce metabolites per se, therefore the need for identification and characterization of such metabolites does not apply.

53. The formation, presence and/or impact of unintentional ingredients should be reported.

4 Biological Properties

4.1. Origin and Examples of Use

54. Baculoviruses are naturally occurring insect-specific dsDNA viruses which have been isolated from several hundred different insect species, predominantly from families of the order Lepidoptera and to a much less extent from Hymenoptera and Diptera (Martignoni and Iwai, 1986). It has been proposed that baculoviruses co-evolved for 250 to 300 million years with their host during which time they developed a unique specificity for insects (Thézé et al., 2011). Baculoviruses comprise one of the largest virus families with hundreds of putative virus species.

55. Due to their high virulence and high specificity for their host insect targets, systematic efforts to use them as agents for insect pest control date back to the mid of last century; first commercial products have been registered in the 1970s, starting with the first commercial product in the USA, containing HzSNPV (species *Helicoverpa armigera nucleopolyhedrovirus)*, for cotton bollworm control (Huber, 1986). Since then, other baculovirus products have been registered in many countries worldwide (Table 1). In the meanwhile, the safety assessments of baculovirus have been continually updated on the basis of newly published scientific literature. In addition, recombinant DNA technology has been employed to insert foreign genes encoding insect-specific toxins, insect hormones, or enzymes into wild-type baculoviruses to improve the speed and efficiency of killing their host insects (Gramkow et al., 2010; Jung et al., 2012; Li and Bonning, 2007; Maeda, 1989). However, none of these recombinants was ever registered or commercially used.

56. At present, approximately 60 baculovirus-based pesticides have been marketed to control diverse pest insects worldwide. Examples of the most prominent marketed baculovirus biopesticides are summarised in Appendix Table A2.

Table 1: Baculoviruses with current registration in OECD countries (December 2020)*.

Virus name	Virus Abbrev.	Registration in OECD countries**
Alphabaculovirus		
Anagrapha falcifera NPV	AnfaNPV	*US*
Autographa californica MNPV	AcMNPV	*CA, US*
Chrysodeixis includes NPV	ChinNPV	*US*
Helicoverpa armigera NPV	HearNPV	*AT, AU, CA, CH, FR, EL, IT, PT, ES, US*
Heliothis zea SNPV	HzSNPV	*MX, US*
Lymantria dispar MNPV	LdMNPV	*CA*
Mamestra brassicae MNPV	MbMNPV	*CL*
Orgyia pseudotsugata MNPV	OpMNPV	*CA,*
Spodoptera exigua MNPV	SeMNPV	*MX, US, EU*
Spodoptera frugiperda MNPV	SfMNPV	*US, CO*
Spodoptera littoralis NPV	SpliNPV	*FR, IT, PT, ES*
Betabaculovirus		
Adoxophyes orana GV	AdorGV	*AT, BE, CH, CZ, DE, DK, FR, PL UK*

Cydia pomonella GV	CpGV	*AT, AU, BE, CA, CH, CL, CZ, DE, EL, FI, FR, HU, IT, LT, LU, LV, MX, NL, NZ, PL, PT, SI, SK, SF, TR, UK, US*
Erynnis ello GV	ErelGV	*CO*
Phthorimaea operculella GV	PhopGV	*pending EU, CO*
Deltabaculovirus		
Neodiprion abietis NPV	NeabNPV	*CA*
Neodiprion lecontei NPV	NeleNPV	*CA*

*Based on OECD member states` pesticide databases and notifications from OECD countries.
** Codes according to ISO-3166-1 ALPHA-2.

57. All registered and commercialized baculoviruses have been applied to protect agricultural annual crops, vegetables, fruit orchards and forests from damage caused by lepidopteran and hymenopteran pests. Looking back on a long history of the safe use of different baculoviruses, the most successful and important examples of commercialized baculovirus insecticides are mentioned here:

- **Anticarsia gemmatalis multiple nucleopolyhedrovirus** (AgMNPV) has been used for control of the velvetbean caterpillar in soybean since the first IPM pilot program in 1982 in Brazil (Haase et al., 2015; Moscardi, 1999). Application of AgMNPV in soybean has increased from 2000 ha treated in 1982/1983 to about one million ha in 1990/1991 and two million ha by 2002/2003 (Szewczyk et al., 2006, 2009). After cropping practices changed in Brazil requiring additional use of herbicides and insecticides, the use of AgMNPV declined to 200,000 ha per year (Haase et al., 2015). Moscardi (2007) estimated that between 1982 and 2005 the cumulative use of AgMNPV amounted to 23,000,000 ha in Brazil.

  After its use to successfully control *A. gemmatalis* in Brazil without causing outbreaks of secondary insect pests, AgMNPV application has been extended to other South and Central American countries, such as in Paraguay where it is applied on about 100,000 ha per year. Eventually, field trials were carried out in Argentina and Colombia, but no registration was sought. Mexico's National Institute for Agriculture, Forestry and Livestock Research (INIFAP) recently started to use AgMNPV in northern Mexico, where regularly an area of over 15,000 ha of soybean is under protection (Williams et al., 2013).
- **Cydia pomonella granulovirus** (CpGV) was the first registered baculovirus to be used in fruit crops, starting in 1987 in Switzerland. Since then, numerous CpGV products have been commercialized for use in many European countries, in North and South America, South Africa, New Zealand and Australia (Lacey et al., 2008). CpGV is highly specific and very virulent for larvae of the codling moth, *C. pomonella*, a major pest in apple, pear and walnut production. Some CpGV isolates also show virulence against oriental fruit moth, *Grapholitha molesta* (=*Cydia molesta*) (Graillot et al., 2017; Lacey et al., 2008). Nowadays, CpGV products are widely used in IPM strategies and are indispensable for organic pome fruit production. Its annual use has been estimated to exceed 150,000 ha worldwide.

  Field isolates of CpGV have been reported from Mexico, Argentina, Canada, Russia, England, South Africa, China, Iran, and other countries. Fully sequenced isolates were phylogenetically grouped into 7 clades, termed genome groups A-G (Fan et al., 2020c; Wennmann et al., 2017). After resistance to CpGV-M (genome group A) was observed, resistance-breaking strains belonging to other genome groups have been registered in Europe (Fan et al., 2020b; Gueli Alletti et al., 2017; Zingg et al., 2011).
- **Phthorimaea operculella granulovirus** (PhopGV) has been used to protect potato from loss caused by the potato tuber moth (PTM), *P. operculella*, not only in the field but also in storage (Espinel-Correal et al., 2012; Mascarin et al., 2010; Rondon, 2010). The known host range of PhopGV is restricted to a few Gelechidae species, such as *P. operculella*, the Guatemalan potato moth *Tecia solanivora*, the tomato leaf miner *Tuta absoluta*, *Symmetrischema tangolias*, *Eurysacca*

quinoae, and *Paraschema detectendum* (cited in Larem et al., 2019b). Isolates of PhopGV originate from Asia, South America, Africa, and Europe.

As PhopGV is a slow-killing GV, infected PTM larvae typically die within 2–3 weeks after the ingestion of virus (Lacey and Kroschel, 2009). Application of PhopGV in the field is complicated by its rapid inactivation due to solar radiation, as is typical for baculoviruses. Half-life times of 1.3 days for PhopGV were determined (Kroschel et al., 1996).

The virus has been commercially produced for PTM control in potato storage in Peru, Bolivia, Egypt, and Tunisia. A variable degree of virulence of PhopGV against different populations of *P. operculella* and alternate hosts has been observed. The selection of geographic isolates of a PhopGV consisting of a mixture of genotypes with efficacy against *P. operculella* and *T. solanivora* was done (Espinel-Correal et al., 2012).

In Colombia, a formulation of PhopGV, "Baculovirus CORPOICA" is registered and recommended for the control of *T. solanivora* in stored potatoes (Haase et al., 2015). Since 2019, emergency registrations of an isolate of PhopGV have been released in different EU countries and a regular registration for control of *T. absoluta* is expected in the EU.

- **Autographa californica multiple nucleopolyhedrovirus** (AcMNPV) has been tested for insecticidal activity against different pest insects, showing an effective ability to infect larvae of species within a relative broad host range that includes *Chloridea (Heliothis) virescens* and *Trichoplusia ni*. AcMNPV has been registered in the USA since 1994. Experimental and commercial use of AcMNPV has been reported from Central America, where it has been applied on several thousand ha of cabbage and broccoli (Hunter-Fujita et al., 1998). Because it is easily propagated in cell culture systems, AcMNPV has served as model of baculoviruses for more than 50 years and is widely used in biotechnological and biomedical applications (see Chapter 5.6). It also served as a model baculovirus for testing strategies to genetically engineer baculoviruses with the aim to increase their speed of kill (Inceoglu et al., 2001). The first reported field test using genetically engineered AcMNPV recombinants expressing the AaHIT toxin protein cloned from the scorpion *Androctonus australis* was conducted in 1993 (Cory et al., 1994). A number of engineered AcMNPV recombinants encompassing foreign genes of toxins and other insecticidal proteins have been constructed (Kroemer et al., 2015). Despite extensive research, including efficacy testing in the laboratory and in the field, none of these genetically modified viruses was ever commercialized (Black et al., 1997; Ishimwe et al., 2015; Shim et al., 2013; Yang et al., 2017).
- **Helicoverpa armigera nucleopolyhedrovirus** (HearNPV), Heliothis zea single nucleopolyhedrovirus (HzSNPV) and Heliothis virescens single nucleopolyhedrovirus (HvSNPV) are viruses belonging to the same species *Helicoverpa armigera nucleopolyhedrovirus* (Adams et al., 2015; Rowley et al., 2011). The cotton bollworm (*H. zea*), African cotton bollworm (*H. armigera*) and tobacco budworm (*C. virescens*) are polyphagous insects occurring worldwide and feeding on numerous crops of cotton, soybean, potato, tomato, pepper, maize, sorghum, sunflower, vegetables and berries (Moscardi et al., 2011; Sun et al., 2002). HzSNPV was first registered in the USA (Elcar®) and successfully commercialized until the early 1980s when synthetic pyrethroids became available (Bell and Hayes, 1994; Hayes and Bell, 1994). After the occurrence of resistance to pyrethroids and other chemicals, commercial interest and re-registration (GemStar™ and others) of HzSNPV was revived. HearNPV-based products are used in many countries worldwide, including US, EU countries, South Africa, Australia Russia, India, and China. In China alone it has been applied on >100,000 ha annually (Sun, 2015). It received emergency registration in Brazil to control the 2013/2014 outbreak of *H. armigera*, where it was applied on 1.3 million ha of different crops, including soybean, cotton and corn (Perini et al., 2016; Sosa-Gómez, 2017).
- **Lymantria dispar multiple nucleopolyhedrovirus** (LdMNPV) is highly pathogenic to larvae of *L. dispar*, a polyphagous forest pest native to Eurasia, which was accidentally introduced to North America in the 19th century. *L. dispar* moth can undergo periodic outbreaks leading to very high

densities of larvae that cause heavy defoliation of infested trees (Reardon et al., 2012). After eruptions of LdMNPV in *L. dispar* were observed, the USDA Forest Service initiated research in the late 1950s to develop a control strategy using LdMNPV, which eventually resulted in the registration of Gypchek in 1987 in the USA, where more than 11,000 ha of forest were treated (Cunningham, 1998; Lewis, 1981; Solter and Hajek, 2009). Since then, LdMNPV formulations have been used in other countries and regions. Gypchek contains the Hamden strain of LdMNPV, which is a mixture of at least 10 different genotypes (Podgwaite et al., 2013; Slavicek, 1991). The safety of Gypchek was carefully evaluated, and was revealed to have no deleterious effects to non-target organisms. However, it was found to be an irritant when applied in massive amounts to eyes of rabbits. The irritation was not caused by the active ingredient, but from parts of ground insects from *in vivo* production present in the technical product (Reardon et al., 2012). It is estimated that from 1974 through 2011 more than 57,000 ha have been sprayed with Gypchek during field experiments, pilot tests, and control programs (Reardon et al., 2012). Product efficacy has been improved by adding sunlight protectants and feeding stimulants and a sticker, augmenting activity when the *L. dispar* population was low in the forest. Podgwaite et al. (1992) studied the control of moderate to high density *L. dispar* in US, and found that two applications of 1.25×10^{12} OB/ha resulted in a reduction of egg masses by 98% and 80% in comparison with control woodlots at different sites. This application rate is currently recommended for the use of LdMNPV preparations in the USA. Field trials using three geographic LdMNPV isolates derived from China, Japan and USA against Chinese *L. dispar* larvae found that a LdMNPV isolate from Japan was remarkably more virulent than the other two isolates over the course of three years (Duan et al., 2012). In summary, due to its high specificity, LdMNPV preparations are currently the only agents that can be used in areas where the application of broad-spectrum pesticides is not suitable, such as natural areas and where conservation of non-target insect species are of particular concern.

- Different NPVs from the genus *Gammabaculovirus,* such as Neodiprion sertifer nucleopolyhedrovirus (NeseNPV), Neodiprion lecontei nucleopolyhedrovirus (NeleNPV) and Neodiprion abietis nucleopolyhedrovirus (NeabNPV) been tested and eventually registered to control the European pine sawfly (*N. sertifer*), the redheaded pine sawfly (*N. lecontei*) and balsam fir sawfly (*N. abietis).* Sawflies are gregarious insects, a behaviour which is conducive to the spread of the virus through a population in a short time, with insects dying 4 to 7 days after infection (Federici, 1997). NeseNPV has been successfully applied for control of the European pine sawfly, which is a serious forest defoliator on pine plantations (Cunningham, 1998). Field work with NeseNPV has been conducted in Canada, Scandinavia, Poland, UK, the former Soviet Union and USA. Commercial products were registered in Finland (1983), UK (1985), USA (1983, but discontinued by the company in 1991) and the former Soviet Union. *N. abietis* is a sporadically occurring defoliator of firs and spruces in North America occurring in periodic outbreaks, usually lasting 3-4 years (Cunningham, 1984). A NeabNPV based product, termed Abietiv™, eventually received registration in 2009 for the control of balsam fir sawfly in Canada. Abietiv™ was used annually for the control of *N. abietis* in Newfoundland on >20,000 ha from 2000-2005, 15,000 ha from 2006 to 2009 and in New Brunswick on 10,000 ha in 2011 (Hajek and van Frankenhuyzen, 2017; Lucarotti et al., 2007). After more than 30 years of research NeleNPV received temporary registration in 1983 and full registration in 1987 in Canada (tradename Lecontvirus) as a restricted class product for control of redheaded pine sawfly. A single application of the virus in pine plantations could suppress the pest populations for several years. Lecontvirus was used between 1983 and 1994 on a total of about 6000 ha of plantations, mostly by private landowners. However, no commercial class product was developed (Hajek and van Frankenhuyzen, 2017).
- **Spodoptera spp. NPV**. Several species of the noctuid genus *Spodoptera* are important pests, causing severe damage on grain crops and vegetable production. Numerous specific viruses have been isolated from different *Spodoptera* species, such as Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV) from the beet armyworm (*S. exigua*), Spodoptera exempta

nucleopolyhedrovirus (SpexNPV) from the African armyworm (*S. exempta*), Spodoptera eridania nucleopolyhedrovirus (SperNPV) from the Southern armyworm (*S. eridania*), Spodoptera frugiperda multiple nucleopolyhedrovirus (SfMNPV) from the fall armyworm (*S. frugiperda*), Spodoptera litura nucleopolyhedrovirus (SpltNPV) from the tobacco cutworm (*S. litura*), and Spodoptera littoralis nucleopolyhedrovirus (SpliNPV) from the Egyptian cotton leafworm (*S. littoralis*). All these viruses belong to different species (Harrison et al., 2018a). Extensive research on some of these viruses and successful field testing resulted in the development and registration of several commercial products (Hunter-Fujita et al., 1998; Moscardi et al., 2011). Several nucleopolyhedroviruses infectious to *Spodoptera* spp. are currently registered and commercially available in different countries worldwide (Table 1, Appendix Table A2). Field isolates of SeMNPV were found in the USA, Thailand and Spain, Pakistan and Mexico etc. (Caballero et al., 1992; Thézé et al., 2014; Zamora-Avilés et al., 2017). A product, based on a North American isolate of SeMNPV, is registered and widely used in greenhouses in the EU and in the USA, where *S. exigua* was found to be tolerant to many chemical insecticides. SfMNPV is being used in Central and South America, in Africa and in Australia where *S. frugiperda* occurs as a major pest, particularly in maize. It was reported that more than 20,000 ha were treated with SfMNPV in Brazil by 1992 (Barrera et al., 2011; de Oliveira, 1998). Isolate SfMNPV-3AP2 (Harrison et al., 2008) has been registered for use in the USA (under brand name Fawligen) and in Brazil (under brand name Cartugen). More than 4 million hectares have been treated with this baculovirus in Brazil since 2018. SfMNPV isolate 6 has been registered in Brazil and Paraguay under the commercial product name Spodovir. Registrations of additional SfMNPV products are under way in different countries in South America (and Mexico), Africa and Asia.

4.2 Host Resistance

58. A successful infection of a given host is dependent on the completion of the baculovirus replication cycle. This means that the virus has to successively complete both primary and secondary infection by passing through peritrophic membrane and initiating infection and virus replication to generate new virus progeny in susceptible tissues. If one of the multiple steps required for a successful infection is suppressed or even blocked, a subpar or completely failed infection will result.

59. Host insects may respond differently when infected with a baculovirus. Susceptibility to infection is not a constant property, but may be subject to the age and developmental stage of the larvae, nutritional factors, genetic background of host population, or genetic resistance.

4.2.1 Developmental Resistance

60. There is a well-documented relationship between the developmental stage, including the intra-stadial (= within stage) age, of insect larvae and their response to baculovirus infection, as documented by numerous studies (Briese, 1986). In general, early instar larvae are much more susceptible than late instars and a considerable variability of developmental resistance, expressed as a LD_{50} or LC_{50} value, is documented for a wide range of baculovirus-host associations (Briese, 1986). A consequence of developmental resistance, or “maturation immunity” as coined by some authors, is that early instars require much lower virus inoculum to develop infections than late instars, whereas the penultimate or final instars often become almost completely refractory to lethal infection (Fuxa and Richter, 1998).

61. Developmental resistance may be caused by midgut-based anti-viral defense mechanisms, including the PM, sloughing of infected midgut cells, and apoptosis of infected cells (Dougherty et al., 2006; Engelhard and Volkman, 1995; Hoover et al., 2000). For intra-stadial age resistance, midgut-based barriers, such as cell sloughing, apoptosis, and the encapsulation and melanisation of infected cells and tissues, were also identified as crucial factors (Haas-Stapleton et al., 2003; Hoover et al., 2002; McNeil et

al., 2010; Washburn et al., 1995). Eberle et al. (2008) demonstrated an instar-specific pattern of decrease in the susceptibility of codling moth larvae to CpGV. Several authors proposed a correlation between dose-mortality response and larval weight (Evans, 1981; Sporleder et al., 2007).

62. These factors add some variability to the field performance of a baculovirus application determined by the age structure of the treated insect population. Because of developmental resistance, it is important to control the early stages of a pest infestation, since late instars may not ingest enough virus inoculum to become infected and generally cause more feeding damage than early instars (Eberle et al., 2012a).

4.2.2 Variations of Susceptibility in Host Populations

63. Different populations of an insect species are not genetically homogenous and hence their response to a pathogen may also exhibit some variability. Variation of susceptibility among insect host populations, measured as LC_{50} or LD_{50}, was occasionally investigated and had been reported for *S. frugiperda, P. operculella, P. brassicae* (for reviews see Briese, 1986; Fuxa, 2004) and *Malacosoma californicum pluviale* populations (Cory and Myers, 2009). It is conceivable when such populations continue to be exposed to virus selection, a population-wide genetic fixation of resistance may develop.

4.2.3 Genetically-based Resistance

64. The FAO defines resistance as “a genetic change in an organism in response to selection by pesticides, which may impair control in the field” (FAO, 2012). It therefore does not refer to pre-existing natural variability of the susceptibility in target populations, but is the result of a selection process by which less susceptible individuals within a pest population transfer their genetic information to the next generation. Repeated rounds of selection from applying the same pesticide results in an increase of resistant individuals and a population-wide decrease of susceptibility. Eventually, resistance allows a pest organism to survive exposure to a pesticide dose that would normally have killed it. Baculoviruses belong to the International Resistance Action Committee (IRAC) mode of action (MoA) group 31 (Sparks et al., 2020).

Laboratory-selected resistance

65. Several cases of resistance in different baculovirus-host systems, which were selected in laboratory experiments, were reported in the literature (for reviews see Briese, 1986; Fuxa, 2004; Siegwart et al., 2015). In these cases, laboratory colonies or field-derived insects with a slightly reduced susceptibility were exposed to repeated rounds of selection with non-lethal baculovirus doses. For example, a 140-fold increase of the LD_{50} was noted when a laboratory colony of the potato tuber moth, *P. operculella,* was repeatedly exposed for six generations to PhopGV (Briese and Mende, 1983). The observed resistance, however, was not stable and decreased after a few generations when virus selection was discontinued (Briese, 1986).

66. Similarly, when a colony of the fall armyworm, *S. frugiperda,* was reared under selection pressure in the form of exposure to SfMNPV, a three-fold increase of the LC_{50} value was achieved within seven generations. The decreased susceptibility was not maintained when rearing was continued without selection pressure (Fuxa et al., 1988; Fuxa and Richter, 1989). In this case, a midgut-related resistance was proposed because injection of BV into hemocoel, thus by-passing the midgut, did not reveal any difference between the susceptible and the resistant colony (Fuxa and Richter, 1990).

67. In selection experiments with a Brazilian colony of *A. gemmatalis* that had been regularly exposed to AgMNPV a more than 1,000-fold increase in resistance ratio was observed within 13-15 generations of virus exposure, whereas similar selection experiments with a US colony gained only a five-fold increase of the LC_{50} value (Abot et al., 1996). Comparisons of the PM of the susceptible and resistant colonies suggested that the PM of the resistant colony may have accounted for its degree of resistance to AgMNPV (Levy et al., 2007). However, although AgMNPV has been widely used for velvet caterpillar control on

thousands to millions of hectares of soybean plantations for more than 20 years, no decrease of susceptibility or development of resistance in the field was noted (Moscardi, 2007).

68. Exposure of the cabbage looper, *T. ni,* to Trichoplusia ni single nucleopolyhedrovirus (TnSNPV) for 27 generations resulted in a 22-fold increase of LC_{50} values as well as cross-resistance to Pieris brassicae granulovirus (PibrGV) and Trichoplusia ni granulovirus (TnGV) (Milks and Myers, 2003; Milks and Theilmann, 2000).

69. A colony of the tea tortrix, *Adoxophyes honmai,* with a high degree of resistance to Adoxophyes honmai nucleopolyhedrovirus (AdhoNPV) was selected from a field-collected colony by repeated exposure to the virus. Fifth-instar larvae of the resistant colony exhibited a >66,000-fold increase of LC_{50} values compared to larvae of the non-selected colony (Iwata et al., 2017). Again, a midgut-based resistance mechanism characterized by a significantly reduced binding and fusion capacity of the ODV was proposed. When selection was continued for >150 generations, the highest degree of acquired resistance, based on the ratio of the LC_{50} values of resistant and susceptible host colonies, was more than 400,000-fold (Nakai et al., 2017).

70. These examples of agricultural pests developing resistance against baculoviruses derive from repeated selection by the virus under laboratory conditions and demonstrate the potential of selection for resistance against baculoviruses in general. Development of field resistance of insects to commercially applied baculovirus biocontrol products has been only reported for codling moth populations in Europe sprayed with CpGV products.

CpGV field resistance

71. The first evidence of field resistance against commercial baculovirus products derived from a survey of organic apple orchards in Germany, which had been regularly treated with CpGV products for codling moth control. LC_{50} values of offspring neonates, which originated from overwintering larvae, were 500- to 1000-fold higher than those of susceptible colonies (Fritsch et al., 2005). Soon after, reports of resistant codling moth populations were reported from France (Sauphanor et al., 2006). After further selection experiments and genetic analyses of a field-collected population, an incomplete dominant, monogenic mode of inheritance that is linked to the Z chromosome was proposed for the CpGV resistance (Asser-Kaiser et al., 2007, 2010). Further field surveys revealed about 50 orchards in Austria, Czech Republic, France, Germany, Italy, the Netherlands and Switzerland, with populations exhibiting significantly reduced susceptibility to the Mexican isolate of CpGV (CpGV-M), the active substance of all registered CpGV products in Europe at that time (Asser-Kaiser et al., 2007; Sauphanor et al., 2006; Schmitt et al., 2013; Zichová et al., 2011). Strikingly, this widely occurring resistance was mainly targeted against CpGV-M (genome group A), whereas other CpGV isolates from phylogenetically different groups were able to break resistance (Berling et al., 2009; Eberle et al., 2008; Gebhardt et al., 2014).

72. After resistance-breaking isolates were discovered, novel active substances based on other sg-strains and ni-strains of CpGV were selected and commercialized (Berling et al., 2009; Gueli Alletti et al., 2017; Zingg et al., 2011). A large number of studies have provided solutions to overcome the lack of activity of CpGV products against resistant codling moth field populations. The two successful ways of obtaining resistance-breaking virus candidates were (i) the discovery of new virus isolates harbouring novel genotypes (Fan et al., 2020b) and (ii) improving efficacy of existing CpGV isolates by selection on resistant codling moth colonies (Graillot et al., 2014; Zingg et al., 2011). After years of using new resistance-breaking sg-strains and ni-strains to control CpGV-resistant codling moth populations, additional types of CpGV resistance with different specificities and inheritance patterns were identified in the field (Jehle et al., 2017; Sauer et al., 2017; Siegwart et al., 2020). For resistance management of CpGV it is recommended to switch to CpGV isolates from other genome groups.

73. Repeated applications of CpGV (up to >12 sprays per season), the use of a sg-strain (CpGV-M) in previous CpGV formulations, and the exclusive use of such products for codling moth control have been

considered as the main reasons for the emergence of resistance in codling moth field populations. In contrast to most alphabaculoviruses, which produce OB containing multiple nucleocapsids, betabaculoviruses such as CpGV carry a single nucleocapsid in an OB, which would be expected to reduce the number of genotypic variants available for replication and propagation in an insect host. Alphabaculoviruses may actually deliver more different genotypes per OB into the host at individual infection than betabaculoviruses (Arrizubieta et al., 2015; Beperet et al., 2020; Bernal et al., 2013; Chateigner et al., 2015; Clavijo et al., 2010).

Regulatory considerations and evaluation

74. Baculoviruses are naturally occurring insect viruses, which are highly virulent and specific for larval stages of their target host insects. They have been developed as biopesticides to be used to control insect pests in agronomic and fruit tree cultures, as well as vegetables and in forests. They have been applied on millions of hectares and have a history of safe use of more than 50 years without reported hazards or adverse environmental effects. Baculoviruses infect only larval stages of the target insect host. Since older instars are less susceptible to virus infection than early instars, control schemes and application strategies predominantly target the early instar larvae. So far, field resistance to baculoviruses (IRAC Mode of Action Group 31) has been only observed in codling moth for CpGV, though laboratory-selected, but reversible resistance has been noted for several baculovirus-host systems. As long as occurrence of field resistance is very rare (so far only noted for CpGV when very intensively used), general resistance management recommendation cannot be applied.

4.3 Mode of Action

4.3.1 Virion Properties

75. Upon coevolution with insect hosts, baculoviruses developed a unique bi-phasic replication cycle during which two morphologically distinct virion phenotypes are produced: the budded virus (BV) and the occlusion-derived virus (ODV) (for review Wang and Hu, 2019). Primary *per os* infection of insect larvae is initiated in midgut epithelial cells by ODV embedded in OB, whereas secondary infection of further larval tissues is initiated by BV (Rohrmann, 2019).

76. The OB shape is either polyhedral for NPV, with a size range of 0.5 to 5 µm in diameter, or ovo-cylindrical for GV, measuring 0.12 to 0.3 by 0.3 to 0.5 µm. The size range of nucleocapsids is 30 to 60 by 250 to 300 nm (Ackermann and Smirnoff, 1983; Herniou et al., 2011; Tanada and Kaya, 1993).

77. Phospholipid and fatty acid compositions of viral envelopes of ODV and BV are also different (Braunagel and Summers, 1994). Both virion phenotypes are sensitive to detergents and organic solvents. ODV buoyant density in CsCl is in the range of 1.18 to 1.25 g/cm^3, and that of the nucleocapsid is 1.47 g/cm^3. BV buoyant density in sucrose is ranged from 1.17 to 1.18 g/cm^3 (Harrison et al., 2018a; Summers and Volkman, 1976).

78. Proteomic analyses based on mass spectrometry and molecular identification revealed that the protein composition of virions consists of approximately 23-73 polypeptides (Deng et al., 2007; Masson et al., 2019; Zhang et al., 2015b). Variable amounts of ODV-specific proteins, BV-specific proteins and ODV- and BV-shared proteins have been identified providing evidence related to the structural and functional differences between both virion phenotypes (Hou et al., 2013). However, the essential proteins involved in nucleocapsid assembly, formation, morphogenesis, production and maturation are shared by ODV and BV (Wang et al., 2010).

Fusion Protein

79. Another distinct morphological feature distinguishing BV from ODV is the presence of the envelope fusion glycoprotein (EFP) located on the surface of the BV virion at one or both polar ends (Monsma et al., 1996; Pearson et al., 2000, Wang et al., 2016b). With the help of BV-specific EFPs, such as GP64 or F proteins, BV can enter and infect surrounding cells by binding and fusion at low pH (Qin et al., 2019).

80. GP64 is found in group I alphabaculoviruses like AcMNPV and produces “Y”-shaped spikes (Wang et al., 2016b). EFP GP64 from AcMNPV and closely related species are well studied and belong to class III penetrenes (Monsma et al., 1996). GP64 shares 74% amino acid identity among its homologues present in group I alphabaculoviruses (Wang and Hu, 2019). When GP64 was deleted, viruses could replicate in primary infected cells but could not bud from and infect adjacent cells (Monsma et al., 1996; Oomens and Blissard, 1999).

81. The EFP of group II alphabaculoviruses, betabaculoviruses and deltabaculoviruses is termed F (fusion) protein, which shares about 20-40% amino acid identity across its homologues; it is “burgee”-shaped and belongs to class I penetrenes (Garry and Garry, 2008; IJkel et al., 2000; Pearson et al., 2000). A study on evolutionary lineages of EFPs among baculoviruses and other virus families has proposed that F protein might be the ancestral baculovirus EFP, while GP64 was acquired by the group I alphabaculoviruses by horizontal gene transfer. GP64 has assumed the original function of the F protein among group I alphabaculoviruses, although a non-active F protein homologue is still retained by these viruses (Rohrmann, 2019).

82. The function of GP64 from group I alphabaculovirus can be rescued by F proteins from different betabaculoviruses with variable efficiencies, e.g. from Agrotis segetum granulovirus (AgseGV), Xestia c-nigrum granulovirus (XecnGV), CpGV, PhopGV and Choristoneura occidentalis granulovirus (ChocGV) (Yin et al., 2008; Yin et al., 2013). The viral envelope of BV is an approximately 6-7 nm bilayer membrane, which is thicker than general lipid bilayers (Wang et al., 2016b).

83. A functional envelope fusion protein encoded by ORF104 of the deltabaculovirus Culex nigripalpus nucleopolyhedrovirus (CuniNPV) facilitates spreading infection quickly to other midgut cells (Wang et al., 2017). Gammabaculovirus infection is naturally limited to midgut tissues, and these viruses lack homologues of either GP64 or F protein.

Polyhedrin/Granulin

84. The major protein of the OB is a single viral-encoded polypeptide, which is named as polyhedrin for NPVs from the genera *Alphabaculovirus, Deltabaculovirus* and *Gammabaculovirus* and granulin for GVs (genus *Betabaculovirus*). Despite these differences in naming of the OB protein, which is related to the scientific history, it needs to be noted that Polyhedrin/Granulin proteins of alpha-, beta- and gammabaculoviruses are genetically homologous 25-33 kDa polypeptides, whereas the Polyhedrin from deltabaculoviruses is unrelated to those from the other three genera and has a different evolutionary origin (Harrison et al., 2018a; van Oers and Vlak, 2007).

85. The atomic structures of OB polyhedrin of AcMNPV and granulin of CpGV have been obtained with high resolution, facilitating exploration of the function of two crystal molecules in biological systems; both polyhedrin and granulin proteins are able to form robust crystals that protect encased virions from environmental damage prior to transmission between insects (Coulibaly et al., 2009; Gati et al., 2017). The resolved crystal structure of NPV and GV polyhedra has elucidated the molecular interactions between the monomers and oligomers that compose the crystal matrix and provided useful explanations on why the matrices are compact but still flexible enough in its arrangements to be able to occlude virions and why it dissolves in high pH, by breaking the salt bridges between oligomers. ODV occlusion and OB dissolution are two crucial processes for horizontal transmission by OB.

4.3.2 Genome Organization

86. The genome of baculoviruses consists of a double-stranded (ds), covalently closed circular (ccc), supercoiled DNA molecule of 80 to 180 kbp, encoding for 90 to 180 proteins. As of 2020, more than 580 baculovirus isolates have been entirely sequenced and deposited in GenBank (Thézé et al., 2018; Wennmann et al., 2018). By convention and for differentiation between DNA and protein sequences, gene names are written in small italic letters (e.g. *lef-8*) whereas the encoded protein is written in normal letters with first or all letters capitalized (e.g. Lef-8 or LEF-8) in scientific literature.

87. Baculovirus genomes encode densely-packed, mostly non-overlapping open reading frames (ORFs) on both strands, as well as short intergenic regions. By convention, baculovirus ORFs downstream of the *polh/gran* gene, which is designated as ORF1, are consecutively numbered. For historic reasons, the only exception of this convention is AcMNPV and variants of AcMNPV, for which ORF1 is the *ptp1* gene. Due to the compact genome, some ORFs overlap with neighboring ones, while some other ORFs appear to be repeated several times or have multiple homologues in a single genome, for instance *baculoviorus repeated orf* (*bro)*, *late expression factor 7* (*lef-7)*, *inhibitor of apoptosis (iap)*, *ecdysteroid-UDP-glucosyltransferase* (*egt)*, and others (Harrison et al., 2018b; van Oers and Vlak, 2007).

88. The vast majority of baculoviruses carry some repetitive genetic elements, such as homologous repeated sequences (*hrs*) and/or multiple copies of some ORFs (e.g. *bro* genes). *Hrs* are comprised of tandemly repeated units present at different genome locations. They are considered to function as transcriptional enhancers as well as origins of DNA replication. In AcMNPV, there are eight *hrs* with 2-8 repeats of a 70-bp unit each containing an imperfect 30-bp palindrome (Rohrmann, 2019). Among baculoviruses, *hrs* are highly variable and there is a very limited level of sequence similarity; some baculoviruses even do not contain *hrs*-like motifs. Baculovirus repeated ORFs, so-called *bro* genes, are widely distributed in alpha-, beta- and deltabaculoviruses but not in gammabaculoviruses. Their number varies from zero in RoMNPV to 16 copies in LdMNPV. The function of *bro* genes is not clear, though a binding activity to viral and/or host DNA was proposed for some Bombyx mori nucleopolyhedrovirus (BmNPV) BRO proteins (Zemskov et al., 2000).

89. So far, a set of 38 conserved genes being essential for gene transcription, DNA replication, nucleocapsid assembly and virion architecture has been found in sequenced baculovirus genomes of all four baculovirus genera and were therefore assigned as baculovirus core genes (Garavaglia et al., 2012; Herniou et al., 2003; Javed et al., 2017; van Oers and Vlak, 2007). For instance, all genes encoding for the ten *per os* infectivity factors (PIFs) of ODV belong to the core gene set (Wang et al., 2019b).

4.3.3 Life Cycle of Baculoviruses

90. Baculovirus OB need to be ingested by susceptible host larvae to initiate infection of the midgut epithelial cells. Lepidopteran midguts have a highly alkaline milieu with a pH ranging from 10 to 11, while the pH in hemolymph is about 6.7, close to neutral level (Dow, 1992). Midguts of hymenopteran and dipteran larvae have also a more neutral pH of 6.8 to 8.9 (Boudko et al., 2001; Terra and Ferreira, 1994). Life cycle is best studied for AcMNPV, which serves as a model for other baculoviruses: after *per os* ingestion of the baculovirus OB, the alkaline condition of the insect midgut triggers the dissolution of OB protein matrix and the release of the ODV (Blissard and Theilmann, 2018); ODV first pass through the PM, then bind to and fuse with the microvilli membrane of columnar cells of the insect midgut epithelium. After membrane fusion, nucleocapsids are released from ODV into the cytoplasm of midgut epithelial cells and then transferred by actin polymerization to the nuclear pore where they enter the nucleus (Au et al., 2016). There, viral DNA is transcribed and replicated and new nucleocapsids are assembled from virus expressed proteins; newly generated nucleocapsids bud from infected cell through plasma membrane to produce BV, which are spread to other cells and tissues via tracheal epithelium, hemocytes, fat body, etc., initiating secondary infection (Blissard and Theilmann, 2018). BV bind to cell plasma membrane and the majority of bound virus particles (90%) enter into the cell by clathrin-mediated endocytosis, while the minority (10%)

enter the cell by direct fusion with the plasma membrane (Qin et al., 2018) and initiate a new round of DNA transcription and replication and nucleocapsid production. At the late stage of infection, baculoviruses switch from BV production to ODV synthesis; newly formed ODV are embedded into the growing polyhedrin or granulin protein matrix to build new OB. When the infected larva is close to death, the outer covering (cuticula) of the larval body may turn to a whitish and milky colour; it becomes fragile and prone to rupture due to the activity of the virally encoded cathepsin and chitinase proteins (Rohrmann, 2019). The larval cadaver liquefies and millions of OB break through the larval cuticle, ready to be distributed in the environment and taken up by other host larvae.

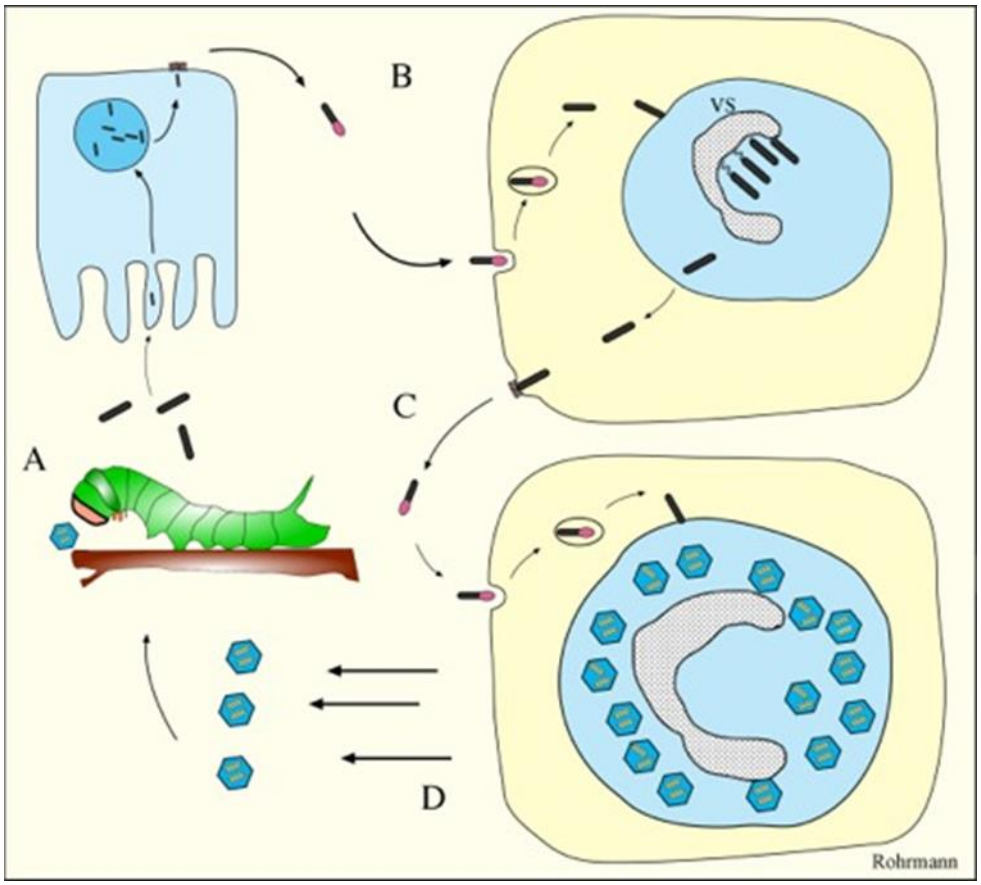

Fig 2. A life cycle of a baculovirus causing systemic infection. Occlusion bodies ingested by an insect dissolve in the midgut, and ODV are released which then infect midgut epithelial cells (A). The virion buds out of the cell in a basal direction and initiate a systemic infection (B). Early in the systemic infection more BV are produced which spread the infection throughout the insect (C). Late in infection occluded virions are produced, and the cell then dies releasing the occlusion bodies (D). The virogenic stroma (VS) is indicated. (Taken from Rohrmann, 2019. © 2019, George Rohrmann (Creative Commons Attribution 4.0 International License)).

91. Members from *Alphabaculovirus* and *Betabaculovirus* genera are able to induce primary and secondary infection in host insects. Members of *Gammabaculovirus* and *Deltabaculovirus* are restricted to host midgut tissue and cause symptoms of “infectious diarrhea” (Arif et al., 2011).

92. On the basis of tissue tropism, betabaculoviruses can be classified into three types: type I GVs comprise slow-killing viruses, which only infect larval midgut cells and fat body of caterpillars, mainly from

the families Noctuidae and Tortricidae; type II GVs are fast-killing, induce a systemic infection and spread over midgut to fat body, epidermis, Malpighian tubule, tracheal matrix, hemocyte, and many other cells and tissues; and type III GVs are restricted to the insect midgut epithelium (Federici, 1997). Similarly, fast- and slow-acting NPVs are also reported.

93. In nearly all lepidopteran insects infected by alpha- or betabaculoviruses, the fat body is the main tissue for OB production. As the nuclear membrane of infected cells remains intact during the replication of alpha-, gamma- and deltabaculoviruses, the ODVs and OB mature within the cell nucleus. During betabaculovirus infection, however, the nuclear membrane is degraded, resulting in an ODV maturation and OB formation in a mixed nuclear-cytoplasmic environment (Federici, 1997). Survival time after infection is affected by different factors and can range from 2-5 days (fast-killing baculoviruses) to 2-3 weeks (slow-killing baculoviruses) (Fuxa, 2004).

Primary Infection of the Midgut

94. Successful baculovirus infection of its host insect depends on the primary infection of the midgut. There, the PM is the first barrier. The PM is composed of chitin microfibrils, proteins and proteoglycan (Wang and Hu, 2019). It is a non-cellular structure of the midgut, which separates the luminal contents into the endoperitrophic and the ectoperitrophic space, in order to protect midgut cells from damage by the food bolus and to a certain extent from infection from pathogens and from other chemical damage. However, there are tiny pores in the PM, which allow the passage of nutrients while blocking pathogens (Wang and Hu, 2019). It is proposed that passage of baculovirus ODV through the PM can be facilitated by viral encoded proteins of the OB that degrade the PM and increase PM permeability (Wang and Granados, 1997). Enhancin, also called synergistic factor, was first found in the granulin matrix of betabaculovirus OB and later also in the ODV envelope of some alphabaculoviruses. It is a metalloproteinase that degrades the insect intestinal mucin and thereby facilitates ODV passage through this barrier to increase access of ODVs to midgut epithelial cells (Slavicek and Popham, 2005; Tanada et al., 1973). Other baculoviral proteins proposed to be involved in the degradation of the PM are ODV-E66 (Ac46), also known as a viral chondroitinase and present in all alpha- and betabaculoviruses, and GP37 (Ac64) (Rohrmann, 2019).

95. Infection of midgut epithelial cells depends on several proteins located in the ODV envelope, namely *per os* infectivity factors (PIFs). Whereas no specific receptor molecule(s) in the midgut epithelial cell membrane has been identified so far, ten PIF proteins are known to be essential for baculovirus *per os* infectivity, including PIF-0 (P74) (Faulkner et al., 1997), PIF-1 (Ac119) (Kikhno et al., 2002), PIF-2 (Ac22) ((Pijlman et al., 2003), PIF-3 (Ac115) (Ohkawa et al., 2005), PIF-4 (AC96) (Fang et al., 2009), PIF-5 (ODV-E56, AC148) (Harrison et al., 2010), PIF-6 (AC68) (Nie et al., 2012), PIF-7 (AC110) (Jiantao et al., 2016), PIF-8 (VP91) (Javed et al., 2017), and PIF-9 (AC108) (Boogaard et al., 2019). All PIF proteins except for PIF-5 form an ODV entry complex with a molecular mass of ~500 kDa, which is required for the infectivity of ODV for midgut cells (Wang et al., 2019b). PIF-0 (P74) is absolutely necessary for ODV binding, while PIF-1 and PIF-2 are essential for the entry of ODV after binding to midgut cells membrane (Mu et al., 2014). Transcriptional levels of known *pif* genes are significantly different from each other and were found to be highest for *pif-5* and lowest for *pif-3* (Chen et al., 2013). During virus replication, the PIF complex is assembled in the cytoplasm and then transported to the nucleus to become part of the ODV envelope. Deletion of any of the ten *pif* genes resulted in a considerable or complete loss of *per os* infectivity (Wang and Hu, 2019). As a defensive strategy, baculovirus-infected midgut cells are sloughed off to prevent further spread of baculovirus beyond the midgut. To achieve a systemic infection, it is essential for baculoviruses to initiate a secondary infection.

Secondary or Systemic Infection

96. Once infection in midgut cells is established, most baculoviruses set on BV transmission between midgut cells as well as from midgut to other types of cells and tissues through the hemocoel and tracheal system, causing a systemic infection across the whole larval body (Engelhard et al., 1994; Granados and Lawler, 1981). Newly generated BV will attach to other types of cells and enter them with the help of actin polymerization-dependent internalization and microtubule-dependent nucleocapsid release. After that, nucleocapsids are released from early endosomes and enter the nuclei through nuclear pores (Ohkawa et al, 2010; Qin et al., 2019).

97. The basal lamina, a thin, flexible, and non-cellular sheet that is secreted on the basal side of epithelial cells, is a further barrier preventing the passage of pathogens that enter the midgut from infecting hemocoelic tissue layers (Passarelli, 2011). The expression of baculovirus-encoded fibroblast growth factor (vFGF) induces a cascade of protease activation, followed by activation of matrix metalloprotease (MMP) which in turn activates effector caspases (Means and Passarelli, 2010). The basal lamina is then degraded by effector caspases, which facilitate BV passage through basal lamina and access to tracheal cells before regeneration of new basal lamina, leading to an enhancement of baculoviral infectivity (Passarelli, 2011). Interestingly, *vfgf* genes have been only found in the genomes of alpha- and betabaculoviruses, whereas the genomes of gamma- and deltabaculoviruses, for which infection is constrained to the midgut, do not carry *vfgf* genes (Passarelli, 2011).

Transcription and DNA Replication: Phases of Virus Infection

98. A common classification of virus gene transcription is a separation into early and late phases, separated by the onset of viral DNA replication (Rohrmann, 2019). Baculovirus genes are further classified into four temporal transcription classes: immediate early (IE), delayed early (DE), late (L) and very late (VL), according to the transcription timing (van Oers and Vlak, 2007). Immediate early and delayed early genes have a canonical promoter motif of a TATA box followed by a CAGT transcription initiation site 25-30 bp downstream of the TATA box, and are transcribed by the host encoded RNA polymerase II. Immediate early genes, such as *ie0*, *ie1*, *ie2*, *me53* and *pe38*, have a transactivation function for the expression of delayed early and late genes.

99. Late and very late genes have a (A/G/T)TAAG promoter motif and are recognized by a virus-encoded RNA polymerase complex, consisting of four proteins (LEF-9 = largest RNA Polymerase subunit, LEF-8 = second largest RNA Polymerase subunit, LEF-4 = RNA capping enzyme, and P47 = unique subunit) (Rohrmann, 2019). The transcript abundance depends on the time line of virus infection; approximately 1/5 of mRNAs are early transcripts (IE and DE), whereas 4/5 of mRNA are transcribed during the late phase (L and VL) (Chen et al., 2013).

100. Viral DNA replication in the cell nucleus employs a large number of virus encoded proteins, including DNAPOL (= DNA polymerase), LEF-3 (= single-stranded DNA binding protein), LEF-1 (= primase), LEF-2 (= primase accessory factor), P143 (= helicase) and perhaps other proteins (Rohrmann, 2019).

101. Baculovirus synthesis mainly occurs in the virogenic stroma (VS) and employs host factors and viral proteins for its replication and assembly. The structure of the VS consists of a filamentous, electron-dense matter and is associated with virion development (Young et al., 1993). No secondary metabolites are produced during baculovirus propagation.

102. The expression of P10 and the OB matrix protein polyhedrin/granulin, both involved in OB formation and release, is the signature of very late gene expression. As both genes, *p10* and *polh/gran*, are highly expressed but not required for virus replication, their promoters have been exploited as part of the baculovirus expression vector system (BEVS) which involves replacing the coding region of these

genes with heterologous ORFs, resulting in the production of high levels of heterologous proteins (Smith et al., 1983; Vlak et al., 1990) (see Chapter 5.6).

103. Transcriptome analysis of baculovirus-infected cell lines and host larval tissues, such as midgut and fat body, have been carried out using microarray analyses and RNA sequencing to elucidate the baculovirus-host interaction at transcription level (Donly et al., 2016; Li et al., 2016; Shrestha et al., 2018; Wang et al., 2015).

104. Transcriptomic and proteomic analyses of fat body of cotton bollworm infected with HearNPV both indicated that cell metabolism of host bollworm larvae is primarily modulated by inhibiting host immune response and inactivating the host´s metabolic activity in favour of HearNPV replication during systemic infection (Xing et al., 2017).

Regulatory consideration and evaluation

105. Like other viruses, baculoviruses can only replicate in a susceptible host. Replication of baculoviruses occurs only in insect larvae but not in other developmental stages. Infection and replication is host-specific. For alpha- and betabaculoviruses, the mode of action can be summarised as follows: Larval infection starts with *per os* ingestion of viral OB which contain ODV embedded in a proteinaceous matrix. The protein matrix is dissolved in the alkaline environment of the larval midgut, ODVs are released and infect the midgut epithelial cells. In the nucleus of the epithelial cells, progeny nucleocapsids are produced, which eventually bud from the cellular plasma membrane producing BV. BV initiate secondary infections in other larval cells and tissues. Late in infection, OB protein is expressed and ODVs are produced and embedded into emerging OB. At the end of the infection, cell lysis occurs and OB are released from insect cadavers into the environment for subsequent ingestion and infection of new hosts. Infection of delta- and gammabaculoviruses is restricted to midgut cells only. Two RNA polymerase enzymes, one host and one viral encoded RNA polymerase and a viral encoded DNA polymerase are involved in gene transcription and DNA replication, respectively.

4.4 Relationship to Animal and Human Pathogens

106. It has been proposed that baculoviruses co-evolved for more than 300 million years with their host during which time they developed a unique specificity for insects (Thézé et al., 2011). Together with arthropod-specific virus families *Nudiviridae* and *Hytrosaviridae,* they constitute the virus order *Lefavirales* in the arthropod-specific virus class *Naldaviricetes* (ICTV, 2021). Baculoviruses are not capable of infecting human, vertebrate and plant cells and are unrelated to human, vertebrate and plant viruses.

Regulatory consideration and evaluation

107. Baculoviruses are not capable of infecting human, vertebrate and plant cells. This is further confirmed by the use of baculoviruses as vectors in human cell therapy (see Chapter 5.6)

4.5. Genetic Stability

108. Genome replication of baculoviruses is accomplished by a viral-encoded DNA polymerase, assisted by Helicase, LEF-1 (DNA primase) and LEF-2 (DNA primase accessory factor) (Rohrmann, 2019; van Oers and Vlak, 2007). Since large dsDNA viruses express proof-reading DNA polymerases, their spontaneous mutation rate during genome replication is very low compared to RNA synthesis of RNA viruses and is estimated to be in the range of 10^{-8} mutations per nucleotide (Duffy et al., 2008); similar rates may apply for baculoviruses. On the other hand, baculovirus genomes show a considerable degree

of genetic variation between viruses from geographically or temporally distinct host populations, as exemplified by REN analyses (Lee and Miller, 1978; Muñoz et al., 1999; Rezapanah et al., 2008) or single nucleotide polymorphism (SNP) analyses (Chateigner et al., 2015; Fan et al., 2020a,c). Such variations between different isolates of a baculovirus might contribute to virulence differences between such isolates towards their target species.

109. Different genetic factors were identified to contribute to the natural variability of baculovirus genomes, including (i) intragenomic recombination (ii) replication-related variation, (iii) homologous recombination between different baculovirus genomes, (iv) horizontal acquisition of genetic material from other organisms, and (v) insertion of host transposable elements (van Oers and Vlak, 2007). Recombination is a natural mechanism resulting in intergenomic exchange of information and has been also reported for baculoviruses. Whereas homologous recombination refers to an exchange of allelic sequences between two different genomes, non-homologous recombination encompasses all events of non-allelic sequence exchange. It is proposed that during the evolutionary history of baculoviruses their genomes have occasionally picked up genetic material from other baculoviruses, other viruses, micro-organisms, and insect hosts (Hughes and Friedman, 2003; van Oers and Vlak, 2007).

110. From genome sequence comparison, it was proposed that regions containing *bro* genes and *hrs* are hotspots for intragenomic recombination and variation (Erlandson, 2009; van Oers and Vlak, 2007). It had been occasionally observed that baculovirus genomes pick up transposable elements (TEs) originating from host genomes, resulting in virus mutants with genotypic and phenotypic changes, such as few polyhedra (FP) mutants (Friesen, 1993). Based on such horizontal influx of TE into genomes of baculoviruses, their possible role as interspecies vectors in the horizontal transmission of insect transposons was proposed (Jehle et al., 1998). Such considerations of horizontal transfer of transposable elements were revived by deep sequencing of AcMNPV, which suggested that baculoviruses may be involved in horizontal DNA or transposon transfer between alternative host insects (Gilbert et al., 2014; 2016).

111. The above mentioned molecular mechanism exemplify the source of heterogeneity of naturally occurring baculoviruses during millions of years of baculovirus evolution. They may occur during replication at the level of single viral genome molecules at very low frequency. For a given baculovirus strain such molecular changes become dominating over time only if there is a selection advantage over the wild-type virus, e.g. as it was observed with FP mutants under specific cell culture conditions (for review see Friesen, 1993).

112. In general, these events do not change the genotypic and phenotypic identity of a given baculovirus strain. Exchange of genes with the host or genetic rearrangement of the genome, as known from other viruses, is not an inherent character of the baculovirus infection and replication process. Because of the known natural mechanism causing genome variability in baculoviruses during replication, it is a good practice to conserve the baculovirus production stocks in a long-term storage facility at a suitable temperature (e.g. -18 ºC) to maintain their activity and genetic identity. New preparations are then generated from the stored production stock to maintain genetic stability.

Regulatory consideration and evaluation

113. Production stocks of baculoviruses should be stored in a suitable or low temperature facility to maintain their genetic stability.

4.6 Population Genetics

4.6.1 Genome Heterogeneity

114. Generally, baculovirus isolates derived from the field carry multiple genotypes, often exhibiting variable virulence (Erlandson, 2009). Early findings of genetic variation of naturally occurring baculoviruses were evidenced by REN analyses, PCR and genome sequencing. With respect to genetic variation of baculoviruses, most research has been carried out with alpha- and betabaculoviruses. There are ample studies identifying genotypic variants of AcMNPV (Lee and Miller, 1978; Yanase et al., 2000), HearNPV (Ogembo et al., 2007), LdMNPV (Harrison et al., 2014, 2016b; Slavicek et al., 1995), Malacosoma disstria nucleopolyhedrovirus (MadiNPV) (Erlandson et al., 2006), SfMNPV (Shapiro et al., 1991; Simón et al., 2004), SeMNPV (Muñoz et al., 1999), Artogeia rapae granulovirus (ArGV) (Smith and Crook, 1988), CpGV (Eberle et al., 2009; Rezapanah et al., 2008), PhopGV (Gómez-Bonilla et al., 2012; Jukes et al., 2014; Zeddam et al., 2013; Zeddam et al., 1999; Vickers et al., 1991), and many others.

115. Among point mutations as well as insertions and deletions, many genotypic heterogeneities are based on variation in the *hrs* regions and *bro* genes, which appear as genomic hotspots of variability. For example, in the genomes of the HearNPV strains C1 and G4, variability was mainly located in *hrs* and *bro* gene regions (Chen et al., 2001; Zhang et al., 2005). Many of these findings have been corroborated by HTS but to unprecedented detail and information depth. HTS methods have been applied to detect the genetic variation in baculoviruses in terms of single nucleotide polymorphisms (SNPs), insertions and deletions including TEs (Thézé et al., 2014; Chateigner et al., 2015; Gilbert et al., 2014).

116. SNP analyses in particular have helped to identify further variation in the population structure of baculovirus isolates without the need to purify single genotypes by *in vitro* or *in vivo* cloning and have been applied to different alpha- and betabaculoviruses, including AcMNPV, SeMNPV, HearMNPV, PhopGV, CpGV (Chateigner et al., 2015; Thézé et al., 2014, Noune and Hauxwell, 2016; Larem et al., 2019a; Fan et al., 2020a,c). HTS approaches have been also applied to decipher the composition of commercial baculovirus preparations and can be used as a powerful tool for strain identification (Gueli Alletti et al., 2017). Recently, application of SNP analyses of different propagation and production batches of CpGV revealed molecular evidence that even highly complex genotype mixtures in ni-strains are stably produced under *in vivo* production conditions (Fan et al., 2020a).

4.6.2 Single vs. Multiple Genotype Isolates

117. *In vitro* plaque purification and *in vivo* cloning are based on serial passages in cell culture or in larvae, respectively, using low virus titers for infection. Both methods, resulting in selection of clonal strains consisting of a single genotype (sg-strains), have been frequently applied to identify genetic variants in baculovirus isolates, elucidating the prevalence of high diversity in wild-type and laboratory-selected baculovirus populations and reflecting the consequences of baculovirus-host interaction (Aguirre et al., 2019; Bernal et al., 2013; Clavijo et al., 2010, 2009; Harrison, 2013; Simón et al., 2005). The application of both methods has generated individual genotypes exhibiting distinguishable virulence as well as morphological differences, e.g. few polyhedral mutants or defective viral genomes (Bull et al., 2003; Cheng et al., 2013; de Rezende et al., 2009). Furthermore, minor genotypes existing in a virus population have been isolated as individual sg-strains after several passages of purification. Co-infections with different baculovirus sg-strains against host larvae were found to increase mortality in laboratory tests and field applications (Del-Angel et al., 2018; Espinel-Correal et al., 2012; Graillot et al., 2016; Hinsberger et al., 2020).

118. Commercially applied baculoviruses need to have highly effective insecticidal activities, appropriate production parameters, and affordable production and application costs, which require that the MPCAs in formulations are highly virulent against their target insect. Before reaching the aforementioned,

it might be useful to purify heterogeneous isolates into individual genotypes (Ferreira et al., 2019; Thézé et al., 2014). *In vitro* plaque purification and *in vivo* cloning may be suitable to select highly pathogenic clones from natural field isolates. For example, a sg-strain of Spodoptera litura nucleopolyhedrovirus (SpltNPV-C3), which was plaque-purified from wild-type isolate of SpltNPV using Spli cells, showed distinct profiles in productivity and virulence on *Spodoptera litura* cultured cells and larvae (Kamiya et al., 2004). SpltNPV-C3 could induce rapid mortality and produce more infectious BV than other clones, demonstrating the possibilities to exploit the inherent variability of field isolates for potential usage in pest control. This approach can improve insecticidal activity by selecting and propagating more virulent strains for further optimization of baculovirus biocontrol agents. However, *in vitro* plaque purification, normally initiated with low titer BV, only includes the secondary infection of the baculovirus life cycle in insects and does not confer any selection for maintenance of the primary infection process, including *per os* infectivity. This relaxed selection pressure occurring during cell culture passage may result in genotypic and phenotypic mutants and virulence reduction (Cory et al., 2005). Mutations within *fp25k*, including point mutations or small insertions/deletions resulting in the decrease of polyhedral production, were observed in continuous passages (Fraser, 1986; Lua et al., 2002; de Rezende et al., 2009; Cheng et al., 2013). Under certain infection conditions, rapid generation of mutants has been noted during cell culture propagation including gene loss and genome shrinkage by deletions of single or multiple genes and/or production of defective interfering particles, which depend on helper genomes to become propagated (Harrison et al., 2008; Pijlman et al., 2001; Simón et al., 2004). Such *in vitro* genotypes often carry large genomic deletions, and with the loss of genes for *per os* infectivity factors, these mutants become deficient to infect insect larvae and are not suitable for biological control purposes. In one instance, a solution to this issue was found when an LdMNPV isolate capable of producing a stable ratio of highly infectious OB was identified that did not accumulate *fp25K* mutants during serial passages *in vitro* (Slavicek et al., 2001).

119. An alternative method for selecting single genotypes from wild-type isolates of baculovirus is *in vivo* cloning in host larvae, during which a baculovirus can complete the primary and secondary infection phase. As a consequence of this stringent selection pressure for maintenance of all aspects of the baculovirus life cycle, the viruses maintain their genomic integrity. During *in vivo* cloning, the OB are diluted to a single infectious unit (being able to induce a mortality of <5-10% on the basis of the single particle theory) (Huber and Hughes, 1984; Muñoz et al., 1999) and fed to the early developmental stages of host larvae, inducing larval death accompanied by the replication of an individual genotype. Such low-dose infections need to be repeated multiple times. The sg-strain obtained after 1-6 passages can be maintained during propagation within host insects (Muñoz et al., 1999). From this process, candidate strains with high virulence can be selected for commercial development of baculoviruses for pest control.

120. Baculovirus strains carrying variable genotypic variants (ni-strains or mg-strains) may have a broader tissue tropism than plaque-purified sg-strains (Erlandson et al., 2006) and are less susceptible to resistance development by the host. Typically, natural and commercial baculovirus isolates contain numerous closely related genotypes. The LdMNPV strain used in Gypchek, a commercial formulation of this virus, is a mixture of LdMNPV genotypes produced *in vivo* (ni-strain) (Slavicek et al., 1995). Similarly, commercial SeMNPV products appear to be genotype mixtures (ni-strain) (Elvira et al., 2013). In a preparation of SpexNPV, at least 17 genetically distinct genotypes could be identified (Redman et al., 2010). In the past, such variants were identified by additional or missing bands or submolar fragments in REN analyses as well as restriction length polymorphisms after cloning and propagation of individual genotypes.

121. Considerations of production quality, insecticidal activity and economic returns have dictated that production of baculovirus biocontrol agents has only occurred *in vivo*, despite intensive research on *in vitro* production (Miller, 1997). Rearing host insects in laboratory or large-scale insect rearing facilities is the most common approach to produce baculoviruses for commercial use. In some cases, if the host insect for virus production cannot be reared on artificial diet, the insects have to be reared on the natural host plant, which may require spraying the virus in insect-accommodating niches in open land or forests.

Collecting moribund larvae has been shown to be an applicable approach to produce the active ingredient, such as Anticarsia gemmatalis nucleopolyhedrovirus (AgMNPV) and *Spodoptera* spp. baculoviruses in South America (Moscardi, 1999, 2007) or Neodiprion abietis nucleopolyhedrovirus (NeabNPV) (Lucarotti et al., 2007). Also, the highly virulent strains of AcMNPV, SeMNPV and CpGV against Lepidoptera pest larvae were isolated *in vivo* from wild-type viruses, becoming the main ingredients in commercial baculovirus products (Moscardi, 2007; Vincent et al., 2007).

122. It becomes more and more evident that the genetic background of the host plays an important role in the genotypic structure of a baculovirus, especially when such genotypic variations of the virus convey biological differences (Cory and Myers, 2003; Kennedy and Dwyer, 2018). Host-mediated selection of baculovirus genotypes have been shown for different baculoviruses serially passaged through different host species, as shown for HearNPV, AcMNPV, MbMNPV, Panolis flammea nucleopolyhedrovirus (PaflNPV) and many others (McIntosh and Ignoffo, 1986; Kolodny-Hirsch and Van Beek, 1997; Hitchman et al., 2007; Belda et al., 2019). Such serial selections have been successfully used to adapt and increase baculovirus virulence to certain hosts (Hitchman et al., 2007). As an example, the host genetic background of CpGV-resistant codling moth strains was successfully employed to select resistant-breaking CpGV strains (Zingg et al., 2011; Graillot et al., 2014) (see Chapter 4.2.3)

4.6.3 Covert Infections

123. Another source of baculovirus genotype heterogeneity may derive from activation of viruses from covertly infected individuals in the insect population or the rearing colony. Covert infections do not result in disease symptoms, but may persist as a sublethal or a latent infection, thereby providing the virus the possibility of vertical transmission from one generation to another (Kemp et al., 2011; Williams et al., 2017) (see Chapter 4.7.2). Ample evidence for sublethal or latent baculovirus infections has been collected for alpha- and betabaculoviruses (Williams et al., 2017). When covertly infected host larvae are inoculated by a *per os* administered baculovirus, the covert genotype may become activated and propagated, altering the genetic composition of the offspring compared to the inoculum. Such activation can be triggered by environmental factors or infection with heterologous or homologous viral genotypes (Cooper et al., 2003; Kemp et al., 2011; Kouassi et al., 2009; Larem et al., 2019a). Covert baculoviruses may suppress replication of an inoculated strain or result in co-infection and mixtures of genotypes different from the inoculum and affecting the genotypic identity of the propagated virus (Larem et al., 2019a). Therefore, the host production colony for a given baculovirus active ingredient is crucial for the quality and identity of the virus produced. Any change of the host production colony may influence the genetic identity and composition of the propagated baculovirus strain and will require additional steps to determine the identity of the produced virus. This is less related to the nature of the virus inoculum, as it was shown the baculovirus strains consisting of genotype mixtures can be stably propagated, (e.g. CpGV or LdMNPV) in their host, rather than to the insect host colony.

124. In summary, baculoviruses employ different mechanisms to generate and maintain considerable genetic diversity within a baculovirus population. Potential mechanisms for maintaining this genetic diversity include (a) trade-offs in relation to infection efficacy, (b) differential selection under certain ecological conditions, (c) infection by multiple genotypes, (d) interspecific competition, and (e) frequency dependent selection (Cory and Myers, 2003).

Regulatory consideration and evaluation

125. Wild-type baculoviruses typically consist of mixtures of genotypes. For strain development, serial propagation is necessary to prove stable inheritance of a strain character. Evidence for stability should be provided within the virus characterization of a 5-batch analysis.

126. Changes of the production host species or host colony requires additional quality control of the MPCA to ensure that the approved baculovirus strain is produced.

4.7. Ecology of Baculoviruses

4.7.1 Host Range

127. Baculovirus propagation depends on the infection of living insect larvae. Thus, the range of insects that can provide replication of a given baculovirus can be regarded as its host range. Studies on baculovirus host range and cross-infectivity date back to the 1960s to 1980s (Gröner, 1986), whereas the molecular basis of host range has been further elucidated with the development of molecular tools and sequencing technologies (Lu and Miller, 1997; Thiem and Cheng, 2009; Ikeda et al., 2015).

128. Extensive infection experiments demonstrated, that baculoviruses are unable to replicate in vertebrates, non-insect invertebrates, plants or other organisms (Burges, 1981; Hunter-Fujita, 1998). It is very well established that baculoviruses have a very narrow host range and can only infect larval stages of three insect orders, namely Lepidoptera, Hymenoptera and Diptera. Host specificity and baculovirus phylogeny are clearly linked (Jehle et al., 2006b; Thézé et al., 2018). Only viruses from the genera *Alphabaculovirus* and *Betabaculovirus* can infect Lepidoptera. Viruses of these genera have been isolated from hundreds of host species from at least 20 different families, many comprising important pest species in agriculture and forestry. Gammabaculoviruses are specific mostly for the families Diprionidae and Tenthredinidae of Hymenoptera, whereas deltabaculoviruses have been isolated from a few Dipterans (Martignoni and Iwai, 1986). Thus, the vast majority of known baculoviruses are specific for lepidopteran larvae (Gröner, 1986; Thiem and Cheng, 2009). Because of the strict host range link between baculovirus genera and host insect order, host range testing in others than the insect host order, classes or phyla has never provided any evidence for virus infection or other adverse effects. Other orders from Arthropoda, previously considered as potential hosts of baculoviruses, have been found to be affected by viruses from phylogenetically related families, such as *Nudiviridae*, *Hytrosaviridae* or *Nimaviridae* (Thézé et al., 2011; Rohrmann, 2019).

129. Cross-transmission experiments have demonstrated that baculovirus host range is restricted to one or few species of the genus or family of the host from which they were isolated (Cory and Myers, 2003; Doyle et al., 1990; Gröner, 1986). There are only a few examples, such as AcMNPV, AnfaNPV and MbMNPV, for which dozens of species from different lepidopteran families have been noted as potential hosts, although except for a few permissive host, very high amounts of OB were needed for infection (Gröner 1986, Doyle et al., 1990; Cory, 2003). The host range of betabaculoviruses (= granuloviruses) appears to be even narrower than that of alphabaculoviruses and often extends to one or a few closely related insect species. A betabaculovirus with a moderately narrow host range extending to hosts from different genera is XecnGV, which has been isolated from noctuid species from six genera (Goto et al., 1992). It was stated that not all cross transmission experiments can be considered as significant since often unrealistic high dosages may be needed to infect secondary hosts (Evans, 1986).

130. Baculoviruses that are infective for different insect species have differing levels of virulence against their host species in laboratory assays, as indicated by differences in LC_{50} or LD_{50} values. AgseNPV and AgseGV infect different cutworm species and closely related Noctuinae (Bourner and Cory, 2004). AnfaNPV has a broad host range in bioassays, showing different levels of virulence against four tortricid apple pests (Lacey et al., 2002). In terms of LC_{50} values, the susceptibility of the oriental fruit moth *Grapholita molesta* (= *Cydia molesta*) to CpGV has been shown to be more than 500 times lower than that of *C. pomonella* (Lacey et al., 2005). Starting with an experimental mixture of different CpGV isolates, the virulence of this mixture for oriental fruit moth could be increased 20- to 50-fold in terms of LC_{50} values during 14 successive passages through this alternative host, whereas the activity against its original host *C. pomonella* decreased (Graillot et al., 2017). Similarly, twenty serial passages of AcMNPV through the diamondback moth, *Plutella xylostella*, resulted in an approximately 15-fold decrease of the LC_{50} value to this host, whereas the virulence to other tested host species remained unchanged (Kolodny-Hirsch and Van Beek, 1997). In both cases a change in the genotype structure of the virus population was noted,

suggesting the forced selection of more virulent variants by the serial passage. The lack of other reporting increase of virulence may be an argument that this effect is rather the exception than the rule. In nature, such passage scenarios can be considered as irrelevant. In the context of field application of baculoviruses, these mechanisms can be neglected because even some infected larvae may propagate certain genotypes better than others, there is no passage selection due to spraying with the same product. It has to be further noted that such selection cannot be considered as a means to extend host range to a refractory species, but is instead useful for increasing virulence against a partially susceptible alternative host.

131. As pointed out by Cory (2003), laboratory host range testing is a “worst case scenario”, since tests are done with young larva cohorts and ideal growth conditions. Under field conditions, however, factors such as population´s age structure, behavioural differences, population density effects, or host plant effects, will considerably reduce the likelihood of infection compared to laboratory assays. In addition, it is highly unlikely that the individual response observed with alternative host species could transfer to an effect on populations scale. Considering the requirement of repeated sprays of baculovirus products to control highly susceptible target insects at high population densities, it appears unlikely that a less susceptible non-target species could be harmed (Cory, 2003)

132. A considerable number of different baculovirus genes have been identified to be involved in tissue tropism and host range, including *pif*s (peroral infectivity factors), *hcf-1* (host cell factor-1), *hrf-1* (host range factor-1), *p35*, *iap* (inhibitor of apoptosis), *p143* (helicase) and *lef-7* (late expression factor 7) (reviewed by OECD, 2002; Thiem and Cheng, 2009; Ikeda et al., 2015). Except for *pif-9*, all *pif* genes and *p143* belong to the baculovirus core genes set, required for ODV entry into host midgut cells and viral DNA replication, respectively. The encoded proteins of these genes are involved in cell entry (PIFs), global protein synthesis shutdown (HCF-1 and HRF-1), inhibition of apoptosis (P35, IAP), and DNA replication (P143, LEF-7). This list of genes and proteins so far identified to be involved in determining the host range of baculoviruses is certainly not complete. It rather demonstrates that the host range is not defined by a single gene or specific determinants but it is the product of a successful cellular entry, viral gene expression and DNA replication, progeny virus production and virus egress and OB production.

Regulatory consideration and evaluation

133. The host range of given baculoviruses is considerably narrow and is usually restricted to one or a few closely related insect species. There are only a few examples of alphabaculoviruses with a host range exceeding to several dozens of known insect species as potential hosts. Considering that highly susceptible hosts need to be frequently sprayed to keep a target insect population at a low level, it is highly unlikely that the population of a non-target insect species with low susceptibility is affected by the virus spray. There is no record of any baculovirus crossing the boundary of the insect order the host species belongs to. Insects from species not belonging to the baculovirus host orders Lepidoptera, Diptera and Hymenoptera are not affected by baculoviruses.

134. To provide some basic information on the host range of a new baculovirus used as active substance, infection studies (alternatively scientific literature records from the same baculovirus species) are recommended to be carried out with early instar larvae of at least five insect species from the same insect order including 1-2 insect species from the same insect family. Infection experiments should be done with a 10- to 100-fold LD_{50} or LC_{50} and a duration of at least 14 days or until pupation of the test insect. Mortality should be recorded in comparison with an untreated control group.

4.7.2 Transmission

135. Baculoviruses, like many other pathogens, adopt mixed-mode transmission routes including vertical and horizontal transmission, which facilitate baculovirus long-term persistence in insect host populations and provide the opportunity for virus host co-evolution (Ebert, 2013). Host fidelity, host range, population and developmental stage structure, and congruent phylogenies act as important factors

involved in the constant adjustment of transmission pathways in order to make sure that baculovirus and host will both survive in nature.

136. Vertical transmission requires host reproduction and is therefore associated with low-virulence infections that result in covert infections, which can be either persistent or latent. In early studies, such covert infections and activation of latent infections had been occasionally reported, though molecular verification was mostly lacking and cross-infections and contamination could not be fully excluded (OECD, 2002). The first molecular evidence for vertical transmission and activation of a latent baculovirus was obtained with *Mamestra brassicae* larvae that were fed with a heterologous baculovirus, but that produced progeny MbMNPV in response (Hughes et al., 1993, 1997). Further molecular studies, mainly based on PCR and qPCR, resulted in more than 30 publications since the early 2000s supporting the occurrence of covert infections and vertical transmission (Williams et al., 2017) (see Chapter 4.6.3). Next-generation sequencing provided additional insight into the prevalence of vertically transmitted alpha- and betabaculoviruses (Larem et al., 2019b; Thézé et al., 2014).

137. Vertical transmission of baculoviruses encompasses both the transovarial and transovum pathways (Kukan, 1999). Whereas most investigations of vertical transmission have been carried out on laboratory colonies, its relevance in field populations is not fully elucidated as there are only a few studies reporting persistent baculovirus infections in field populations of Lepidoptera (Cory, 2015). Such persistent infections have been discovered in field populations of African armyworm (*S. exempta*), western tent caterpillars (*Malacosoma californica*), *Lymantria dispar*, Indian meal moth (*Plutella xylostella*) etc., and also in insect cell lines (Burden et al., 2002; Cooper et al., 2003; Fang et al., 2016; Kennedy and Dwyer, 2018; Vilaplana et al., 2010). Cases of vertical transmission via the transovarial route (infected eggs) from one generation to next generation could be increased by horizontal transmission when the parental moths were exposed to the virus during the larval phase (Vilaplana et al., 2008).

138. Horizontal transmission initiated by the release of OB from infected cadavers and their ingestion by larvae during feeding is surely the main strategy of baculovirus infection transmission. The formation of the OB itself is the evolutionary adaptation that facilitates this horizontal transmission strategy. In addition, modifications of host behaviour favouring environmental spread of OB, such as hyperactivity and tree-top disease, are caused by (or correlated with) the expression of baculoviral genes *protein tyrosine phosphatase* (*ptp*) and *ecdysteroid uridine 5'-diphosphate (UDP)-glucosyltransferase* (*egt*) (Han et al., 2018; Hoover et al., 2011; Houte et al., 2014; Kamita et al., 2005; Ros et al., 2015). Larvae are induced by baculovirus infection to climb towards the top of plant, from which progeny OB are released upon death of the infected larvae and disperse to the ambient environment in a radial pattern, allowing for highly efficient transmission (Rebolledo et al., 2015).

139. Spraying baculovirus OB to control pest insect larvae employs the horizontal transmission mode via the OB phenotype. When baculovirus biocontrol agents are sprayed, uptake of sprayed OB by feeding larvae is essential. Due to plant growth, UV inactivation or dislodgement of OB by rain, repeated applications per season are normally required. The contribution to horizontal transmission by infected larvae depends on host density. Horizontal transmission between agricultural or forestry pest hosts has been noted for open, colonial feeders, such as *Helicoverpa zea*, *Anticarsia gemmatalis*, *L. dispar*, and *Neodiprion sertifer* (Fuller et al., 2012; Silva and Moscardi, 2002; Zhou et al., 2005). SeMNPV was found to cause higher mortality in a host population via horizontal transmission from larvae infected by way of autodissemination and transovum transmission (Yu and Brown, 1997).

140. However, for cryptic, low-density hosts such as the codling moth, it was concluded that horizontal transmission may be an unimportant factor for efficacy in the field and may not play a role in management of infestations in orchards where high larval densities are never reached (Steineke and Jehle, 2004).

141. Environmental spread of baculovirus OB may be further promoted by predators feeding on virus-infected prey and dispersing virus through faeces (reviewed by Abbas, 2020). Studies on this subject are

mostly derived from research with alphabaculoviruses and betabaculoviruses, as reports on gammabaculoviruses and deltabaculoviruses with respect to sawflies and mosquitoes are very few.

142. Combining vertical and horizontal transmission modes enables baculoviruses to persist in host insects and induce covert infections with a low degree of pathogenicity, or to cause overt infections characterized by high mortality. Though evidence for the capacity of vertical transmission has accumulated during the last decades, horizontal transmission is still considered to be the main transmission mode. When baculoviruses are used as pest control agents, repeated sprays are needed, indicating their limitations with respect to reducing host populations below an economic threshold.

Regulatory consideration and evaluation

143. Baculoviruses are ubiquitously present in insect populations as natural pathogens. Natural transmission between host individuals occurs either horizontally or vertically. No further data on transmission is needed on strain level.

4.7.3 Baculovirus Epizootics and Environmental Occurrence

144. An outbreak of disease in an insect population requires the presence of pathogen and a sufficient number and density of susceptible hosts to allow for an increased rate of infection (Cory and Myers, 2003). Such epizootics, caused by baculoviruses, naturally occur from time to time in forest and agricultural pest populations (Cory and Myers, 2003; Weiser, 1987). Natural and application-induced baculovirus epizootics of pest insects in forestry and field crops have been studied to identify the parameters that regulate epidemic disease in insect populations (Il'inykh, 2007): (1) Baculovirus virulence and quantity in biocoenosis; (2) behaviour, density and age structure of the host, (3) nutrition-mediated effects, (4) environmental factors, and (5) presence of latent virus in host populations.

Baculovirus Virulence and Quantity

145. Baculoviruses have been shown to be highly virulent to their insect hosts and, as demonstrated by laboratory assays, only a few OB might be sufficient to initiate lethal infection in early instar larvae, whereas increasingly larger doses are required to infect later instars (Bianchi et al., 2000b; Evans, 1986; Kunimi et al., 1997). Infection with non-fatal doses may result in a clearance of infection or in sublethal effects, such as delayed development, low weight or deformation of pupae, reduction of reproduction, and decreased life-span (Evans, 1986; Fuxa, 2004).

146. Baculoviruses, however, carry an upper limit of virulence against host insects due to fitness costs in virus isolates, hinting that baculovirus-induced mortality is possibly lower than 100% in general. The surviving insect cohort could develop and produce a large generation of progeny for a subsequent round of infection in the field. Conventional applications of baculovirus formulation cause viral epizootics in forest and agriculture systems, protecting crops from insect pest damage (Huber, 1998; Mcleod et al., 1982). The released OB from liquefied cadavers can serve as inoculum for peroral infection of host insects. These OB can either disperse on the plant surface to form multiple foci for subsequent infections or translocate to the soil to serve as an inoculum for another outbreak. When baculovirus multiplicity of infection is lower than that is required for the epidemic outbreak or OB become inactivated by environmental factors, the host population can recover. As the amount of OB progeny produced by early infected instars is much lower than that of older instars, there is an equilibrium established between infection susceptibility and OB production contributing to virus epizootics. Such natural epizootics usually occur only at very high host densities, such as with forest defoliators, but can be usually neglected for agricultural crops due to low damage thresholds requiring baculovirus applications at low host densities and control of early instars.

147. Instances of seasonal or sporadic baculovirus epizootics were observed in different pest-plant systems, in which the infection rate exceeded enzootic levels and caused a death rate of 10% or higher

(Gelernter and Federici, 1990; Il'inykh, 2007). In populations of *Spodoptera exigua,* virus epizootics often caused >90% mortality in an area of about 1000 km^2 and was dominated by single SeMNPV strain and its genotypic variants (Federici, 1978; Gelernter and Federici, 1990). Epizootics are frequently observed in forest pests, such as *L. dispar, Lymantria monocha, N. sertifer* and others. Estimates of LdMNPV OB quantities during epizootics were in the range of 10^{11} to 10^{13} OB/ha (Il'inykh, 2007). After a massive outbreak of *N. sertifer* in Sweden, NeseNPV concentration in soil was 10^5 OB/mL one year after an epizootic (Olofsson, 1988). Similarly, in agricultural systems, OB concentrations were determined in the range between 10^4 to 10^5 OB/g soil for SfMNPV and >10^3 OB/g soil for AgseNPV (reviewed by Fuxa, 2004).

148. In addition to virus quantity, the spatial distribution of virus OB in the soil and the plant canopy may play a crucial role during epizootics, but solid quantitative data are very difficult to obtain. A few studies concluded that the pattern of virus distribution in the environment influences the likelihoods of transmission and epizootics, such that the number of OB-containing foci is more important than the amount of OB within foci (Cory and Myers, 2003; Steineke and Jehle, 2004).

Behaviour, Density and Age Structure of Host

149. Epizootics of baculoviruses are further affected by host behaviour. Host larvae that aggregate to high density, carry out open feeding on the plant canopy and perhaps indulge in cannibalism are more likely to be exposed to virus infection than larvae feeding in protected situations (Fuxa, 2004). For example, egg masses of *L. dispar* may contain 300 to 800 eggs (sometimes even more than 1000) and larvae feed in large groups and in close proximity to each other, whereas the codling moth, *Cydia pomonella,* lays eggs individually on leaves and fruits and only produce more than one larva within a single fruit at high codling moth densities.

150. Many baculoviruses induce the so-called tree-top climbing phenomenon ("Wipfelkrankheit") in infected larvae (Goulson, 1997) (see also Chapter 4.7.2). Baculovirus-induced changes of the behaviour of infected larvae have been reported for several baculovirus host species and are thought to contribute to a rapid dissemination of progeny OB, thus increasing the probability that a susceptible host will encounter OB in the environment (Cory and Evans, 2007). It could be demonstrated for larvae of *S. exigua* that infection with SeMNPV triggers a positive phototactic response prior to death, causing larvae to die at elevated positions and thereby increase the local dissemination of virus progeny (Han et al., 2018; Houte et al., 2014). An opposite (tree-down) behaviour has also been noted: insects infected with Operophtera brumata nucleopolyhedrovirus (OpbrNPV) exhibited an altered behaviour characterized by moving down the plants, distributing progeny OB on plant stems in the process. Since neonate larvae were able to acquire infections from tree stems contaminated with a low level of virus, it was proposed that this change in behaviour of winter moth larvae could enhance viral persistence by increasing local OB deposition (Raymond et al., 2005).

151. Cannibalistic behaviour in which healthy larvae feed on infected ones is reported as an additional route of infection in some virus-host systems, e.g. SfMNPV, HearNPV, and others (Chapman et al., 1999; Dhandapani et al., 1993; Valicente et al., 2013).

152. Developmental resistance of host larvae is a universal feature of all baculoviruses (see Chapter 4.2.1). For example, in the case of MbMNPV, the difference of susceptibility between L1 and L5 *Mamestra brassicae* larvae can vary by a factor of over 34,000 (Evans, 1983). Thus, the age structure of a host population also affects the development of baculovirus epizootics.

Nutrition-mediated Effects

153. Host response to baculovirus infection under field conditions can be further influenced by quality of the foliage that is ingested along with the virus, which is determined by the plant species, the defensive status of the plant, plant phenology and the ingested plant part (reviewed in Shikano et al., 2017). Mortality

of *L. dispar* larvae feeding on leaves from different tree varieties varied by a factor of >2, and leaf phenolics, such as hydrolyzable tannins, were proposed to inhibit Galleria mellonella multiple nucleopolyhedrovirus (GmMNPV) activity (Keating et al., 1990). Nutritional modulations caused by the consumed diet or plant foliage that affect the baculovirus infection process have been reported for different insect hosts, including *C. virescens*, *L. dispar*, *Orgyia leucostigma*, *Pieris brassicae*, *Spodoptera frugiperda*, *T. ni* and others (Brodersen et al., 2012; Chen et al., 2018; Cory and Hoover, 2006; Hoover et al., 2000; Keating and Yendol, 1987; Shikano et al., 2018). Elderd et al. (2013) proposed that induced hydrolyzable-tannin defenses in red oak reduce heterogeneity among *L. dispar* larvae in terms of the risk of virus infection and correlated the severity and uniformity of LdMNPV epizootics with the frequency of oaks in the forest. These plant-mediated effects may influence the population dynamics of the virus and the host insect.

Environmental Factors

154. Once OB are released from the insect cadaver, they become dispersed and/or inactivated in the environment by biotic and abiotic factors. Factors influencing their environmental persistence are discussed in detail in Chapter 7.

Regulatory considerations

155. Baculoviruses are naturally occurring insect viruses which are ubiquitously present in the environment. Natural transmission between host individuals occurs either horizontally or vertically. No further data on the ecology is relevant on strain level.

5 Human Health Considerations

156. The major difference between baculoviruses and other cellular microbial pesticides are that baculoviruses do not produce any toxins, metabolites or degradation products of metabolites and cannot reproduce without susceptible host insect. Baculoviruses do not produce substances with antibiotic activity and do not carry genes encoding for the production or spread of resistance to antibiotics of medical importance (Burges et al., 1980; Gröner, 1986; OECD, 2002; Mudgal et al., 2013).

157. During the past decades, extensive safety testings of baculoviruses to invertebrate and vertebrate species, including humans, have been carried out (reviewed by Ignoffo, 1975; Burges et al., 1980; Gröner, 1986). The safety of baculoviruses to humans has been extensively studied not only for their use as biological control agents but also for the application of baculoviruses in vaccine production and human cell therapy (see Chapter 5.6) (OECD, 2002; Lapointe et al., 2012; Fabre et al., 2020). The general safety of baculoviruses for humans and other vertebrates has been emphasized in numerous studies and documents (OECD, 2002). A recent literature study, performed by Hackl et al. (2015) did not reveal any recent evidence of adverse effects to human or animal health.

158. The EU European Food Safety Authority (EFSA) established the concept of Qualified Presumption of Safety (QPS) that has been applied for micro-organism safety assessments in food and feed (EFSA, 2005). The QPS document proposed a system similar in concept and purpose to the GRAS (Generally Recognized As Safe) definition used in the USA. The QPS approach can meet the need for different EFSA panels and establish a list of taxonomic units that is reviewed and updated every three years (EFSA, 2020). Baculoviruses were added on the QPS list in 2009 on the family level (*Baculoviridae*) as the highest taxonomic unit (EFSA, 2009). The list of QPS baculoviruses has been reviewed on the basis of newly published literature related to safety assessments and public health concerns. At present, continued and updated scientific findings in both short- and long-term investigations reviewed by the EFSA has confirmed that baculoviruses cause no hazard to non-target organisms because they cannot replicate or induce detrimental effects in other living animals and plants, indicating their safety for animal feed and human consumption (EFSA, 2010, 2011, 2012, 2013, 2017, 2020; Leuschner et al., 2010).

159. It has to be stated that most published literature on systematic safety studies on baculoviruses biocontrol agents derived from the early 1970s to the late 1980s and is covered by the OECD (2002) consensus document. Recent studies since 2000 referring to biosafety were mainly done with genetically engineered baculoviruses, though none of these viruses was ever further developed as a pesticide. The absence of any adverse effects caused by baculoviruses has been noted in numerous scientific and regulatory publications (Burges et al., 1980; Gröner, 1986; OECD, 2002; EFSA, 2009; Hauschild, 2011). Therefore, the findings are briefly summarised here:

5.1 History of Exposure

160. Baculoviruses have co-evolved with insects during the last 300 million years and are natural pathogens of insects and natural components of the environment. Heimpel et al. (1993) demonstrated a high load of baculovirus OB on untreated cabbage leaves from supermarkets, which derived from natural epizootics and concluded a high natural exposure of humans to naturally occurring baculovirus OB in the

environment (see Chapter 4.7.3. and Chapter 7). Many baculoviruses belonging to the genera *Alphabaculovirus, Betabaculovirus* and *Gammabaculovirus* have been commercially produced and sprayed for biological control, some for half a century. There is no evidence that any of these viruses ever caused disease or harm in humans and other vertebrates. Despite numerous registered baculovirus products produced in insect rearing facilities worldwide, no evidence of toxicity, irritation, sensitisation, or other exposure related health effects have ever been reported. According to the US Incident Data System (IDS) “there have been no reports of adverse human, domestic animal, or environmental incidents for pesticides containing NPVs and GVs” (EPA, 2011).

5.2 Acute Toxicity, Pathogenicity and Infectiveness

161. Toxicity studies of baculovirus to mammals (rat, guinea pig, rabbit, mouse, sheep, dog, monkey) have not detected any deleterious effects in short- and long-term tests via various routes of exposure using much higher doses than typically achieved by field application.

162. From injection studies of OB of different NPVs and GVs to determine the intraperitoneal, subcutaneous or intravenous toxicity, no symptoms or pathological findings were observed in animals. In a recent study, Ashour et al. (2007) administered different wild-type NPVs as well as a genetically engineered AcMNPV recombinant either orally or by intraperitoneal injection to male and female albino rats. Some slight to moderate differences in hematological parameters depending whether the OB were orally exposed or injected could be detected; no mortality or indications of disease was observed over a 21 day-long test and the authors concluded that the viruses provided no increased threat to mammals.

163. The latest safety evaluation of HearNPV, extensively used in more than 20 countries, with experimental rats intravenously injected with virus indicated that injected viruses can be gradually cleared in a short time while tissues and organs do not become infected (Zhao et al., 2019). This new method of safety testing, distinct from oral inoculation with virus, corroborated previous findings of the inability of baculoviruses to infect mammals.

164. Histopathological screenings of baculoviruses on non-target animals indicated no teratogenic and carcinogenic occurrence in acute toxicity-pathogenicity experiments (reviewed by Burges, 1980; Gröner 1986).

5.3 Cell Culture Studies

165. As viruses need cells for replication, a cell culture study provides information on whether the baculovirus can infect, replicate in, transform or cause toxicity in the mammalian cell system. Another concern is that genes in the mammalian cell, which might be “silent” during normal cell growth, may be activated if a baculovirus enters the cell. As reviewed by Arif and Pavlik (2013), at least 35 different baculoviruses have been propagated in established insect cell lines, most of which are NPVs belonging to the genus *Alphabaculovirus*. Only a very few cell lines were permissive for GVs of the genus *Betabaculovirus*, most of them lost permissiveness after a couple of passages (Granados et al., 1986). The only repeatedly reported cell currently permissive to a betabaculovirus was established for CpGV and was derived from embryonic cells of *C. pomonella* (Winstanley and Crook, 1993). This cell line is very slow-growing because it must be kept at temperatures between 22-24 °C to avoid loss of its capacity to replicate CpGV.

166. In their natural state, OB are not infectious to cell cultures because culture media are typically slightly acidic, precluding the release of virions from the OB matrix (Lynn, 2003). Even infection with ODVs, released from OB by an alkali treatment is highly ineffective. It was estimated that ODVs are 1,700-1,900-fold less infectious to TN368 cells, in some cases trypsinization of ODVs improved their infectivity to cell

cultures (reviewed by Lynn, 2003). ODVs are specialized in infection of insect midgut cells and lack envelope fusion components of the BV, which is either the F protein (group II alphabaculoviruses and betabaculoviruses) or GP64 (group I alphabaculoviruses) (Braunagel et al., 2003; Hou et al., 2013). For these reasons, cell culture studies can only be done with baculoviruses for which a permissive cell line exists, as this is necessary for sufficient BV production as well as a positive control in the infection experiment with the mammalian cell(s). For all those baculoviruses intended to be used as plant protection agent, for which no cell line is available, such studies cannot be performed according to the OECD ENV/JM/MONO(2018)19 guidance document.

167. Most cell culture studies of baculoviruses have been done with AcMNPV, which is known for its very broad insect host range and which is widely used in gene therapy. Thus, studies with AcMNPV (and other NPVs) can be assumed as a worst case scenario that clearly demonstrates that no genetic hazards have been observed. AcMNPV is able to penetrate and deliver genes into non-target cells, but in the non-target cells, the virus is incapable for replication or proper viral gene expression and is thus nontoxic for the vertebrate cells (reviewed by Airenne et al., 2010, 2013). No or very limited viral DNA transcription, no replication or reproduction were observed in human and mammalian cell lines, nor the persistence of viral DNA or fragments of viral DNA were detected in them as well, although baculovirus could enter into these cells (Gröner, 1986; Via et al., 1983; Fujita et al., 2006). Recombinant baculoviruses could not express foreign genes in an inserted cassette unless it was driven by a mammalian promoter (Hu, 2005). Based on the weight of evidence, there is no indication that baculoviral DNA or DNA fragments persist in or harm mammalian cell cultures.

168. For baculoviruses used as plant protection agents, it is extremely unlikely that a mammalian cell could even get in contact with a free BV. Also, ODV can hardly enter any other cells than insect midgut cells, hence it is questionable if the limited experimental outcome of such cell culture tests warrant the efforts of such assays for each baculovirus strain to be registered as pesticide. Some countries accept a universal baculovirus waiver rationale in lieu of cell culture studies.

5.4 Sensitisation/Allergenicity

169. In general, micro-organisms may provoke sensitisation reactions through dermal exposure and by inhalation. Early guidelines were developed by EPA in the 1970s and required Guinea Pig Maximization Test as well as inhalation test. In these tests, no allergenic reactions have been reported for registered baculoviruses (Burges, 1981).

170. Sensitisation studies belong to the data requirement for micro-organisms, including baculoviruses, for some OECD countries. It needs to be emphasised that no validated methods for testing sensitisation caused by micro-organisms are currently available, neither for dermal nor for inhalation sensitisation. On the other hand, no publications or occupational reports indicate that viruses (as well as bacteria and yeasts) are causing allergies (Martel et al., 2010, Hackl et al., 2015, OECD, 2022a). Although there is no evidence that baculoviruses active substances cause sensitisation (EFSA. 2015), a general warning phrase on the label (“Contains [Baculovirus XY Strain Z]. Micro-organisms may have the potential to provoke sensitising reactions”) is applied in the EU regarding the potential for sensitisation for microbials. Other OECD countries use a similar approach or do not use such warning phrases due to the lack of evidence of sensitisation. More important than testing the active substance for sensitisation might be to test the final product because this may contain larval residues from production or co-formulants (see Chapter 3.3).

5.5 Baculovirus Biotechnological and Biomedical Application

171. Baculoviruses have been coined as the most beneficial viruses to mankind (Miller, 1997) for the potential of their use as biocontrol agent (Chapter 4.1) as well as the possibility to use them as expression vector system and in human gene therapy. Smith et al. (1983) and Pennock et al. (1984) were the first who expressed foreign coding sequences inserted into a recombinant AcMNPV under the control of the *polh* promoter in the *Spodoptera frugiperda* cell line IPLB-SF-21. Subsequently, the baculovirus expression vector system (BEVS) was further developed and improved to satisfy the requirements of scientific interest and for use by commercial developers all over the world (Chambers et al., 2018). To date, applications of the BEVS fall into four groups: (i) recombinant proteins expression, (ii) baculovirus surface display using BV surface protein GP64 as antigen carriers, (iii) viral vectors for gene therapy, (iv) gene delivery vehicles for mammalian cells (Airenne et al., 2013; van Oers et al., 2015).

172. Recombinant proteins produced in the BEVS can be used for enzymatic analysis, crystallography, diagnostics, subunit vaccines and virus-like particles (VLPs), biosensors, and protein microarray. The most important utilization of these expressed proteins, particularly protein subunits and VLPs, is the production of veterinary and human vaccines used for disease prevention. Seven approved vaccine products, five veterinary vaccines for pigs and two human vaccines, have been produced on the basis of BEVS technology (Airenne et al., 2013; van Oers et al., 2015).

173. Baculovirus vectors can also be used to produce other viral vectors such as recombinant adeno-associated viruses (AAVs) that house therapeutic DNA. Such vectors have been approved by European authorization in 2012, becoming the first AAV-based gene therapy product (Haddley, 2013). More than twenty AAV-based gene therapy product candidates have been processed in clinical development (reviewed by Felberbaum, 2015). Since baculovirus cannot replicate when it enters into mammalian cells, these baculovirus-based vectors have been developed into gene delivery vehicles for mammalian cells (Trianti et al., 2018). The application of gene delivery vectors using BEVS has been reviewed by van Oers et al. (2015). From the extensive body of research using baculoviruses in gene therapy, low cytotoxicity in vertebrate cells even at very high virus load was noted (Airenne et al., 2009).

174. There are numerous publications noting that “baculoviruses” can enter mammalian cells but without replicating, integrating genomically or producing viral progeny. This principle is used in human gene therapy for mammalian cell transduction, though its efficiency is low (Mansouri and Berger, 2018; Ono et al., 2018; Amalfi et al., 2020). Several of such studies even report a non-specific immune response triggered by the viral vector which is commonly based on BV of AcMNPV. It has to be emphasized here that solely BV are used as vectors in these applications but not OB or ODV. The unspecific antiviral response caused by BV might be even strong enough to non-specifically protect animals from lethal doses of pathogenic homologous viruses (Gronowski et al, 1999; Abe et al., 2003; Molinari et al. 2010; Ono et al., 2014).

175. These findings may not be miss-interpreted that baculoviruses used as plant protection agents may be able to generate immune-stimulating effects. In the BEVS and for gene therapy the BV phenotype is used as vector for insect cell infection and transduction of mammalian cells, respectively. In this application, AcMNPV BV are injected at high doses into humans. For plant protection purposes, however, only OB are sprayed. The structural components of ODV released from the OB in the midgut of insect host are very different from BV (Braunagel et al., 2003; Hou et al., 2013). During the baculovirus life-cycle, BV occur only as intracellular or intralarval phenotype for spreading the infection within the host larvae, whereas OB are sprayed and eventually released from succumbing larvae. Thus, there is hardly any possibility of exposure of humans to BV in infected larvae, especially as BV are environmentally highly unstable. Furthermore, the major envelope fusion protein GP64 of AcMNPV is essential in mediating receptor binding and membrane fusion during BV entry into mammalian (as well as insect cells). GP64 is only encoded by group I alphabaculoviruses, such as AcMNPV, but not by group II alphabaculoviruses or other baculovirus genera (Rohrmann, 2019). Thus, the successful use of AcMNPV in human cell therapy

provides additional evidence that baculoviruses are not able to replicate or infect or are pathogenic to human cells.

Regulatory consideration and evaluation

176. Baculoviruses are obligate intracellular replicating microbials and do not produce toxins, toxic metabolites or degradation products. Baculoviruses are specific for defined target insects and do not infect (cells of) non-target insects or other organisms including humans and animals. No baculovirus-related adverse effects were observed in individuals involved in the research, development, manufacturing and formulation of baculovirus pest control products. There is no evidence of genotoxicity, carcinogenicity, teratogenicity or reproductive toxicity of baculoviruses.

177. In view of the established evidence of the safety of baculoviruses, tests for acute toxicity as well as cell culture tests appear to be not necessary anymore. Exposure estimates of operators, workers, residents and bystanders are not relevant. There is no evidence that baculoviruses cause allergies, therefore, it should be considered to dispense with precautionary sentences, as used in the EU.

6 Residues

178. Baculoviruses are typically sprayed at application rates of about 10^{10} OB/ha to 10^{13} OB/ha, depending on the virus-target insect system (Federici, 1999). Repeated sprays per growing season may be needed. Thus, the total application per season may reach up to 10^{14} OB/ha. At the same time, the sprayed OB are dramatically inactivated by UV radiation.

179. As noted in Chapters 5 to 9, there is no risk arising from exposure of baculoviruses to the environment, humans and other vertebrates or other non-target organisms. Toxins, toxic metabolites and degradation products are not produced. The application of baculoviruses is considered not relevant in terms of consumer health protection.

180. In its most recent QPS update of the family *Baculoviridae*, the BIOHAZ Panel of the EU confirmed that no new safety concern was identified and that therefore the current QPS status remained unchanged (EFSA, 2020). In the EU, baculoviruses plant protection products are exempted from restrictions regarding maximum residue levels and included in Annex IV of Regulation (EC) No 396/2005 (MRL). In the US, baculoviruses in plant protection products are typically exempted from the requirement of a tolerance for residues (EPA, 2014).

Regulatory consideration and evaluation

181. Neither analytical methods for residues of baculoviruses nor results of supervised residue trials are relevant. Since acute or chronic risks to consumers arising from exposure to baculoviruses do not exist, no assessment of the intake of baculoviruses by consumers is necessary.

7 Environmental Fate

182. As pointed out in Chapter 4.7.2, baculoviruses most likely employ vertical and horizontal transmission strategies to persist in insect host populations. Since baculoviruses are naturally occurring pathogens of insects and can cause epizootics in insect populations, they are considered to be ubiquitously present in the environment (see Chapter 4.7.3). By analyzing the occurrence of Trichoplusia ni nucleopolyhedrovirus on untreated cabbage after natural epizootics of the cabbage looper, Heimpel et al. (1973) estimated that the residue was about 7 million viable OB per square inch (approx. 6.5 cm^2) and concluded that exposure of consumers to OB arising from natural epizootics was much higher than that from field application of baculovirus treatments.

183. After baculovirus agents are sprayed on the surface of plants, virus OB will be either consumed by their target insect hosts starting an infection cycle, or will remain on the plant canopy or on the soil where they become inactivated by a set of environmental factors.

7.1 Persistence on Surface of Crop Plant/UV Inactivation

184. After baculovirus OB are sprayed and deposited on the surface of the crop plant, their persistence is mainly affected by the UV component of solar radiation, but also high temperature and plant exudates are reported to interfere with baculovirus activity.

Solar radiation

185. UV radiation between 290 and 400 nm, as a natural component of the sunlight, is the most destructive environmental factor affecting the field persistence of baculoviruses. UV inactivation of baculovirus OB has been extensively studied. In general, baculovirus OB can be drastically inactivated by sunlight in less than 24 hours, mean half-life times in the field are typically between 2 to 5 days (reviewed by Benz, 1987; Fuxa, 2004; Grzywacz, 2017; Ment et al., 2017). Average persistence time of purified OB of LdMNPV on oak, TnNPV on cabbage, HearNPV on cotton were typically between 0.4 to 1.5 days (half-life) (reviewed by Williams, 2018). In open field tomato plantations half-life activity of HearNPV was 2 to 4 days, whereas in greenhouse grown (or protected) tomato crops with ~55% reduced exposure to UV radiation the virus' half-life activity was extended to 7 to 8 days emphasizing the crucial role of UV inactivation (Arrizubieta et al., 2016). In these experiments, residual activity of HearNPV was about 13% in greenhouse and below 0.1% in open field plantations. Also, Jeyarani et al. (2013) reported residual activity of HearNPV on cotton plants of 49% after 3 days and 0% after 9 days when exposed to UV radiation.

186. Similarly, in field experiments SeMNPV lost 50% activity in <2 days, after 7 days residual activity of only 5% was left (Shapiro et al., 2012). However, experiments with Agrotis ipsilon multiple nucleopolyhedrovirus (AgipNPV) revealed residual activity to larvae of *Agrotis ipsilon* of more than 40% even after 28 days post application of 7×10^9 OB/m^2 onto the turfgrass (Prater et al., 2006). This field persistence might be related to shading effects and temporary deposits on the soil, which is the habitat of *A. ipsilon.* OB of AgMNPV sprayed on the upper surfaces of soybean leaves lost >60% activity within

2 days, but when sprayed on the underside of the foliage, loss of activity was less than 13% (Peng et al., 1999). For PhopGV, half-life times of 1.3 days were determined (Kroschel et al., 1996).

187. These findings support previous observations that baculovirus OB environmentally applied as biopesticides persist only for a short time in the environment. To achieve successful control of insect pests, multiple applications of baculovirus formulation may be needed during the period of pest occurrence. UV protective substances added in the formulation may delay but not prevent UV inactivation and OB can remain moderately effective for 8 to 14 days (Arthurs and Lacey, 2004; Shapiro et al., 2012).

Heat

188. Most studies on the direct impact of ambient temperature on the stability of baculoviruses outside of their hosts were conducted in the 1960s to 1980s (for detailed review see Benz, 1987). Whereas OB remain active frozen and at 4 °C for decades, their activity is gradually lost with increasing temperature. Exposure to temperatures beyond 40 °C can inactivate baculoviruses by the degradation of OB and a decrease of the biological efficacy of baculovirus products in the field (Fuxa, 2004).

Plant effects

189. The crop can have a certain influence on OB persistence on plant surfaces. The pH value on the leaf surface as well as plant exudates, e.g. peroxidase activity, organic acids, isoflavonoids, can limit the persistence of OB or interfere with baculovirus-midgut interaction; cotton, chickpea and amaranth have been reported as host plants with negative impact on baculovirus activity compared to others (reviewed by Williams, 2018; Stevenson et al., 2010; Lasa et al., 2018). Another factor influencing OB persistence is plant phenology and plant architecture which changes during the season. Depending on host plant to be protected, spray schemes need to be developed.

7.2 Persistence in Soil

190. Sprayed OB that have not been previously inactivated by exposure to UV solar radiation (Jones et al., 1993) or chemically inactivated by plant secondary chemicals (Hoover et al., 1998) are likely to persist in the soil for extended periods (Jaques, 1975; Thompson et al., 1981). The soil is thought to be the most important reservoir for OB, which can be transported to the soil compartment by rain wash-off or windblown dusts or sprays which do not hit the plant. Another route of OB from plant can be via infected cadavers which fall to earth or are washed off by rain (Williams, 2018).

191. It needs to be emphasised that the number of OB progeny deriving from infected larvae after spray application is likely to be much lower than the number originating from natural baculovirus epizootics. Outbreaks of natural epizootics typically start at high host density and with older larvae, whereas commercial baculoviruses are sprayed on early instars which are highly susceptible but produce much less OB progeny than the older ones. The importance of soil as a reservoir for baculoviruses was reviewed by Evans (1987) and later by Fuxa (2004). The persistence of baculovirus OB in soil is strongly influenced by moisture, pH, and temperature extremes but also by human activity such as tillage (Fuxa, 2004). Also soil type is important; OB bind readily to clay components and are then difficult to recover as shown for HearNPV (Christian et al., 2006). Besides soil type and pH, microbial activity also affects the persistence of baculovirus OB (OECD, 2022).

192. In a systematic study carried out in 1997 and 1998 (Fuxa et al., 2001), HearNPV was sprayed on 5 days at a total concentration of 2.5 x 10^{13} OB/ha, equaling 2.5 x 10^{4} OB/cm^2 on a mixed population of bollworm, *H. zea*, and tobacco budworm, *C. virescens*. Active OB were determined using bioassays and could be detected in soil depths down to 35 cm. The maximum mean concentrations of OB were found 5 weeks after the last application, with an estimate of 322 OB per gram soil and 30 OB per gram at 0-2 cm

depth and 26-38 cm depth, respectively, whereas 11 months after the last application mean numbers were down to 13 OB/g and zero OB/g, respectively. These finding suggested a clearance of OB in this system within one year. In the same field experiment, a complete lack of HearNPV on cotton plants or in host insects was also noted one year after the application. A different situation was noted for AgMNPV in soybean, where a single spray of *A. gemmatalis* with AgMNPV induced disease in target insects for 3-4 years (Fuxa and Richter, 1999). In conclusion of these quantitative experiments, it appears that only minimum amounts of sprayed baculovirus inoculum are deposited in soil and that field persistence of sprayed viruses appears to be strongly dependent on the cropping system, agricultural practice and the biology of the target host. In soil, baculovirus OB become rapidly adsorbed to soil particles and are not leached to deeper soil layers, as shown for OB from alphabaculoviruses and betabaculoviruses (Lopez-Pila, 1988).

7.3 Persistence in the Aquatic Compartment

193. Baculovirus OB do not dissolve in water and are rather stable in aqueous suspensions; stability is influenced by pH and salt concentration. From lysimeter studies, a good retention of baculoviruses by soils was concluded and this property was tentatively attributed to the particular protein envelope of virus particles consisting of polyhedrin and granulin, respectively (OECD, 2002). It was concluded that no risks of pollution of surface or groundwater is expected, due to the high level of retention of the OB by the ground. If baculovirus OB become introduced into a water body, they are inactivated by UV radiation in surface water or tend to deposit and are absorbed by sediments, where they will be mineralized by microbial activity in water and sediment.

7.4 Persistence in Air

194. Sprayed baculovirus OB are suspended solid particles of >0.5 µm diameter that are non-volatile. Therefore, a distribution of baculoviruses, except by drifting by the wind, can be excluded.

Regulatory consideration and evaluation

195. There are ample studies and confirmations from literature that baculoviruses have a restricted field persistence due to inactivation by solar radiation. Sun light is considered the most important factor contributing to a loss in efficacy by time.

196. Baculovirus OB may persist in soil for long periods. Soil can be a potential OB reservoir, inactivation rate not only depends on soil type and pH, but also on microbial activity (OECD, 2002). In soil, baculovirus OB rapidly adsorb to soil particles and are not leached to deeper soil layers. This characteristic is not baculovirus strain/isolate specific, but is due to the baculovirus OB particles. If not inactivated, baculovirus OB may persist in the upper soil and remain accessible to further host generations, leading to a sustainable effect on the host insects.

197. Baculovirus OB or virion particles are not soluble and do not dissolve in water. There are no risks of pollution of surface or groundwater expected, due to the high level of retention of the viral particles by the soil. Again, this is a general characteristic of all members of the *Baculoviridae* family not of a specific strain/isolate.

198. In summary, baculoviruses based active substances are considered to be safe due to lack of toxicity, pathogenicity and infectivity to mammals and other non-target organisms. Therefore, their fate and behaviour are not relevant to environmental compartments considered.

8 Effects on Non-Target Organisms

199. Baculoviruses are specific for insects from three orders and have been exclusively isolated from lepidopteran species (members of the genera *Alphabaculovirus* and *Betabaculovirus*) as well as a limited number of Hymenopterans (*Gammabaculovirus*) and Dipterans (*Deltabaculovirus*). Baculoviruses neither infect non-insect animals, nor any other organisms. Furthermore, they do not have any phylogenetic relationship with viruses being infective for non-arthropods (see Chapter 4.4).

200. Extensive published infection experiments with numerous baculoviruses administered to birds, aquatic organisms including fish, freshwater invertebrates and algae, plants, bees, parasitoids and predatory mites as well as earthworms were carried out from early 1970s until 1980s and did not provide any evidence that any of these organisms could get harmed by the application of baculovirus-based plant protection products (Burges, 1980; Gröner, 1986).

8.1 Birds

201. Regarding effects on birds non-target laboratory studies on viral toxicity and pathogenicity of different NPVs from the genera *Alphabaculovirus* and *Gammabaculovirus* with chicken, turkey, pheasant, dove, duck, sparrow and quail did not reveal any adverse effect (reviewed by Gröner, 1986). No published literature is available for GVs (genus *Betabaculovirus*) but considering their close genetic and monophyletic relationship with NPVs of the genus *Alphabaculovirus* as well as their even higher host specificity there is no reason to assume they could cause any harm to birds.

8.2 Aquatic Organisms

202. No adverse effect was noted for OB of the genus *Alphabaculovirus* and *Gammabaculovirus* exposed to aquatic organisms including rainbow trout, brown trout, steelhead trout, Chinook salmon, white sucker, black bullhead, killifish, sheepshead minnow and goldfish, as well as Panaeid shrimp, brown shrimp, grass shrimp, oyster (reviewed by Gröner, 1986).

203. In a more recent study, trout fingerlings were fed with OB of either LdMNPV or CfMNPV at a total dose of 1.4×10^7 OB/per fish (Kreutzweiser et al., 1997). The authors considered the study protocol as representing worst case dose rates based on the effective regulatory guidelines. After 21 days experimental period, there were no significant differences in feeding rates or growth and no adverse effects on fish behaviour and survival when treated and control fish were compared. When the internal organs of all fish were examined, no signs of lesions, discoloration, swelling, haemorrhaging, or other aberrations were noted. Also, there was no indication of NPV infection in the stomach and intestinal tract tissues of treated fish by using DNA hybridization techniques.

204. In another study by Ashour et al. (2007), a dose of 1×10^9 OB of either AcMNPV, a genetically engineered AcMNPV (AcAaIT) expressing a scorpion neurotoxin, and SpliNPV was administered with their standard diet to groups of 10 Nile Tilapia for two days. No mortality was induced in treated and untreated fish during the experimental period of 28 days after initial treatment. After 30 days, phagocytic activity of

macrophages prepared from anterior part of the kidney was determined. Though macrophage phagocytic activity seemed to be lower in virus-treated groups after 30 minutes of the assay, the experiment suffered from high variability, and no statistical analysis were given. Over the subsequent time period phagocytic activity increased in all samples and after 180 minutes hardly any difference between the samples was noted.

205. Tests with freshwater invertebrates, such as shrimp and the water flea (*Daphnia*), were reported in the literature for members of the genera *Alphabaculovirus* (AcMNPV, HzSNPV, LdMNPV) and *Gammabaculovirus* (NeleNPV), without any adverse effect (Gröner, 1986). In safety studies with *Daphnia*, the green algae *Selenastrum capricornutum* and the duckweed *Lemna valdiviana*, as a result of exposure to 1000 times the maximum calculated pesticide concentration of an AgMNPV formulation (Jonsson et al., 1995). No other recent public literature is available.

206. In addition, numerous unpublished studies provided to regulatory authorities during the registration process of many baculoviruses to be used as plant protection products in many OECD countries never revealed evidence that these viruses are toxic, pathogenic or infective risk to aquatic organisms. It can be concluded that there are no effects to fish, aquatic Crustaceae or algae.

207. There is no evidence for any risk of baculoviruses to aquatic or terrestrial plants. As stated in OECD (2002), no member of the *Baculoviridae* family is infective for plants, it is therefore highly unlikely that baculoviruses would be toxic or pathogenic for plants.

8.3 Effects on Bees

208. Since many baculoviruses were intended to be used in the open field on crops as well as on fruit trees and forests, the safety of pollinators and other beneficial insects, such as parasitoids and predators was always a major concern. Early studies were reviewed by Gröner (1986). Studies reported from at least 15 NPVs and five GVs exposed or fed to honey bee (*Apis mellifera*), but also tests with bumble bee (*Bombus terrestris*) and the alfalfa leaf cutting bee (*Megachile rotundata*) were stated (Table 2, modified from Erler et al., 2022). These tests were done with either baculovirus OB or formulated products. None of these studies showed an adverse effect on bees, in terms of direct mortality, larval development, foraging activity or colony health (egg production, brood rearing, drone production, bee mortality) and it was concluded that baculoviruses are safe to bees (Erler et al., 2022).

8.4 Safety to other Terrestrial Invertebrates other than Bees

Predators and Parasitoids

209. The host range of baculoviruses used for pest control is restricted to species from the genera Lepidoptera (members of the genera *Alphabaculovirus* and *Betabaculovirus*) and to Hymenoptera (*Gammabaculovirus*). No deleterious effects on beneficial insects resulting from baculovirus treatment were found, though there may be the indirect effect that a larval parasitoid cannot complete its development when the host individual succumbs because of viral infection (Gröner, 1986). But these are indirect effects depending on density of the shared host individual of the baculovirus and the parasitoid. The occurrence of such indirect effects depends on the time-point when a larval individual is parasitized and when it is infected by the virus. When larvae were concurrently infected and parasitized, survival of the parasitoid was hampered because of virus induced larval death. With increasing time between parasitisation and subsequent baculovirus infection, there seems to be only a small window of interference (Escrabino et al., 2000, 2001; Mathews et al., 2004).

210. Evidence of lack of any detrimental effect on parasitoids and predators derive from laboratory and field experiments (reviewed by Abbas, 2020). Numerous predator species, such as *Coccinella undecimpunctata, Chrysoperla carnea, Orius albidipennis, Labiduria riparia* and others, reared on NPV-infected prey did not show deleterious effects (Ruberson et al., 1991; Fuxa et al., 1993; Heinz et al., 1995; Li et al., 1999; Abbas, 1987), though De Nardo et al. (2001) proposed some effect of inert components in the formulation, when the predator *Podisus nigrispinus* was reared for several generations on *Anticarsia gemmatalis* treated with commercial AgMNPV products. Laboratory experiments testing whether parasitoids could transmit baculoviruses are ambiguous and might depend on the experimental design and the host-virus-parasitoid system (Abbas, 2020).

211. On the other hand, the safety of baculoviruses to beneficials is used in integrated control strategies when applications of baculoviruses and parasitoid or predator action are combined to control an insect pest. Additive effects of combined use of HearNPV and the parasitoid *Trichogramma pretiosum* to control *H. armigera* larvae were reported by Balasubramanian et al. (1989).

212. Gröner (1990) concluded from the natural occurrence of baculoviruses that beneficial insects have always had contact with these natural regulatory agents. Deleterious effects of baculoviruses to predators and adult parasitoids have never been reported. It can be concluded that baculoviruses are safe to beneficials.

Earthworms

213. Sprayed baculovirus plant protection products as well as OB released from infected insect cadavers ultimately arrive at the soil surface, where OB can persist. Studies on effects on earthworms were part of safety guidelines since 1980 (Burges et al., 1980) and have been performed as a regular required data point during the registration process of many baculoviruses. No detrimental effects were ever reported with earthworms.

214. In fact, earthworms may contribute to a vertical distribution of OB populations in soil habitats as reported by Infante-Rodirguez et al. (2016) from microcosm experiments with SfMNPV. Since earthworm intestine is slightly acidic, OB do not dissolve in the intestinal tract, and it was proposed by the authors that earthworms could mediate dispersal of OB in the soil habitat.

Table 2: Observation of effects on honey bee, bumble bee and leafcutter bee after application of baculoviruses purified OB or as commercial products (Modified from Erler et al., 2022). Given are the tested viruses and bee species, the assay type, application and administration form, test duration, age of test animals, observed effect and country where the study was performed.

Microbial organism	Bee species **	Assay	Application	Administration	Concentration	Duration (days)	Age	Effect (on treated bees)	Country ***	References
Alphabaculovirus* and *Gammabaculovirus										
AcMNPV	AM	cage, colony	food	sucrose solution	$7.5 \times 10^{4-5}$ OB/g	60	newly emerged	minimum or no effect on longevity, behaviour, brood production	US	Morton et al. 1975
AcMNPV (wildtype and recombinant)	AM	cage	injection	culture medium	5×10^{4} BV/bee	9	newly emerged	no effect on bees	US	Heinz et al. 1995
AcMNPV, AgMNPV, CfMNPV, HvSNPV*, HzSNPV, OpSNPV, TnNPV, PsseNPV*, MbMNPV, SfMNPV, TnSNPV, TnMNPV, NeseNPV	AM (Africanized and Italian strain), ACI	cage, observation hive, colony	food, spray, contact, inhalation, in-forest spray	water, sucrose, honey solution	5×10^{7} - 10^{9} OB/mL or g; 10^{3-8} OB/bee; 1.3 to 10×10^{9} OB/colony; 5.5 to 247.5×10^{9} OB/ha	3, 10-22	newly emerged, young adults, forager, winter bees	no effect on mortality, bees, foraging activity, colony health (egg production, brood rearing, bee mortality), no infected bees	BR, CA, DE, IN, UK, US	Alves et al. 1996; Buckner et al. 1975; Cantwell & Lehnert 1979; Cantwell et al. 1966; Dhaduti & Mathad 1980; Doyle et al. 1990; Gröner et al. 1978; Kingsbury et al. 1978; Knox 1970
LdMNPV, NeleNPV	MR	cage	food	sucrose solution, pollen, nectar provision	1.2×10^{5} OB/bee; 10^{4-6} OB/bee	7	young larvae, newly emerged males	no effect on bees, larval development, no infected bees	CA	Barber et al. 1993; Goerzen et al. 1990
Betabaculovirus										
ArveGV*, CpGV, DisaGV, EsacGV	AM, AM (Africanized)	cage, observation hive	food, spray, contact, inhalation	sucrose, honey solution	5×10^{6} OB/bee; 10^{8} OB/mL; 10^{10-12} OB/mL or g; 10 and 50×10^{9} OB/colony	0, 3, 10	-, 3-13 day, forager, winter bees	no effect on mortality, bees, no infected bees	BR, DE, US	Alves et al. 1996; Cantwell et al. 1966; Gröner et al. 1978; Knox 1970
AdorGV, CpGV	BT	microcolony, colony	topical, food	pure product, pollen, sprayed pollen, water, sugar solution	6.6×10^{12} OB/L; 5 to 6.6×10^{13} OB/L	0, 77	-, newly emerged	no effect on mortality, drone production, reproduction	BE	Mommaerts et al. 2009; Sterk et al. 2002

* ArveGV = Argyrotaenia velutenana granulovirus; EsacGV = Estigmene acrea granulovirus; HvSNPV = Heliothis virescens single nucleopolyhedrovirus; PsseNPV = Pseudaletia separata nucleopolyhedrovirus;
** AM = *Apis mellifera*; ACI = *Apis cerana indica*; MR = *Megachile rotundata*; BT = *Bombus terrestris*
*** Two-letter country code: BE = Belgium; BR = Brazil; CA = Canada; DE = Germany; IN = India; UK = United Kingdom; US = United States of America;

Regulatory consideration and evaluation

215. Baculoviruses are highly specific to insect species. A given baculovirus may have an effect on only a very few host insect species. Baculovirus-based active substances are considered to be safe to non-target organisms due to lack of toxicity, pathogenicity and infectivity to birds, aquatic organism, bees and other invertebrates. Therefore, birds, aquatic organism, bees and other invertebrates are not at risk and non-target studies should not be regarded as necessary for baculovirus active substances.

9 Conclusions for Safety Assessment

216. Based on a tremendous body of research, the following key characteristics of baculoviruses have been considered in evaluating the potential risks on human health and environment posed by baculoviruses as well as criteria for facilitating registration of baculovirus biocontrol agents (EPA, 1978, 1996; OECD, 2002; European Commission, 2008; Mudgal et al., 2013;):

1. Baculoviruses are highly host-specific, with their host range limited to one or a few species of the same genus. Larger host ranges covering different genera or even different host families are very rare. Baculoviruses represent one of the most specific insecticidal agents. The vast majority of baculovirus products is registered for control of a single or very few closely related insect pest species (Appendix Table A2) restricting the use of a product to only a few applications.
2. Baculoviruses only infect insects belonging to the orders Lepidoptera, Diptera, and Hymenoptera.
3. Baculoviruses are not infective for mammals and replication does not occur in mammalian cells.
4. No pathogenic, genotoxic, mutagenic, or carcinogenic effects of baculoviruses were ever observed in mammals.
5. Baculoviruses do not produce any metabolites or toxins. They have no metabolism independent from that of the host cell and cannot reproduce outside the host.
6. Effects on non-target species can be widely excluded, especially for vertebrates, micro-organisms, and plants.

217. Based on a tremendous body of research and as supported by the present document, baculoviruses have been the only micro-organism taxon to be included on the species level in Annex I of the EU Directive 91/414/EEC in the registration process of the European Union (European Commission, 2008). This guidance is also followed under the current EU regulation EC 1107/2009 concerning the placing of plant protection products on the market. Related to this regulation, baculoviruses have been recognized as a candidate for microbial low-risk active substance on the family level unless at strain/isolate level they have demonstrated adverse effects on non-target insects (EU, 2017, 2018, 2022). The evaluation and the risk assessment are conducted for a strain/isolate of a baculovirus species, and other isolates can be approved as active substances with a reduced data set and evaluation procedure.

218. Based on the weight of evidence, deriving from (1) the narrow host range, which is - for commercially used baculoviruses of the genera *Alphabaculovirus*, *Betabaculovirus* or *Gammabaculovirus* - restricted to single or few insect species of the same insect genus, or members of a single or few families of either Lepidoptera or Hymenoptera (Chapter 4.2), (2) the narrow genetic relationship to each other as expressed by the shared conserved core genes involved in peroral infection, DNA transcription and replication, as well as the highly conserved structural proteins (Chapter 4.3), (3) the unique OB morphology confining the ODV from the environment as well as the ODV´s insect midgut specificity, (4) their safety record from other biotechnological applications (Chapter 4.3), (5) the uniform and invariable safety record of all baculoviruses used for plant protection (Chapter 4.1), and (6) their long history of safe use for more than 50 years without any report of adverse human, domestic animal, or environmental incidents (Chapters 5,6,7,8) it appears that their safety to humans and the environment, as well as their residual fate is not strain-specific but refers to the whole family of *Baculoviridae*. Specificity for certain host species appears

to be baculovirus species-specific, whereas virulence differences towards their host species are strain-specific.

219. Given that all published reviews unequivocally state that baculoviruses are safe and support their use as low-risk biological control agents for the control of insect pests, it is proposed that human and environmental toxicity tests of baculoviruses as well as studies related to their residual fate should no longer be required at strain level for the registration of baculoviruses.

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Appendix

Table A1. A total of 84 baculoviruses have been assigned to species (ICTV 2020*).

Species Name (until 2023)	Species name*** (from 2023)	Virus name	Virus Abbrev.	Isolate**	Accession number	RefSeq number	Reference
Genus: *Alphabaculovirus*							
Adoxophyes honmai nucleopolyhedrovirus	*Alphabaculovirus adhonmai*	Adoxophyes honmai nucleopolyhedrovirus	AdhoNPV	ADN001	AP006270	NC_004690	Nakai et al., 2003
Agrotis ipsilon multiple nucleopolyhedrovirus	*Alphabaculovirus agipsilonis*	Agrotis ipsilon multiple nucleopolyhedrovirus	AgipMNPV	Illinois	EU839994	NC_011345	Harrison, 2009
Agrotis segetum nucleopolyhedrovirus A	*Alphabaculovirus agsegetum*	Agrotis segetum nucleopolyhedrovirus A	AgseNPV-A	Polish	DQ123841	NC_007921	Jakubowska et al., 2006
Agrotis segetum nucleopolyhedrovirus B	*Alphabaculovirus alteragsegetum*	Agrotis segetum nucleopolyhedrovirus B	AgseNPV-B	English	KM102981	NC_025960	Wennmann et al., 2015
Antheraea pernyi nucleopolyhedrovirus	*Alphabaculovirus anpernyi*	Antheraea pernyi nucleopolyhedrovirus	AnpeNPV	Liaoning	DQ486030	NC_008035	Nie et al., 2007
Anticarsia gemmatalis multiple nucleopolyhedrovirus	*Alphabaculovirus angemmatalis*	Anticarsia gemmatalis multiple nucleopolyhedrovirus	AgMNPV	2D	DQ813662	NC_008520	Oliveira et al., 2006
Autographa californica multiple nucleopolyhedrovirus	*Alphabaculovirus aucalifornicae*	Autographa californica multiple nucleopolyhedrovirus	AcMNPV	C6	L22858	NC_001623	Ayres et al., 1994

		Galleria mellonella multiple nucleopolyhedrovirus	GmMNPV				
		Plutella xylostella multiple nucleopolyhedrovirus	PlxyNPV				
Bombyx mori nucleopolyhedrovirus	*Alphabaculovirus bomori*	Bombyx mori nucleopolyhedrovirus	BmNPV	T3	L33180	NC_001962	Gomi et al., 1999
Buzura suppressaria nucleopolyhedrovirus	*Alphabaculovirus busuppressariae*	Buzura suppressaria nucleopolyhedrovirus	BuzuNPV	Hubei	KF611977	NC_023442	Zhu et al., 2014
Catopsilia pomona nucleopolyhedrovirus	*Alphabaculovirus capomonae*	Catopsilia pomona nucleopolyhedrovirus	CapoNPV	416	KU565883	NC_030240	Wang et al., 2016a
Choristoneura fumiferana DEF multiple nucleopolyhedrovirus	*Alphabaculovirus alterchofumiferanae*	Choristoneura fumiferana DEF multiple nucleopolyhedrovirus	CfDEFMNPV		AY327402	NC_005137	Lauzon et al., 2005
Choristoneura fumiferana multiple nucleopolyhedrovirus	*Alphabaculovirus chofumiferanae*	Choristoneura fumiferana multiple nucleopolyhedrovirus	CfMNPV	Ireland	AF512031	NC_004778	de Jong et al., 2005
Choristoneura murinana nucleopolyhedrovirus	*Alphabaculovirus chomurinanae*	Choristoneura murinana nucleopolyhedrovirus	ChmuNPV	Darmstadt	KF894742	NC_023177	Rohrmann et al., 2014
Choristoneura rosaceana nucleopolyhedrovirus	*Alphabaculovirus chorosaceanae*	Choristoneura rosaceana nucleopolyhedrovirus	ChroNPV	NB_1	KC961304	NC_021924	Thumbi et al., 2013
Chrysodeixis chalcites nucleopolyhedrovirus	*Alphabaculovirus chrychalcites*	Chrysodeixis chalcites nucleopolyhedrovirus	ChchNPV		AY864330	NC_007151	van Oers et al., 2005
Chrysodeixis includens nucleopolyhedrovirus	*Alphabaculovirus chrincludentis*	Pseudoplusia includens single nucleopolyhedrovirus	PsinSNPV	IE	KJ631622	NC_026268	Craveiro et al., 2015
Clanis bilineata nucleopolyhedrovirus	*Alphabaculovirus clabilineatae*	Clanis bilineata nucleopolyhedrovirus	ClbiNPV	DZ1	DQ504428	NC_008293	Zhu et al., 2009
Ectropis obliqua nucleopolyhedrovirus	*Alphabaculovirus ecobliquae*	Ectropis obliqua nucleopolyhedrovirus	EcobNPV	A1	DQ837165	NC_008586	Ma et al., 2007
Epiphyas postvittana nucleopolyhedrovirus	*Alphabaculovirus eppostvittanae*	Epiphyas postvittana nucleopolyhedrovirus	EppoNPV		AY043265	NC_003083	Hyink et al., 2002

Euproctis pseudoconspersa nucleopolyhedrovirus	*Alphabaculovirus eupseudoconspersae*	Euproctis pseudoconspersa nucleopolyhedrovirus	EupsNPV	Hangzhou	FJ227128	NC_012639	Tang et al., 2009
Helicoverpa armigera nucleopolyhedrovirus	*Alphabaculovirus helarmigerae*	Helicoverpa armigera nucleopolyhedrovirus	HearNPV	G4	AF271059	NC_002654	Wang et al., 2001
		Heliothis zea single nucleopolyhedrovirus	HzSNPV	F16	AF334030	-	Chen et al., 2001
Hemileuca species nucleopolyhedrovirus	*Alphabaculovirus heleucae*	Hemileuca sp. nucleopolyhedrovirus	HespNPV	MEM	KF158713	NC_021923	Rohrmann et al., 2013
Hyphantria cunea nucleopolyhedrovirus	*Alphabaculovirus hycuneae*	Hyphantria cunea nucleopolyhedrovirus	HycuPV	N9	AP009046	NC_007767	Ikeda et al., 2006
Hyposidra talaca nucleopolyhedrovirus	*Alphabaculovirus hytalacae*	Hyposidra talaca nucleopolyhedrovirus	HytaNPV	India001	MH261376	NC_055453	Nguyen et al., 2018
Lambdina fiscellaria nucleopolyhedrovirus	*Alphabaculovirus lafiscellariae*	Lambdina fiscellaria nucleopolyhedrovirus	LafiNPV	GR15	KP752043	NC_026922	Rohrmann et al., 2015b
Leucania separata nucleopolyhedrovirus	*Alphabaculovirus leseparatae*	Leucania separata nucleopolyhedrovirus	LeseNPV	AH1	AY394490	NC_008348	Xiao and Qi, 2007
Lonomia obliqua nucleopolyhedrovirus	*Alphabaculovirus lonobliquae*	Lonomia obliqua multiple nucleopolyhedrovirus	LoobNPV	SP/2000	KP763670	NC_043520	Aragão-Silva et al., 2016
Lymantria dispar multiple nucleopolyhedrovirus	*Alphabaculovirus lydisparis*	Lymantria dispar multiple nucleopolyhedrovirus	LdMNPV	43621	AF081810	NC_001973	Kuzio et al., 1999
Lymantria xylina nucleopolyhedrovirus	*Alphabaculovirus lyxylinae*	Lymantria xylina multiple nucleopolyhedrovirus	LyxyNPV	5	GQ202541	NC_013953	Nai et al., 2010
Mamestra brassicae multiple nucleopolyhedrovirus	*Alphabaculovirus mabrassicae*	Mamestra brassicae multiple nucleopolyhedrovirus	MbMNPV	K1	JQ798165	NC_023681	Choi et al., 2013
Mamestra configurata nucleopolyhedrovirus A	*Alphabaculovirus maconfiguratae*	Mamestra configurata nucleopolyhedrovirus A	MacoNPV-A	90/2	U59461	NC_003529	Li et al., 2002b
Mamestra configurata nucleopolyhedrovirus B	*Alphabaculovirus altermaconfiguratae*	Mamestra configurata nucleopolyhedrovirus B	MacoNPV-B	96B	AY126275	NC_004117	Li et al., 2002a
Maruca vitrata nucleopolyhedrovirus	*Alphabaculovirus mavitratae*	Maruca vitrata nucleopolyhedrovirus	MaviNPV	MV-8	EF125867	NC_008725	Chen et al., 2008

Mythimna unipuncta nucleopolyhedrovirus A	*Alphabaculovirus myunipunctae*	Mythimna unipuncta nucleopolyhedrovirus A	MyunNPV-A	#7	MF375894	NC_043530	Harrison et al., 2018b
Mythimna unipuncta nucleopolyhedrovirus B	*Alphabaculovirus altermyunipunctae*	Mythimna unipuncta nucleopolyhedrovirus B	MyunNPV-B	KY310	MH124167	-	Harrison et al. 2019
Operophtera brumata nucleopolyhedrovirus	*Alphabaculovirus opbrumatae*	Operophtera brumata nucleopolyhedrovirus	OpbuNPV	MA	MF614691	NC_040621	Harrison et al., 2017b
Orgyia leucostigma nucleopolyhedrovirus	*Alphabaculovirus orleucostigmae*	Orgyia leucostigma nucleopolyhedrovirus	OrleNPV	CFS-77	EU309041	NC_010276	Thumbi et al., 2011
Orgyia pseudotsugata multiple nucleopolyhedrovirus	*Alphabaculovirus orpseudotsugatae*	Orgyia pseudotsugata multiple nucleopolyhedrovirus	OpMNPV		U75930	NC_001875	Ahrens et al., 1997
Oxyplax ochracea nucleopolyhedrovirus	*Alphabaculovirus oxochraceae*	Oxyplax ochracea nucleopolyhedrovirus	OxocNPV	435	MF143631	NC_043529	Wang et al., 2018
Peridroma saucia nucleopolyhedrovirus	*Alphabaculovirus pesauciae*	Peridroma species nucleopolyhedrovirus	PespNPV	GR167	KM009991	NC_024625	Rohrmann et al., 2015a
Perigonia lusca nucleopolyhedrovirus	*Alphabaculovirus peluscae*	Perigonia lusca single nucleopolyhedrovirus	PeluSNPV		KM596836	NC_027923	Ardisson-Araújo et al., 2016a
Spodoptera eridania nucleopolyhedrovirus	*Alphabaculovirus speridaniae*	Spodoptera eridania nucleopolyhedrovirus	SperNPV	251	MH320559	-	Harrison & Rowley, 201)
Spodoptera exempta nucleopolyhedrovirus	*Alphabaculovirus spexemptae*	Spodoptera exempta nucleopolyhedrovirus	SpexNPV	244.1	MH717816	NC_055455	Escasa et al., 2019
Spodoptera exigua multiple nucleopolyhedrovirus	*Alphabaculovirus spexiguae*	Spodoptera exigua multiple nucleopolyhedrovirus	SeMNPV	US1	AF169823	NC_002169	IJkel et al., 1999
Spodoptera frugiperda multiple nucleopolyhedrovirus	*Alphabaculovirus spofrugiperdae*	Spodoptera frugiperda multiple nucleopolyhedrovirus	SfMNPV	3AP2	EF035042	NC_009011	Harrison et al., 2008
Spodoptera littoralis nucleopolyhedrovirus	*Alphabaculovirus splittoralis*	Spodoptera littoralis nucleopolyhedrovirus	SpliNPV	AN1956	JX454574	NC_038369	Breitenbach et al., 2013
Spodoptera litura nucleopolyhedrovirus	*Alphabaculovirus spliturae*	Spodoptera litura nucleopolyhedrovirus	SpltNPV	G2	AF325155	NC_003102	Pang et al., 2001
Sucra jujuba nucleopolyhedrovirus	*Alphabaculovirus sujujubae*	Sucra jujuba nucleopolyhedrovirus	SujuNPV	473	KJ676450	NC_028636	Liu et al., 2014

Thysanoplusia orichalcea nucleopolyhedrovirus	*Alphabaculovirus thorichlaceae*	Thysanoplusia orichalcea nucleopolyhedrovirus	ThorNPV	p2	JX467702	NC_019945	Wang et al., 2012
Trichoplusia ni single nucleopolyhedrovirus	*Alphabaculovirus trini*	Trichoplusia ni single nucleopolyhedrovirus	TnSNPV		DQ017380	NC_007383	Willis et al., 2005
Urbanus proteus nucleopolyhedrovirus	*Alphabaculovirus urprotei*	Urbanus proteus nucleopolyhedrovirus	UrprNPV		KR011717	NC_029997	Santos et al., 2018
Wiseana signata nucleopolyhedrovirus	*Alphabaculovirus wisignatae*	Wiseana signata nucleopolyhedrovirus	WisiNPV		AF016916	NC_038370	Sadler et al., 1998
Genus: *Betabaculovirus*							
Adoxophyes orana granulovirus	*Betabaculovirus adoranae*	Adoxophyes orana granulovirus	AdorGV	English	AF547984	NC_005038	Wormleaton et al., 2003
Agrotis segetum granulovirus	*Betabaculovirus agsegetum*	Agrotis segetum granulovirus	AgseGV	DA	KR584663	NC_039213	
Artogeia rapae granulovirus	*Betabaculovirus arrapae*	Pieris rapae granulovirus	PiraGV	Wuhan	GQ884143	NC_013797	Zhang et al., 2012
Choristoneura fumiferana granulovirus	*Betabaculovirus chofumiferanae*	Choristoneura occidentalis granulovirus	ChocGV		DQ333351	NC_008168	Escasa et al., 2006
Clostera anachoreta granulovirus	*Betabaculovirus clanachoretae*	Clostera anachoreta granulovirus	ClanGV	HBHN	HQ116624	NC_015398	Liang et al., 2011
Clostera anastomosis granulovirus A	*Betabaculovirus clanastomosis*	Clostera anastomosis granulovirus A	ClasGV-A	Henan	KC179784	NC_022646	Liang et al., 2013
Clostera anastomosis granulovirus B	*Betabaculovirus alterclanastomosis*	Clostera anastomosis granulovirus B	ClasGV-B		KR091910	NC_038371	Yin et al., 2015
Cnaphalocrocis medinalis granulovirus	*Betabaculovirus cnamedinalis*	Cnaphalocrocis medinalis granulovirus	CnmeGV	Enping	KU593505	NC_029304	Zhang et al., 2015a
Cryptophlebia leucotreta granulovirus	*Betabaculovirus cryleucotretae*	Cryptophlebia leucotreta granulovirus	CrleGV	CV3	AY229987	NC_005068	Lange and Jehle, 2003
Cydia pomonella granulovirus	*Betabaculovirus cypomonellae*	Cydia pomonella granulovirus	CpGV	Mexican 1	U53466	NC_002816	Luque et al., 2001
Diatraea saccharalis granulovirus	*Betabaculovirus disaccharalis*	Diatraea saccharalis granulovirus	DisaGV	Parana-2009	KP296186	NC_028491	Ardisson-Araújo et al., 2016b
Epinotia aporema granulovirus	*Betabaculovirus epaporemae*	Epinotia aporema granulovirus	EpapGV		JN408834	NC_018875	Ferrelli et al., 2012

Erinnyis ello granulovirus	*Betabaculovirus erellonis*	Erinnyis ello granulovirus	ErelGV	S86	KJ406702	NC_025257	Ardisson-Araújo et al., 2014
Harrisina brillians granulovirus	*Betabaculovirus habrilliantis*	Harrisina brillians granulovirus	HabrGV	M2	AF142425	NC_038372	Bideshi et al., 2000
Helicoverpa armigera granulovirus	*Betabaculovirus helarmigerae*	Helicoverpa armigera granulovirus	HearGV		EU255577	NC_010240	Harrison and Popham, 2008
Lacanobia oleracea granulovirus	*Betabaculovirus lacoleraceae*	Lacanobia oleracea granulovirus	LaolGV	Scottish-S1	Y08294	NC_038868	
Mocis latipes granulovirus	*Betabaculovirus molatipedis*	Mocis latipes granulovirus	MolaGV	Southern Brazil	KR011718	NC_029996	Ardisson-Araújo et al., 2018
Mythimna unipuncta granulovirus A	*Betabaculovirus myunipunctae*	Pseudaletia unipuncta granulovirus	PsunGV	Hawaiian	EU678671	NC_013772	Harrison et al., 2017a
Mythimna unipuncta granulovirus B	*Betabaculovirus altermyunipunctae*	Mythimna unipuncta granulovirus	MyunGV	8	KX855660	NC_033780	Harrison et al., 2017a
Phthorimaea operculella granulovirus	*Betabaculovirus phoperculellae*	Phthorimaea operculella granulovirus	PhopGV	T	AF499596	NC_004062	Espinel-Correal et al., 2010
Plodia interpunctella granulovirus	*Betabaculovirus plinterpunctellae*	Plodia interpunctella granulovirus	PiGV	Cambridge	KX151395	NC_032255	Harrison et al., 2016a
Plutella xylostella granulovirus	*Betabaculovirus pluxylostellae*	Plutella xylostella granulovirus	PlxyGV	K1	AF270937	NC_002593	Hashimoto et al., 2000
Spodoptera frugiperda granulovirus	*Betabaculovirus spofrugiperdae*	Spodoptera frugiperda granulovirus	SpfrGV	VG008	KM371112	NC_026511	Cuartas et al., 2015
Spodoptera litura granulovirus	*Betabaculovirus spliturae*	Spodoptera litura granulovirus	SpltGV	K1	DQ288858	NC_009503	Wang et al., 2008
Trichoplusia ni granulovirus	*Betabaculovirus trini*	Trichoplusia ni granulovirus	TnGV	LBIV-12	KU752557	NC038375	de los Ángeles Bivian-Hernández et al., 2017
Xestia c-nigrum granulovirus	*Betabaculovirus xecnigri*	Xestia c-nigrum granulovirus	XecnGV	alpha4	AF162221	NC_002331	Hayakawa et al., 1999
Genus: *Gammabaculovirus*							
Neodiprion lecontei nucleopolyhedrovirus	*Gammabaculovirus nelecontei*	Neodiprion lecontei nucleopolyhedrovirus	NeleNPV		AY349019	NC_005906	Lauzon et al., 2004
Neodiprion sertifer nucleopolyhedrovirus	*Gammabaculovirus nesertiferis*	Neodiprion sertifer nucleopolyhedrovirus	NeseNPV		AY430810	NC_005905	Garcia-Maruniak et al., 2004

Genus: ***Deltabaculovirus***							
Culex nigripalpus nucleopolyhedrovirus	*Deltabaculovirus cunigripalpi*	Culex nigripalpus nucleopolyhedrovirus	CuniNPV	Florida1997	AF403738	NC_003084	Afonso et al., 2001

*****Species updates can be found on https://ictv.global/msl; ** refers to isolate used in genome sequencing; *** van Oers et al. (2023)

Table A2. Examples of baculovirus biopesticides that have been registered in different countries all over the world. Note: Some of these viruses are no longer in use.

Virus Name	Virus Abbrev.	Target Pest Species	Crops	Product Name(s) (examples)
Alphabaculovirus				
Anticarsia gemmatalis MNPV	AgMNPV	*Anticarsia gemmatalis*	Soybean	Baculo-soja, Multigen, Baculovirus Nitral, Coopervirus SC, Protege
Autographa californica MNPV	AcMNPV	*Autographa californica, Trichoplusia ni, Manduca sexta, Chloridea (Heliothis) virescens, Spodoptera exigua,*	Alfalfa, cotton, vegetables, fruits, ornamentals	Gusano Biological, VPN-80^{TM}, VPN Ultra 1,6 WP, Lepigen, Loopex
Autographa californica MNPV + Spodoptera albula NPV	AcMNPV + SpalNPV	*Autographa californica, Trichoplusia ni, Pseudoplusia includens, Chloridea (Heliothis) virescens, Spodoptera exigua, Estigmene acrea, Plutella xylostella*	Alfalfa, cotton, vegetables, fruits, ornamentals	VPN-ULTRA
Chrysodeixis includens NPV	ChinNPV	Chrysodeixis includes, *Trichoplusia ni*	Soybean, vegetables	Chrysogen, Loopovir
Helicoverpa armigera NPV	HearNPV	*Helicoverpa armigera, Helicoverpa zea, Chloridea (Heliothis) virescens*	Maize, tomato, cotton and tobacco, sweet pepper, soybean, vegetables, crops	Gemstar, Heligen, Armigen, Vivus Max, Diplomata, Helicovex, Virin-HS, DOA BIO V2, Biotrol, Elcar, Biovirus-H
Hyphantria cunea NPV	HycuNPV	*Hyphantria cunea*	Forestry	Virin-ABB
Lymantria dispar MNPV	LdMNPV	*Lymantria dispar*	Forestry	Disparvirus, Gypchek, Virin-ENSH,
Mamestra brassicae MNPV	MbMNPV	*Mamestra brassicae, Helicoverpa armigera, Plutella xylostella, Phthorimaea operculella, Lobesia botrana*	Vegetables	Mamestrin, Virin-EKS
Mamestra configurata NPV	MacoNPV	*Mamestra configurata*	Oilseed rapes	Virosoft
Orgyia pseudotsugata MNPV	OpMNPV	*Orgyia pseudotsugata*	Forest	Biocontrol I, Virtuss

Spodoptera exigua MNPV	SeMNPV	*Spodoptera exigua*	Tomato, chili, eggplant	SPOD-X LC, Vir-ex, Spexit
Spodoptera frugiperda MNPV	SfMNPV	*Spodoptera frugiperda*	Corn, rice, maize, sorghum	Fawligen, Cartugen, Spodovir
Spodoptera littoralis NPV	SpliNPV	*Spodoptera littoralis*	Cotton, corn	Spodopterin; Littovir
Spodoptera litura NPV	SpltNPV	*Spodoptera litura*	Tobacco, vegetables	DOA BIO V3, Biovirus-S
Syngrapha falcifera NPV	SyfaNPV	*Syngrapha falcifera*	Vegetables	Protus WG
Betabaculovirus				
Adoxophyes orana GV	AdorGV	*Adoxophyes orana*	Apple, pears	Capex 2
Agrotis segetum GV	AgseGV	*Agrotis segetum, Agrotis ipsilon*	Vegetables	Agrovir
Cydia pomonella GV	CpGV	*Cydia pomonella, Grapholita molesta*	Pome fruit (Apple, pear etc.) and walnut, stone fruits	Carpovirusine, Carpovirusine EVO2, Carpovirus Plus, Cyd-X, Cyd-X HP, Granupom, Madex, MadexPlus, MadexTwin, MadexMax, MadexTop, Madex HP, Virosoft CP4
Cryptophlebia leucotreta GV	CrleGV	*Cryptophlebia leucotreta*	Citrus	Cryptex, Cryptogran
Erinnyis ello GV	ErelGV	*Erinnyis ello*	Cassava, Rubber trees	Baculovirus erinnyis
Phthorimaea operculella GV	PhopGV	*Phthorimaea operculella, Tecia solanivora*	Potato	Baculovirus Corpoica, PTM baculovirus, Matapol, Matapol Plus
Plodia interpunctella GV	PlinGV			NutGuard-V, FruitGuard-Ve, BioGuard-V
Plutella xylostella GV	PlxyGV	*Plutella xylostella*	Vegetables	Plutella xylostella GV SC
Deltabaculovirus				
Neodiprion lecontei NPV	NeleNPV	*Neodiprion lecontei*	Forest, ornamentals	Lecontvirus WP
Neodiprion sertifer NPV	NeseNPV	*Neodiprion sertifer*	Forest, ornamentals	Monisärmiövirus

It is a collection of several reviews from Arthurs and Dara (2019), Beas-Catena et al. (2014), Erlandson (2008), Prasad and Srivastava (2016), Sun (2015); Haase et al., 2015, and notifications from OECD member states.