Introduction and Objective

Microbial pest control agents are increasingly being investigated for use as alternatives to conventional pesticides because they are thought to pose a lower potential risk to human health and the environment, compared with conventional pesticides. These new biological products, however, are not without their unique risks.

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 (e.g., baculoviruses) as hosts. These manufacturing processes are more environmentally sound since few organic solvents or other harsh chemicals are required during manufacturing. However, they all have the potential of producing unwanted microbial organisms in addition to the desired microbial pest control agent. Depending on the growing conditions, these unwanted or contaminating microorganisms could include pathogens, and toxins or other toxic metabolites derived from contaminating microorganisms. As a result, a contaminated microbial pest control product could pose a risk if it is applied over populated areas, habitats frequented by susceptible non-target organisms or other sensitive areas (e.g., drinking water sources).

Many regulatory authorities have recognized the risk posed by contaminating microorganisms 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. 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 since most regulatory authorities provide little guidance. Consequently, applicants/notifiers might encounter different regulatory requirements that could ultimately delay or prevent registration/authorization. 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.
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 harmonization. This issue paper focuses only on regulatory requirements for Canada, the U.S. and the EU.

In Canada, Health Canada’s Pest Management Regulatory Agency (PMRA) establishes data requirements for microbial pest control products. Data requirements, including those for manufacturing and quality assurance, are outlined in Regulatory Directive, DIR2001-02. 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. The PMRA 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 agent 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 Regulatory Directive recommends that suitable indicator organisms be routinely monitored in production samples to assess the hygienic state of the production facility and manufacturing process. Potential microbial hazards 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 5 production or pilot-scale batches are required.

U.S. regulatory requirements for microbial pest control products are currently outlined in 40 CFR 158.740. Please note that new data requirements have been proposed by the U.S. EPA (see Federal Register Vol. 71 No. 45, March 8, 2006), however, the data requirements relating to manufacturing and quality assurance remained unchanged. Details on these requirements are provided in two separate test guidelines, namely U.S. EPA Microbial Pesticide Test Guidelines OPPTS 885.1200, and 885.1300. According to these 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 an analysis of all human or animal pathogens that might be present at potentially hazardous levels in unformulated product and should propose methods to detect and/or eliminate them from the unformulated product.

In the EU, data requirements for plant protection products and biocides are outlined in Commission Directives 1991/414/EEC and 1998/8/EC, respectively. Both documents have similar requirements but there are some important differences in quality control requirements. Both Commission Directives require detailed information on how the microbial pest control agent is produced, and the methods to ensure the integrity of the active ingredient and the microbiological purity of the final product. Both regulatory documents require a detailed analysis of composition of the final product, including the identity of microbial contaminants if possible and appropriate. Products should be free from microbial contaminants, if possible; otherwise,
they should be controlled to acceptable levels. The acceptable levels are not specified in either
document; however, according to Commission Directive 1991/414/EEC, the nature and
acceptable levels of contaminants should be judged from a risk assessment point of view and be
established by the competent authority. Commission Directive 1991/414/EEC also states that
both production and product must be subject to continuous quality control by the
applicant/notifier to monitor contaminating microorganisms and the integrity of the microbial
pest control agent. All techniques must be described and specified.

**International Microbiological Specifications in Food and Drinking Water**

Much 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 organizations for
food and drinking water could be adapted for establishing acceptable contaminant limits in
microbial pest control products.

The presence of microorganisms 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 sterilized. Some of these contaminating microorganisms 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 organizations such as the International
Commission on Microbiological Specifications for Foods (ICMSF) and the Joint FAO/WHO
Codex Alimentarius Commission 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.

Microorganisms as components of microbiological specifications in food and water can
be grouped into two categories: pathogens and indicator organisms. According to the
Subcommittee on Microbiological Criteria (1985), suitable pathogens are those likely to be found
in the commodity, which thereby becomes a potential vehicle for its transmission to consumers.
Suitable indicators are those whose presence in food or water indicates (Subcommittee on
Microbiological Criteria 1985):

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

Food-borne microbial pathogens have been classified in one of three categories based on hazard
(Subcommittee on Microbiological Criteria 1985, ICMSF 1986), namely: i) severe hazards; ii)
moderate hazards with potentially extensive spread; and iii) moderate hazards with limited
spread. The microorganisms listed as moderate hazards generally cause illnesses that are milder
than those listed in the severe hazards group.
Pathogens

a. Severe hazards (Subcommittee on Microbiological Criteria 1985, ICMSF 1986, ICMSF 1978)

i. *Brucella melitensis, Brucella abortus, Brucella suis,* and *Mycobacterium bovis*

Brucellosis and tuberculosis are serious diseases often resulting in long-term illnesses and serious 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. Apparently, the methods for detecting *Brucella* and *Mycobacterium* are insensitive, time-consuming and are generally unsuited to routine screening. Furthermore, brucellae are highly infectious, even in very small numbers. Laboratory personnel can contract the disease while working with these organisms, and such work should only be carried out in specially constructed laboratories with appropriate containment facilities.

ii. *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 whose spores are heat-resistant. The disease is characterized 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.

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

*Salmonella* species produce one of two types of clinical symptoms: i) typhoid and paratyphoid fevers caused by *Salmonella typhi* and *Salmonella paratyphi* A, B and C; or ii) enteric infections caused by other *Salmonella* species. Typhoid and paratyphoid fevers are characterized by septicemia without enteritis. All *Salmonella* are considered pathogenic to humans and standard methods are available for routine testing.

iv. *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 characterized 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 subculturing onto a variety of selective media.

v. *Vibrio cholerae*
*Vibrio cholerae* causes an acute diarrheal 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 cholera* in foods involves an enrichment procedure followed by subculturing onto various selective media. Suspect colonies are verified using biochemical and serological procedures and other tests, including a mouse adrenal cell assay. Routine testing for *Vibrio cholera* is not practical as detection methods are too insensitive and time-consuming.

b. Moderate hazards with potentially extensive spread (Subcommittee on Microbiological Criteria 1985, ICMSF 1986, ICMSF 1978)

i. 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 have been linked in the past 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.

ii. 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. Methods for the recovery of *Escherichia coli* and pathogenic biotypes are available, but the standard enrichment and plating methods for enumerating *Escherichia coli* that are routinely applied in the food industry cannot differentiate pathogenic biotypes from other *Escherichia coli*. Recognition of pathogenic *Escherichia coli* requires serological and physiological tests as well as pathological tests with laboratory animals. Routine testing for pathogenic biotypes is impractical.

iii. *Salmonella typhimurium* and other *Salmonella* serovars

As noted in the severe hazards section, *Salmonella* produce one of two types of clinical symptoms: 1) typhoid and paratyphoid fevers caused by *Salmonella typhi* and *Salmonella paratyphi* A, B and C; or 2) enteric infections caused by *Salmonella typhimurium* and other *Salmonella* serovars. Enteric infections are characterized by fever, diarrhea, intestinal cramps and vomiting. All *Salmonella* species are considered pathogenic to humans and standard methods are available for routine screening.

iv. *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, however, 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.

c. Moderate hazards with limited spread (Subcommittee on Microbiological Criteria 1985, ICMSF 1986, ICMSF 1978)

i. *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 are reported from this organism, but this observation may be due to misdiagnosis since symptoms resemble those of other microorganisms. Furthermore, *Bacillus cereus* is often regarded as harmless since it is ubiquitous in many environments. Methods for the detection and enumeration of *Bacillus cereus* are apparently available to the food industry, but few include additional steps to distinguish it from other species in the Bacillus cereus-group such as *Bacillus thuringiensis*.

ii. *Campylobacter fetus* subsp. *jejuni*

This microorganism is a common cause of gastroenteritis in humans. Methods for detecting *Campylobacter fetus* subsp. *jejuni* are available, but its accurate detection requires: 1) large sample sizes; 2) suitable microaerophilic conditions; 3) selective broth enrichment; 4) filtration to separate the small filterable campylobacters from other non-filterables in the enrichment cultures; and 5) selective agar plating. Routine analysis is not practical in the food industry.

iii. *Clostridium perfringens* type A

*Clostridium perfringens* is one of the most common causes of enteritis. The spores of this microorganism 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 which increases stomach pH and therefore survivability of the cells. Small numbers of this microorganism are unavoidable in foods, but its presence in large numbers may be indicative of poor sanitary conditions or mishandling of food by workers. Illnesses are caused by an enterotoxin that is released in the intestines when the microorganism undergoes sporulation and is characterized by diarrhea and abdominal pain. Standard methods are available from the Association of Official Analytical Chemists (AOAC) and the International Organization of Standardization (ISO).

iv. *Staphylococcus aureus*

Illnesses caused by this microorganism are attributed to several heat-stable enterotoxins produced by certain strains of this species. Nausea, vomiting, diarrhea, general malaise and weakness characterize the disease. The presence of enterotoxins is the principal concern rather than the organism itself. Small numbers of *Staphylococcus aureus* are to be expected in foods that have been exposed to or handled by food handlers, but small 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.
v. Vibrio parahaemolyticus

Illnesses by this microorganism are often erroneously identified as salmonellosis or dysentery. Illness usually begins with a violent epigastric pain accompanied by nausea, vomiting, and diarrhea. 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 the method for identifying pathogenic strains is complicated. Routine analysis is not recommended in the food industry.

vi. Yersinia enterocolitica

This microorganism typically causes gastroenteritis and terminal ileitis. Methods for the recovery of Yersinia enterocolitica are available, however, no single method is suitable for the recovery of all types of this species from various substrates. Since not all strains are pathogenic, isolates must be tested for pathogenicity. Routine analysis is not recommended in the food industry.

d. Other pathogens considered in drinking water quality (World Health Organization 2002)

i. Aeromonas

Mesophilic species of this genus have been implicated in a wide range of infections (wounds, respiratory tract, 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.

ii. Legionella

Legionnaires’ disease is type of pneumonia caused by many serogroups of Legionella pneumophila (most common), Legionella micdadei and many other species of Legionella. Legionnaires’ disease is frequently accompanied with extrapulmonary manifestations such as renal failure, encephalopathy, and pericarditis. The International Organization of Standardization has prepared a standard procedure for the isolation, culture and identification of Legionella species. The difficulty and complexity of this method was not provided. Continuous monitoring of Legionella is only advocated when antimicrobial measures must be verified.

Indicator Microorganisms

The use of pathogens in microbial specifications is limited and 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 1997). As a result, many microbial specifications in water and food make use of indicator organisms or agents. These groups of indicators typically fall in one of four categories (Subcommittee on Microbiological Criteria 1985): i) those that assess microbial numbers or activity; ii) indicators of potential human pathogens or fecal contamination; iii) indicators of post heat processing contamination (mainly
food-related); and iv) metabolic products of pathogens that indicate the presence of a pathogen
(not discussed in this issue paper).

a. Assessment of microbial numbers and/or activity (Subcommittee on Microbiological Criteria

i. Aerobic plate count (APC)

The aerobic plate count 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 clumps of cells will form visible colonies 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. Although
this test can potentially measure a large population of a sample, it only measures the
fraction of living microorganisms that is able to produce colonies on the given medium
and growth conditions. Unexpected high values for this indicator may be attributed to
contamination so 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).

ii. Anaerobic plate count

The practice of using aerobic plate counts instead of anaerobic plate counts developed
because it was much easier to incubate under aerobic conditions. Anaerobic growth
chambers are now available to measure obligate anaerobes such as clostridia and
facultative anaerobes such Enterobacteriaceae, enterococci and staphylococci.

iii. Thermoduric, psychrotrophic, thermophilic, proteolytic and lipolytic counts

Minor modifications can be made to the APC to enumerate specific groups of
microorganisms. These counts are usually used to measure microbial activity under
specific circumstances. For instance, thermoduric 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 microorganisms 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.

iv. Direct microscopic counts (DMC)

Direct microscopic counts 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 microorganisms, 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 microorganisms and the small number
of examined samples usually limits its precision.

v. Microscopic mould counts (MMC)
Microscopic mould counts 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.

vi. 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 become predominant when bacterial growth is less favourable such as lower water activity, low pH, high salt, or high sugar content. Satisfactory methods are available and use either acidified media or media with added antibiotics to inhibit bacterial growth (APHA 1976, 1978, 1984).

vii. Heat-resistant moulds

Some moulds such as *Byssochlamys fulva* and *Aspergillus fischeri* 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).

viii. Thermophilic spore count

The canning industry often monitors the quality of ingredients such as sugar, starch, flour, spices, mushrooms, nonfat 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 sporal heat resistance and their ability to grow in foods held at elevated temperatures. Methods to determine these spores are available (AOAC 1980, APHA 1976 and 1984, NCA 1968).

b. Indicators of potential human pathogens or fecal contamination

i. 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 or handled by workers. Large numbers of staphylococci may be indicative of the presence of toxins. Methods for the detection and enumeration of *Staphylococcus aureus* are available for routine analysis (AOAC 1980, APHA 1976 and 1984).

ii. *Escherichia coli*

*Escherichia coli* conforms 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 microorganisms 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 because it is time consuming, costly, and inhibitory to injured cells.

iii. Thermotolerant (fecal) coliforms

Fecal coliforms are a group of organisms selected by incubating an inoculum derived from a coliform enrichment broth at higher temperatures (44–45.5°C) than those used for incubating coliforms. 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, and 1984), 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.

iv. 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 feces 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. Many media have been proposed (APHA 1976, 1984; ICMSF 1978) for the selective isolation and enumeration of enterococci, these have definite 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.

v. *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is a common contaminant of the environment (including water) and enters the environment with fecal wastes of humans or of animals associated with humans. This microorganism is an opportunistic pathogen and may be of particular concern for infants who may become infected when contaminated water or equipment is used to make baby formulas. In some countries, this organism is used as an indicator of
contamination in bottled water. Methods for detecting \textit{Pseudomonas aeruginosa} are described by APHA (1981).

c. Indicators of post-heat processing contamination

i. Coliform bacteria

Coliform bacteria are members of the \textit{Enterobacteriaceae} that are capable of fermenting lactose with the production of acid and gas within 48 hours at 35°C. Some coliforms (e.g., \textit{Escherichia coli}) are common in feces of humans and other animals, but others are commonly found in soil, water, and grains (e.g., \textit{Enterobacter}, \textit{Klebsiella}, \textit{Serratia}, \textit{Erwinia}, \textit{Citrobacter}, and \textit{Aeromonas}). Small numbers of these microorganisms are normal in many food commodities and are considered to be of limited value in monitoring food quality. Coliforms are easily killed by heat therefore their usefulness is limited to contamination following heat processing. Its use in other areas requires a thorough understanding of the production, processing, and distribution practices to which a commodity is subjected. Methods for enumerating coliform bacteria are available for routine analysis (AOAC 1980; APHA 1978, 1984; ICMSF 1978).

ii. Enterobacteriaceae

Enterobacteriaceae counts are very similar to coliform counts. The enrichment-plating procedure, which employs violet-red bile agar with 1% glucose, allows a greater spectrum of members of the Enterobacteriaceae than usual procedures that select for the lactose-positive members only. Similarly to coliform counts, its usefulness is mainly limited to post-processing contamination. The presence of Enterobacteriaceae does not imply fecal contamination.

Recommended OECD Limits on Microbiological Contamination

Several points must be considered when establishing OECD limits on microbiological contamination of microbial pest control agents and their associated end-use products:

i. Will the chosen limits on microbiological contamination satisfy the data requirements of all OECD member countries?

ii. Is the list of contaminants to be monitored practical or feasible for registrants/notifiers?

iii. Will the chosen indicators and/or pathogens provide meaningful data to assess the overall acceptability and risk of the microbial pest control product?

iv. Are appropriate standard methods available for each of the selected indicators and/or pathogens?

v. Will any of the selected indicators and screening methods put laboratory technicians at risk of infection or outbreak, if performed outside an accredited laboratory?

Obviously, the chosen list of limits on microbial contamination must satisfy the data requirements of all OECD member countries, but the list must also be practical and not present an unnecessary burden for registrants/notifiers as well. Furthermore, the chosen list of limits is useless unless relatively simple standard methods are available for routine analysis. The standard methods must also be sensitive and provide meaningful data on estimating risk from pathogens in order to avoid unnecessary testing. Furthermore, the recommended tests must not potentially endanger the laboratory technicians. Authorities must recognize that manufacturers may perform microbial
testing in-house rather than employ the services of accredited laboratories. As previously noted, the WHO does not recommend the isolation of certain pathogens.

Based on the above considerations, the following list of indicator microorganisms and contaminant limits in microbial pesticides are offered for consideration by regulatory authorities:
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<tr>
<th>Type of Indicator</th>
<th>Indicator</th>
<th>Limit*</th>
<th>Rationale</th>
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<tbody>
<tr>
<td>Pathogen</td>
<td><em>Salmonella</em></td>
<td>Absence in 25 g or 25 mL</td>
<td>- U.S. EPA test guideline requirement</td>
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<td>- many standard methods available</td>
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<td>- often used in the food industry</td>
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<td><em>Vibrio</em></td>
<td>Absence in 25 g or 25 mL</td>
<td>- U.S. EPA test guideline requirement</td>
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<td>- the isolation of specific species or pathogens (e.g., <em>Vibrio cholerae</em>)</td>
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<td>however, is not recommended</td>
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<td><em>Shigella</em></td>
<td>Absence in 25 g or 25 mL</td>
<td>- U.S. EPA test guideline requirement</td>
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<td>- isolation of specific species or pathogens (e.g., <em>Shigella dysenteriae</em>)</td>
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<td>however, is not recommended</td>
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<tr>
<td>Microbial Activity</td>
<td>Aerobic Plate Count</td>
<td>&lt; 1 × 10^7 CFU/g or mL</td>
<td>- quick indicator of aerobic bacterial contamination</td>
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<td>- many standard methods available</td>
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<td>Yeast and mould Count</td>
<td>&lt; 1000 CFU/g or mL</td>
<td>- many standard methods available</td>
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<td>- general indication of yeast and mould contamination, and potential for</td>
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<td>mycotoxins</td>
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<td>Human, fecal and</td>
<td><em>Escherichia coli</em> or</td>
<td>Absence in 1 g or mL</td>
<td>- indicator of fecal contamination</td>
</tr>
<tr>
<td>environmental</td>
<td>thermophilic (fecal)</td>
<td>&lt;10 CFU/g or mL</td>
<td>- many standard methods available</td>
</tr>
<tr>
<td>contamination</td>
<td>coliform</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Staphylococci</em></td>
<td>Absence in 1 g or mL</td>
<td>- indicator of contamination due to improper handling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- many standard methods available</td>
</tr>
<tr>
<td></td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Monitoring**</td>
<td>- indicator of environmental contamination</td>
</tr>
<tr>
<td>Other tests</td>
<td>Mouse IP Test</td>
<td>See Appendix I</td>
<td>- as described in 40 CFR 180.1011 (see Appendix I for details)</td>
</tr>
<tr>
<td>(case-by-case)</td>
<td></td>
<td></td>
<td>- required by EPA and PMRA for <em>Bacillus thuringiensis</em>-based and</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>baculovirus-based products</td>
</tr>
</tbody>
</table>

* The recommended limits are provided for discussion only, and are based on PMRA experiences (see Appendix I) and/or other limits included in Appendix I.

** Evaluation will be based on levels that occur.

References


Appendix I - PMRA Limits on Contaminating Microorganisms

Please note that for both the mouse intraperitoneal (IP) 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 Batch Analysis (For Bacillus thuringiensis and baculovirus preparations)

The US Environmental Protection Agency 40 CFR 180.1011 provides guidelines for the mouse IP batch analysis. Each batch of technical active ingredient (prior to the addition of formulation ingredients) must be tested by IP 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. Any 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 Bt 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 recognized 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.

**Bt formulations:**

Currently, the PMRA has officially established microbial contaminant limits for Bt-based formulations only (as per a 1988 Agriculture Canada notice directed to registrants). The following limits were established in 1988 and are still applied to all Bt formulations, though a number of registrants have committed to lowering the target limit for enterococci by one order of magnitude to $10^4$ CFU/g or mL:

<table>
<thead>
<tr>
<th>Selected Organisms Bt Formulations</th>
<th>Target Values (1988)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella</em> spp.</td>
<td>0 CFU/25 g or mL*</td>
</tr>
<tr>
<td>Streptococci/Enterococci</td>
<td>$10^4$ CFU/g or mL*</td>
</tr>
<tr>
<td>Coliforms (Total)</td>
<td>$10^3$ CFU/g or mL*</td>
</tr>
<tr>
<td><em>Clostridium perfringens</em></td>
<td>monitoring**</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>monitoring**</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>monitoring**</td>
</tr>
</tbody>
</table>
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.

**Fungal and Actinomycete formulations:**

No fixed or pre-set limits for microbiological contaminants have been established by the PMRA. Applicants have voluntarily set their own acceptance limits for various microbes of concern and have been accepted by PMRA if deemed appropriate. However, some registrants have not volunteered to set contaminant limits. In such cases, PMRA requires that, in the event of microbial contamination, registrants comply with the following criteria for harmful or pathogenic microorganisms and for the total number of mesophile contaminants:

<table>
<thead>
<tr>
<th>Microbiological Contaminant</th>
<th>Limit of Contamination (CFUs/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>total mesophiles (aerobic plate count at 30°C)</td>
<td>&lt; 10^5</td>
</tr>
<tr>
<td>Coliforms</td>
<td>&lt; 100</td>
</tr>
<tr>
<td>fecal coliforms</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>fecal streptococci/enterococci</td>
<td>&lt; 100</td>
</tr>
<tr>
<td>yeasts and moulds</td>
<td>&lt; 1000</td>
</tr>
<tr>
<td>Staphylococci</td>
<td>&lt; 100</td>
</tr>
<tr>
<td>Salmonellae</td>
<td>0 CFU/25 g</td>
</tr>
</tbody>
</table>

These limits of microbiological contamination are comparable to those recognized 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 addition, production batches must be absent of human pathogenic organisms; the presence of any primary human pathogenic bacteria should result in destruction of the batch.

In some cases, registrants have voluntarily lowered acceptance limits for some contaminant organisms as follows:

<table>
<thead>
<tr>
<th>Test</th>
<th>Allowable Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Aerobic</td>
<td>&lt; 1000 CFU/g or mL</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>&lt; 10 CFU/g or mL</td>
</tr>
<tr>
<td>Fecal Streptococci/Enterococci</td>
<td>Absence in 1 g or mL</td>
</tr>
<tr>
<td>Fecal Coliforms</td>
<td>Coliforms: &lt; 10 CFU/g or mL</td>
</tr>
<tr>
<td>Fecal Coli</td>
<td>E. coli: Absence in 1 g or mL</td>
</tr>
<tr>
<td>Staphylococci</td>
<td>Absence in 1 g or mL</td>
</tr>
<tr>
<td>Salmonella</td>
<td>Absence in 1 g or mL</td>
</tr>
</tbody>
</table>
**Baculoviruses (produced *in vivo*):**

For baculovirus preparations manufactured *in vivo* using insect larvae, the PMRA requires that manufacturers include tests (microbe-specific selection media, and mouse IP assay) to insure that no potential human pathogens are present in each lot of the final unformulated preparation and that populations of certain other microorganisms do not exceed acceptable limits.

The following guidance has been provided to prospective registrants of baculovirus preparations: 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 HPB Compendium of Analytical Methods (http://www.hc-sc.gc.ca/food-aliment/mh-dm/mhe-dme/compendium/e_index.html) 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 streptocci/enterococci, a summary is given of a reference method that is not available on-line.

<table>
<thead>
<tr>
<th>Microbial Contaminant</th>
<th>Contaminant Limit</th>
<th>Suggested Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Aerobic Bacteria (Mesophilic)</td>
<td>$&lt; 1 \times 10^7$ CFU/g or mL</td>
<td>Determination of the Aerobic Colony Count in Foods, MFHPB-18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aerobic Plate Count, Chapter 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td><a href="http://www.cfsan.fda.gov/~ebam/bam-3.html">http://www.cfsan.fda.gov/~ebam/bam-3.html</a></td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>$&lt; 1 \times 10^6$ CFU/g or mL</td>
<td>Determination of <em>Bacillus cereus</em> in Foods, MFLP-42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OR</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Bacillus cereus</em>, Chapter 14</td>
</tr>
<tr>
<td></td>
<td></td>
<td><a href="http://www.cfsan.fda.gov/~ebam/bam-14.html">http://www.cfsan.fda.gov/~ebam/bam-14.html</a></td>
</tr>
<tr>
<td>Other Aerobic Sporeformers</td>
<td>$&lt; 1 \times 10^7$ CFU/g or mL</td>
<td>Determination of Aerobic and Anaerobic Sporeformers, MFLP-44</td>
</tr>
<tr>
<td>Bacterial Group</td>
<td>Absence Condition</td>
<td>Methodology</td>
</tr>
<tr>
<td>-----------------</td>
<td>-------------------</td>
<td>-------------</td>
</tr>
</tbody>
</table>
| Fecal Streptococi - 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; faecal streptococci will appear as pink or dark red or maroon colonies  
- streak presumptive faecal streptococci colonies on trypticase soy agar and incubate at 35°C for 24 hours; colonies negative for catalase are confirmed as faecal streptococci (include a positive control culture of *S. faecalis*)  
| Total Coliforms  
Fecal coliforms/ *Escherichia coli* | < 100 CFU/g or mL; Absence in 1 g or mL | Determination of Coliforms, Faecal Coliforms and of *E. coli* in Foods, MFHPB-19  
OR  
Enumeration of *Escherichia coli* and the Coliform Bacteria, Chapter 4  
http://www.cfsan.fda.gov/~ebam/bam-4.html |
| *Staphylococcus* | Absence in 1 g or mL | Enumeration of *Staphylococcus aureus* in Foods, MFHPB-21  
OR  
*Staphylococcus aureus*, Chapter 12  
http://www.cfsan.fda.gov/~ebam/bam-12.html |
| **Salmonella** | Absence in 25 g or mL | Isolation and Identification of *Salmonella* from Foods, MFHPB-20  
**OR**  
*Salmonella*, Chapter 5  
http://www.cfsan.fda.gov/~ebam/bam-5.html |
|---|---|---|
| **Shigella** | Absence in 25 g or mL | *Shigella*, Chapter 6  
http://www.cfsan.fda.gov/~ebam/bam-6.html |
| **Vibrio** | Absence in 25 g or mL | The Isolation and Identification of *Vibrio cholerae* 01 and non-01 from Foods, MFLP-72  
**OR**  
*Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and Other *Vibrio* spp.  
| **Yeasts and Moulds** | < 1000 CFU/ g or mL | Enumeration of Yeasts and Moulds in Foods, MFHPB-22  
**OR**  
Yeasts, Moulds and Mycotoxins, Chapter 18  
http://www.cfsan.fda.gov/~ebam/bam-18.html |