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## Issue Paper on Microbial Contaminant Limits for Microbial Pest Control Products

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Issue Paper on Microbial Contaminant Limits for Microbial Pest Control Products

**IOMC**

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

**Environment Directorate**  
**ORGANISATION FOR ECONOMIC COOPERATION AND DEVELOPMENT**  
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# Foreword

The OECD work on agricultural pesticides (i.e., chemical and biological pesticides) aims to help member countries improve the efficiency of pesticide control, share the work of pesticide registration and re-registration, minimise non-tariff trade barriers and reduce risks to human health and the environment resulting from their use. In support of these goals, the OECD Pesticides Programme has undertaken work to:

- identify and overcome obstacles to work-sharing;
- harmonise data requirements and test guidelines; and
- harmonise hazard/risk assessment approaches.

With the primary goal of facilitating the sharing of national review reports, OECD's work initially focused on ways to harmonise the format/structure of reviews that are exchanged. The OECD dossier and monograph guidance provide a general layout and standardised formats for industry reporting (dossier) and government reviews (monographs). They were developed with the aim of facilitating the exchange of reviews among countries.

The OECD Expert Group on BioPesticides (EGBP) was established by the Working Party on Pesticides (WPP) in 1999 to help member countries harmonise the methods and approaches used to assess biological pesticides and improve the efficiency of control procedures. Biological pesticides include: microbials, pheromones and other semiochemicals, and invertebrates as biological control agents. The first tasks of the EGBP consisted of: (i) reviewing regulatory data requirements for the three categories of biopesticides; and (ii) developing formats for dossiers and monographs for microbials, and pheromones and other semiochemicals. This was achieved in 2004.

The EGBP then decided to concentrate its efforts on scientific and technical issues that remain as barriers to harmonisation and work-sharing. Five areas were identified: taxonomy; genetic toxicity; operator and consumer exposure; residues in treated food crops; and efficacy evaluation. The objective was to develop a "working document" to guide government and industry risk assessors and scientists involved in the registration and regulation of microbial pest control products (MPCPs) and their active agents (MPCAs). The resultant document titled: "*Working Document on the Evaluation of Microbials for Pest Control*" was published as ENV/JM/MONO(2008)36 in OECD Series on Pesticides No. 43.

The EGBP regards its work as "dynamic" intended to address scientific issues as they arise, and which may be impediments to harmonisation and work-sharing of microbial dossiers and monographs. Consequently, the EGBP has endeavoured to address and develop guidance on other issues as needed. The present document represents one such area, namely the establishment of acceptable limits of microbiological contamination in microbial pest control products. Microbiological contamination was originally identified by the EGBP as an issue requiring guidance in 2006 and Canada assumed the lead for developing a document in consultation with other member countries and the regulated industry. The document was then updated in 2022 by experts led by Canada and Germany.

The present guidance document received final approval from the OECD EGBP at its meeting in March 2023 and from the OECD WPP by written procedure in May 2023.

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

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# 1 Introduction and Objective

Microbial pest control agents are increasingly being developed for use in plant protection and they are thought to pose a lower risk to human health.

Microbial pest control products are manufactured using various methods depending on their unique characteristics. Most biological agents are produced in some type of submerged culture or solid-state substrate unless they are obligate intracellular parasites that require cell cultures, whole animals or other living forms as hosts. These manufacturing processes have environmental benefits since few organic solvents or other harsh chemicals are required during manufacturing. However, they all have the potential of producing unwanted micro-organisms in addition to the desired microbial pest control agent. Depending on the growing conditions, these unwanted or contaminating micro-organisms could include pathogens, their associated microbial compounds and other metabolic by-products that could be of health concern. As a result, a contaminated microbial pest control product could pose a risk if it is applied over human populated areas, habitats frequented by susceptible non-target organisms or other sensitive areas (e.g., drinking water sources) as well as to food crops up to, or near, the time of harvest.

The discussion herein is limited mainly to microbial contaminants of human and animal concern, including primary human pathogens, and does not consider the presence of other micro-organisms of concern, for example the presence of plant pathogens. The implications of such contaminants are intended to be addressed under subsequent sections of the dossier (e.g., non-target plant testing) and is beyond the scope of this paper. Nevertheless, it is understood that by limiting the number of human and animal pathogens, the potential presence of other contaminants is also reduced.

Many regulatory authorities have recognised the risk posed by certain contaminating micro-organisms in microbial pest control products and, as a result, have drafted appropriate regulations and/or regulatory guidelines to minimize this risk. For instance, detailed information on the manufacturing process and quality assurance procedures (including microbial contaminant screening) are required by the European Union (EU), the United States (U.S.) and Canada for each application/dossier to register a microbial pest control product. Few differences in manufacturing and quality assurance data requirements were noted among all three jurisdictions. However, some differences could potentially occur with respect to microbial contaminant screening as most regulatory authorities provide little guidance to applicants/notifiers. Consequently, applicants/notifiers might encounter different regulatory requirements that could ultimately delay or prevent registration/authorisation. The purpose of this issue paper is to highlight current international microbial contaminant criteria on food and drinking water and to promote a dialogue among OECD member countries on the acceptable levels of microbial contamination in microbial pest control products. A compilation of methods available for screening microbial pest control products for the presence of pathogens and other micro-organism contaminants are referenced for guidance to regulatory authorities and applicants/notifiers alike.

# **2** Existing Regulatory Requirements on Manufacturing and Microbial Contaminants

A good understanding of each OECD member country's regulatory requirements is essential before initiating formal discussions on harmonisation. This issue paper focuses only on regulatory requirements for Canada, the U.S. and the EU.

In Canada, Health Canada establishes data requirements for microbial pest control products, including those for manufacturing and quality assurance, which are outlined in Regulatory Directive DIR2001-02, Guidelines for the Registration of Microbial Pest Control Agents and Products (Health Canada 2001). Applicants/notifiers must clearly describe all the individual steps in the manufacturing process, with particular emphasis on critical process points and measures taken to ensure consistent quality and to limit extraneous contamination. Health Canada also requires a discussion on the formation or presence of unintentional ingredients, including microbial contamination that is likely to occur for a particular microbial pest control product during manufacturing. The impact of these unintentional ingredients on product quality, the integrity of the active ingredient and possible effects on human health and environmental safety must be discussed. If there is a likelihood that contamination can occur, data must be submitted showing that such contamination either does not occur or occurs at levels too low to represent a risk in the product. The DIR2001-02 directive recommends that suitable indicator organisms be routinely monitored in production samples to assess the hygienic state of the production facility and manufacturing process. The presence and level of potential microbiological contamination should be assessed by the applicant/notifier, using methods and criteria that are consistent with international standards for food or related microbial products, e.g., supplements, and probiotics. International standards set by the International Commission on Microbiological Specifications for Foods are recommended. The approaches, methods and rationales for detection and quantification of contaminants must be described in detail and representative data from five production or pilot-scale batches are required.

The U.S. EPA's regulatory requirements for microbial pest control products are currently outlined in 40 CFR 158.2120 (U.S. Federal Register 2007). The U.S. EPA's revised data requirements state that an analysis of samples (i.e., batch data) is required to support registration of each manufacturing-use product, and each end-use process after which there is presumed to be no potential for microbial contamination or microbial re-growth. For full registration, generally an analysis of samples is considered to be a compilation of batches, over a period of time, depending on the frequency of manufacturing. Details on these requirements are provided in two separate test guidelines, namely U.S. EPA Series 885 Microbial Pesticide Test Guidelines OCSPP (Office of Chemical Safety and Pollution Prevention) 885.1200 (U.S. EPA 1996a) and 885.1300 (U.S. EPA 1996b).

According to these U.S. EPA guidelines, applicants/notifiers must describe the basic manufacturing process, the starting and intermediate materials, and the steps taken to limit extraneous contamination. Applicants/notifiers must also submit a theoretical discussion on the formation of unintentional ingredients, including microbial contaminants, and include a list of procedures to ensure the purity of unformulated

product. Human or other non-target animal pathogens such as, but not limited to, Shigella, Salmonella, and Vibrio must not be present at hazardous levels in the technical grade of the active ingredient. Each submission/dossier must also include a batch analysis of all human or animal pathogens that might be present at potentially hazardous levels in unformulated product, including proposed methods to detect and/or eliminate them from the unformulated product. The amount of batch analyses required for each product is discussed at a pre-registration meeting with the potential registrant. There are two areas of potential concern: the growth of pathogens to high numbers in the production process; and the possibility of contaminants growing in the end-use product. The nature of the manufacturing process is a key element for consideration and includes factors such as handling of the seed stock, the type of fermentation technology (e.g., autoclaved, steam-treated), the potential for contaminants in the growth medium, the ratio of pure inoculant culture to the growth medium, product variability, and potential for pathogen contamination throughout the manufacturing process. For instance, if pure cultures of the active ingredient are always produced in fully sterilised growth media within sterilised equipment, then routine batch monitoring data could be omitted from the quality assurance programme. The anticipated level of exposure to potential contaminants is also considered. For example, products with direct food applications could require more stringent batch data analyses, as humans would have a greater level of dietary exposure than if the product were soil-incorporated.

The U.S. EPA is careful to consider each registration application independently with respect to the need for batch data and recognises that it is not unequivocally needed for each registration. Once a clear understanding of the potential for contamination by human pathogens is better understood, U.S. EPA can choose, as needed, to request additional batch data if modification to the manufacturing process had been suggested to exclude contaminants, or if the original data were not definitive. Only in rare instances is batch monitoring required for determining hazardous levels of specific contaminants or indicator organisms. These monitoring provisions could be part of a conditional registration which would be removed once met, but if the potential for certain microbial contamination is judged to be random and/or not amenable to fixing with modifications to the manufacturing process, could remain in place indefinitely.

In the EU, data requirements for microbial active substances and products for plant protection, and biocidal active substances and biocidal products are outlined in Regulations (EC) No283/2013 Part B, (EC) No284/2013 Part B and (EU) No528/2012, respectively. Both legislations have similar requirements, but there are some important differences. Both Commission Regulations require detailed information on how the microbial pest control agent (MPCA) is produced, and the methods to ensure the integrity of the MPCA as manufactured and the microbiological purity of the final product (MPCP). Both regulatory documents require a detailed analysis of composition of the microbial pest control agent and of the final product. This includes the identity and maximum content of pathogenic contaminating micro-organisms, expressed in the appropriate unit. Both MPCA and Products should be free from pathogenic microbial contaminants, if possible; otherwise, they should be controlled to acceptable levels. The acceptable levels of contaminants are not specified in either document; however, according to Regulation (EU) No 546/2011 and Regulation (EU) No 528/2012, the nature and acceptable levels of contaminants should be judged from a risk assessment point of view and be established by the Competent Authority.

Regulation (EU) No 546/2011 also states that both production and product must be subject to continuous quality control by the applicant to monitor pathogenic contaminating micro-organisms and the integrity of the MPCA as manufactured. All techniques shall be described, specified, and validated, if not following an already validated one (e.g., ISO).

Article 65(2) of Regulation (EU) No 528/2012 requires that Member States shall make the necessary arrangements for official controls to be carried out in order to enforce compliance with this Regulation. This includes the monitoring of the manufacturing process and the level of impurities and contaminants in the active substance or biocidal products. The analytical methods for monitoring used by the applicant and enforcement offices to determine the concentration of the impurities and contaminants should be highly specific and fully validated.

# 3 International Microbiological Specifications in Food and Drinking Water

Significant time and effort has been invested in establishing acceptable limits of microbial contamination in drinking water and in food to protect the safety of consumers. Wherever possible, the same principles and/or criteria developed by various international organisations for food and drinking water could be adapted for establishing acceptable contaminant limits in microbial pest control products. That being said, /while it is worthwhile to reflect on the experiences gained from food and drinking water, certain fundamental differences in these regulatory fields must be recognised. Firstly, the intended use of food and drinking water are remarkably different from those of microbial pest control products. The purpose of microbial pest control products is to eliminate a target pest by killing or suppressing it, which is an inherently noxious use. Products from the food-industry and drinking water, on the other hand, are intended for human consumption and are therefore, for all intents and purposes, entirely innocuous. The exposure scenarios are also very different. Microbial pest control products are applied as large amounts of micro-organisms to an area in the environment. This use pattern includes occupational exposure during manufacturing/formulating and mixing/loading and applying the product, and bystander exposure during and after application. Additionally, this use pattern results in an inadvertent dietary exposure by residues left on edible commodities. While there is also occupational exposure in the use pattern for food and drinking water during its manufacturing, formulating and processing, dietary exposure is by far the main exposure route of concern for microbial pest control products. Consequently, a different approach to regulating/monitoring contaminants may be appropriate.

Furthermore, the regulations for ensuring safety of food and drinking water and microbial pest control products are different. Microbial pest control products undergo an evaluation of the safety of the micro-organism prior to market, part of which includes a product-specific assessment of the potential for microbial contamination where various factors must be considered. Following this evaluation is a regulatory decision on the acceptability of the product and its intended use(s). The issue of microbial contamination in the context of the food-industry and drinking water regulation, on the other hand, is straightforward as there is an obligation for human pathogens to be absent from all products and for all contaminant levels to be closely monitored with adherence to acceptance standards. For these reasons, certain variations in the acceptable levels and type of contaminants in food/drinking water and microbial pest control products may be appropriate.

The presence of micro-organisms in a commodity is not necessarily an indicator of hazard to a consumer or of inferior quality. Moulds, yeasts, and bacteria are almost always found in food and water unless they are sterilised. When these are contaminants, micro-organisms may be innocuous, others may cause spoilage, and still, others may cause disease. The possibility of commodities becoming hazardous to consumers increases significantly if sanitation or hygiene is compromised. As a result, many international organisations such as the International Commission on Microbiological Specifications for Foods (ICMSF) and the Joint FAO/WHO Codex Alimentarius Commission as well as regional/country jurisdictions (e.g.,

European Commission Regulation) have established hygienic practices, sampling plans and microbiological specifications as well as other composite programs such as the Hazard Analysis Critical Control Point System (HACCP) to help prevent food- and water-borne diseases. Hygienic practices and sampling plans are both considered essential to food safety but will not be further discussed as they fall outside the scope of this issue paper.

Micro-organisms as components of microbiological specifications in food and water can be grouped into one of the following two categories of pathogens or indicator organisms. A brief description of these groups follows.

## Pathogens

According to the Subcommittee on Microbiological Criteria ([www.icmsf.org](http://www.icmsf.org)), suitable pathogens are those likely to be found in the commodity, which thereby becomes a potential vehicle for its transmission to consumers. Food-borne microbial pathogens have been classified in one of the following categories based on hazard (Subcommittee on Microbiological Criteria [www.icmsf.org](http://www.icmsf.org)), namely:

- severe hazards;
- moderate hazards with potentially extensive spread;
- moderate hazards with limited spread; and
- other pathogens considered.

The following is a summary of potential food-borne pathogens, based largely on the Subcommittee on Microbiological Criteria ([www.icmsf.org](http://www.icmsf.org)). The list is offered as a basis for the discussion on the microbial contaminant screening requirements for microbial pest control products.

### **Severe hazards (Subcommittee on Microbiological Criteria, [www.icmsf.org](http://www.icmsf.org))**

#### *Brucella melitensis, Brucella abortus, Brucella suis, and Mycobacterium bovis*

Brucellosis and tuberculosis are serious diseases often resulting in long-term illnesses and major health complications. Brucellosis is primarily an occupational disease of workers of meat-processing and livestock industries but can also be transmitted in raw milk. Similarly, tuberculosis may be shed in milk from infected cattle and goats and be transmitted to humans. The methods for detecting *Brucella* and *Mycobacterium* are insensitive, time-consuming and generally unsuited to routine screening. Furthermore, brucellae are highly infectious, even in very small numbers. Because of their high pathogenicity and their ability to spread in aerosols, laboratory personnel can contract the disease while working with these organisms, and such work should only be carried out in specially constructed laboratories (Level 3) with appropriate containment facilities.

#### *Clostridium botulinum types A, B, E, and F*

Botulism is intoxication resulting from the consumption of botulinum toxin produced during the growth of *Clostridium botulinum*. *Clostridium botulinum* is an anaerobic Gram-positive rod-shaped bacterium whose spores are heat-resistant. The disease is characterised by paralysis with abdominal disturbances and a generally high mortality rate. Its isolation in pure cultures may be an intricate and time-consuming process. Current methods of detecting the organism and identifying it are based on the detection of the toxin and the protection of test animals from specific toxins using monovalent antisera. Routine analysis of *Clostridium botulinum* is not advised.

*Salmonella typhi, Salmonella paratyphi A, B, and C, Salmonella sendai, and Salmonella cholerae-suis*

*Salmonella typhi* and *Salmonella paratyphi* A, B and C cause typhoid and paratyphoid fevers, respectively, which are characterised by septicemia without enteritis. All *Salmonella* are considered pathogenic to humans and standard methods are available for routine testing; other *Salmonella* species can cause enteric infections and are included under Part *Moderate hazards with potentially extensive spread*.

*Shigella dysenteriae*

Shigellosis is an infectious disease transmitted most commonly by close person-to-person contact via the fecal-oral route. Shigellae are invasive and penetrate the intestinal mucosa. The disease is characterised by the sudden onset of abdominal pain, tenesmus, pyrexia, and prostration. Bloody stools can quickly become composed of mainly blood and mucus. *Shigella dysenteriae* is host adapted to humans and higher primates and the infectious dose can be as low as 10 organisms. The method for its detection is, however, not sensitive and quantification is rarely performed. Detection is usually performed on suspect food using an enrichment medium followed by sub-culturing onto a variety of selective media.

*Vibrio cholerae*

*Vibrio cholerae* live in brackish water and naturally inhabit coastal waters. These halophilic organisms are present in high concentrations in the summer months. *Vibrio cholerae* causes an acute diarrhoeal disease called cholera. This species also includes strains that can cause epidemics, namely *Vibrio cholera* O group 1. *Vibrio cholera* O1 produces an enterotoxin that causes excretion and severe loss of fluids and electrolytes. Detection of *Vibrio cholerae* in foods involves is complex and requires a number of steps including enrichment, plating on selective media and performing a mouse adrenal cell assay on suspect colonies. Routine testing for *Vibrio cholerae* is not practical as detection methods are too insensitive and time-consuming.

***Moderate hazards with potentially extensive spread (International Commission on Microbiological Specifications for Foods, [www.icmsf.org](http://www.icmsf.org))***

The micro-organisms listed as moderate hazards generally cause illnesses that are milder than those listed in the severe hazards group.

*β-hemolytic Streptococcus (groups A, C, and G)*

Hemolytic streptococci have been associated with some of the world's most serious and devastating human diseases. Most streptococcal disease is spread by direct or indirect contact and many serious epidemics in the past have been linked to raw milk. Streptococci can be separated into different serological groups based on the existence of a hapten known as the 'C' substance which is attached to the outer cell wall. Group A streptococci are the most dangerous, but groups C, E, F and G have also been implicated in human infections. Methods that are sufficiently selective and quantitative for routine examination are not available for differentiating the various groups.

*Toxigenic and pathogenic Escherichia coli*

Certain toxigenic (cholera-like symptoms) and/or invasive (*Shigella*-like symptoms) biotypes of *Escherichia coli* can cause gastroenteritis in humans and other animals. Verotoxin-producing *Escherichia coli* infections cause severe illnesses such as hemorrhagic colitis and hemolytic uremic syndrome. Symptoms may be mild or severe, and include abdominal pain, diarrhoea, and blood in stool. *Escherichia coli* infections can spread from many food sources, such as undercooked ground beef, unpasteurised milk, sandwich meat,

and raw vegetables. Infections can spread from person to person by hand to mouth contact. Standard methods for rapid detection of enterohemorrhagic *Escherichia coli* O157 are available in the food.

#### *Salmonella typhimurium and other Salmonella serovars*

Enteric infections caused by *Salmonella typhimurium* and other *Salmonella* serovars are characterised by fever, diarrhoea, intestinal cramps and vomiting. All *Salmonella* species are considered pathogenic to humans and standard methods are available for routine screening; other *Salmonella* species which cause typhoid and paratyphoid fever are included under Part Severe hazards.

#### *Shigella flexneri, Shigella boydii, and Shigella sonnei*

As noted in the severe hazards section, shigellosis is an infectious disease transmitted most commonly by close person-to-person contact via the fecal-oral route. The diseases caused by *Shigella flexneri*, *Shigella boydii*, and *Shigella sonnei* tend to be intermediate in severity but show considerable variation. As previously noted, the detection method for *Shigella* pathogens is not sensitive and quantification is rarely performed. Detection is usually performed on suspect food using an enrichment medium followed by subculturing onto a variety of selective media.

### **Moderate hazards with limited spread (International Commission on Microbiological Specifications for Foods, [www.icmsf.org](http://www.icmsf.org))**

#### *Bacillus cereus*

*Bacillus cereus* produces one of two types of clinical symptoms: one that closely resembles those of *Staphylococcus aureus* (gastroenteritis) and another that closely resembles those of *Clostridium perfringens* (enterocolitis). Few outbreaks of disease are reported from this organism, but this observation may be due to misdiagnosis since symptoms resemble those of other micro-organisms. Although *Bacillus cereus* is often regarded as harmless since it is ubiquitous in many environments, it is a common cause of food poisoning and is often isolated as a contaminant of rice, spices, meat, eggs and dairy products. *Bacillus cereus*-associated gastroenteritis is mediated by a variety of enterotoxins, a group of heat-labile proteins causing abdominal pain and diarrhoea after incubation for 8–16 hours and vegetative growth of the bacteria in the intestine. Three enterotoxins are currently recognised: hemolysin BL (HBL), non-hemolytic enterotoxin (NHE) and cytotoxin K (CytK or EntK). Enterocolitis, or the emetic syndrome, is caused by an acid resistant cyclic dodecadeptide, cereulide, and is characterised by nausea and vomiting only a few hours after a meal. Unlike diarrhoeal strains of *Bacillus cereus*, emetic strains are not heterogeneous and consist of a single, distinct cluster of isolates. Most detection methods for *Bacillus cereus* involve plating onto a selective agar followed by confirmatory testing of presumptive colonies. A rapid detection method was recently approved by ISO that involves chromogenic medium enabling enumeration without any subculture or confirmation of *Bacillus cereus*.

#### *Campylobacter fetus subsp. jejuni*

This micro-organism is a common cause of gastroenteritis in humans. Methods for detecting *Campylobacter fetus subsp. jejuni* are available, but routine analysis is not practical in the food industry. Although molecular methods for *Campylobacter fetus* have recently been developed the methods have not yet been validated. Nevertheless, testing for *Campylobacter* is not considered necessary as they are microaerophilic and cannot multiply outside of intestinal tracts.



*Clostridium perfringens* type A

*Clostridium perfringens* is one of the most common causes of enteritis. The spores of this micro-organism can survive in water for long periods of time and vegetative cells can pass through the stomach to the small intestine when consumed with proteins. This increases stomach pH and creates a favourable environment for cell survival. Illnesses arise when an enterotoxin is released in the intestines when the micro-organism undergoes sporulation. Symptoms include diarrhoea and abdominal pain. Small numbers of this micro-organism are unavoidable in foods, but its presence in large numbers may be indicative of poor sanitary conditions or mishandling of food by workers. Methods for the detection and enumeration of *Clostridium perfringens* involve plating on selective agar media and confirmation tests on presumptive isolates.

*Staphylococcus aureus*

Illnesses caused by this micro-organism are attributed to several heat-stable enterotoxins produced by certain strains of this species. The presence of enterotoxins is the principal concern rather than the organism itself. Nausea, vomiting, diarrhoea, general malaise and weakness characterise the disease. Small numbers of *Staphylococcus aureus* are to be expected in foods that have been exposed to food handlers. However, even low numbers do not assure safety because the organism can grow and produce enterotoxin then die off or be killed. The heat-stable enterotoxin will remain active in the commodity. Methods for the detection and enumeration of *Staphylococcus aureus* are available for use in routine analysis.

*Vibrio parahaemolyticus*

As with *Vibrio cholerae*, these halophilic organisms live in brackish water. Most *Vibrio parahaemolyticus* infections occur from eating raw or undercooked shellfish, particularly oysters. Symptoms generally occur within 24 hours. The illness is self-limiting and usually lasts three days (source: U.S. CDC website). Illnesses caused by this micro-organism are often erroneously identified as salmonellosis or dysentery. Illness usually begins with a violent epigastric pain accompanied by nausea, vomiting, and diarrhoea. In severe cases, mucus and blood occur in the stool. Mild fever and headaches frequently occur as well. The method for enumerating *Vibrio parahaemolyticus* is considered time-consuming and identification of pathogenic strains is also complicated.

*Yersinia enterocolitica*

This micro-organism typically causes gastroenteritis and terminal ileitis. Infection is most often acquired by eating contaminated food, especially raw or undercooked pork.

Certain molecular methods which detect virulence genes are available for rapid characterisation of *Yersinia enterocolitica* isolates. The methods have not yet been validated. Routine analysis is not recommended in the food industry. *Yersinia* seems not to be a problem contaminant associated with microbial pest control products.

**Other pathogens considered in drinking water quality (World Health Organization 4th Edition, 2017)***Aeromonas*

Mesophilic species of this genus have been implicated in a wide range of infections (wounds, respiratory tract, and eye) in humans and are commonly isolated from patients with gastroenteritis although their role

in disease causation is still unclear. Some species produce extracellular toxins (hemolysins) and enzymes. Routine monitoring in water is not recommended because the methods for identifying specific phenospecies and genospecies are very complex and have not yet been validated.

### *Legionella*

Legionnaires' disease is a type of pneumonia caused by many serogroups of *Legionella pneumophila* (most common), *Legionella micdadei* and many other species of *Legionella*. As *Legionella* grow best in warm water, most people contract Legionnaires' disease when they breathe in mist or vapour that has been contaminated with the bacteria. Legionnaires' disease is frequently accompanied with extrapulmonary manifestations such as renal failure, encephalopathy, and pericarditis. The International Organization of Standardization (ISO) developed (but has not yet published) a PCR method for detection and quantification of *Legionella* and/or *Legionella pneumophila*. Continuous monitoring of *Legionella* is only advocated when antimicrobial measures must be verified.

## Indicator Micro-organisms

Recovering pathogens themselves for microbiological specifications presents certain difficulties. Firstly, screening is subject to availability and ease of detection methods. Also, the World Health Organization does not recommend the isolation of specific pathogens unless accredited laboratories perform the isolation for the purposes of investigating and controlling outbreaks (World Health Organization 2017). For these reasons, many microbiological specifications in water and food make use of indicator organisms in lieu of screening for the pathogens themselves. The principle is that levels of indicator organisms verify whether microbial levels are properly excluded during manufacturing, thereby alerting manufacturers of a potential concern for contamination and possibly, a need for further action.

Suitable indicators are those whose presence in food or water indicates (Subcommittee on Microbiological Criteria, [www.icmsf.org](http://www.icmsf.org)):

- the likelihood that a pathogen(s) or harmful toxin of concern may also be present;
- the likelihood that faulty practices occurred during production, processing;
- distribution may adversely affect safety or shelf-life; or
- the commodity may be unsuited for its intended use.

These types of indicators typically fall into one of the following four categories (Subcommittee on Microbiological Criteria, [www.icmsf.org](http://www.icmsf.org); WHO/OECD 2003):

- indicators that assess microbial numbers or activity;
- indicators of potential human pathogens or fecal contamination;
- indicators of post heat processing contamination<sup>1</sup> (not discussed in this issue paper); and
- metabolic products of pathogens that indicate the presence of a pathogen (not discussed in this issue paper).

A brief review of these categories follows.

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<sup>1</sup> Generally only applicable in the food industry, as heat processing is not conducive for manufacturing of microbial pest control products.

## **Indicators which assess microbial numbers and/or activity**

### *Aerobic plate count*

The aerobic plate count (APC) is used as a microbiological criterion in dairy products such as raw and pasteurized milk, and drinking water. The test is based on the assumption that each microbial cell or cell clump will form a single visible colony when incubated for a period of time in an aerobic atmosphere under specific conditions. The results of this test are often misused for estimating the entire microbial population in a sample. As a total aerobic colony count, this test potentially measures a large proportion of the total sample population of a sample; however, the count may reflect only a fraction of living micro-organisms that are able to produce colonies on the given medium and growth conditions. Nevertheless APC is a good general method to judge the overall hygiene of food or drinking water. Unexpected high values for this indicator may be attributed to contamination which suggests that an examination of critical control points should be made. The two most widely used APC methods are the AOAC method (AOAC 1980) and the standard plate count (SPC) method described in the Standard Methods for the Examination of Dairy Products (APHA 1978) as well as in Health Canada's Compendium of Analytical Methods (MFHPB-18, MFHPB-33, MFLP-10, MFLP-17, MFLP-56) and the U.S. Food and Drug Administration's (FDA) Bacteriological Analytical Manual (BAM; Chapter 3).

Although widely used to determine the overall purity of microbial pest control products, there are obvious challenges in requiring manufacturers to employ APC in quality assurance programmes especially if the active ingredient is an aerobic bacterium. Including APC batch analysis of such products would be of little utility to determine the presence of other (i.e., contaminant) aerobes and should not be required by regulators for routine testing purposes.

### *Anaerobic plate count*

Anaerobic growth chambers are available to measure obligate anaerobes such as clostridia and facultative anaerobes such as Enterobacteriaceae, enterococci and staphylococci. Similar to concerns raised above for aerobic MPCAs, if the active ingredient is known to be micro-aerophilic, then discretion should be exercised by regulators in requiring anaerobic plate counts in quality assurance programmes involving these MPCAs. Methods for detecting anaerobic bacteria/spore-formers are available in Health Canada's Compendium of Analytical Methods (MFHPB-16, MFHPB-23, MFLP-44, MFLP-50).

### *Thermotolerant, psychrotrophic, thermophilic, proteolytic and lipolytic counts*

Minor modifications can be made to the APC to enumerate specific groups of micro-organisms with particular growth requirements, and hence, are useful to measure microbial activity under specific circumstances. For instance, thermotolerant and thermophilic counts are often used to measure excessive activity following heat pasteurization. Psychrotrophic counts are used to measure the potential shelf-life of products stored under refrigeration conditions. Proteolytic and lipolytic micro-organisms can be responsible for a variety of flavour and odour problems in foods. Such counts are often used to maintain food quality in various commodities.

### *Direct microscopic counts*

Direct microscopic counts (DMC) are used as a component of microbiological criteria of raw milk, dried milks, liquid and frozen eggs, and dried eggs. The DMC is a rapid method that gives an estimate of the total number of micro-organisms, viable and non-viable, in a sample as well as their morphological characteristics. This method, however, is only suitable for samples containing large numbers of micro-organisms and the small number of examined samples usually limits its precision.

### *Microscopic mould counts*

Microscopic mould counts (MMC) are used to assess the soundness of raw horticultural products and the sanitary conditions of processing lines. Examples of such counts include Howard Mold Count (APHA 1976, 1984), the Rot Fragment Count (AOAC 1980, APHA 1976 and 1984), and “Machinery Mold” (AOAC 1980, APHA 1976 and 1984). The Howard Mold and Rot Fragments counts are used to assess the quality of raw products whereas the “Machinery Mold” count is used to verify the sanitation of equipment in vegetable and fruit processing plants.

### *Yeast and mould counts*

Yeasts and moulds are ubiquitous in the environment and can contaminate food through inadequately sanitized equipment or as airborne contaminants. Yeast and mould counts frequently predominate when conditions for bacterial growth are less favourable, such as lower water activity, low pH, high salt, or high sugar content. Satisfactory screening methods are available and use either acidified media or media with added antibiotics to inhibit bacterial growth (APHA 1976, 1978, 1984; Health Canada Compendium of Analytical Methods MFHPB-22, MFHPB-32; U.S. FDA BAM Chapter 18).

### *Heat-resistant moulds*

Some moulds such as *Byssochlamys fulva* and *Aspergillus fisheri* produce ascospores that are sufficiently heat resistant to survive thermal processing. Limits on these moulds are sometimes set for fruit and fruit products. Satisfactory methods for detecting and enumerating these moulds are available (APHA 1976, 1978, 1984).

### *Thermophilic spore count*

The canning industry often monitors the quality of ingredients such as sugar, starch, flour, spices, mushrooms, non-fat dry milk, and cereals that are intended for low-acid heat processed foods. Concern for thermophilic organisms in these foodstuffs is related to their high spore heat resistance and their ability to grow in foods held at elevated temperatures. Methods to determine these spores are available (AOAC 1980, APHA 1976, 1984; NCA 1968).

### *Alternative methods*

A variety of alternative methods for detection and quantification of microbial contaminants are available for the majority of pathogens. These include, but are not limited to, nucleic acid-based methods such as real-time polymerase chain reaction (RT-PCR), quantitative real-time PCR (qRT-PCR), DNA-microarray, and isothermal amplification techniques such as loop-mediated isothermal amplification (LAMP). In general, protein-based methods such as enzyme-linked immunosorbent assay (ELISA) and lateral flow assays have also been developed. Of these methods, PCR based analytics appear to be the most widely used at the time of drafting of this document.

By employing these methods, the detection of only components of micro-organisms (e.g., DNA or proteins) may indicate positive results. Moreover, it is important to take into consideration that such methods may detect both viable micro-organisms, or non-viable micro-organisms. For this reason, it is suggested to confirm a positive result in a nucleic acid-based method, by a method that indicates viability. This could be done with methods that combine a check for presence using a nucleic acid-based method with a test for viability (e.g. qRT-PCR with dead-cell selective dyes) or using qRT-PCR protocols that integrate both detection and CFU enumeration (Abdullah et al 2020; Baymiev et al 2020). Such methods are new and their usefulness therefore will depend on whether they are available and validated. To also note, the determination of colony-forming units via plate test, as described in the chapters above, remains possible.

All negative results will be considered as valid provided appropriate controls are included in the test (see Appendix III).

In general, absence of DNA of contaminating micro-organism allows conclusion on absence of contamination. Specifically for those micro-organisms for which absence in the manufacturing batch is required (see Tables 1.1 and 1.2), a positive test result triggers the check for viable cells of this species. For those contaminating micro-organisms for which presence in the manufacturing batch is tolerated, but must be below a certain threshold (see Tables 1.1 and 1.2), confirmation of viability must include enumeration of this micro-organism species.

When used for the analysis of microbial contaminants in MPCP, these alternative methods must be validated to be fit-for-purpose (see Appendix III) according to appropriate standards. If ISO or AOAC validated test kits are used for the qRT-PCR analysis, no further validation is necessary if the controls are used according to the test kit protocols. Non-certified alternative methods may also be validated and used on a case-by-case approach, where appropriate (see Appendix III), taking into consideration that, positive and negative controls should be used, and that level of detection sought to simulate either infectious doses, or the ability of a pathogen to grow to larger numbers.

Method descriptions for molecular detection of micro-organisms using qRT-PCR are available (e.g. Stromgren, 2019). Furthermore, descriptions of the necessary validation of molecular detection of micro-organisms using qPCR are available (including but not only, ISO 16140, ISO 20395:2019, ISO 20837:2006, ISO 22174:2005, EPPO PM 7/098 Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity).

### ***Indicators of potential human pathogens or fecal contamination***

#### *Staphylococci*

Staphylococci originate from the nasal passages, skin, and lesions of humans and other mammals. Staphylococci are usually killed during heat processing. In heat-processed commodities, their reappearance indicates contact with contaminated equipment or air. Small quantities of *Staphylococcus aureus* are expected in foods that have been exposed to, or handled by, workers. Large numbers of staphylococci may indicate the presence of toxins. Methods for the detection and enumeration of *Staphylococcus aureus* are available for routine analysis (AOAC 1980; APHA 1984; Health Canada Compendium of Analytical Methods MFO-14, MFHPB-21, MFHPB-28, MFLP-21; U.S. FDA BAM Chapter 12).

#### *Escherichia coli*

*Escherichia coli* conform to the definition of “Enterobacteriaceae”, “coliforms” and “thermotolerant (fecal) coliforms”. Its natural habitat is the intestines of vertebrate animals thus its presence indicates the possibility that fecal contamination has occurred and that other micro-organisms of fecal origin, including pathogens, may be present. The presence of *Escherichia coli* in a sample signifies a more positive assumption of hazard than the presence of other coliforms. The failure to detect *Escherichia coli*, however, does not assure the absence of other enteric organisms. Routine methods for the detection and enumeration of *Escherichia coli* are available (AOAC 1980, APHA 1984). Direct plating methods are preferable to the most probable number (MPN) method, which is time consuming, costly, and inhibitory to injured cells. Health Canada’s Compendium of Analytical Methods (Volumes 2 and 3) includes a number of isolation and enumeration methods - as does U.S. FDA’s BAM (Chapter 4).

### *Thermotolerant (fecal) coliforms*

Fecal coliforms are a group of organisms selected for by incubating an inoculum derived from a coliform enrichment broth at higher temperatures (44 – 45.5°C) than those used for incubating coliforms (35°C). Thermotolerant counts usually contain a high proportion of *Escherichia coli* and some strains of *Enterobacter*, *Klebsiella*, and *Citrobacter*. The thermotolerant coliforms have a higher probability of containing organisms of fecal origin than do coliforms that have received no further differential tests and are thus useful indicators of fecal contamination. Routine MPN methods are available (AOAC 1980; APHA 1976, 1981, 1984; Health Canada Compendium of Analytical Methods MFHPB-17, MFHPB-19, MFLP-55), but these suffer from the same limitations as those identified for *Escherichia coli* MPN methods. Since rapid direct plating methods for *Escherichia coli*, including provisions for resuscitation of injured cells, are available it may be advantageous to employ *Escherichia coli* counts rather than thermotolerant coliforms as an indicator of fecal contamination.

### *Enterococci (“fecal streptococci”)*

All enterococci bear the Lancefield group D antigen and consist of species within the genera *Enterococcus* and *Streptococcus*. Enterococci have certain features that make them unique as indicator organisms. All are facultatively anaerobic, grow well at 45°C (except for *Streptococcus bovis* and *Streptococcus equinus*), and, unlike *Escherichia coli*, they are resistant to freezing. Also, most enterococci are quite salt-tolerant and can grow in the presence of 6.5% NaCl. Enterococci originate from faeces of both warm-blooded and cold-blooded animals, but can also originate from plants and insects. Many food commodities normally contain small to large numbers of enterococci, especially *Enterococcus (Streptococcus) faecalis* and *Enterococcus (Streptococcus) faecium*. Enterococci counts in foods are not considered a reliable index of fecal contamination. A thorough understanding of the role and significance of enterococci in a food is required before any meaning can be attached to their presence and population numbers. While many media have been proposed (APHA 1976, 1984; ICMSF 1978) for the selective isolation and enumeration of enterococci, they each have shortcomings in selectivity, quantitative recovery or differential ability. Enterococci counts have limited use in the food industry, but if they are to be used as an indicator of poor manufacturing processes, it is necessary to establish the normal population levels at different stages of manufacturing with a standardized method.

### *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is commonly found in the environment, including aquatic ecosystems. This micro-organism is mainly an opportunistic pathogen of immunocompromised individuals, but it is also a concern for infants who may become infected when contaminated water or equipment is used to prepare baby formula. In some countries, this organism is used as an indicator of contamination in bottled drinking water. Methods for detecting *Pseudomonas aeruginosa* are described by APHA (1981) and Health Canada (Compendium of Analytical Methods MFLP-61, MFLP-61B).

# 4 Criteria for establishing OECD Limits on Microbiological Contamination in microbial pest control products

The following criteria were considered for establishing proposed OECD limits on microbiological contamination in microbial pest control products:

- The chosen limits on microbiological specifications and screening requirements must satisfy the data requirements of all OECD member countries.
- The list of contaminants has to be practical and feasible for registrants to monitor in their product(s).
- Appropriate standard screening methods have to be available for each of the selected indicators and/or pathogens.
- The chosen indicators and/or pathogens and corresponding screening methods must provide meaningful data to assess the overall acceptability and risk of the microbial pest control product in order to avoid unnecessary testing and placing an unreasonable burden on applicants/notifiers.
- In the event that the chosen screening requirements may put laboratory technicians at risk of infection, or outbreak, if performed outside an accredited laboratory, authorities must be willing to dictate that applicants/notifiers without accredited laboratories employ the services of accredited laboratories to satisfy the requirements.

## Microbial pest control products (excluding baculoviruses manufactured in vivo)

### ***Proposed microbiological specifications in microbial pest control products (excluding baculoviruses manufactured in vivo)***

The list of microbiological specifications for microbial pest control products (excluding baculovirus preparations) presented in Table 1.1 is offered for further consideration by regulatory authorities.

For comparative purposes, Tables I-III in Appendix I provide examples of the types and levels of microbiological contaminants that have been established by regulatory authorities or proposed by registrants.

**Table 1.1: Proposed OECD microbial contamination screening requirements for microbial pest control products  
(excluding baculoviruses manufactured in vivo)**

Type of Indicator	Indicator	Limit*	Rationale
Pathogen	<i>Salmonella</i>	Absence in 25 g or 25 mL	<ul style="list-style-type: none"> <li>- U.S. EPA and Health Canada requirement</li> <li>- many standard methods available</li> <li>- often used in the food industry</li> </ul>
	<i>Listeria monocytogenes</i>	Absence in 25 g or 25mL	<ul style="list-style-type: none"> <li>- recent health concern with respect to contaminated fruits/vegetables and processed meat products</li> <li>- can survive/multiply on foods stored under refrigerated temperatures</li> <li>- optional requirement particularly if screens for other hygiene indicators consistently demonstrate acceptably low levels of contamination. Regulatory authorities must also have a high degree of confidence in the manufacturer's quality assurance programme when deciding whether to waive routine screening for this micro-organism</li> </ul>



Type of Indicator	Indicator	Limit*	Rationale
	<i>Vibrio</i>	Absence in 25 g or 25 mL	<ul style="list-style-type: none"> <li>- U.S. EPA test guideline requirement, therefore testing is mandatory for U.S. registration/authorisation</li> <li>- not endemic in many countries</li> <li>- basic food handling precautions/personal hygiene habits exclude these organisms during manufacturing</li> <li>- isolation of specific species or pathogens (e.g., <i>Vibrio cholerae</i>) is NOT recommended unless the analytical laboratory follows appropriate biohazard protocols</li> <li>- optional requirement and recommended ONLY if there is a high potential for contamination or if species of <i>Vibrio</i> are known to naturally occur at the geographical location of the manufacturing site</li> </ul>
	<i>Shigella</i>	Absence in 25 g or 25 mL	<ul style="list-style-type: none"> <li>- U.S. EPA test guideline requirement, therefore testing is mandatory for U.S. registration/authorisation</li> <li>- not endemic in many countries</li> <li>- most commonly related to undercooked shellfish rather than manufacturing processes; basic food handling precautions/personal hygiene habits exclude these organisms during manufacturing</li> <li>- isolation of specific species or pathogens (e.g., <i>Shigella dysenteriae</i>) is NOT recommended unless the analytical laboratory follows appropriate biohazard protocols</li> <li>- optional requirement and recommended ONLY if there is a high potential for contamination or if species of <i>Shigella</i> are known to naturally occur at the geographical location of the manufacturing site</li> </ul>

Type of Indicator	Indicator	Limit*	Rationale
Microbial Activity	Aerobic Plate Count	< 10 <sup>5</sup> CFU/g or mL	<ul style="list-style-type: none"> <li>- indicator of aerobic bacterial contamination</li> <li>- often used in the food industry</li> <li>- many standard methods available</li> <li>- optional requirement if MPCA is an aerobic bacterium</li> </ul>
	Anaerobic spore-formers	< 10 <sup>5</sup> CFU/g or mL	<ul style="list-style-type: none"> <li>- cause of health concerns in the food industry; indicates hygiene failures during processing</li> <li>- anaerobic spore-forming organisms have potential to persist in soil/water for long periods of time</li> <li>- standard methods for anaerobic spore-formers available</li> <li>- optional requirement if other hygiene indicators (i.e., <i>Escherichia coli</i> and <i>Staphylococcus aureus</i>) are screened during product manufacture and if the MPCA is a known micro-aerophile</li> </ul>
	Yeast and Mould Count	< 1000 CFU/g or mL	<ul style="list-style-type: none"> <li>- many standard methods available</li> <li>- general indication of yeast and mould contamination, and potential presence of mycotoxins</li> <li>- may be optional requirement if MPCA is a fungus</li> </ul>

Type of Indicator	Indicator	Limit*	Rationale
Human, fecal and environmental contamination	<i>Escherichia coli</i>	Absence in 1 g or mL	- indicator of fecal contamination - recent health concern involving contaminated fruits/vegetables; certain sensitive sub-populations are particularly at risk - can survive/multiply on plants and in soil and water - many standard methods available
	<b>OR</b>  Thermotolerant (fecal) coliforms	< 10 CFU/g or mL	
	<i>Staphylococcus aureus</i>	Absence in 1 g or mL	- indicator of contamination due to improper handling - many standard methods available
	<i>Pseudomonas aeruginosa</i>	Monitoring*	- indicator of environmental contamination - optional requirement recommended ONLY if screening results for other hygiene indicators suggest possible presence of pseudomonads
Other tests  (product-by-product basis)	Mouse IP/SC assay	No evidence of infection or injury in test animals	- optional requirement recommended ONLY for products where there is a significant potential for contamination by a primary mammalian pathogen, especially if closely related to the MPCA, or when there is significant potential for production of a toxin of concern that cannot be ruled out by appropriate analysis by any other means  - unnecessary if intravenous/intraperitoneal (IV/IP) toxicity and pathogenicity (infectivity) test data on the pre-formulated product shows no positive results for toxicity or if toxicity may be attributed to an ingredient other than the MPCA

### ***Rationale for proposed microbial contaminant screening requirements***

Inclusion of screening for *Shigella* spp. and *Vibrio* spp.: While *Shigella* and *Vibrio* are not considered endemic in many parts of the world, species of these genera are pathogens often transmitted by food contaminated by infected individuals and thus merit consideration as potential contaminants in microbial pest control products.

Shigellosis is most common in settings where hygiene is poor; many cases are related to the spread of illness in child-care settings involving the fecal-oral route. Food may also become contaminated with *Shigella* by infected food handlers who neglect basic handwashing or hand-sanitizing habits. In general, basic food safety precautions and disinfection of drinking water generally prevents shigellosis from food and drinking water. In the developing world, shigellosis is far more common and is present in most communities most of the time (U.S. CDC website) Methods for the isolation and identification of *Shigella* are described by Health Canada (Compendium of Analytical Methods MFLP-25, MFLP-26) and the U.S. FDA (BAM Chapter 6).

Infections from *Vibrio* spp. usually arise from eating raw or undercooked shellfish (particularly oysters for *Vibrio parahaemolyticus* infections) since *Vibrio* spp. are naturally occurring organisms in seawater (U.S. CDC website). Other food commodities are rarely associated with infections. Methods for the isolation and identification of *Vibrio* are described by Health Canada (Compendium of Analytical Methods MFLP-23, MFLP-37, MFLP-72, MFLP-73) and the U.S. FDA (BAM Chapter 9).

Although the risk of contamination of microbial pest control products by *Shigella* and *Vibrio* spp. is not entirely clear, it seems unlikely that these organisms present a high risk for contamination. Therefore, screening for these organisms may present an unnecessary burden on applicants/notifiers. Furthermore, screening methods are reported to be insensitive and time-consuming.

This requirement should be considered optional as some authorities may decide screening for *Shigella* and *Vibrio* spp. in microbial pest control products is not of concern particularly if the manufacturing site is not located in a region where these organisms are found.

Addition of screening for *Salmonella* spp.: Although contamination by enteric micro-organisms is addressed in screens for *Escherichia coli* or thermotolerant (fecal) coliforms, the incidence of food-borne illness from ingestion of *Salmonella* represents a major public health concern. A variety of isolation and identification methods, as well as immunological and molecular screening techniques (both requiring cultural confirmation), are readily available to manufacturers. Health Canada's HPFB Compendium of Analytical Methods serves as a valuable source of internationally recognised as well as other scientifically valid methods for detecting and enumerating species of *Salmonella* (Health Canada Compendium of Analytical Methods MFHPB-20, MFLP-75; U.S. FDA BAM Chapter 5).

Addition of screening for *Listeria monocytogenes*: Listeriosis is an infection caused by eating food contaminated with *Listeria monocytogenes*. Symptoms include fever, muscle aches, nausea and/or diarrhoea. In some instances, these symptoms may be followed by meningitis encephalitis and/or septicemia, either of which can result in death. The infection may also spread through the nervous system, causing symptoms such as headaches, stiff neck, confusion, loss of balance, or convulsions. Infections during pregnancy can lead to miscarriage or stillbirth, premature delivery, or infections in newborns. Recently, outbreaks of contamination in the food industry have been associated with *Listeria monocytogenes*, which identifies this organism as a public health concern. It has been associated with raw milk, pasteurized fluid milk, soft-ripened cheeses, other processed dairy products such as ice cream, raw vegetables, raw meats, fermented raw-meat, cooked poultry and smoked fish. Certain sensitive sub-populations, such as the elderly, pregnant women, newborns and adults with weakened immune systems are particularly at risk of *Listeria* infections (source: Canadian PHAC website, U.S. CDC website). As this

micro-organism is able to grow on animal and plant substrates, and under refrigerated conditions, its presence in microbial pest control products could pose a concern if they are applied to food crops especially at, or near, the time of harvest. Methods of isolation and identification for *Listeria* spp. and *Listeria monocytogenes* in foods and environmental samples are available from Health Canada (Compendium of Analytical Methods MFHPB-07, MFHPB-30, MFLP-31, MFLP-39, MFLP-53, MFLP-54, MFLP-74, MFLP-78, MFLP-79) and the U.S. FDA (BAM Chapter 10).

Regulatory authorities may, on a product-by-product basis, determine that routine analysis for *Listeria monocytogenes* in microbial-based products is unwarranted particularly if screens for other hygiene indicators consistently demonstrate acceptably low levels of contamination. Regulatory authorities must also have a high degree of confidence in the manufacturer's quality assurance programme when deciding whether to waive routine screening for this micro-organism.

Addition of an anaerobic plate count to monitor anaerobic spores-formers: Although certain anaerobic spore-formers, such as *Clostridium botulinum* (types A, B, E and F) and *Clostridium perfringens* (type A) are common causes of health concern in the food industry, contamination of microbial pest control products by these organisms is not expected to be problematic given their anaerobic nature. However, their presence would be an indication of hygiene failures during processing. As anaerobic spore-forming bacteria can survive in soil and water for long periods, their presence in microbial pest control products is undesirable. Therefore, screening may be warranted if manufacturing processes indicate the potential for contamination.

Mouse IP/SC bioassay for *Bacillus thuringiensis* products with food uses (U.S. EPA 40 CFR 180.1011): Historically, the U.S. EPA and Health Canada have required each batch of technical active ingredient (prior to the addition of formulation ingredients) of *Bacillus thuringiensis* preparations be tested by intraperitoneal (IP) or subcutaneous (SC) injection at  $10^6$  CFU in mice to test primarily for the presence of *Bacillus anthracis*. This test has been used to show no evidence of infection or injury to test animals.

In the U.S., the mouse injection bioassay was initially required to address the concern for high levels of pathogens with respect to the food tolerance exemption for *Bacillus thuringiensis* in 1971. The assay was suggested from consultations with experts who advised that this assay was the best method for detecting *Bacillus anthracis*. The mouse assay has not been required for any other subsequently registered *Bacillus* species or other type of microbial pest control product, with the exception of baculovirus preparations manufactured in vivo. As part of the *Bacillus thuringiensis* re-registration process, batch monitoring requirements have been put into each active ingredient's manufacturing process agreement, as needed (e.g., for food uses only), and the mouse IP/SC bioassay may no longer be required by the EPA to support continued registration.

The EU does not specify the requirement for animal testing to ensure absence of mammalian pathogens given the microbial pest control product has demonstrated a lack of infectivity in mammals.

While the likelihood of contamination by *Bacillus anthracis* is low, the resultant risk to humans if it is present in *Bacillus thuringiensis* products is high, and therefore authorities should consider requiring the mouse IP/SC injection assay if certain criteria are met. For example, the bioassay should be required for any product that is intended for direct application to food crops or aerial application to human populated areas and the site of manufacture does not have an established record of demonstrating complete absence of *Bacillus anthracis* contamination.

Diagnosis via PCR is a modern technique that was established and used during a recent outbreak of anthrax in Sweden. The method of Lewerin et al. (2010) is based on two genetic markers on the pXO1 plasmid (*lef* and *cap*) and a third on the chromosome (*rpoB*). A multiplex PCR technique designed to distinguish *Bacillus anthracis* from closely related *Bacillus cereus* and *Bacillus thuringiensis* was developed recently (Wielinga et al. 2010) based on the conserved chromosomal region of the lambda pro-phage type 3 (PL3).

## Baculovirus-based pest control preparations manufactured in vivo

### **Existing regulatory approaches**

The following regulatory perspectives on the issue of microbial contamination in baculovirus-based products are offered for consideration by regulatory authorities.

In Canada, the historical acceptance limits for microbial contaminants in baculovirus preparations produced in vivo are listed in Table IV of Appendix I. Consistent with other microbial pest control products, Health Canada requires that contaminant screening of baculovirus products include microbe-specific selection media to ensure absence of human pathogens in every batch of the final unformulated preparation, and that populations of certain other micro-organisms do not exceed specified limits. If hygiene cannot be adequately controlled and there is high batch-to-batch variability, Health Canada may require that every batch of technical active ingredient be tested by the mouse IP/SC injection assay to confirm the absence of primary pathogens and any toxins of concern that cannot be addressed by standard microbiological screening methods.

The U.S. EPA's regulatory requirements for contaminant screening of baculovirus products are not directly presented, but are presumably the same as those for standard microbial pest control products; that is: batch analyses for human or other non-target animal pathogens (such as, but not limited to, *Shigella*, *Salmonella*, and *Vibrio*) for each manufacturing-use product, and each end-use process. Although the U.S. EPA has not explicitly stated that it requires the mouse IP/SC assay for baculovirus preparations manufactured in vivo, it has required it for products that were assessed jointly with Canada under the North American Free Trade Agreement (NAFTA) Technical Working Group on Pesticides.

The EU conducted an extensive review of all publicly available information relevant to safety assessments of baculoviruses, including information on the issue of microbial contaminants (REBECA 2006, 2007). The particular issue of microbial contamination in baculovirus preparations is complicated by the unique production methods of these products. Viral preparations are produced in vivo using unsterile insect larvae which makes it impossible to exclude the larvae's natural microflora during manufacturing. For example, the spore-forming bacterium *Bacillus cereus* is part of the intestinal flora of *Cydia pomonella* larvae, and consequently, *Cydia pomonella* granulovirus (CpGV) preparations regularly contain *Bacillus cereus*. Overall, REBECA concluded that separation of the virus from contaminants during manufacturing is not feasible, and consequently, contamination in baculovirus preparations cannot be avoided. Fortunately, since viruses cannot grow on artificial media, standard methods for detection of contaminants are well-suited for monitoring. The maximum contamination levels that have been accepted by REBECA for baculovirus preparations are presented in Table I of Appendix II.

### **Proposed microbiological specifications in baculovirus-based pest control products manufactured in vivo**

Authorities generally agree that microbial contamination presents an inherent hazard associated with baculovirus-based products, and consequently, microbial screening should be required to ensure that the products meet certain microbiological specifications. When assessing the risk of contaminants in a baculovirus-based product, it is important to consider whether its use pattern includes food crops for which contamination may result in significant levels in/on the treated food commodities and to consider that pre-harvest intervals are generally not required and that the product may be applied right up to the day of harvest.

Regulatory authorities must also consider requiring a mouse IP/SC assay to detect the presence of primary mammalian pathogens in baculovirus-based products. Depending on the quality assurance programme in the manufacturing process, the likelihood of contamination by primary human and mammalian pathogens may be low, but if present the risk to humans can be very high. The risk increases for products with direct

food uses and for products applied aurally to communities. Therefore, authorities should consider requiring a mouse IP/SC assay for baculovirus-based products that are to be applied directly to food crops or if the product is to be applied aurally over inhabited areas (e.g., in urban forest or park pest management programmes). Even if food uses or aerial application is proposed for a baculovirus product, the mouse IP/SC assays may not be necessary. Regulatory authorities should consider the potential presence of unknown pathogens in the product based on the hygienic practices of the manufacturing site, including the types and levels of contaminants present in the insect colonies used to produce the baculovirus. If the manufacturer can demonstrate through contaminant screens on selective media that contamination of the product is low and the contaminants are well characterised, then the mouse IP/SC assay would be unwarranted. If, however, the baculovirus is produced in such a way that there is high batch-to-batch variability and the end-use product may contain unknown contaminants (i.e., not likely to be detected using screens in Table 1.2), then requiring the mouse IP/SC assay would be appropriate. In Canada, two sawfly baculovirus products are manufactured using infected insect larvae collected from the wild rather than in laboratory-reared larvae. Because the potential for unknown contaminants is high in these products, Health Canada's PMRA requires that each batch pass the mouse IP/SC assay before it is formulated and released for use.

Based on these considerations, the proposed microbiological specifications for baculovirus-based products are presented in Table 1.2.

**Table 1.2: Proposed OECD microbiological contamination screening requirements for baculovirus-based pest control products manufactured in vivo**

Type of indicator	Indicator	Limit	Rationale
Severe pathogen	<i>Salmonella</i>	Absence in 25 g  or 25 mL	<ul style="list-style-type: none"> <li>- U.S. EPA and Health Canada requirement</li> <li>- many standard methods available</li> <li>- often used in the food industry</li> </ul>
	<i>Vibrio</i>	Absence in 25 g  or 25 mL	<ul style="list-style-type: none"> <li>- U.S. EPA test guideline requirement, therefore testing is mandatory for U.S. registration/authorisation</li> <li>- not endemic in many countries</li> <li>- basic food handling precautions/personal hygiene habits exclude these organisms during manufacturing</li> <li>- isolation of specific species or pathogens (e.g., <i>Vibrio cholerae</i>) is NOT recommended unless laboratory follows appropriate biohazard protocols</li> </ul>



Type of indicator	Indicator	Limit	Rationale
			<ul style="list-style-type: none"> <li>- optional requirement and recommended ONLY if there is a high potential for contamination or if species of <i>Vibrio</i> are known to naturally occur at the geographical location of the manufacturing site</li> </ul>
	<i>Shigella</i>	Absence in 25 g or 25 mL	<ul style="list-style-type: none"> <li>- U.S. EPA test guideline requirement, therefore testing is mandatory for U.S. registration/authorisation</li> <li>- not endemic in many countries</li> <li>- most commonly related to undercooked shellfish rather than manufacturing processes; basic food handling precautions/personal hygiene habits exclude these organisms during manufacturing</li> <li>- isolation of specific species or pathogens (e.g., <i>Shigella dysenteriae</i>) is NOT recommended unless laboratory follows appropriate biohazard protocols</li> <li>- optional requirement and recommended ONLY if there is a high potential</li> </ul>

Type of indicator	Indicator	Limit	Rationale
			for contamination or if species of <i>Shigella</i> are known to naturally occur at the geographical location of the manufacturing site
Moderate with limited spread	<i>Bacillus cereus</i>	10 <sup>7</sup> CFU/ g or mL	- part of natural intestinal microflora of insect larvae
	<i>Staphylococcus aureus</i>	Absence in 1 g or mL	- indicator of contamination due to improper handling - many standard methods available
Indicators of human, fecal or environmental contamination	Total coliforms	< 100 CFU/g or mL	-optional requirement if host insect larvae are laboratory reared and rearing facility is able to maintain a reasonably hygienic colony where likelihood of contamination by non-fecal coliforms is low or limit for fecal coliforms/ <i>E.coli</i> is satisfied
	<i>Escherichia coli</i>  <b>OR</b>	Absence in 1 g or mL	- indicator of fecal contamination - recent health concern involving contaminated fruits/vegetables; certain sensitive sub-populations are particularly at risk

Type of indicator	Indicator	Limit	Rationale
	Thermotolerant (fecal) coliforms	< 10 CFU/g or mL	<ul style="list-style-type: none"> <li>- can survive/multiply on plants and in soil and water</li> <li>- many standard methods available</li> </ul>
	Total aerobic bacteria (mesophiles)	10 <sup>8</sup> CFU/g or mL	<ul style="list-style-type: none"> <li>- indicator of aerobic bacterial contamination</li> <li>- often used in the food industry</li> <li>- many standard methods available</li> </ul>
	Yeast and Mould Count	Visually monitored <sup>a</sup>	<ul style="list-style-type: none"> <li>- many standard methods available</li> <li>- optional requirement recommended ONLY if screening results for other hygiene indicators suggest possible presence of yeasts or moulds</li> </ul>
Other tests (case-by-case basis)	Mouse IP/SC assay	No evidence of infection or injury in test animals	<ul style="list-style-type: none"> <li>- required by U.S. EPA and Health Canada if microbiological contamination at the insect rearing facility is high and not well characterised or if baculovirus is produced in such a way that there is high batch-to-batch variability and may contain unknown contaminants which may not be readily detected on microbe-specific media (e.g., baculovirus is produced in wild</li> </ul>

Type of indicator	Indicator	Limit	Rationale
			larvae) - optional requirement; unnecessary if intravenous/intraperitoneal (IV/IP) toxicity and pathogenicity (infectivity) test data on the pre-formulated product shows no positive results for toxicity

<sup>a</sup> evaluation based on levels that occur

### ***Rationale for the proposed microbiological specifications of baculovirus preparations***

Deletion of screening for fecal streptococci and enterococci: Screening for fecal streptococci and enterococci is unnecessary as other indicators of human pathogens and fecal contamination remain part of the screening requirements (e.g., fecal coliform/*Escherichia coli*, *Staphylococcus aureus*). Despite the fact that these bacteria are unique indicator organisms in that they are facultatively anaerobic, grow at higher temperatures (45°C), and resist freezing enterococci counts in foods are not considered a reliable index of fecal contamination and many media that have been proposed for the selective isolation and enumeration of enterococci have definite shortcomings. Therefore screening for these organisms would present an unnecessary burden to registrants/notifiers and is not recommended.

Inclusion of *Bacillus cereus* and deletion of screening for other aerobic spore-formers: The proposed microbiological specifications include screening for the aerobic spore-former, *Bacillus cereus*. Since this organism is most likely to be the dominant aerobic spore-forming contaminant in baculovirus-based products, it should be an adequate indicator of these bacteria. Also, due to the resistance of their spores to environmental factors such as temperature extremes and UV light, this group of organisms is ubiquitous in the environment, particularly in soil. Contamination of microbial pest control products by other aerobic spore-formers is not likely to considerably increase exposure to humans or the environment. Based on this information, routine screening for these organisms would present an unnecessary burden to applicants/notifiers and is not recommended.

Most methods for isolating and enumerating *Bacillus cereus* require additional confirmatory tests for distinguishing presumptive isolates from other species in the *Bacillus cereus*-group, including *Bacillus mycoides*, *Bacillus thuringiensis* and *Bacillus anthracis*. Health Canada's Compendium of Analytical Methods (MFLP-42) and the U.S. FDA's BAM (Chapter 14) describe such methods. Additional immunological and molecular methods have been developed to confirm presumptive isolates as *Bacillus cereus*. Two commercial kits based on antibody detection are available for detecting HBL and NHE. The BCET-RPLA kit from Oxoid Ltd. (UK) detects the L2 component of HBL, while the TECRA-BDE kit (Tecra International Pty Ltd., Australia) detects the A component of NHE (Stenfors Arnesen et al. 2008). For nonspecific detection of enterotoxins, Vero cell or Chinese hamster ovary cells have been used to assess cytotoxicity of strains (Damgaard et al. 1996, Hsieh et al. 1999). Furthermore, several PCR methods have been developed to detect the genes involved in enterotoxin production (Stenfors Arnesen et al. 2008). For detection of the emetic toxin, a boar sperm bioassay has been developed for screening strains. The toxin can also be identified by the more costly HPLC-MS analysis and the genes can be detected using PCR-based techniques (Stenfors Arnesen et al. 2008). However, for rapid screening purposes of microbial pest control products, a highly selective chromogenic plating method was developed and is commercially available for enumerating *Bacillus cereus* (Tallent et al., 2012).

Further clarification on the proposed limit of  $10^7$  CFU/g or mL of *Bacillus cereus* in baculovirus preparations is warranted given the ubiquity and elevated levels of this bacterium in the larval gut of certain insect species used in the manufacture of baculoviruses (e.g., CpGV). *Bacillus cereus* is also frequently isolated as a contaminant of various foods. The consumption of foods that contain more than  $10^5$  CFU/g may result in food poisoning. In Germany, for example, the contamination limit for *Bacillus cereus* in food items is  $10^3$  for baby food to  $10^5$  CFU/g for other foods. *Bacillus cereus* is a predominant contaminant in baculovirus products containing CpGV achieving levels of  $10^7$  CFU/g of baculovirus product. It is not possible to reduce the contamination of *Bacillus cereus* without reducing the viability of the active ingredient at the same time. If rejecting authorisation or registration of a baculovirus product on the basis of such elevated contaminant levels for *Bacillus cereus* is unreasonable or undesirable, then authorities can refine the human health risk assessment. Following a worst-case scenario of multiple seasonal applications of CpGV product to apples, the residue levels of *Bacillus cereus* on apples can be estimated. For example, one hectare of orchard with 9 applications (3 applications on each of 3 generations of *Cydia pomonella*) of 100 mL of CpGV product

contaminated with  $10^7$  CFU/mL of *Bacillus cereus* will result in an overall contamination over the entire season of  $9 \times 10^9$  CFU/ha/season. Assuming that the surface of a standard apple is  $200 \text{ cm}^2$  and the apple has a weight of 150 g, contamination with *Bacillus cereus* would be  $1.2 \times 10^2$  CFU/g apple, or 10 times lower than the limit established for baby food. Estimation of residue levels under field conditions can be refined still if one considers the effect environmental factors have on the viability of *Bacillus cereus* such as UV irradiation, ambient temperatures and relative humidity.

## Discussion on the amount of batch data required for microbial contamination assessments

The following points were considered with respect to the amount of batch data required on microbial contaminants:

A detailed description of the manufacturing methods is a compulsory requirement of any submission package/dossier. As the potential for microbial contamination in microbial pest control products is directly dependent on the production method and formulation of the product, this description should provide ample information to make an assessment of whether a risk for contamination exists, and whether that risk is higher than that in the food industry or in handling of other organic materials. For example, some bacterial-based and many fungal-based pest control products are manufactured under strict sterile conditions which virtually excludes extraneous contamination during production, with the possible exception of post-culture harvesting and formulating steps. In such cases, requiring the full battery of contaminant screening tests described in this issue paper would understandably present an unreasonable burden to applicants/notifiers and consideration could be given to reducing the number of contaminants to be screened.

Authorities could reduce the amount of contaminant screening required for commercial production to organisms which are actually likely to be present in the product if the applicant/notifier is able to clearly demonstrate that certain microbes in Table 1.1 or Table 1.2 are consistently (e.g., using results from five-batch analyses) absent in their manufactured product. Authorities may also wish to consider requiring testing on a scheduled basis (i.e., on every so many production runs) rather than on each batch of product if the applicant/notifier can reasonably demonstrate their QA/QC programme is consistently of a high standard.

Furthermore, it is in the best interest of the manufacturer to exclude or minimize contamination to ensure that each production lot successfully satisfies the quality control criteria and reaches the marketplace. Experience has shown that, in general, manufacturers follow standard industry procedures to limit contamination during the production process consistent with good hygienic practices (and HACCP principles) in the manufacture of foodstuffs. Therefore, if such practices are routinely followed then contamination during manufacturing will likely be kept to a minimum. Here, the mandatory batch data serves to confirm that the quality control measures are adequate at limiting microbial contamination, and also establishes the variability between batches.

Potential sources of microbiological contamination include manufacturing equipment, growth media constituents, ambient air, and the water supply as well as the added formulation ingredients and post-manufacture handling. While low levels of contaminating microbes may be unavoidable, this is not considered problematic as long as growth of the contaminants is suppressed. Manufacturers may adopt certain measures to avoid growth of contaminating micro-organisms in order to maintain product quality and ensure an adequate shelf-life. For example, some products are dried to reduce the water activity below a level which allows growth of micro-organisms thereby ensuring that levels of contaminants do not increase after packaging. Similarly, liquid formulations may be stabilized by lowering the pH, or by keeping them cold to avoid fungal and bacterial growth. Alternatively, preservatives can be added, although this practice could exclude the formulation from use in organic agriculture. Growth of aerobic micro-organisms is also prevented when products are formulated or packed in oxygen-limiting conditions.

As noted, the addition of formulation ingredients is recognised as a possible source of contamination during manufacture. It has been suggested that an analysis of the microbial contaminants of the formulating ingredients could accompany registration documents to ensure that the formulation ingredients are uncontaminated.

Based on the above-noted considerations, microbiological contamination in microbial pest control products is not generally expected to present an unreasonable risk to human health (or to the environment) if applicants/notifiers can demonstrate to regulatory authorities that their products conform to the specified limits for quality. Submitting the results from analysis of five representative batches for microbial contaminants (as listed in Table 1.1 or Table 1.2) should suffice to confirm adequate quality assurance measures have been put in place during the manufacturing process to prevent or minimize contamination. Authorities may, however, request additional action be taken, based on the review of the manufacturing methods and quality control measures, use patterns, and exposure scenarios associated with the product. Should microbial contamination present a concern, various regulatory options may be considered to help mitigate those concerns. For example, additional batch monitoring data may be requested of the manufacturer, or additional quality control measures may be implemented to the manufacturing process, or an expiration date of the product could be required on the product label. In some jurisdictions continued screening for contaminants in commercial production batches may be required as a condition of registration/authorisation to ensure adherence to product quality standards.

## Other important considerations for determining acceptance limits for microbial contaminants

### ***Dietary exposure***

The risk posed by potential contamination must also be considered, starting with the level of dietary and occupational/by-stander exposure to microbial pest control products. Since microbial pest control products are not intended for direct consumption by humans, dietary exposure to microbial pest control products (and to potential contaminants therein) is limited mainly to the indirect exposure to residues left on edible commodities post-application. Certain use patterns, however, would be reasonably expected to result in higher dietary exposure to these organisms. For example, products with direct food applications up to the day of crop harvest would have a higher potential for dietary exposure, than for example, a soil-incorporated product. On the other hand, the use pattern of a microbial pest control product may not include food uses in which case the risk to consumers from potential contaminants is reduced even further since dietary exposure is not a factor.

### ***Occupational and by-stander inhalation exposure***

Occupational exposure to microbial pest control products (and potential contaminants therein) may arise during manufacturing/formulating, and during mixing/loading/application of the microbial pest control product. Manufacturers generally follow standard quality control measures to reduce the likelihood of human contamination during manufacturing (i.e., personnel wear gloves, masks, coveralls; follow basic personal hygiene, etc.) which equally limits exposure to potential contaminants in the production line. Similarly, pesticide workers may be required to wear personal protective equipment, including a dust/filtering mask, for all or some mixing/loading/application activities which significantly reduces exposure. It is expected that these precautions will adequately mitigate the risks from occupational exposure to any low levels of potentially harmful contaminants. Unless the formulation is comprised of fine particulates/dusts (e.g., dry powder), granular or suspension concentrates will not pose an inhalation risk to mixers and loaders. Granular formulations (i.e., comprised of large particles) would be the only formulation type that would not pose a direct inhalation risk to applicators during application. For application

of other formulations where fine mists/aerosols/particles are generated inhalation by applicators not wearing suitable protective equipment (respirator/dust mask) is possible. Diluted product applied with equipment not generating fine mists/aerosols or particulates will undoubtedly pose a lower risk to applicators through the inhalation route as it is unlikely that such air-suspended particles will reach the lungs but rather will be trapped in mucous. If this mucous is not expectorated but is instead swallowed it is unlikely to pose any more risk to the applicator than ingesting freshly treated produce. If acceptance limits for microbial pest control products are established based on food safety criteria, then additional risk through unintentional or accidental inhalation is not expected to be of concern.

It is also worthwhile considering the inhalation risk posed by the application of microbial pest control products contaminated by other micro-organisms relative to background air levels of micro-organisms in both agricultural and non-agricultural areas.

Above land surfaces, almost 25 % of the total airborne particulate matter may be made up of biological materials (Jones & Harrison, 2004). Brodie et al. (2007) demonstrated by a comprehensive molecular analysis of airborne bacteria, that urban aerosols contained at least 1,800 diverse bacterial types, a diverse assemblage of micro-organisms representing the amalgamation of numerous point sources.

A review of the literature on microbial load in air is hampered by the sampling methods used and the growth media used to establish the CFU/m<sup>3</sup>. Many of the studies are specific for certain kinds of bacteria or fungi, or moulds. Thus, it is important when trying to establish microbial loads of air to carefully evaluate how much of the microbial airborne load is actually being depicted. Any of these specific methods will give a lower limit to the total bioburden measured.

A review of the literature clearly indicates that airborne microbial load is actually higher in dwellings and buildings than it is outside. Humans themselves have been shown to increase the microbial loads in buildings. The literature indicates that human populations are routinely exposed to a large and varied airborne microbial load.

#### *Agricultural uses of microbial products*

To determine if the proposed microbiological contaminant limits for microbial products present inhalation (respirable) risks to operators and bystanders it is important to first consider typical airborne levels of micro-organisms in agricultural and greenhouse settings.

Lis et al. (2008) found that in farmhouses (defined as residential buildings on farms), the concentration of indoor bacterial aerosols ranged from 587 to 9752 CFU/m<sup>3</sup> (mean 3235 CFU/m<sup>3</sup>), and respirable fractions from 325 to 4176 CFU/m<sup>3</sup> (mean 1759 CFU/m<sup>3</sup>). In urban dwellings, the corresponding values were in the range of 271 to 4858 CFU/m<sup>3</sup> (mean 1792 CFU/m<sup>3</sup>) and 218 to 2088 CFU/m<sup>3</sup> (mean 1013 CFU/m<sup>3</sup>), respectively.

Radon et al. (2002) examined microbial loads in air in animal houses in Switzerland and Denmark and greenhouses in Spain. In animal houses sampled for 30 or 60 minutes, the total bacteria counts ranged between  $4 \times 10^8$  and  $4 \times 10^9$  CFU/m<sup>3</sup>. In greenhouses, bacterial levels were at  $1.5 \times 10^7$  CFU/m<sup>3</sup> after 64 minutes of sampling.

Blomquist et al. (1987) described airborne fungal spores of more than  $1 \times 10^8$  cells per m<sup>3</sup> in pig houses, but only between  $1 \times 10^3$  –  $1 \times 10^5$  cells per m<sup>3</sup> in greenhouses.

Hansen et al. (2010) found that in untreated greenhouses, the highest concentration of total mesophilic bacteria,  $1.1 \times 10^6$  CFU/m<sup>3</sup>, was detected in a cucumber greenhouse. The authors also concluded that there was no significant difference in exposure to mesophilic bacteria between tomato greenhouses and vegetable fields.

Dutkiewicz et al. (2001) examined herb processing plants and found that the values of the respirable fraction of airborne microflora in the facilities studied varied between 14.7 and 67.7%. The dominant micro-



organisms in the air were mesophilic bacteria, among which endospore-forming bacilli (*Bacillus* spp.) and actinomycetes of the species *Streptomyces albus* were most numerous.

#### *Estimated contaminant concentrations in air from applied product*

Like other pest control products, microbial products are formulated such that when they are sprayed, droplets will land on the targeted crop and not linger as respirable fractions in air. Hansen et al. (2010) measured mesophilic bacterial counts in greenhouses that were sprayed with *Bacillus thuringiensis* and concluded that the already present airborne bacteria in the greenhouses might have a greater influence on growers' health than the applied biocontrol strains.

Continuing with *Bacillus thuringiensis* as an example, the additional airborne loading of contaminants can be calculated. First, assume the product contains the limit for bacterial contamination (see Appendix I, Table I), i.e., streptococci/enterococci at  $10^5$  CFU/g and coliforms at  $10^3$  CFU/g, and is applied at a maximum application rate of 1 kg/ha (equivalent to  $0.1 \text{ g/m}^2$ ). Second, assume a **worst case scenario** where all the microbial contaminants from the applied product remain in the air and conservatively estimate a  $2 \text{ m}^3$  minimum air volume a person would be surrounded by (1 square meter by 2 meters high). Thus, a  $0.1 \text{ g/m}^2$  application would give a theoretical maximum contaminant concentration within the breathing air volume of about  $5000 \text{ CFU/m}^3$  [ $(100,000 \text{ CFU/g of product} \times 0.1 \text{ g applied product}) \div 2 \text{ m}^3 \text{ breathing air volume} = 5000 \text{ CFU/m}^3$ ]. Assuming further that all the bacterial contamination in the product is respirable and available to the lungs, the levels expected to be found in air would be in line with a normal exposure scenario in an agricultural setting.

# **5** **Conclusions for microbial pest control products**

The proposed OECD microbial contamination screening requirements (Table 1.1 and Table 1.2), which employ standard methods from the food industry, provide practical microbiological specifications for microbial pest control products, and will provide meaningful data to assess the overall acceptability of microbial pest control products without posing an unreasonable burden on applicants/notifiers. Authorities can be reasonably assured that products which meet these microbiological specifications do not contain microbial contaminants which will pose a risk to human health or to the environment.

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## Appendix I - The following are excerpts from Health Canada's existing guidance document on acceptable limits for contaminating micro-organisms in microbial pest control products.

### Health Canada Limits on Contaminating Micro-organisms

Please note that for both the mouse intraperitoneal (IP)/subcutaneous (SC) batch analysis and the contaminant screening, the test substance may be prepared on a small or pilot scale but must be produced in a manner identical to that intended for large-scale batch productions (i.e., same fermentation medium, pH, stabilizers, etc.).

#### **Mouse IP/SC Batch Analysis** (For *Bacillus thuringiensis* and baculovirus preparations)

The U.S. EPA's 40 CFR 180.1011 provides guidelines for the mouse IP/SC batch analysis. Each batch of technical active ingredient (prior to the addition of formulation ingredients) must be tested by IP or SC injection of at least  $10^6$  colony forming units (CFUs) into each of five laboratory mice weighing between 17 and 23 grams. The test animals must be observed for 7 days following injection for signs of infection or injury (i.e., any indication of either systemic or localized infectivity or toxicity). Mice should be weighed at the time of dosing and at the end of the 7-day observation period. Animals that exhibit adverse effects or die during the observation period must be necropsied for gross pathological changes.

#### **Contaminant Screening** (For all microbial preparations including *Bacillus thuringiensis* and baculovirus products)

The purpose of using microbe-specific media for contaminant testing is to identify and determine the levels of primary human pathogens and other potential pathogens of concern to human health and safety. Protocols for contaminant testing should be based on guidelines recommended by the International Commission on Microbiological Specifications for Foods (ICMSF), the Association of Official Analytical Chemists (AOAC) or other internationally recognised body.

Screening for potential microbiological contaminants including total aerobes, total coliforms, fecal coliforms, fecal streptococci, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella* spp., and yeast and moulds are considered routine. The following is a listing of the microbiological contaminants and their corresponding allowable limits that have been approved in recently registered microbial pesticide products in Canada.

#### i) *Bacillus thuringiensis* formulations:

Currently, Health Canada has only officially established microbial contaminant limits for *Bacillus thuringiensis*-based formulations (as per a 1988 memorandum to manufacturers issued by Agriculture Canada, the then Canadian federal pesticide regulatory authority).

The limits described in Table I were established in 1988 and are still applied to all formulations, though a number of registrants have voluntarily lowered the target limit for enterococci by one order of magnitude to  $10^4$  CFU/g or mL.

**Table I: Health Canada's microbiological specifications for *Bacillus thuringiensis* formulations**

Selected Organisms Bt Formulations	Target Values (1988)*
<i>Salmonella</i> spp.	0 CFU/25 g or mL*
Streptococci/Enterococci	$10^5$ CFU/g or mL*

Selected Organisms Bt Formulations	Target Values (1988)*
Coliforms (Total)	10 <sup>3</sup> CFU/g or mL*
<i>Clostridium perfringens</i>	monitoring**
<i>Staphylococcus aureus</i>	monitoring**
<i>Pseudomonas aeruginosa</i>	monitoring**

\* Recommended Target Values (a.k.a. bioburden limits, acceptable limits, allowable limits) expressed as colony forming units (CFU) are based on average values for samples taken per lot (batch means).

\*\* Evaluation will be based on levels that occur.

#### ii) Fungal and Actinomycete formulations:

No fixed or pre-set limits for microbiological contaminants have been established by Health Canada for fungal or actinomycete based products. Many registrants have voluntarily set their own acceptance limits for various microbes of concern which have been accepted by Health Canada. However, some registrants have not voluntarily set contaminant limits for their products. In such cases, Health Canada requires that, in the event of microbial contamination, registrants comply with the limits for harmful or pathogenic microorganisms and for the total number of mesophile contaminants in Table II.

**Table II: Health Canada's microbiological specifications for fungal and actinomycete formulations**

Microbiological Contaminant	Limit of Contamination (CFUs/g or mL)
Total mesophiles (aerobic plate count at 30°C)	< 10 <sup>5</sup>
Coliforms	< 100
Fecal coliforms	< 10
Fecal streptococci/enterococci	< 100
Yeasts and moulds	< 1000
Staphylococci	< 100
Salmonellae	Absence in 25 g or 25 mL

These limits of microbiological contamination are comparable to those recognised as safe for food products such as cheese and other milk products. Batches that exceed the acceptable quality limits for contaminants are to be destroyed.

In some cases, registrants of fungal and actinomycete products have voluntarily lowered acceptance limits (Table III) for some contaminant organisms.

**Table III: Registrant proposed limits for fungal and actinomycete formulations**

Test	Allowable Limits
Total aerobes	< 1000 CFU/g or mL
Enterobacteriaceae	< 10 CFU/g or mL
Fecal streptococci/enterococci	Absence in 1 g or mL
Fecal coliforms	Coliforms: < 10 CFU/g or mL
Fecal coli	<i>E. coli</i> : Absence in 1 g or mL
Staphylococci	Absence in 1 g or mL
<i>Salmonella</i>	Absence in 1 g or mL

iii) Baculoviruses (produced in vivo):

For baculovirus preparations manufactured in vivo using insect larvae, Health Canada requires that manufacturers include tests (microbe-specific selection media, and mouse IP assay) to ensure that no potential human pathogens are present in each lot of the final unformulated preparation and that populations of certain other micro-organisms do not exceed acceptable limits as detailed in Table IV.

The presence of any potential human pathogen must result in the destruction of the production lot or batch. The level of extraneous microbial contamination in the final unformulated preparation and the methods that may be used to isolate and enumerate each class of contaminant are indicated in the table below. For most of the contaminants listed, a choice is given between federally-approved Canadian and U.S. analytical methods. These methods are available on-line at Health Canada's HPFB Compendium of Analytical Methods (<http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/index-eng.php>) and at the U.S. Food and Drug Administration's CFSAN Bacteriological Analytical Manual (<http://www.cfsan.fda.gov/~ebam/bam-toc.html>) web sites. For fecal streptococci/enterococci, a summary is given of a reference method that is not available on-line.

**Table IV: Health Canada's microbiological specifications for baculovirus-based products (historical)**

Microbial Contaminant	Contaminant Limit	Suggested Method
Total Aerobic Bacteria (Mesophilic)	< 10 <sup>7</sup> CFU/g or mL	Determination of the Aerobic Colony Count in Foods, MFHPB-18 <a href="http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume2/mfhp18-01-eng.php">http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume2/mfhp18-01-eng.php</a>  <b>OR</b>  Aerobic Plate Count, Chapter 3 <a href="http://www.cfsan.fda.gov/~ebam/bam-3.html">http://www.cfsan.fda.gov/~ebam/bam-3.html</a>
<i>Bacillus cereus</i>	< 10 <sup>6</sup> CFU/g or mL	Determination of <i>Bacillus cereus</i> in Foods, MFLP-42 <a href="http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume3/mflp42-01-eng.php">http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume3/mflp42-01-eng.php</a>  <b>OR</b>  <i>Bacillus cereus</i> , Chapter 14 <a href="http://www.cfsan.fda.gov/~ebam/bam-14.html">http://www.cfsan.fda.gov/~ebam/bam-14.html</a>
Other Aerobic Sporeformers	< 10 <sup>7</sup> CFU/g or mL	Determination of Aerobic and Anaerobic Sporeformers, MFLP-44 <a href="http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume3/mflp44-01-eng.php">http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume3/mflp44-01-eng.php</a>
Fecal Streptococci - Enterococci	Absence in 1 g or mL	1) M-Enterococcus Agar 2) Trypticase Soy Agar  -plate appropriate dilutions of test sample on M-Enterococcus agar and incubate M-Enterococcus plates at 35°C for 48 hours; fecal streptococci will appear as pink or dark red or maroon colonies -streak presumptive fecal streptococci colonies on trypticase soy agar and incubate at 35°C for 24 hours; colonies negative for catalase are confirmed as fecal streptococci (include a positive control culture of <i>S. faecalis</i> )  American Public Health Association, Standard Methods for Examination of Water and Wastewater, 18 <sup>th</sup> Edition, 1992. Method 9230A and 9230C.

Microbial Contaminant	Contaminant Limit	Suggested Method
Total Coliforms  Fecal coliforms/ <i>Escherichia coli</i>	< 100 CFU/g or mL;  Absence in 1 g or mL	Determination of Coliforms, Faecal Coliforms and of <i>E. coli</i> in Foods, MFHPB-19 <a href="http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume2/mfhp19-01-eng.php">http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume2/mfhp19-01-eng.php</a>  <b>OR</b>  Enumeration of <i>Escherichia coli</i> and the Coliform Bacteria, Chapter 4 <a href="http://www.cfsan.fda.gov/~ebam/bam-4.html">http://www.cfsan.fda.gov/~ebam/bam-4.html</a>
<i>Staphylococcus</i>	Absence in 1 g or mL	Enumeration of <i>Staphylococcus aureus</i> in Foods, MFHPB-21 <a href="http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume2/mfhp21-01-eng.php">http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume2/mfhp21-01-eng.php</a>  <b>OR</b>  <i>Staphylococcus aureus</i> , Chapter 12 <a href="http://www.cfsan.fda.gov/~ebam/bam-12.html">http://www.cfsan.fda.gov/~ebam/bam-12.html</a>
<i>Salmonella</i>	Absence in 25 g or mL	Isolation and Identification of <i>Salmonella</i> from Foods, MFHPB-20 <a href="http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume2/mfhp20-01-eng.php">http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume2/mfhp20-01-eng.php</a>  <b>OR</b>  <i>Salmonella</i> , Chapter 5 <a href="http://www.cfsan.fda.gov/~ebam/bam-5.html">http://www.cfsan.fda.gov/~ebam/bam-5.html</a>
<i>Shigella</i>	Absence in 25 g or mL	<i>Shigella</i> , Chapter 6 <a href="http://www.cfsan.fda.gov/~ebam/bam-6.html">http://www.cfsan.fda.gov/~ebam/bam-6.html</a>

Microbial Contaminant	Contaminant Limit	Suggested Method
<i>Vibrio</i>	Absence in 25 g or mL	<p>The Isolation and Identification of <i>Vibrio cholerae</i> 01 and non-01 from Foods, MFLP-72  <a href="http://www.hc-sc.gc.ca/fn-an/alt_formats/hpfb-dgpsa/pdf/res-rech/mflp72-eng.pdf">http://www.hc-sc.gc.ca/fn-an/alt_formats/hpfb-dgpsa/pdf/res-rech/mflp72-eng.pdf</a></p> <p><b>OR</b></p> <p><i>Vibrio cholerae</i>, <i>V. parahaemolyticus</i>, <i>V. vulnificus</i>, and Other <i>Vibrio</i> spp.  <a href="http://www.cfsan.fda.gov/~ebam/bam-9.html">http://www.cfsan.fda.gov/~ebam/bam-9.html</a></p>
Yeasts and Moulds	< 1000 CFU/ g or mL	<p>Enumeration of Yeasts and Moulds in Foods, MFHPB-22  <a href="http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume2/mfhp22-eng.php">http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume2/mfhp22-eng.php</a></p> <p><b>OR</b></p> <p>Yeasts, Moulds and Mycotoxins, Chapter 18  <a href="http://www.cfsan.fda.gov/~ebam/bam-18.html">http://www.cfsan.fda.gov/~ebam/bam-18.html</a></p>

## Appendix II- The following information was extracted from REBECA (2007)

The maximal contamination levels for baculovirus preparations accepted by REBECA at the REBECA Conference in 2006 (REBECA 2007) are presented in Table I. These levels are in accordance with the current state of the art production methods and application rates of virus products.

**Table I: Maximal contamination levels for baculovirus preparations accepted by REBECA<sup>1</sup>**

<b>Microbial contaminant</b>	<b>Accepted contaminant limit</b>
Mesophiles	10 <sup>8</sup> CFU/mL
<i>Bacillus cereus</i>	10 <sup>7</sup> CFU/mL
<i>Escherichia coli</i>	0 in 1 g
<i>Staphylococcus aureus</i>	0 in 1 g
<i>Salmonella</i> spp.	0 in 25 g
Yeasts and moulds	Visually monitored; evaluation based on levels that occur

<sup>1</sup> REBECA (2007)



### Appendix III - Minimum validation criteria to be considered for alternative methods of detection and quantification

The minimum requirement for a validation includes factors such as:

- a) Demonstration of the specificity of the method to the contaminant of interest
- b) Use of an appropriate analytical unit (e.g., a 25 g sample size is required for detection of contaminants for which the limit is "Absence in 25 g")
- c) Use of appropriate positive and negative controls (e.g., not only positive and negative controls for the reaction overall, but for specificity to target organism), where possible.
- d) Demonstration of precision, repeatability, reproducibility, limit of detection, and/or limit of quantification

It should be noted that each key step of the methods (extraction/quantification) should be validated. A pre-enrichment and/or selective enrichment may be needed prior to the testing (as necessary depending on the pathogen).