Final Screening Assessment for *Aspergillus oryzae* ATCC 11866

Environment Canada

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Synopsis

Pursuant to paragraph 74*(b)* of the *Canadian Environmental Protection Act, 1999* (CEPA), the Minister of the Environment and the Minister of Health have conducted a screening assessment on *A. oryzae* strain ATCC 11866.

A. oryzae ATCC 11866 is a fungus that is a member of the *Aspergillus flavus* group and has characteristics in common with two members of that group, *A. oryzae* and *A. flavus.*

A. oryzae occurs where it is used in food fermentation facilities, primarily in Japan and China, but it may also be found sporadically in soil or on decaying plant materials. *A. oryzae* is considered by some taxonomists as a group of domesticated strains of *A. flavus* that have lost the ability to produce aflatoxins, exhibit sparse sporulation and have floccose aerial mycelia. Although genetically almost identical to *A. flavus*, *A. oryzae* is classified as a separate species to indicate strains that are suitable for food production. There is no evidence in the scientific literature indicating that *A. oryzae* is a plant or animal pathogen, although a few cases of infection in animals with predisposing conditions have been reported. Under normal circumstances, it is unlikely to be a serious hazard to healthy livestock or to other organisms in the environment. Information from the scientific literature indicates that *A. oryzae* is unlikely to cause infection in healthy or debilitated humans.

A. flavus is generally considered to be ubiquitous in nature, and able to thrive in a variety of terrestrial and aquatic habitats. *A. flavus* is a plant pathogen and is known to cause disease in maize, cottonseed, tree nuts, and oilseed crops. *A. flavus* has also been reported as an opportunistic animal pathogen, causing mycosis (i.e., infection) mostly in birds and mycotoxicosis (i.e., disease from ingestion of toxin-contaminated feed), which triggers a range of symptoms that can debilitate the host. *A. flavus* can cause sinus and eye infections in otherwise-healthy humans, and potentially fatal lung disease and systemic infections in susceptible groups (i.e., infants and the elderly, the immunocompromised and individuals with debilitating comorbidities).

Both species have been reported infrequently to cause allergic reactions in humans and animals, including hypersensitivity in susceptible individuals.

A. oryzae ATCC 11866 was originally selected for high protease production. This property makes it of possible commercial and industrial interest for fermentation, enzyme production, chemical production, as a livestock probiotic, for bioremediation, biodegradation, industrial effluent treatment, municipal wastewater treatment (particularly grease traps and sewer lines), organic waste treatment, biosorption of environmental contaminants and use as a host organism for recombinant protein and enzyme production.

The morphological characteristics of *A. oryzae* ATCC 11866, particularly small conidial size, which is associated with virulence, more closely resemble those of *A. flavus*; however, *A. oryzae* ATCC 11866 is not known to produce aflatoxin, and it is susceptible to the major clinical antifungals used to treat aspergillosis. These are mitigating factors in this risk assessment.

This assessment considers the aforementioned characteristics of *A. oryzae* ATCC 11866 with respect to human health and environmental effects associated with product use and industrial processes subject to CEPA, including releases to the environment through waste streams and incidental human exposure through environmental media. A conclusion under CEPA on this living organism is not relevant to, nor does it preclude, assessment of products produced by or containing *A. oryzae* ATCC 11866 as prescribed under the purview of the *Food and Drugs Act*. To update information about current uses, the Government launched a mandatory information-gathering survey under section 71 of CEPA as published in the *Canada Gazette*, Part I, on October 3, 2009 (section 71 Notice). Information submitted in response to the section 71 Notice indicates that *A. oryzae* ATCC 11866 was not imported into or manufactured in Canada in 2008. Based on the information available, *A. oryzae* ATCC 11866 is not currently in commerce in Canada.

Based on the information available, it is concluded that *A. oryzae* ATCC 11866 does not meet the criteria under paragraph 64(*a*) or (*b*) of CEPA as it is not entering the environment in a quantity or concentration or under conditions that have or may have an immediate or long-term harmful effect on the environment or its biological diversity or that constitute or may constitute a danger to the environment on which life depends. It is also concluded that *A. oryzae* ATCC 11866 does not meet the criteria under paragraph 64(*c*) of CEPA as it is not entering the environment in a quantity or concentration or under conditions that constitute or may constitute a danger in Canada to human life or health.

Table of Contents

Synopsis..iii

Introduction..viii

Decisions from Domestic and International Jurisdictions........................ix

Domestic..ix

International..ix

1. Hazard Assessment..1

1.1 Characterization of *Aspergillus oryzae*..1

1.1.1 Taxonomic identification and strain history..1

1.1.2 Biological and ecological properties..6

1.1.3 Effects..13

1.2 Hazard Severity..20

1.2.1 Environment..21

1.2.2 Human Health..22

2. Exposure Assessment..22

2.1 Sources of exposure..22

2.2 Exposure characterization..25

2.2.1 Environment..25

2.2.2 Human..26

3. Risk Characterisation..27

4. Conclusion..28

5. References..30

Appendices..53

Appendix A: Growth of *Aspergillus oryzae* ATCC 11866 in various media........53

Appendix B: Secondary metabolites produced by *Aspergillus oryzae* and *Aspergillus flavus* 55

List of Tables

Table 1-1 Comparison of morphological characteristics of *Aspergillus oryzae* ATCC 11866 with the type strain for *A. oryzae* and the type strain for the closest pathogenic strain, *A. flavus.* 4

Table 1-2: Minimal Inhibitory Concentration (MIC) for *A. oryzae* ATCC 11866 9

Table A-1 Growth of *A.oryzae* ATCC 11866 in liquid media at various temperature [a] 53

Table A-2 Growth Characteristics of *A. oryzae* ATCC 11866 on Solid Media at various temperature[a] 54

Table B-1 List of toxins and secondary metabolites produced by *Aspergillus oryzae/flavus* 55

List of Figures

Figure 1-1 Terminology used to describe conidiophore morphology for the identification of *Aspergillus* species. 4

Introduction

Pursuant to paragraph 74*(b)* of the *Canadian Environmental Protection Act, 1999* (CEPA), the Minister of the Environment and the Minister of Health are required to conduct screening assessments of living organisms added to the *Domestic Substances List* (DSL) by virtue of section 105 of the Act to determine whether they present or may present a risk to the environment or human health (according to criteria as set out in section 64 of CEPA).[1] *A. oryzae* ATCC 11866 was added to the DSL under subsection 25(1) of CEPA 1988 and to the DSL under subsection 105(1) of CEPA because it was manufactured in or imported into Canada between January 1, 1984 and December 31, 1986.

This screening assessment considers hazard information obtained from the public domain and from unpublished research data generated by Health Canada[2], as well as comments from scientific peer reviewers. Exposure information was obtained from the public domain and from a mandatory CEPA section 71 Notice published in the *Canada Gazette*, Part I, on October 3, 2009. Further details on the risk assessment methodology used are available in the Risk Assessment Framework document entitled "Framework on the Science-Based Risk Assessment of Micro-organisms under the *Canadian Environmental Protection Act, 1999*" (Environment Canada and Health Canada 2011).

In this report, data that are specific to the DSL-listed *Aspergillus oryzae* ATCC 11866 are identified as such. Strain-specific data are limited and are from five sources: the Nominator, the American Type Culture Collection (ATCC), unpublished data generated by Health Canada[3], and two published papers i.e., Baens-Arcega et al. (1956) and Wei and Jong (1986). Where strain-specific data were not available, surrogate information from literature searches was used. Surrogate information was considered in this report relating to other strains of the species *A. oryzae*, as well as information on the species *A. flavus*, given that the morphological characteristics of *A. oryzae* ATCC 11866 may make its behaviour more similar to *A. flavus* than to other *A. oryzae* strains. When applicable,

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

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

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

literature searches conducted on the organism included synonyms, and common and superseded names. Surrogate organisms are identified in each case to the taxonomic level provided by the source. Literature searches were conducted using scientific literature databases (i.e., SCOPUS, CAB Abstracts, Google Scholar and NCBI PubMed), web searches, and key search terms for the identification of human health and environmental hazards. Information identified up to May 2014 was considered for inclusion in this screening assessment.

Decisions from Domestic and International Jurisdictions

Domestic

A. oryzae ATCC 11866 is a Risk Group 1 pathogen for humans and terrestrial animals (low individual risk, low community risk) according to the Public Health Agency of Canada (PHAC) (personal communication, PHAC 2015). It is not considered to be a regulated plant pest in Canada by the Canadian Food Inspection Agency (CFIA) (personal communication, CFIA 2013).

A. oryzae, as a taxonomical synonym for *Aspergillus flavus* var. *oryzae*, is listed in the Natural Health Products Ingredients Database (NHPID) with a medicinal role and as a source material for alpha-amylase, catalase, cellulase, fungal protease, glucoamylase, hemicellulase, lactase, leucyl aminopeptidase, malt diastase, oligopeptidase B, polygalacturonase, protease, and lipase (NHPID 2015). Although *A. oryzae* is found in the Licensed Natural Health Products Ingredients Database as being present as medicinal ingredient in currently licensed natural health products (NHPs), these products are not expected to contain or provide live *A. oryzae*, as consistent with the Food Chemicals Codex and the information found in the NHPID. *A. oryzae* (strain unknown) is used as the production organism for the various enzymes currently found in licensed NHPs (LNHPD 2015; FCC 2012).

International

A. oryzae and the enzymes it can produce are accepted as constituents of food (Barbesgaard et al. 1992). The Organisation for Economic Co-operation and Development (OECD) has assigned *A. oryzae* a Good Industrial Large-Scale Practice (GILSP) host status (OECD 1992), and the United States Food and Drug Administration (U.S. FDA) has accepted *A. oryzae* as a safe recipient organism for the purposes of enzyme production (Olempska-Beer et al. 2006). A risk assessment of *A. oryzae* was conducted under the *Toxic Substances Control Act* (TSCA) by the biotechnology program of the United States Environmental Protection Agency (U.S. EPA), and this species is included as a recipient micro-organism at õ 725.420 (recipient organism) for the tiered exemption (U.S. EPA 1997a; U.S. EPA 1997b), allowing it to be used in containment in the U.S. for production of enzymes and organic acids without notifying the U.S. EPA.

1. Hazard Assessment

1.1 Characterization of *Aspergillus oryzae*

1.1.1 Taxonomic identification and strain history

Binomial name: *Aspergillus oryzae*

Taxonomic designation:

Kingdom:	Fungi
Phylum:	Ascomycota
Class:	Eurotiomycetes
Order:	Eurotiales
Family:	*Trichomaceae*
Genus:	*Aspergillus*
Subgenus:	*Circumdati*
Section:	*Flavi*
Species:	*oryzae* (Ahlburg) Cohn 1884, anamorph
Strain:	ATCC 11866 (equivalent to NRRL A-5777)

Synonyms and common names:

The most common synonyms associated with the species *A. oryzae* include: *A. flavus* Link 1809 (Roskov et al. 2014); *A. flavus* var. *oryzae* (Kurtzman et al. 1986); and *Eurotium oryzae* (Machida et al. 2008). A more exhaustive list of synonyms is available in the Catalogue of Life (Roskov et al. 2014).

The species *A. oryzae* can be described by the common name of koji mould (Machida et al. 2008). *A. oryzae* ATCC 11866 is also referred to as the Philippine strain of *A. oryzae* (Baens-Arcega et al. 1956).

Strain history: *A. oryzae* ATCC 11866 was originally isolated as a contaminant of indoor air in the Institute of Science and Technology in Manila, Philippines and was deposited to the ATCC by L. Baens-Arcega (Baens-Arcega et al. 1956). The strain was of interest for its high protease activity and for possible use in the production of soy sauce from copra meal (a coconut by-product). The isolate was identified as a typical strain of *A. oryzae* by Drs. D. I. Fennell and C.W. Hesseltine at the Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois after the isolate was sent to ATCC for identification. No information on the basis of their identification of the isolate as *A. oryzae* was provided.

1.1.1.1 Phenotypic and molecular characteristics

A. oryzae is a filamentous, aerobic fungus belonging to the *Aspergillus* Section *Flavi*, which is commonly referred to as the *A. flavus* group. This group also includes

Aspergillus sojae, *Aspergillus tamarii, Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus nomius.* The original *A. flavus* group comprised nine species and two varieties distinguished by the colour and ornamentation of the conidial heads (Raper and Fennell 1965). Currently, the *A. flavus* group contains 18 accepted species (reviewed in Samson et al. 2006).

A. oryzae is the name given to the domesticated strains of *A. flavus* used as koji moulds in Japan and China for the fermentation of rice and soy products (Barbesgaard et al. 1992). The species *A. oryzae* may have emerged as a natural variant of *A. flavus* through long-term successive cultivation on *Oryza sativa* (rice) (Wicklow 1984a). Domestication is thought to have reduced the selective pressure required by *A. flavus* to maintain genes associated with spore formation and dispersal, and those associated with the production of aflatoxins (Jorgensen 2007) resulting in sparse sporulation, floccose aerial mycelia and production of few or no scleriotia (Chang and Ehrlich 2010). Furthermore, the loss or inactivation of these genes through domestication created morphological differences between the domesticated and wild type species, namely: differences in colony texture, conidiophore length and texture, conidia texture, and in the arrangement of sterigmata, as well as the loss of some metabolic capabilities, such as the formation of aflatoxins (Barbesgaard et al. 1992; Kurtzman et al. 1986; Wicklow 1984a). Nevertheless, the lack of production of aflatoxins alone is not a criterion to differentiate *A. oryzae* from *A. flavus* since some strains of *A. flavus* do not produce aflatoxins (Jorgensen 2007).

Because they are almost genetically identical, it was once proposed to reclassify *A. oryzae* as a variant of *A. flavus* (reviewed in Blumenthal 2004).The two species remain difficult to differentiate based on morphological, physiological or genotypic characteristics (Jorgensen 2007). According to Ehrlich (2008), it is becoming clear that *A. oryzae* is not a separate species, but rather a group of non-aflatoxigenic variants of *A. flavus*. However, *A. oryzae* continues to be classified as a different species for economic and food safety reasons (Chang and Ehrlich 2010).

Since *A. oryzae* and *A. flavus* are so closely related, information on both species is presented in this report.

Morphology:

As for many fungi, the taxonomy of *Aspergillus* species is primarily based on morphological features. The conidiophore morphology and associated terminology used for the identification of *Aspergillus* species are illustrated in Figure 1-1.

In spite of subtle morphological differences, *A. oryzae* and *A. flavus* have a very broad range of overlapping characteristics. As such, several characteristics must be used simultaneously to distinguish them accurately (Barbesgaard et al. 1992; Klich and Pitt 1988). Morphologically:

- Raper and Fennell (1965) used the colour change in aged colonies to differentiate *A. oryzae* from *A. flavus*;
- Murakami (1971) identification key discriminates between the two species based on conidial diameter, and colour on anisaldehyde medium; and
- Compared to *A. flavus*, *A. oryzae* has reduced sporulation, produces conidiophores and conidia that are often less conspicuously roughened and more variable in size (usually larger). *A. oryzae* also produces a flocculent aerial mycelium, does not produce aflatoxins and most strains does not produce sclerotia (Jorgensen 2007; Kurtzman et al. 1986; Wicklow 1984a).

The morphological characteristics of the DSL strain, ATCC 11866, are consistent with those of the group of yellow-green moulds belonging to the *A. flavus* group. Using Raper and Fennell's (1965) identification keys, the identifying characteristics of the *A. flavus* group include: biseriate sterigmata in most species; non clavate vesicles; bright yellow-green conidial heads in young cultures, becoming brown with time; loosely radiate conidial heads; spore chains usually separate, sometimes forming poorly defined columns; and conidiophores usually presenting as roughened and colourless. At the time of its isolation, Baens-Arcega et al. (1956) could not determine whether the DSL strain was *A. flavus* or *A. oryzae* based on its morphology (Table 1-1). Based on the length and diameter of the conidiophores, the size and form of the vesicles, the manner of colouring the agar, and the appearance of the conidial heads and conidia, it appeared closer to *A. flavus* than to *A. oryzae*; however, it could not be identified as *A. flavus* with certainty because the colour of the colony was not as deep a green as the colour of the control *A. flavus* culture used. The basis of the identification of the isolate as *A. oryzae* by Drs. D.I. Fennell and C.W. Hesseltine was not provided by Baens-Arcega et al. (1956).

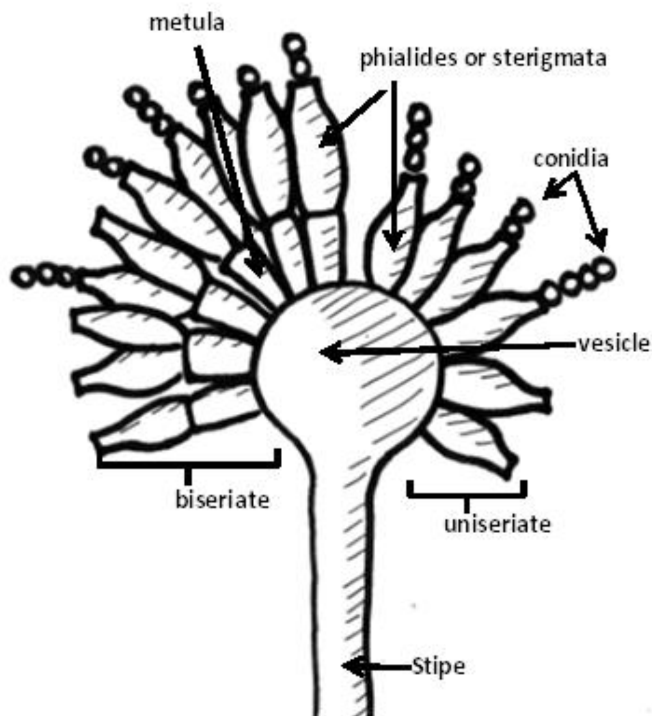

Figure 1-1 Terminology used to describe conidiophore morphology for the identification of *Aspergillus* species.

Table 1-1 Comparison of morphological characteristics of *A. oryzae* ATCC 11866 with the type strain for *A. oryzae* and the type strain for the closest pathogenic strain, *A. flavus.*

Characteristic	***A. oryzae*** **ATCC 1011 (type strain)[a]**	***A. flavus*** **ATCC 16883 (type strain)**	***A. oryzae*** **ATCC 11866 (DSL strain)[c]**	***A. oryzae*** **ATCC 11866 (DSL strain)[d]**
Growth temperature	Optimal at 34°C	Optimal at 37° C [b]	Not reported by the source	15-42°C (not tested below 15°C)
Colony color	Young colonies yellow, maturation giving yellow green, green/olive; brown in aged cultures	Yellow-green to leaf green [b] Green colonies and white or lighter green edges and black specks at the point of the colony radiating away from the center (7 days growth on CYA at 25°C). [d]	Colony turning yellow, then russet green as it matures; surface of the colony becoming radiantly wrinkled	White colonies with yellowish centres (7 days growth on CYA at 25°C)
Conidial head	Globose, radiate or columnar	Shape is radiate to loosely columnar; aged heads split into columns [b]	Subglobose or nearly globose, radiate	NA
Conidial head size (µm)	Not reported by the source	60 [d]	30 to 50 (horizontal diameter)	72.2 +/- 8.6

Characteristic	***A. oryzae*** **ATCC 1011 (type strain)[a]**	***A. flavus*** **ATCC 16883 (type strain)**	***A. oryzae*** **ATCC 11866 (DSL strain)[c]**	***A. oryzae*** **ATCC 11866 (DSL strain)[d]**
Conidiophore/ stipe	Colorless and roughened	No coloration, nearly smooth to echinulate [b]	Colourless conidiophores with wart-roughened walls	NA
Conidiophore (µm)	1000 and longer in length	Not reported by the source	350-750 (length) 5.0-8.0 (diameter)	NA
Vesicle	Subglobose to clavate	Elongate, subglobose to globose [b]	Subglobose vesicles	globose
Vesicle diameter (µm)	Not reported by the source	Not reported by the source	10.0 -20.0	NA
Sterigmata/ phialides	20% biseriate sterigmata, mostly uniseriate	Phialides biseriate (20-80%); uniseriate when on small vesicles [b]	Arranged in single series, greenish and closely packed over one-half to two-thirds of the vesicular surface	NA
Sterigmata size (µm)	Not reported by the source	Not reported by the source	6.4-9.6 (length) 3.2-4.8 (diameter)	NA
Conidia	Often globose or subglobose, or elliptical to pyriform. Slightly rough	Subglobose to globose; smooth to definitely echinulate [b]	In chains, greenish, mostly globose, wall roughened with spinules or warts	NA
Conidia diameter (µm)	5 – 6 and larger	3-7.5 [b] 4.6 [d]	4.0- 4.8	3.9 +/- 0.8
Presence of sclerotia	Rare; globose, purple-brown or black when mature	NA	No sclerotia	NA

[a] Data for *A. oryzae* ATCC 1101 are from Murakami (1971)
[b] Data for *A. flavus* ATCC 16883 are from Christensen (1981)
[c] Data for *A. oryzae* ATCC 11866 are from Baens-Arcega et al. (1956)
[d] Data generated by Health Canada's Environmental Health Science and Research Bureau
NA = Not available.

Genotype:

The close evolutionary proximity of the species *A. flavus* and *A. oryzae* is reflected in their genomes, which are nearly identical in size and gene content (Payne et al. 2006). Comparison of *A. oryzae* and *A. flavus* 18S rRNA and 26S rRNA gene sequences and ITS regions, and DNA/DNA hybridization studies, show the species to be almost identical (Nakamura et al. 2011; Kurtzman et al. 1986). Amplified fragment length polymorphism (AFLP) cannot differentiate *A. oryzae* from *A. flavus* (Montiel et al. 2003). Nonetheless, *A. oryzae* and *A. flavus* can be distinguished using *Sma*I digestion of total DNA and multilocus sequence analysis (MLSA) using four aflatoxin biosynthetic gene loci (*aflR*, *aflT*, *norA*, and *vbs*) (Klich and Mullaney 1987; Nakamura et al. 2011).

The genome of *A. oryzae* RIB40 has been sequenced, and the strain has a high gene content related to metabolism and transport (Machida et al. 2005). *A. oryzae* strain RIB40 is considered to be representative of the species (but is not a type strain), as its characteristics are typical of industrial strains used for sake brewing, including morphology, growth, amylase production, and potent protease activity, which are important properties for soy sauce fermentation (Abe et al. 2006). The DSL strain ATCC 11866 shares the property of potent protease activity with strain RIB40, but it is not

known to what degree further properties of the DSL strain are common to RIB40 (Baens-Arcega et al. 1956). The genome of *A. oryzae* ATCC 11866 has not been sequenced.

1.1.2 Biological and ecological properties

Other than Baens-Arcega et al. (1956) and Wei and Jong (1986), there are no additional publications in the literature on the DSL strain ATCC 11866. Therefore, this section relies on surrogate information relating to other members of the species *A. oryzae*. This section also includes surrogate information on *A. flavus*, given that *A. flavus* is considered to be the wild type of domesticated *A. oryzae* (Blumenthal 2004; Kurtzman et al. 1986); the behaviour of *A. oryzae* in the environment (i.e., outside a fermenter) is not well understood, and its potential to revert to the wild type phenotype is unclear; morphological characteristics of the DSL strain (e.g., small conidia) may make its behaviour more similar to *A. flavus* than to other *A. oryzae* strains, as described below.

1.1.2.1 Natural occurrence

A. oryzae is mainly found in Japan and China where it has been intensively used in food fermentation. Outside those countries, the fungus may be found sporadically in soil or on decaying plant materials (Barbesgaard et al. 1992).

Researchers have also reported isolating this fungus from:

- cultivated soil, grassland and forest soils in India, the former USSR, Czechoslovakia, Japan, Tahiti, Peru, Syria, Italy, the air of the U.S. and the British Isles (reviewed in Domsch et al. 1980), and agricultural soil in the U.S. (Mothapo et al. 2013);
- wild coffee leaves in Columbia (Vega et al. 2010), onion seeds in Sudan (El-Nagerabi and Abdalla 2004), and medicinal plants and spices imported from India (Aziz et al. 1998);
- stored maize grains and poultry feeds in Nigeria (Adebajo 1992; Amadi and Adeniyi 2009) and stored wheat grains in Egypt (Atalla et al. 2003);
- grain mill, poultry house, and swinery indoor air (Lugauskas et al. 2004);
- Indian mangrove soils and rhizosphere (Behera et al. 2012; Gayatri and Hasmukh 2013);
- a wastewater-polluted site in the Mediterranean Sea (El-Kassas and El-Taher 2010);
- a marine algae (Qiao et al. 2010); and
- the beetle *Chilocorus bipustulatus* and the beet armyworm *Spodoptera (Laphygma) exigua* in Israel (Kenneth and Olmert 1975).

Nevertheless, some specialists argue that there are no wild strains of *A. oryzae* and that these environmental isolates are, in fact, *A. flavus*. *A. flavus* is distributed worldwide in soil and litter (Horn 2003; Wicklow 1984a). It is most commonly found at tropical and subtropical latitudes, and is uncommon above 45 degrees latitude (Klich 2007; Klich

2002). Soils containing high levels of organic matter, and abundant nitrate, phosphate, and potassium are linked to denser populations of *A. flavus* (Zablotowicz et al. 2007). *A. flavus* is also common in marine environments (Zuluaga-Montero et al. 2010).

A. flavus has been isolated from a diversity of terrestrial, aquatic, and marine habitats. Terrestrial sources of isolation include cotton (Klich 1986), corn and maize (Shearer et al. 1992; Wicklow et al. 1998), plants and leguminous trees (Boyd and Cotty 2001), peanuts (Pinto et al. 2001), fresh date fruits (Shenasi et al. 2002), bee pollen (Gonzalez et al. 2005), and from bird and mammal tissues (Gallagher et al. 1978). In a survey on the overall diversity of the Mahanadi River in India, *A. flavus* comprised 2.5% of the fungal population; more dominant species were *A. niger* (15%) and *A. vesicolor* (9.5%). It was also found to be among the dominant fungi associated with common house mosquito (*Culex pipiens*) larvae, pupae, and adult life stages collected from a fresh water pond in Egypt (Parveen et al. 2011; Badran and Aly 1995). In marine environments, *A. flavus* has been isolated from sponges, sea fans, and corals (Zuluaga-Montero et al. 2010). *A. flavus* has also been isolated in hospitals together with *A. fumigatus, A. oryzae,* and *A. niger* (Allo et al. 1987; Arnow et al. 1991; Buffington et al. 1994; Grossman et al. 1985; Hahn et al. 2002; Humphreys et al. 1991; Iwen et al. 1994; Lai 2001; Leenders et al. 1996; Loo et al. 1996; Lutz et al. 2003; Opal et al. 1986; Pegues et al. 2002; Rotstein et al. 1985; Sherertz et al. 1987; Singer et al. 1998; Thio et al. 2000).

1.1.2.2 Growth Conditions

Under laboratory conditions, optimal growth of *A. oryzae* occurs within a temperature range of 32°C to 36°C and a pH range between 2 and 8, and it requires ions of the trace elements Fe and Zn (Domsch et al. 1980) for growth. The optimum growth temperature of *A. flavus* is 37°C, within a growth temperature range of 12°C to 48°C (Hedayati et al. 2007). The culture method recommended for *A. oryzae* ATCC 11866 is on potato dextrose agar at 24°C (ATCC 2014).

Characterization of the growth kinetics of *A. oryzae* ATCC 11866 was performed at Health Canada laboratories at different temperatures in liquid media (Appendix A, Table A-1), and growth on a variety of solid media at 28°C and 37°C was also tested (Appendix A, Table A-2). *A. oryzae* ATCC 11866 can grow at 37°C, but grows poorly at 42°C.

1.1.2.3 Life cycle, persistence and survival

A sexual life cycle has not been observed in *A. oryzae* (Abe et al. 2006; Jorgensen 2007). However, the presence of mating loci, recombination and meiosis-associated genes in *A. oryzae* all indicate that a yet to be identified heterosexual generation exists (Goffeau 2005; Rokas 2009); the sexual stage of *A. flavus* is *Petromyces flavus* (Horn et al. 2009).

In the asexual life cycle, asexual spores (conidia) are readily formed by *A. oryzae* and released into the air (Barbesgaard et al. 1992; Klich and Pitt 1988). Although *A. oryzae* sporulation is sparser, its conidia typically germinate 3 hours earlier than those of *A. flavus,* which could be a competitive advantage in some environments such as fermentation processes, which may favour selection of conidia that germinate rapidly (Wicklow 1984b). *A. oryzae* conidia are also typically larger. Larger conidia contain greater endogenous spore reserves, which could increase the species' colonization capacity and have a positive impact on its competitive ability; however, greater size might also be a disadvantage for dispersal in nature, and this may be the more important selective factor for wild-living *A. flavus* (Wicklow 1984b).

The DSL strain, ATCC 11866, has conidia that are within the size range described for *A. flavus*, and smaller in size than the range described for *A. oryzae*; thus, this strain might be expected to behave more like *A. flavus* in the environment.

A wide range of factors, including temperature, pH, water activity, presence of preservatives, and gaseous atmosphere may affect sporulation and spore germination in the fungal life cycle. In *A. flavus*, sporulation is optimal at an atmospheric water activity (a_w) range between 0.86 and 0.96 (Vujanovic et al. 2001). When compared to some mycotoxin-producing *Aspergillus, Cladosporium,* and *Penicillium* species, *A. flavus* may require more time to germinate at a lower a_w (Vujanovic et al. 2001). In addition, *A. flavus* spore germination declines by 45% with an increase in temperature from 37°C to 41°C (Pasqualotto 2009). No germination of *A. flavus* spores is observed at 18°C or 12°C (Vujanovic et al. 2001).

All tested strains of *A. flavus* produce sclerotia, which are hardened, thick-walled spherical structures formed for survival under adverse conditions (Vujanovic et al. 2001). The majority of *A. flavus* sclerotia buried in sandy soils in the U.S. survived at least 36 months burial, while buried *A. flavus* conidia survived for a maximum of 24 months (Wicklow et al. 1993). Although most *A. oryzae* strains, including the DSL strain ATCC 11866, have lost the ability to produce sclerotia (reviewed in Wada et al. 2014), some strains such as *A. oryzae* RIB40 produce large and abundant sclerotia (Rank et al. 2012). In a study testing the persistence of *A. oryzae* CCFC008014 live cells inoculated into intact soil microcosms, a reduced quantity of *A. oryzae* DNA within the soil was seen by day 46 post-inoculation, and by day 126, the concentration of DNA had declined approximately 32 fold relative to the concentration on day 2. Both qualitative and quantitative PCR analyses indicated that *A. oryzae* declined in abundance initially, but survived for the full test period of 126 days (Hynes et al. 2006). *A. oryzae* can survive in soil for several months – including under cold conditions – which indicates that *A. oryzae* used in industrial applications could survive in highly competitive soil environments, and if released into the Canadian environment, are likely to persist for at least one growing season (Hynes et al. 2006). However, information that *A. flavus* and *A. oryzae* are uncommon above 45 degrees latitude suggests that introduced populations are unlikely to overwinter successfully enough to be maintained in many parts of Canada. In addition, maintenance of high numbers of introduced micro-

organisms beyond background levels is unlikely due to natural competition with naturally occurring micro-organisms in the environment (Leung et al. 1995).

1.1.2.4 Antifungal susceptibility

Overall, antifungal resistance in cases of human mycoses is increasing (Hedayati et al. 2007). Until recently, aspergilloses have mainly been treated with itraconazole or amphotericin B, but now, other antifungal drugs such as voriconazole, posaconazole and caspofungin have been approved to treat aspergillosis (Hedayati et al. 2007). The antifungal susceptibility profile of *A. oryzae* ATCC 11866 generated by Health Canada scientists (Table 1-2) shows that the DSL strain is susceptible to amphotericin B, itraconazole and voriconazole, which is consistent with susceptibility profiles reported for clinical isolates of *A. flavus*. Voriconazole is considered the most effective antifungal agent against *A. flavus* (Misra et al. 2011), and caspofungin (Denning 2006; Maertens et al. 2004) and terbinafine (Li et al. 2008) have been proposed for use when other drugs fail to control infection. Futhermore, the DSL strain is resistant to anidulafungin, caspofungin, fluconazole and miconazole for which resistance has been reported in isolates of *A. flavus* (Eschertzhuber et al. 2008; Fattahi et al. 2012; Li et al. 2008).

Table 1-2: Minimal Inhibitory Concentration (MIC) for *A. oryzae* ATCC 11866[a]

Antifungal Drug	MIC after 48h (µg/mL)	Breakpoint[b]
Amphotericin B	0.5	*A. flavus* IE+; *A. fumigatus* S≤1 R >2
Anidulafungin	>8	IE
Caspofungin	>8	IE
Fluconazole	256	-
5-Flucytosine	>64	NA
Itraconazole	0.12	S≤1 R >2
Micafungin	>8	IE
Posaconazole	0.06	*A. flavus* IE+; *A. fumigatus* S≤0.12 R >0.25
Voriconazole	0.5	*A. flavus* IE+; *A. fumigatus* S≤1 R >2

IE: insufficient evidence that *A. flavus* is a good target for therapy with the drug.
IE+: insufficient evidence that *A. flavus* is a good target for therapy with the drug and the epidemiological cut-off values for A*. flavus* are in general one step higher than for *A. fumigatus*.
-: susceptibility testing is not recommended as the species is a poor target for therapy with the drug
S; Susceptible, R: Resistant, NA: Not Available
[a] Data generated by Health Canada's Environmental Health Science and Research Bureau. Work conducted by using Sensititre Assay Kit (TREK Diagnostic Systems) and by following manufacturer's protocol. The MIC was interpreted as the lowest concentration corresponding to a blue-coloured well, indicating no growth.

[b] Breakpoint for interpretations of Minimal inhibitory concentration from EUCAST 2014.

The fungal cell targets of antifungal drugs such as polyenes, nystatin and azole are structurally analogous to some components of human cells (reviewed in Lamb et al. 2000); and as a result, some antifungal drugs are relatively toxic to humans. However, antifungals such as echinicandins (which include caspofungin, micafungin and anidulafungin) target fungal cell wall components that are lacking in humans; and therefore, have few side effects (S. Perkhofer, personal communication). In lieu of antifungal therapy, less commonly employed treatments for fungal infection include surgical debridement of the infected tissue and the use of steroid sprays. In refractory cases, the use of a combination of these treatments with antifungal therapy may be required; however, these procedures are also damaging.

1.1.2.5 Pathogenesis

In *Aspergillus* species, small conidial size is one of the most significant factors affecting pathogenicity in humans (Denning 1998; Speth and Rambach,2012) and presumably also in dogs, horses and in particular birds, as in all, the most common route of infection is through the inhalation of conidia (Denning 1998; Speth and Rambach 2012). It is generally recognized that particles between 0.01 and 5 microns in diameter can be deeply inhaled into the pulmonary alveoli (Witschi et al. 2008). Pathogenic *Aspergillus* species have smaller conidia compared to non-pathogenic species; the conidial size of *A. flavus* ranges from 3 to 7.5 microns in diameter (Christensen 1981; Larone 2011), while the conidia of *A. oryzae* are typically larger, ranging from 5 to over 6 microns in diameter. However, the DSL strain, ATCC 11866, has conidia between 4.0 and 4.8 microns in diameter, which are within the size range that can be deeply inhaled into the pulmonary alveoli.

After conidia have gained access to the pulmonary alveoli within the lungs, they germinate and grow hyphae (Denning 1998). The hyphal tip secretes extracellular enzymes into the pulmonary tissue, including aspartyl proteinase, serine proteinase, metalloproteinase, alkaline proteinase and lipases. These enzymes act as virulence factors, by breaking down complex host protein and lipid molecules, the subunits of which are used as fungal nutrients. This process also digests the extracellular matrix of the host to facilitate tissue penetration (Krishnan et al. 2009). Other hydrolases, such as α-amylases, pectinases, and other proteases and lipases are also involved in fungal virulence (reviewed in Amaike and Keller 2011). The DSL strain ATCC 11866 was originally selected for its high proteolytic activity, but it is unclear whether this affects its virulence.

Pectinases produced by plant pathogens macerate plant tissues thereby facilitating plant invasion and capture of nutrients (reviewed in Mellon et al. 2007). Polygalacturonase, P2c, is a major player in the maceration of host tissues in *A. flavus* and is correlated with virulence to plants (reviewed in Mellon et al. 2007). Other hydrolases such as amylase can breakdown the plant storage polysaccharides, creating intermediates such as glucose, maltose and maltotriose that have a role in the induction

of aflatoxins biosynthesis (reviewed in Mellon et al. 2007). It is not known whether the DSL strain of *A. oryzae*, ATCC 11866, produces these hydrolases.

Elastase produced by *A. flavus* is also considered to play a role in the invasion of host tissues because elastin is a major structural component of the lung. Of 23 *A. flavus* strains isolated from human patients suffering from invasive aspergillosis, 19 had elastase activity (Alp and Arikan 2008). It is not known whether the DSL strain of *A. oryzae*, ATCC 11866, produces elastase.

A. oryzae has haemolysin (Nayak et al. 2013) and haemolysin-like (Bando et al. 2011) coding genes, with a promoter for one of these genes possessing high activity. Haemolysins are potential virulence factors produced by *A. terreus* and *A. fumigatus* (Nayak et al. 2011; Wartenberg et al. 2011) that lyse erythrocytes and other cells (Nayak et al. 2011). Hemolysis was not observed when the DSL strain, ATCC 11866, was grown on sheep blood agar (Appendix A, Table A-2).

A. flavus can synthesize and release a complement inhibitor (CI) molecule within the human host, which inhibits both the activation of the complement cascade and the resulting fungal opsonisation. CI prevents activation of the alternative pathway, and interferes with complement protein C3b-induced phagocytosis and fungal cell killing (reviewed in Speth and Rambach 2012). It is not known whether the DSL strain ATCC 11866 produces CI.

Various *Aspergillus* species, including *A. flavus*, produce dihydroxynaphthalene (DHN) – melanin (Thywißen et al. 2011), a pigment that covers the conidia and protects them from ingestion by macrophages, as well as heat, ultraviolet radiation, and extremes of pH (Krishnan et al. 2009). There is limited information on DHN-melanin in *A. oryzae*. Genes for DHN-melanin synthesis are present in the genome of *A. oryzae* RIB 40 but gene expression has not been shown (Baker 2008). DHN-melanin has not been reported for the DSL strain ATCC 11866.

In vitro tests conducted by Health Canada scientists to evaluate the potential of *A. oryzae* ATCC 11866 cells to cause cytotoxicity and adverse immune effects demonstrated that ATCC 11866 was non-cytotoxic to moderately cytotoxic towards human colonic epithelial cells (HT29) and macrophage cells (J774A.1) after 4- and 24-hour exposures.[4]

[4] Unpublished data generated by Health Canada's Environmental Health Science and Research Bureau.

1.1.2.6 Toxigenesis

Species in the *A. flavus* group are known to produce secondary metabolites, including mycotoxins. Secondary metabolites are compounds produced by an organism that are not required for a physiological function (i.e., growth, development or reproduction of the organism), some of which are presented here because they have been reported to have negative effects. Mycotoxins, a subset of these, are small organic molecules produced by filamentous fungi that can cause disease and death in humans and animals through a natural exposure route (Bennett 1987). Mycotoxins enter the human food chain when the fungus grows and produces the toxin in foods such as vegetables or grains or when food animals ingest the toxins in contaminated animal feed. Toxins may also be inhaled along with spores when handling infected material. Mycotoxins and some secondary metabolites produced by *A. oryzae* and *A. flavus* are listed in Appendix B (Table B-1). For *A. oryzae*, these include: cyclopiazonic acid, kojic acid, maltoryzine and 3-nitropropionic acid (Blumenthal 2004; Samson et al. 2006). It is unclear whether the DSL strain, ATCC 11866, produces these or other mycotoxins associated with the *A. flavus* group, including: aflatrem, aspergillic acid, aspertoxin, gliotoxin, or sterigmatocystin (Appendix B, Table B-1).

Some strains of *A. flavus* produce the potent, carcinogenic mycotoxins, aflatoxins, which is regulated in numerous countries (reviewed in Klich 2007). The DSL strain ATCC 11866 did not produce aflatoxins when tested on rice, peanut or YES medium after incubation at 25 °C for 8-10 days, whereas strains of *A. flavus*, the wild type counterpart of *A. oryzae*, produced aflatoxins under the same conditions (Wei and Jong 1986). *A. oryzae* is considered by some taxonomists as a group of domesticated strains of *A. flavus* that have lost the ability to produce aflatoxins (Jorgensen 2007). A full range of intraspecific variation exists within the *A. oryzae* aflatoxins biosynthetic pathway, from deletion of 0.8 kb to 1.5 kb of the aflatoxins gene cluster to no deletions (reviewed in Jorgensen 2007). In many strains of *A. oryzae*, all the genes necessary for aflatoxins biosynthesis are present, yet inactive (Chang et al. 1995; Klich et al. 1995; Kusumoto et al. 1998; Lee et al. 2006; Nakamura et al. 2011; Watson et al. 1999). No data are available on the completeness of the aflatoxins synthesis gene cluster in the DSL strain of *A. oryzae*, ATCC 11866.

The production of secondary metabolites (including toxins) in *A. oryzae* is strain-specific and environment dependent (Park et al. 2008). Environmental factors which are known to have an impact on toxin production by strains of *A. oryzae* include: growth temperature, where optimal toxin production is achieved in the range of 25-35°C; the growth medium or substrate composition; and incubation time, where toxin production is favoured by longer incubation periods (Adebajo 1992; Blumenthal 2004; Jorgensen 2007). In the practice of koji fermentation, the normal fermentation time period is short (2 to 3 days), whereas secondary metabolites are produced by *A. oryzae* typically after 3 days of incubation. This may explain, in part, why mycotoxins are not detected in the final fermented products. Some secondary metabolites, including kojic acid and cyclopiazonic acid, could also be degraded during the fermentation process (reviewed in Jorgensen 2007).

It is reasonable to assume that if the DSL strain, ATCC 11866, encounters suitable conditions in environments to which it is released, it may produce any of the above-mentioned mycotoxins that it is able to produce.

1.1.3 Effects

1.1.3.1 Environment

Since *A. oryzae* and *A. flavus* are essentially indistinguishable by most molecular techniques and the DSL strain is morphologically similar to *A. flavus*, environmental effects caused by both *A. oryzae* and *A. flavus* have been considered in this assessment. Available information on *A. oryzae* is presented first, since few cases are reported in the literature, followed by information on *A. flavus*.

A) *A. oryzae*

Enzymes secreted by *A. oryzae* (e.g., xylanase, polygalacturonase, cellulase and α-amylase) can degrade plant cell walls (reviewed in Al-Hindi et al. 2011; Chang et al. 2012b; Um and Walsum 2010), and may allow it to act as a saprophyte on decaying plant material; however, there are no reports of negative effects on living terrestrial or aquatic plants in the scientific literature.

Although *A. oryzae* is not considered to be an animal pathogen, a few cases of infection have been reported. *A. oryzae* caused one case of spontaneous keratomycosis of a previously injured cornea in a horse (Marolt et al. 1984). Several cases of spontaneous fatal aspergillosis in various species of parrot were associated with *A. oryzae*, confirmed through morphological and histopathological examination (Kaplan et al. 1975). All the parrots were predisposed to infection because of stress associated with capture and shipment to the U.S., co-infection with psittacosis, and treatment with either antibiotics or corticosteroids. Histologic investigations identified hyphal structures and conidia in the pulmonary tissues (Kaplan et al. 1975). Possible allergic effects in animals have also been reported (Lugauskas et al. 2004).

A. oryzae was also reported as a parasite of the Beet Armyworm (*Spodoptera exigua*) (Kenneth and Olmet 1975), and in pathogenicity tests (inoculation of food, spraying of spore suspensions and dusting of spores), *A. oryzae* caused mortality in all life stages of the Maize Stem Borer (*Chilo partellus*) and was pathogenic to the Codling Moth larvae (*Cydia pomonella*) (Gardezi 2006).

No reports were found in the scientific literature of *A. oryzae* having negative effects on aquatic vertebrate or invertebrate species.

Given that it is difficult to differentiate *A. oryzae* from *A. flavus*, it is possible that the causative agent in some of the cases described above was *A. flavus*, erroneously identified as *A. oryzae*.

B) *A. flavus*

A. flavus has been widely reported in the literature to infect many terrestrial and aquatic species of organisms:

Plants

As a known plant pathogen, *A. flavus* commonly affects maize, cottonseed, tree nuts, oilseed crops, and peanut plants (reviewed in Klich 2007). Insect damage to plant tissues increases the susceptibility of plants to fungal infection (reviewed in Klich 2007). In maize, *A. flavus* infection causes ear rot, while in peanuts, infection causes a disease termed yellow mould of seedlings (also known as aflaroot), which manifests as chlorosis and necrotic lesions of the plant's upper tissues, rot of mature peanuts, and developmental failure of secondary roots (reviewed in Klich 2007). In cotton plants, *A. flavus* infection can lead to boll rot and infection of the fibre, causing yellow spot disease, although infection is most likely in bolls under water stress during maturation (reviewed in Klich 2007).

Plant embryos can be damaged by mycotoxins in *A. flavus*-contaminated grain crops, resulting in germination failure or reduced fitness of the seedling (Hasan 1999). Experimental challenges reported in the literature indicate that *A. flavus* infection is associated with lower seed viability of cottonseed plants (Klich 2007), peanut plants (Pitt et al. 1991), and Himalayan Cedar (*Cedrus deodara*) (Mittal 1983), and that viability further decreased in the presence of aflatoxins (Klich 2007).

No reports of negative effects on aquatic plants from *A. flavus* were found in the scientific literature.

Vertebrates

A. flavus is the second most common cause of avian aspergillosis after *A. fumigatus*, and frequently kills 5-10% of birds in domestic poultry flocks (reviewed in Richard et al. 1984). The primary route of *A. flavus* infection in birds is inhalation of large amounts of spores (Richard et al. 1984). Immunocompromised birds are generally at the greatest risk of infection and turkeys are reported to be more susceptible than chickens (Richard et al. 1984). Young waterfowl (ducks and geese) are often affected by *A. flavus* after exposure to high levels of spores in a hatchery environment (Morishita 2004).

Fatal outbreaks of aspergillosis caused by *A. flavus* within commercial poultry houses have been reported:

- One outbreak of localized tracheal aspergillosis in 6- to 8-week-old leghorn chickens was associated with predisposing health conditions among the flock, including recent live vaccine administration and parasitic infection (Barton et al. 1992);
- An outbreak of disseminated aspergillosis was reported in immunocompromised 5- to 10-week-old broiler chickens (Martin et al. 2007); and

- A fatal case of *A. flavus* pulmonary aspergillosis was reported in a 2-year-old Eclectus Parrot (*Eclectus roratus*) living in captivity, and presenting with no clinical signs of infection prior to death (Gornatti Churria et al. 2012).

A. flavus is known to cause keratomycosis in horses. In one case, *A. flavus* was recovered from the affected cornea of a horse in Japan. Fungal hyphae were observed in the corneal scrapings (Wada et al. 2013). *Aspergillus* species (*A. niger*, *A. flavus*, and unidentified species) were the most common isolates (64%) in equine keratomycosis in California (Reed et al. 2013).

Additional cases have been reported involving the successful treatment of *A. flavus* infection in animals using antifungal therapy:

- a case of equine respiratory aspergillosis was resolved through treatment with itraconazole (Cafarchia et al. 2012); and
- a case of ulcerative keratomycosis in a cat with previous feline herpes virus conjunctivitis and ulcerative keratitis was resolved through the off-label use of a topical 1% voriconazole solution (Labelle et al. 2009).

There are a few reports of experimental infections induced using *A. flavus* in terrestrial vertebrate species:

- Intratracheal exposure of 2-week old quail (*Coturnix japonica*) to a dose of 0.1 mL saline spore suspensions containing 1.23×10^7 spores/mL, triggered the development of respiratory difficulty and anorexia within 48 hours of exposure, and an overall mortality of 25%. Gross and microscopic lesions typical of aspergillosis were observed in the lungs post-exposure (Pandita et al. 1991);
- Inoculation of 0.5 mL of a 3×10^9 colony forming units (CFU)/mL *A. flavus* spores culture suspension into the orbit of immunocompromised and diabetic rabbits resulted in proptosis of the eyes and clinically significant orbital infection (Mahajan 1988);
- No clinical signs of infection were observed up to 75 days after inoculation of 0.5 mL of a suspension of 1.2×10^7 *A. flavus* spores/mL into the orbits of either immunocompromised or healthy rhesus monkeys (Mahajan et al. 1978); and
- Intratracheal and intravenous inoculation of goats with *A. flavus* administered in two doses, two days apart (1×10^8 spores in 10 mL, followed by 1.5×10^8 spores in 15 mL) resulted in respiratory difficulty and fever, with congestion of the lungs, liver, spleen, brain, and kidneys observed upon necropsy (Chattopadhyay et al. 1996).

A. flavus is ubiquitous in marine environments; however, little is known about the behavior of fungi in marine ecosystems (Zuluaga-Montero et al. 2010). An outbreak of aspergillosis caused by *A. flavus* and *A. niger* was reported among tilapia fish (*Sarotherodon* species) in a Kenyan fish farm (Olufemi et al. 1983).

Invertebrates

A. flavus is the most common *Aspergillus* species to infect insects (reviewed in Foley et al. 2014). It is associated with different stages of a variety of insects and is very potent in decomposing chitin (Badran and Aly 1995; reviewed in Ismail and Abdel-Sater 1993). It is a rare facultative pathogen of honey bees (*Apis mellifera*), in which it is the primary cause of stonebrood disease, causing mummification of larvae and infecting adult bees in colonies weakened by predisposing factors (Vojvodic et al. 2011). *A. flavus* infection in the bee species *Tetralonia lanuginose* can cause up to 50% decay of pupae and nymphs (Mohamed and El-Khadem 1976, referred to in the abstract of a German-language journal article). *A. flavus* was found at an incidence of 10% in a population of grasshopper (*Zonocerus variegatus*) in Nigeria with a 76% total fungal incidence (Balogun and Fagade 2004), and was found to cause natural infection in 6% of a Southern Cattle Tick (*Rhipicephalus {Boophilus} microplus*) population surveyed in Mexico (Miranda-Miranda et al. 2012).

Experimental infections with *A. flavus* in terrestrial invertebrate species at unspecified challenge doses had the following effects:

- Adult German Cockroaches (*Blattella germanica*) became hypoactive and died within 72 hours (Kulshrestha and Pathak 1997);
- Grasshoppers (*Atractomorpha crenulata*) presented with paralysis and inactivity prior to death at 5 days post-inoculation (Mahalingam and Muralirangan 1996);
- Eggs of the greater wax moth (*Galleria mellonella*) were penetrated and damaged by *A. flavus* mycelium, to the point of conidiophore and mycelial protrusion outward from the egg chorion 2 days after the eggs were dusted with *A. flavus* spores (Behnke and Yendol 1969);
- Two species of Velvet Mite (*Trombidium gigas* and *Dinothrombium giganteum*) lost the outer scarlet-red colouration of their integument and 50% died after 5 days of infection (Sannasi 1968; Sannasi and Amirthavalli 1970);
- Infection of Small Hive Beetle (*Aethina tumida*) pupae by placement of an *A. flavus* culture agar plate in a container with the pupae caused mortality at a rate of 38% (Richards et al. 2005); and
- Healthy queen and drone termites (*Odontotermes obesus*) exposed to sporulating *A. flavus* by rolling the insects over the surface of the culture media resulted in an average mortality of 85% (Sannasi 1968).

Effects of experimental infections with specified doses of *A. flavus* were reported as follows:

- All oral doses between 50 and 25,000 conidia were pathogenic in adult honey bees (Foley et al. 2014);
- Honey Bee (*Apis mellifera*) larvae fed 5 µL of *A. flavus* spore suspensions at concentrations between 1.0×10^5 and 2.0×10^6 spores/mL showed an overall mortality of approximately 58% at the lowest dosage, to approximately 90% at the highest dosage (Vojvodic et al. 2011);

- *A. flavus* was pathogenic to the larvae of the wasp *Vespula germanica* by surface inoculation with 5 µl of a spore suspension of 5.25 x 10^7 spores/mL and *Vespula vulgaris* (by surface inoculation with 5 µl of a spore suspension of 3.5 x 10^7 spores/mL) (Glare et al. 1996); and
- Infection of silkworm (*Bombyx mori*) larvae by topical application of an unknown volume of a 1 x 10^6 conidia/mL inoculum resulted in death after 5 days, followed by larval mummification by 6 days post-inoculation (Kumar et al. 2004).

A. flavus is also known to affect marine invertebrates, as a common isolate from the tissue of diseased sea fans (*Gorgonia ventalina*), indicating its potential role in sea fan aspergillosis (reviewed in Zuluaga-Montero et al. 2010). No reports were found in the scientific literature of *A. flavus* having negative effects on fresh water invertebrate species.

C) Mycotoxicosis

Consumption of food or feed that is contaminated with mycotoxins may cause a variety of symptoms, depending on the type of mycotoxin, quantity and duration of exposure (Kanora et al. 2009), animal species, its age, and nutritional and health status at the time of exposure to contaminated feed (Prelusky et al. 1994). Mycotoxicosis can affect a wide range of susceptible animal species, including livestock, poultry and fish (Marasas and Nelson 1987; Moss 1996). Grains, cereals or products made from such grains are common sources of mycotoxin exposure (Binder 2007; Richard 2007; Sweeney and Dobson 1998). The signs elicited by mycotoxin consumption vary with the amount ingested. Mycotoxin concentrations occurring under field conditions lead to reduced animal performance or immune suppression without causing overt clinical signs (Oswald and Comera 1998; Marquardt 1996). Clinically overt disease results from ingestion of higher concentrations. Signs of mycotoxin intoxication include diarrhea, liver and kidney damage, pulmonary edema, vomiting, hemorrhaging and tumours (Binder 2007; Bryden 2012). The following are examples of the case reports related to mycotoxins produced by *A. oryzae* and *A. flavus* found in the literature:

- Food poisoning in cattle due to maltoryzine produced by a variant of *A. oryzae* was reported by Iizuka and Iida (1962). Possible adverse health effects in farm animals due to allergenicity and toxicosis from contaminated feed have also been reported (Lugauskas et al. 2004; Kharchenko and Yatsyshin 1984);
- An outbreak of pulmonary aspergillosis concurrent with aflatoxicosis in a flock of 5-week-old turkey poults and a group of 2-week-old goslings was linked to heavy contamination of the birds' litter and feed with *A. flavus* and aflatoxin B_1 (Okoye et al. 1989); and
- Natural field outbreaks among fowl and turkeys in the Ukraine between 1979 and 1985 were attributed to toxins, particularly kojic acid, formed by *A. flavus* which was isolated from wheat bran and crushed soybeans (Burdock et al. 2001).

1.1.3.2 Human health

Infection:

In spite of the long-term traditional use of *A. oryzae* in fermentation, no reports of infection are associated with occupational or household exposure to *A. oryzae* fermentation strains. However, members of the species *A. oryzae* have caused a small number of infections in immunocompromised, debilitated, or injured patients:

- One fatal case of aspergillosis in the brain of a farmer who had been injured in a fall (Ziskind et al. 1958);
- One episode of *A. oryzae* meningitis (Gordon et al. 1976; also reviewed in Antinori et al. 2013) in a drug addict who then experienced an *A. oryzae* eye infection six years later (Stenson et al. 1982);
- One paranasal sinus aspergillosis in a patient undergoing chemotherapy for leukemia (Byard et al. 1986). The authors referred to two similar case reports caused by *A. oryzae*;
- One case of pulmonary aspergillosis (attributed to a strain of *A. oryzae* from a hospital in Shanghai) (Liao et al. 1988). The identification of the isolated strain as *A. oryzae* appears problematic;
- Four cases of "bronchial stump aspergillosis", as a bizarre complication of pulmonary resection using silk thread sutures (Sawasaki et al. 1969);
- Two cases of superficial mycosis of the external ear (Reviewed in Barbesgaard et al. 1992);
- One case of fungal peritonitis in an immunocompromised patient (Schwetz et al. 2007); and
- One case of ulcerative keratomycosis (eye infection) after the patient was pricked in the eye by a leaf (Sukumaran 1991).

Given that it is difficult to differentiate *A. oryzae* from *A. flavus*, it is possible that the causative agent in some of the cases described above was *A. flavus*, erroneously identified as *A. oryzae*. *A. flavus*, which is considered to be the wild type of *A. oryzae*, is a human opportunistic pathogen, and systemic infection is often fatal in affected immunocompromised individuals (reviewed in Amaike and Keller 2011). *A. flavus* is the second most common cause of aspergillosis after *A. fumigatus* (Guarro et al. 2010).

In the following cases, *A. flavus* was identified as a cause of infection in patients with predisposing health conditions including nephritis, uremia, hepatitis, cholecystitis, tuberculosis, haemoptysis, diabetes, heart disease, hypertension, leukemia, lung cancer, and neuroblastoma. Many of these patients had undergone chemotherapy, surgery, or organ transplants as treatments for these diseases, and were thereby immunocompromised, debilitated, or were exposed to *A. flavus* through significant breaches in normal barriers to infection:

- Invasive *Aspergillus* sinusitis (Verschraegen et al. 1997; Xavier et al. 2009);

- Invasive pulmonary aspergillosis (Russo et al. 2011; Tendolkar et al. 2005; Zhirong et al. 1999);
- Invasive *Aspergillus* tracheobronchitis (Wu et al. 2010);
- Aspergilloma (Khan et al. 1995; Pasqualotto and Denning 2008);
- Pneumonia (Offner et al. 2004; Rao and Saha 2000);
- Osteomyelitis (Beluffi et al. 2008; Chang et al. 2012a; Chi et al. 2003; Nicolle et al. 2013; Stodulski et al. 2006; Verghese et al. 2009; Zhu et al. 2011);
- Articular aspergillosis (Yu et al. 2010);
- Venous catheter tunnel infection (Kriván et al. 2006);
- Necrotic lesions (Galimberti et al. 1998; Koss et al. 2002);
- Tuberculoid granulomas (Galimberti et al. 1998);
- Invasive otitis (Finer et al. 2002);
- *Aspergillus* myositis (Li et al. 2008);
- Endocarditis (Khan et al. 1997; Shoar et al. 2004);
- Orbital aspergillosis (Kumar et al. 2013); and
- Polymicrobial infections (Eschertzhuber et al. 2008; Frank et al. 1988; Rao and Saha 2000).

Hospital-acquired infections associating *A. flavus* have also been reported:

- Lower respiratory tract infection (Burwen et al. 2001; Sarubbi Jr. et al. 1982);
- Stomatitis (Myoken et al. 2003);
- Surgical site infection (Heinemann et al. 2004); and
- Vascular graft infection (Florio et al. 2004);

There are a few reports of *A. flavus* infections in otherwise-healthy individuals:

- Chronic granulomatous invasive fungal sinusitis (Rupa and Thomas 2013);
- Frontal sinus aspergillosis (Panda and Reddy 2005);
- Keratomycosis (Upadhyay et al. 1980); and
- Scleritis (Howell et al. 2005).

Allergy:

There have been multiple reports in the literature of allergy to alpha–amylase produced by *A. oryzae*, which are often linked to breadmaking as baker's asthma. Hypersensitive pneumonitis in brewers exposed to *A. oryzae* has also been reported (Tsuchiya et al. 1993).

Allergic bronchopulmonary aspergillosis (ABPA) has been associated with *A. oryzae*, which is a form of aspergillosis related to the inhalation of *Aspergillus* spores. Five similar cases of *A. oryzae* ABPA were reported in Japan related to the production of soybean products (Akiyama et al. 1987), and a case of ABPA was reported in which immunological tests indicated that both *A. fumigatus* and *A. oryzae* were involved, but fungal material was not isolated from the patient (Kurosawa et al. 1990). In Japan, it is

estimated that more than 30,000 individuals are exposed to high concentrations of *A. oryzae* spores from the fermentation of soy products in small family workshops (Akiyama et al. 1987); given this context, Kurosawa et al. (1990) stressed the fact that surprisingly only a few cases of ABPA due to *A. oryzae* have been reported.

A. flavus caused allergic fungal sinusitis (Chhabra et al. 1996) and allergic *Aspergillus* pneumonitis (Bakri et al. 2010) in two patients with either a history of asthma, or a weak immune system resulting from leukemia. *A. flavus* also caused allergic reactions or hypersensitivities among people in India with no predisposing conditions, including allergic fungal rhinosinusitis (Taj-Aldeen et al. 2004; Taj-Aldeen et al. 2003).

In vivo testing of *A. oryzae* ATCC 11866 using BALB/c mice as a model for human infection were done at Health Canada [5]. Four replicate BALB/c mice per time point exposed to 1 x 10^6 conidia/25 µL by endotracheal instillation for 2 hours to 1 week showed that there were no changes in behaviour or physical appearance. In addition, significantly elevated levels of proinflammatory cytokines, IL-1beta, IL-6, and TNF-alpha and KC in the lungs were observed, maximally between 4 and 48 hours post-exposure, declining to control levels by 4 days to 1 week post-exposure. These observations were accompanied by an elevation in levels of pulmonary granulocytes between 4 and 48 hours post-inoculation. These results are indicative of transient local inflammation resolving within one week; furthermore, there was clearance of *A. oryzae* ATCC 11866 from the lungs by 4 days post-exposure, with the lungs remaining clear at one week.

1.2 Hazard Severity

A. oryzae strains have a long history of use in the production of enzymes for brewing and baking, and as “koji moulds” for the fermentation of soy sauce, miso and sake (Barbesgaard et al. 1992; Blumenthal 2004; Jorgensen 2007). Until 1965, cultivation of *A. oryzae* in Europe took place in open trays, and massive amounts of conidia were released into the environment (Barbesgaard et al. 1992). Despite its long history of use in food production, there have been no reported safety concerns associated with exposure to *A. oryzae*.

A. oryzae is considered to be a domesticated strain of the wild type species *A. flavus*, and is thought, through domestication, to have lost, inactivated or modified some genes related to spore formation and dispersal, sclerotium formation and aflatoxins production (Jorgensen 2007; Kurtzman et al. 1986; Wicklow 1984a).These changes also appear to make *A. oryzae* less hazardous to environmental species and humans than *A. flavus*; however, the DSL strain ATCC 11866 has characteristics more typical of *A. flavus*. This is relevant especially with respect to conidial size, which in the DSL strain could favour infectivity in organisms in which inhalation is the principal route of infection. Futhermore,

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[5] Unpublished data generated by Health Canada’s Environmental Health Science and Research Bureau.

as with *A. flavus*, the DSL strain can grow at 37°C. Nevertheless, the DSL strain, ATCC 11866, differs from *A. flavus* in ways that reduce the estimation of hazard: ATCC 11866 is non-hemolytic and non-aflatoxigenic.

Sources of uncertainty in the assessment of hazard include a lack of information on the ability of *A. oryzae* ATCC 11866 to produce mycotoxins other than aflatoxins, a lack of information on the potential for *A. oryzae* strains to regain characteristics favouring fitness and infectivity under selective pressures encountered in the environment, and an absence of a history of safe use of *A. oryzae* ATCC 11866.

To help prevent the formation and consumption of mycotoxins, the livestock feed industry has established internal monitoring methods. Similarly, government regulatory agencies, including the CFIA, regulate mycotoxin levels in livestock feeds; non compliance with the CFIA *Feeds Regulations* is subject to the compliance and enforcement policies of that agency (Bennett and Klich 2003; CFIA 2014).

1.2.1 Environment

A. oryzae is implicated in a small number of animal infections, whereas some *A. flavus* isolates are pathogenic to plants, birds, and insects, and to a lesser extent, mammals. Most mammals and birds that were reported to be naturally infected by *A. oryzae* and *A. flavus* had predisposing health conditions and were exposed to high concentrations of spores. No negative effects were reported when *A. oryzae* was used as a probiotic in mammals and birds; therefore, ingestion of *A. oryzae* would not appear to be of concern. In the event of an infection, antifungal treatments are available to mammals. However, terrestrial invertebrates seem to be highly susceptible to *A. oryzae* and *A. flavus* pathogenicity.

Given that the DSL strain, ATCC 11866, shares characteristics with *A. flavus* that could favour its infectivity (i.e., small conidia, growth at 37°C), but lacks other determinants of pathogenicity and toxicity, and taking uncertainties into account, its hazard severity is estimated to be intermediate between that of *A. flavus* and ‘typical’ *A. oryzae*. Based on surrogate information relating to the species *A. oryzae* and *A. flavus*, the DSL strain ATCC 11866 may pose a potential hazard to plants, birds, insects, and mammals, especially those that are predisposed to infection.

Thus, based on these considerations, and on the uncertainties described above, the environmental hazard severity for *A. oryzae* ATCC 11866 is estimated to be medium.

1.2.2 Human Health

Hazards related to micro-organisms used in the workplace should be classified accordingly under the Workplace Hazardous Materials Information System (WHMIS). [6]

In spite of a long history of safe use of *A. oryzae* in food production (both commercially and domestically), there are very few case reports of *A. oryzae* infection in humans. In addition, the human body temperature (37°C) is optimal for the growth of *A. flavus*, but *A. oryzae* grows optimally at a lower temperature (34°C). The DSL strain can grow at normal, but not at febrile (42°C) human body temperature. The conidial size of the DSL strain could favour infectivity, making its hazard potential closer to that of *A. flavus* for pulmonary infections. Since there are no reports of gastrointestinal illness caused by *A. oryzae* or *A. flavus*, ingestion of *A. oryzae* ATCC 11866 is not of concern.

The vast majority of *A. flavus*-related diseases in healthy humans are mild, self-resolving and usually treatable, but there have been mortalities in immunocompromised individuals.

A lower estimation of hazard is supported by experimental testing conducted by Health Canada in a mouse model of human infection, in which no adverse effects were observed following endotracheal exposure to *A. oryzae* ATCC 11866. Although there was an inflammatory cytokine response, it was transient, and *A. oryzae* was cleared from the lungs within one week.

Based on these considerations, and taking into account the uncertainties described above, the human health hazard severity for *A. oryzae* ATCC 11866 is estimated to be low for healthy individuals and medium for immunocompromised individuals.

2. Exposure Assessment

2.1 Sources of exposure

The focus of this assessment is to characterize exposure to *A. oryzae* ATCC 11866 from its deliberate addition to consumer or commercial products or its use in industrial processes.

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[6] A determination of whether one or more criteria of section 64 of CEPA 1999 are met is based on an assessment of potential risks to the environment and/or human health associated with exposure in the general environment. For humans, this includes, but is not limited to, exposure from air, water and the use of products containing the substances. A conclusion under CEPA 1999 on *A. oryzae* ATCC 11866 is not relevant to, nor does it preclude, an assessment against the hazard criteria for WHMIS that are specified in the *Controlled Products Regulations* or the *Hazardous Products Regulations* for products intended for workplace use.

A. oryzae ATCC 11866 was nominated to the DSL in 1997 for its use in consumer and commercial products. In 2007, a voluntary questionnaire was sent to a subset of key biotechnology companies. These results, combined with information obtained from other federal government regulatory and non-regulatory programs, indicate that 10,000 to 100,000 kg of products potentially containing *A. oryzae* ATCC 11866 (formulation and concentration unknown) were imported into or manufactured in Canada in 2006.

To update information about current uses, the Government of Canada conducted a mandatory information-gathering survey under section 71 of CEPA, as published in the *Canada Gazette*, Part I, on October 3, 2009 (hereafter referred to as the section 71 Notice). The section 71 Notice applied to any persons who, during the 2008 calendar year, manufactured or imported *A. oryzae* ATCC 11866, whether alone, in a mixture, or in a product. No commercial or consumer activities using *A. oryzae* ATCC 11866 were reported in response to the section 71 Notice.

The 2007 and 2009 surveys differed significantly in target and scope. In this assessment, results from the 2009 survey were used to estimate exposure from current uses because it required that requested information on uses of the micro-organism strain that is listed on the DSL be provided, whereas the 2007 survey asked about uses of the products that had been associated with the micro-organism at the time it was nominated to the DSL. Because product formulations may have changed, information from the 2009 mandatory survey may more accurately represent current uses. Uses reported in the 2007 voluntary survey were also considered in the assessment of potential uses.

The DSL strain ATCC 11866 was originally selected for its high protease activity. This characteristic could be applied for preparing bates for the tanning industry and for hydrolyzing fish, animal, and vegetable proteins into food (Baens-Arcega et al. 1956). A search of the public domain (material safety data sheet, literature and patents) revealed the following consumer, commercial and industrial applications for *A. oryzae* ATCC 11866:

- Use in food industry:
    - Process for producing soy sauce or Miso (Noda,F. 1983, patent US 4382964 A); and
    - Process for producing solid koji (Noda, F. 1982, patent US 4329370 A).

A. oryzae is metabolically diverse, which makes it of commercial interest in a variety of industries, particularly in enzyme production and food fermentation. Although no uses were reported for ATCC 11866 during the mandatory survey, it is available for purchase from the ATCC. As it is on the DSL, and so can be used in Canada without prior notification, it could be an attractive choice for commercialization.

A search of the public domain (material safety data sheet, literature and patents) revealed the following consumer, commercial and industrial applications of other strains *of A. oryzae.* These represent possible uses of the DSL strain, as strain ATCC 11866 is

likely to share the characteristics (modes of action) with other commercialized *A. oryzae* strains:

- Use as a production organism for the following:
    - enzymes, and recombinant enzymes (Abe et al. 2006) and specialty chemicals, including α-amylase, β-galactosidase, β-glucosidase, B-N-acetylhexosaminidase, diatase, lactase, lipase, nuclease, phospholipase A1, proteinase 2A, protease, ribonuclease, taka-diastase (Product Sheet A, 2014; Belli et al. 1994, patent CA 2125793; Bhargava et al. 2005, patent CA 2566252; Boel et al. 2009, patent CA 1341593; Foley and Jones 2000, patent CA 2183745; Hattori et al. 2002, patent CA 2098421;Huffman 2001, patent CA 2188280; Kiuchi and Tanaka 1977, patent CA 1016092; Kurita et al. 1979, patent CA 1059051; Riou and Gunata 1999, patent CA 2326496; Van Den Broek and Affolter 1999, patent CA 2263947); fucose-specific lectin protein (Product Sheet B 2014), fermentation extracts used in animal feed (Product Sheet C 2014) and lipid biodiesel from potato-processing wastewater (Muniraj et al. 2013);
- Use in Asian traditional food fermentation industries, producing soy sauce, miso, alcoholic beverages, and vinegar (Kumeda and Asao 2001; Luh 1995);
- Use as probiotics in pigs (Choi et al. 2011), chickens (Shim et al. 2010), sheep (Jouany et al. 1998), dairy cows (Chiquette 2009; Miranda et al. 1996) and other ruminants (reviewed in Yoon and Stern 1995);
- Use for treatment of grease traps and sewer lines:
    - *A. oryzae* with other micro-organisms are used to dispose of organic waste materials (Higa, T. 1997, patent CA 2098969);
- Use in bioremediation of the following contaminants:
    - chromium (Sepehr et al. 2005);
    - organic substrates in phenol-contaminated wastewater (Abd El-Moneim et al. 2013; Zhang et al. 2013);
    - melanoidin polymers (Chavan et al. 2013); and
    - dyes in textile effluents (Corso and Maganha De Almeida 2009; Corso et al. 2012); and
- Use of dead *A. oryzae* cell in biosportion of heavy metals (Huang and Huang 1996).

These uses are largely industrial, but its ability to degrade organic wastes makes it of possible interest for use in certain consumer products, including septic tank treatments, drain cleaners and degreasers.

2.2 Exposure characterization

2.2.1 Environment

The environmental exposure for *A. oryzae* ATCC 11866 is estimated to be low based on the absence of responses to the 2009 section 71 Notice, suggesting that this strain is no longer used in consumer or commercial products or for industrial processes in Canada.

Nevertheless, environmental exposure scenarios, in the event that consumer, commercial or industrial activities with *A. oryzae* ATCC 11866 resume, have been considered along with the persistence and survival properties of this micro-organism. Due to the expanding commercialization of microbial-based products, some potentially containing *A. oryzae* ATCC 11866, there is a likelihood of an increase in the use and release of this micro-organism in the environment (Chatzipavlidis et al. 2013).

Former and possible future uses of the DSL strain are described in Section 2.1. These would likely introduce *A. oryzae* ATCC 11866 into both aquatic and terrestrial ecosystems. For example, uses in bioremediation or biodegradation would involve direct application to soils, and subsequent rainfall events could introduce *A. oryzae* ATCC 11866 into waterways. In addition, the potential uses of *A. oryzae* ATCC 11866 in waste water treatment facilities and for the production of biofuels, organic acids or enzymes could lead to direct input into waterways in wastewater effluent.

The magnitude of plant and animal exposure to *A. oryzae* ATCC 11866 will depend on the nature of the use and on its persistence and survival in the environments to which it is released. Hynes et al. (2006) demonstrated that *A. oryzae* can persist 4 months after inoculation into soil microcosms; the ability of *A. oryzae* to survive wastewater treatment from industrial discharges, probably due to its resistant conidia, suggests that it is likely to persist after introduction into aquatic environments (U.S. EPA 1997a).

On the basis of its larger conidial size, *A. oryzae* is generally considered less fit to disperse in nature than *A. flavus* (Horn 2003; Wicklow 1984a); however, the conidia of *A. oryzae* germinate earlier and produce a larger mycelium than wild relatives. In an environment where it is in competition with its wild-type counterparts, it could provide a competitive advantage over its wild-type counterparts in accessing available substrates at a particular site (Wicklow 1984b). The DSL strain, ATCC 11866, shares morphological characteristics with *A. flavus,* such as smaller conidia, that could favour its dispersal in the environment, but reduce its competitiveness. In addition, the DSL strain does not produce sclerotia, and this limits its ability to persist under unfavourable conditions in soil relative to *A. flavus*. Considering that *A. oryzae* and *A. flavus* are uncommon above 45 degrees latitude, it is unlikely that introduced populations of these species can overwinter successfully enough to be maintained in many parts of Canada.

2.2.2 Human

Human exposure to *A. oryzae* ATCC 11866 is estimated to be low based on responses to the 2009 section 71 Notice, which indicated that this strain was no longer used in consumer or commercial products or for industrial processes in Canada in 2008.

Nevertheless, human exposure scenarios in the event that consumer, commercial or industrial activities with *A. oryzae* ATCC 11866 resume have been considered. These are based on former and probable future uses as described in Section 2.1. Should potential uses of *A. oryzae* ATCC 11866 be realized, human exposure would be expected primarily through direct contact with consumer products containing *A. oryzae* ATCC 11866. The handling and application of such products would be expected to result in direct exposure to the skin, as well as exposure through inhalation of aerosolized droplets or dusts containing *A. oryzae* ATCC 11866. Secondary to product application, residual *A. oryzae* ATCC 11866 on surfaces or in reservoirs such as treated drains could result in dermal exposure; secondary exposure through inadvertent ingestion could occur where the organism persists on food preparation surfaces; inhalation may occur where aerosols are generated (e.g., from kitchen garbage disposal units), or where spores are formed in reservoirs such as treated drains. If custodial products containing *A. oryzae* ATCC 11866 were used in hospitals, clinics, or long-term care facilities, there would be an increased risk that susceptible individuals could be exposed. Since *A. oryzae* ATCC 11866 is expected to persist in organic-rich sites following application, such exposures may be temporally distant from the time of application.

The general population could be exposed to *A. oryzae* ATCC 11866 as bystanders during the application of commercial products containing this strain. The route and extent of exposure will depend on the nature of the product, the application method, the concentration of *A. oryzae* ATCC 11866 in the product, the amount of product applied, and the proximity of the bystander to the site of application, but in general is expected to be moderate to low. The general population could also come into contact with residual *A. oryzae* ATCC 11866 on surfaces treated with commercial products.

Indirect exposure to *A. oryzae* ATCC 11866 in the environment subsequent to its use in bioremediation and biodegradation, bioleaching, textile processing, municipal or industrial wastewater treatment or in disposal of waste from its use in the production of enzymes and fermentation extracts is likely to occur. Certain uses in waste and wastewater treatment or in industrial processes may introduce *A. oryzae* ATCC 11866 into bodies of water. Human exposure to this strain through recreational activities is expected to be low. Drinking water treatment processes might not eliminate this micro-organism (Sisti et al. 2012); however, ingestion of this micro-organism is not of concern. The micro-organism could possibly be inhaled from water droplets (e.g., while showering), but this would be expected to occur only in minute quantities due to dilution and because growth conditions for *A. oryzae* are not optimal in drinking water.

The exposure route of primary concern for human infection with *A. oryzae,* as for *A. flavus*, is through the inhalation of conidia; of secondary concern is infection through contact with broken skin or wounds, and the contamination of intravenous solutions and wound dressings (Kagen et al. 1983). Inhalation of fungal spores through smoking contaminated tobacco or marijuana has been reported (reviewed in Amaike and Keller 2011), but is an unlikely route of exposure from foreseeable future uses of *A. oryzae* ATCC 11866.

In the event that consumer, commercial or industrial activities resume, the human exposure to *A. oryzae* ATCC 11866 could change based on the exposure scenarios described above.

3. Risk Characterisation

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

Hazard has been estimated for *A. oryzae* ATCC 11866 to be medium for the environment, and for human health, low for healthy individuals, and medium for immunocompromised individuals. Environmental and human exposure to *A. oryzae* ATCC 11866 from its deliberate use in industrial processes or consumer or commercial products in Canada is not currently expected, so the risk associated with current uses is estimated to be low for both the environment and human health.

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

Risks to the environment from foreseeable future uses:

This assessment concludes that the hazard associated with *A. oryzae* ATCC 11866 is likely to be intermediate between the hazard typically associated with *A. oryzae* and that associated with *A. flavus*.

A. flavus is a plant pathogen commonly affecting maize, cottonseed, tree nuts, oilseed crops, and peanut plants (Klich 2007). It is also an opportunistic animal pathogen, causing mycosis, particularly in poultry, but also affecting insects, including bees and grasshoppers, and in marine environments, sea fans.

Wild plants and animals (including birds and insects) could be exposed to elevated concentrations of *A. oryzae* ATCC 11866 if it were used in bioremediation, biotransformation or biodegradation in contaminated sites; however, such releases would be areas already affected by contamination with toxic compounds such as textile dyes, phenols and chromium, which are also likely to affect plants and animals, and these sites are expected to be small relative to the total Canadian land mass. Crops and

domestic animals, especially poultry, could be affected if such sites were adjacent to farms or pastures, but this is expected to be an exceedingly rare occurrence.

Aquatic or marine animals could inadvertently be exposed to *A. oryzae* ATCC 11866 through its use in waste water treatment, through runoff from terrestrial applications or release in industrial effluents. *A. oryzae* could persist and survive in the aquatic environment due to its conidia; however, no adverse effects have been found in the literature regarding freshwater species. In the marine environment, few effects were found in sea fans and sea farm fishes; even these effects are unlikely to be realized from commercial uses of *A. oryzae* products because of the expected moderate extent of their use, and because of conidial dilution in receiving waters. Furthermore, the Canadian climate would limit survival of the conidia in the receiving water.

Therefore, the use of *A. oryzae* ATCC 11866 in bioremediation, biodegradation, and waste water treatment or in industrial processes is unlikely to have a serious impact on terrestrial or aquatic populations and trends over an entire ecosystem or an ecozone, and the risk from foreseeable future uses remains low.

Risks to human health from foreseeable future uses:

A. flavus is a human opportunistic pathogen leading to a wide array of infections including lung, sinus, skin, bone, eyes, ear, heart and systemic infections.

Industrial uses are not of significant concern from a human health perspective; however, its ability to degrade organic wastes makes *A. oryzae* ATCC 11866 of possible interest for use in certain consumer products, including septic tank treatments, drain cleaners and degreasers. Susceptible individuals could be exposed to *A. oryzae* ATCC 11866 during the application of consumer products containing *A. oryzae* ATCC 11866, or from contaminated surfaces or reservoirs such as drains. If custodial products containing *A. oryzae* ATCC 11866 were used in hospitals, clinics, or long-term care facilities, there would be an increased risk that susceptible individuals could be exposed and that medical devices could become contaminated. These risks are expected to increase with growth in the market for “greener” microbial-based cleaning products (Spök and Klade 2009; Vandini et al. 2014)), and so the risk from foreseeable future uses of *A. oryzae* ATCC 11866 is considered to be medium for human health.

4. Conclusion

Based on the information presented in this screening assessment, it is concluded that *A. oryzae* ATCC 11866 is not entering the environment in a quantity or concentration or under conditions that:

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

- constitute or may constitute a danger in Canada to human life or health.

Therefore, it is concluded that *A. oryzae* ATCC 11866 does **not** meet the criteria as set out in section 64 of the CEPA.

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Appendices

Appendix A: Growth of *Aspergillus oryzae* ATCC 11866 in various media

Table A-1 Growth of *A. oryzae* ATCC 11866 in liquid media at various temperatures[a]

Medium	28°C	32°C	37°C	42°C
Sabouraud Liquid Medium	+	+	+	~
100% Fetal Bovine Serum	~	+	+	–
Dulbecco's Modified Eagles Medium (mammalian cell culture)	(+)	(+)	(+)	–
10 % Sheep Blood Serum	~	~	~	–

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

[a] Data generated by Health Canada's Environmental Health Science and Research Bureau. Growth of *Aspergillus oryzae* ATCC 11866 in broth culture, as measured by increase in absorbance at 500 nm, in four different growth media and over a range of temperatures: Measurements were taken every 15 minutes over a 24-hour period with a multi-well spectrophotometer.

Table A-2 Growth Characteristics of *A. oryzae* ATCC 11866 on Solid Media at various temperatures[a]

Medium	28°C	37°C
Blood Agar [b] growth	+	+
Blood Agar [b] hemolysis	-	-
Czapek Agar [c]	+	+
Dermatophyte test agar [d]	-	-
Mycosel Agar [e]	-	-
Potato Dextrose Agar [f]	+	+
Sabouraud Dextrose Agar [g]	+	+
Staib's Hivey Bird Seed Agar [h]	+	+
Yeast Mould Agar [i]	+	+

(+) Positive for growth (-) Negative for growth
[a] Data generated by Heath Canada's Environmental Health Science and Research Bureau
[b] Sheep Blood Agar. Used to detect lysis of blood cells (hemolysis).
[c] Czapek-Dox Agar is recommended in Standard Methods for the Examination of Water and Wastewater for the isolation of *Aspergillus*, *Penicillium*, *Paecilomyces* and other types of fungi with similar physiological requirements (Hardy Diagnostics).
[d] Dermatophyte test agar. Selective medium used for the isolation of pathogenic fungi from cutaneous specimens.
[e] Mycosel Agar. For the isolation of pathogenic fungi from materials having a large flora of other fungi and bacteria.
[f] Potato Dextrose Agar. Used for cultivation and isolation of yeasts and molds
[g] Sabouraud Dextrose Agar (SAB). A standard medium for the isolation and maintenance of a wide variety of fungi commonly encountered in a clinical setting.
[h] Staib's Hivey Bird Seed Agar. For the selective isolation of Crytococcus neoformans from other yeasts.
[i] Yeast Mould Agar. Cultivation of yeasts, molds and other aciduric micro-organism

Appendix B: Secondary metabolites produced by *Aspergillus oryzae* and *Aspergillus flavus*

Table B-1 List of toxins and secondary metabolites produced by *Aspergillus oryzae/flavus*

Toxins	Actions
Aflatoxins	• Produced by some strains of *A. flavus* (Klich 2007). • Multiple forms: B1, B2, G1, G2, M1 and M2 which differ slightly in structure (reviewed in Amaike and Keller 2011). • Link to crop and feed contamination (Adebajo 1992; Jaime-Garcia and Cotty 2004). • In mice, aflatoxin B1 decreases DNA synthesis in lymphocyte cultures, peripheral leukocyte counts, DNA, protein and RNA synthesis in splenic lymphocytes, and concanavalin A-induced suppressor cells, suggesting a direct and complex effect on lymphocytes (Reddy and Sharma 1989). • Daily intake of low dose of aflatoxins can cause chronic aflatoxin poisoning resulting in anorexia, stunted growth, immune suppression and possible liver cancer development (reviewed in Amaike and Keller 2011). • Acute aflatoxin poisoning with high doses can kill humans and animals (reviewed in Amaike and Keller 2011). • *A. oryzae* ATCC 11866 does not produce aflatoxin (Wei and Jong 1986).
Aflatrem	• Produced by some strains of *A. flavus* (Nicholson et al. 2009). • Potent tremorgenic toxin, part of a group of fungal secondary metabolites known as indole-diterpenes (Nicholson et al. 2009). • In mice, gavage doses between 0.5 g to 1.0 mg of the partially purified toxin induced tremors, and when the dose was increased to 2 mg, tremors were followed by convulsions. Lower doses produced either no effect or mild tremors for a short period (Wilson and Wilson 1964). • No LD 50 available. • No indication if *A. oryzae* ATCC 11866 produces this mycotoxin.
Aspergillic acid	• Produced by some strains of *A. flavus* (Masaki et al. 1966).

	• A hydroxypyrazine derivative secondary metabolite with bactericidal properties (Masaki et al. 1966). • Induces acute severe toxicity in mice at 150 mg/kg (intraperitoneal), however no chronic toxicity is indicated at sublethal doses, unlike aflatoxins (MacDonald 1973). • No LD 50 available. • No indication if *A. oryzae* ATCC 11866 produces aspergillic acid.
Aspertoxin	• Produced by some strains of *A. flavus* (Rodricks et al. 1968). • Hydroxy derivative of another metabolite produced by *A. flavus*, O-methylsterigmatocystin. • The structure resembles aflatoxin M_1, aflatoxin B_1 and sterigmatocystin (Rodricks et al. 1968). • No LD 50 or information on toxicity available. • No indication if *A. oryzae* ATCC 11866 or any *A. oryzae* strains produce aspertoxin.
Cyclopiazonic Acid (CPA)	• Produced by some strains of *A. flavus* and *A. oryzae* (Benkhemmar et al. 1985; Chang et al. 2009; Matsudo and Sasaki 1995; Tokuoka et al. 2008). • Associated with contamination of crop and animal foods (Chang et al. 2009; Gallagher et al. 1978). • An indole-tetramic acid neurotoxin (Chang et al. 2009). • Causes the inhibition of Ca^{2+}-ATPase which in turn causes the sarcoplasmic reticulum (Smooth ER) to lose function (reviewed in Blumenthal 2004). • LD 50: 2 mg/kg in rat (intraperitoneal) 13 and 64 mg/kg in mouse (intraperitoneal and oral) and 12 mg/kg in chickens (oral) (reviewed in Blumenthal 2004). • Caused leukocytosis and lesions in gastrointestinal tract, liver and kidney of pigs (NOEL between 0.1 and 0.01 mg/kg). Altered renal or hepatic function was not determined (Lomax et al. 1984). • Some *A. oryzae* strains used in food fermentation are capable of CPA production (Chang and Ehrlich 2011; Orth 1977), but in most, domestication has likely resulted in a loss of biosynthetic pathways for CPA because it is of no adaptive value to the fungus under conditions of domestication (Wicklow 1984a). • No indication if *A. oryzae* ATCC 11866 produces cyclopiazonic acid.
Gliotoxin	• Produced by some strains of *A. flavus* (reviewed in Hedayati et al. 2007). • An immunosuppressive toxin (Mullbacher et al. 1985;

	Sutton et al. 1994). • Inhibits macrophage and polymorphonuclear cell function and generation of alloreactive cytotoxic T cells; inhibits the transcription factor of activated B cells; toxic to mitochondria by reducing the production of ATP, which leads to apoptosis (Pardo et al. 2006). • LD 50 (intraperitoneal): 25mg/kg in mice (Johnson et al. 1943; Vigushin et al. 2004). • No indication if *A. oryzae* ATCC 11866 or any other *A. oryzae* strains produce gliotoxin.
Kojic Acid (KA)	• Produced by some *A. oryzae* and *A. flavus* strains (Blumenthal 2004; Manabe et al. 1984). • Inhibits melanosis by hindering the uptake of oxygen required for enzymatic browning (reviewed in Burdock et al. 2001). • Inhibits the oxidation of D-amino acids, xanthine, L-phenylalanine, and L-methionine in the liver of rats in vitro and inhibits 4-nitroanisole O-demethylation in vitro and in vivo in *Helicoverpa zea* (moth) and *Spodoptera frugiperda* (Army worm) (reviewed in Burdock et al. 2001). • Insecticidal, antibacterial and antifungal properties (Chaves et al. 2012). • Can cause toxicity in mice, rats, dogs, and poultry (Burdock et al. 2001). • LD 50 (intraperitoneal): 250 mg/mouse (Blumenthal 2004). • Considered a contaminant and is present at negligible levels in fermented foods. The incubation period for sake, shoyu and miso is about two days and no kojic acid is found at that time (Tanaka et al. 2002). • No indication if *A. oryzae* ATCC 11866 produces kojic acid.
Maltoryzine	• Produced by *A. oryzae* var. *microsporus* and possibly by very few other strains of *A. oryzae* (Ciegler and Vesonder 1983). • Highly toxic to dairy cows (Iizuka and Iida 1962). • LD 50 (mouse): 3mg/kg and causes muscular narcotism (Iizuka and Iida 1962). • No indication if *A. oryzae* ATCC 11866 produces maltoryzine.
3-Nitropropionic Acid (NPA)	• Produced by some strains of *A. oryzae* (reviewed in Blumenthal 2004) and *A. flavus* (Doxtader and Alexander 1966). • Considered neurotoxic by irreversibly inhibiting

	succinate dehydrogenase (reviewed in Blumenthal 2004). • Can cause energy impairment, excitotoxicity (apoptotic neuronal death) and oxidative stress in rats and mice (Doxtader and Alexander 1966; Kim and Chan 2002). • LD 50: 67 mg/kg in rat (intraperitoneal), 22 mg/kg in rat (subcutaneous) 140 and 50 mg/kg in mouse (intraperitoneal), 68.1 mg/kg in mouse (oral) and 25.1mg/kg in chickens (oral) (reviewed in Blumenthal 2004). • No indication if *A. oryzae* ATCC 11866 produces 3-Nitropropionic acid.
Sterigmatocystin	• Produced by some strains of *A. flavus* (reviewed in Hedayati et al. 2007). • An intermediate in aflatoxin synthesis (Blumenthal 2004). • Caused lung tumours in mice, liver tumours in rats after oral administration, and skin and liver tumours in rats after application to the skin, and is therefore considered carcinogenic in mice and rats (reviewed in IARC 1976). • LD 50 in mice (oral) 166mg/kg in males (dimethylformamide as vehicle) and 120 mg/kg in females (wheat-germ oil as vehicle) and LD 50 (intraperitoneal) in males were 60 and 65 mg/kg using respectively dimethylformamide and wheat-germ oil as vehicle (Purchase and Van der Watt 1969). • No indication if *A. oryzae* ATCC 11866 produces sterigmatocystin.