Environment Canada Environnement Canada

Draft Screening Assessment for *Aspergillus awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *Aspergillus brasiliensis* ATCC 9642

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Synopsis

Pursuant to paragraph 74(b) of the *Canadian Environmental Protection Act, 1999* (CEPA 1999), the Ministers of Environment and of Health have conducted a screening assessment of two micro-organism strains: *Aspergillus awamori* (ATCC 22342) (also referred to as *Aspergillus niger* ATCC 22342) and *Aspergillus brasiliensis* (ATCC 9642).These strains were added to the Domestic Substances List (DSL) under subsection 105(1) of CEPA 1999 because they were manufactured in or imported into Canada between January 1, 1984 and December 31, 1986 and entered or were released into the environment without being subject to conditions under CEPA 1999 or any other federal or provincial legislation.

Recent publications have demonstrated that the DSL strain ATCC 22342 is a strain of *A. niger* and not *A. awamori*. However, both names are still being used. Therefore, in this report we will use the name "*A. awamori* ATCC 22342 (=*A. niger* ATCC 22342)".

The *A. niger* group is generally considered to be ubiquitous in nature, and is able to adapt to and thrive in many aquatic and terrestrial niches; it is common in house dust. *A. brasiliensis* is relatively a rarely occurring species; it has been known to occur in soil and occasionally found on grape berries. These two species form conidia that permit survival under sub-optimal environmental conditions.

A. awamori ATCC 22342 (=*A. niger* ATCC 22342) is known to produce ochratoxin A and fumonisins (mainly B_2) which are potential carcinogens that can affect humans and animals. *A. brasiliensis* ATCC 9642 does not produce these mycotoxins.

A. niger and A. brasiliensis are commonly found as saprophytes. In particular *A. niger,* which is a well-studied organism, is considered a weak plant pathogen and not a major cause of plant disease. *A. niger* secretes extracellular enzymes that may cause damage to agricultural crops. Despite its occurrence in nature, there is no evidence in the scientific literature to suggest that *A. brasiliensis* has any ecological effects at a population level for plants. *A. niger* has been reported as an opportunistic animal pathogen, causing mycosis (infection) and mycotoxicosis (from ingestion of toxin-contaminated feed), which triggers a range of symptoms that can debilitate the host. However, under normal circumstances, it is unlikely to be a serious hazard to healthy livestock or to other organisms in the environment. Government regulatory agencies, including the Canadian Food Inspection Agency, regulate mycotoxin levels in livestock feeds.

Information from the scientific literature indicates that *A. niger* and *A. brasiliensis* can cause ear and eye infections in otherwise-healthy humans, and potentially fatal lung disease in susceptible groups (i.e., infants and the elderly, the immunocompromised and individuals with debilitating comorbidities). *A. niger* and *A. brasiliensis* are resistant to some clinical antifungals, which could, in some circumstances, compromise the effectiveness of treatment of *A. niger* and *A. brasiliensis* infections. Furthermore, because of the adverse effects associated with antifungal treatment, the availability of clinically relevant drugs is not strongly weighted as a mitigating factor in this assessment.

This assessment considers human and environmental exposure to *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 from their deliberate use in consumer or commercial products or in industrial processes in Canada. The government launched a mandatory information-gathering survey (Notice) under section 71 of CEPA 1999 as published in the Canada Gazette Part I on October 3rd, 2009. Information submitted in response to the Notice indicates that *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 were not imported or manufactured in Canada in 2008, except for limited quantities for academic research, teaching, and research and development activities.

Considering all available lines of evidence presented in this draft screening assessment, there is low risk of harm to organisms and the broader integrity of the environment from *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642. It is proposed to conclude that *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 do not meet the criteria under paragraphs 64(*a*) or (*b*) of CEPA 1999 as they are 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.

Based on the information presented in this draft screening assessment, it is also proposed to conclude that *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 do not meet the criteria under paragraph 64(*c*) of CEPA 1999 as they are 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.

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Introduction

Pursuant to paragraph 74(b) of the *Canadian Environmental Protection Act, 1999* (CEPA 1999), the Ministers of Environment and of Health are required to conduct screening assessments of those living organisms listed on the Domestic Substances List (DSL) 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 1999). *Aspergillus awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *Aspergillus brasiliensis* ATCC 9642 were nominated and added to the DSL under Section 105 of CEPA 1999 because they were manufactured in or imported into Canada between January 1, 1984 and December 31, 1986 and they entered or were released into the environment without being subject to conditions under CEPA 1999 or any other federal or provincial legislation.

This screening assessment considers hazard information obtained from the public domain, from unpublished research data and comments from researchers in related fields. Exposure information was obtained from the public domain, from the nominator, as well as from a mandatory CEPA 1999 s.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 “Framework on the Science-Based Risk Assessment of Micro-organisms under the *Canadian Environmental Protection Act, 1999*”.

A. awamori ATCC 22342 (=*A. niger* ATCC 22342) was recently demonstrated to be *A. niger* and *A. brasiliensis* ATCC 9642 has been identified as *A. niger*, and various authors refer to them as *A. niger* in the literature. For this reason, the two strains are grouped in the same risk assessment, and literature searches on the species *A. awamori*, *A. brasiliensis, A. niger* , and *Aspergillus* section *Nigri* were used. Data specific to DSL-listed *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) or *A. brasiliensis* ATCC 9642 are identified as such. Surrogate organisms are identified to the taxonomic level provided by the source. Literature searches were conducted using scientific literature databases (SCOPUS, CAB Abstracts, and NCBI), web searches, and key search terms for the identification of human health and environmental hazards of each of the DSL strains assessed in this report. Information identified as of May 2013 was considered for inclusion in this report.

1. Hazard Assessment

1.1 Characterization of *Aspergillus awamori* and *A. brasiliensis*

1.1.1 Taxonomic Identification and Strain History

Binomial Name	*Aspergillus awamori* (*A. niger*)	**Binomial name**	*Aspergillus brasiliensis*
Kingdom	Fungi	**Kingdom**	Fungi
Phylum	Ascomycota	**Phylum**	Ascomycota
Class	Eurotiomycetes	**Class**	Eurotiomycetes
Order	*Eurotiales*	**Order**	*Eurotiales*
Family	*Trichomaceae*	**Family**	*Trichomaceae*
Genus	*Aspergillus*	**Genus**	*Aspergillus*
Subgenus	*Circumdati*	**Subgenus**	*Circumdati*
Section	*Nigri*	**Section**	*Nigri*
Species	*awamori (niger)*	**Species**	*brasiliensis*
Strain	ATCC 22342	**Strain**	ATCC 9642

Common/Superseded Names: For *A. awamori* ATCC 22342, which is also referred *as A. niger* ATCC 22342, different names have been used as synonyms or to identify alternate states. Since the strain was mainly known until recently as *A. awamori*, the following synonyms have been used in the literature review: *Aspergillus awamorii*; *Aspergillus niger* var. *awamorii; Aspergillus niger* var. *awamori*; *Aspergillus inuii* Sakaguchi et al., anamorph*; Aspergillus luchuensis Inui, anamorph; Aspergillus usamii* Sakaguchi et al., anamorph*, Aspergillus niger* var. *fusca Blochwitz,* anamorph and *A. welwitschiae.* There is no synonym for *A. brasiliensis*; however, until 2007, it was called *Aspergillus niger*.

Strain History: *A*. *awamori* ATCC 22342 (=*A. niger* ATCC 22342) was originally isolated from bran by H. Ono, deposited to the NRRL by J. van Lanen and later on deposited to the ATCC as *A. awamori*. This strain is still referred to as *A. awamori* in culture collections, including ATCC and NRRL. *A. brasiliensis* ATCC 9642 was originally isolated as a contaminant from wireless radio equipment in New South Wales, Australia and was deposited to the ATCC by W.H. Weston.

The taxonomy of the genus *Aspergillu*s is complicated by a lack of suitable taxonomic criteria that consistently discriminate between the different species. Many authors, such as Raper and Fenell (1965), Al-Musallam (1980) and Kozakiewicz (1989), have arrived at different groupings based on phenotypic characteristics (Abarca et al., 2004). Currently, the genus *Aspergillus* contains approximately 250 species in eight subgenera (*Aspergillus, Fumigati, Circumdati, Candidi, Terrei, Nidulantes, Warcupi,* and *Ornati*), which are further divided into sections or species complexes (Alastruey-Izquierdo et al., 2012; Samson and Varga, 2012).

Both *A. niger* and *A. brasiliensis* are part of the *Aspergillus* subgenus *Circumdati,* section *Nigri.* Despite their importance in medical, agricultural and industrial settings, the taxonomy of members of the *Aspergillus* section *Nigri* is poorly defined (Howard et al., 2011), and is still evolving, with new species being accepted (Varga et al., 2011). *Aspergillus* section *Nigri* includes 26 taxa that produce a black pigment, and share the production of citric acid and one or more of the following secondary metabolites: pyranonigrins, naphtho-γ-pyrones, malformins, antafumicins and kotanins (Samson et al., 2004; Varga and Samson, 2008). Of those, several belong to the *A. niger* ``aggregate`` (Perrone et al., 2011), which includes *A. acidus*, *A. awamori*, *A. brasiliensis*, *A. niger* and *A. tubingensis*, all of which are morphologically indistinguishable (Howard et al., 2011; Samson et al., 2007a). Members of this section are often called black aspergilli or ``*A. niger* `` without regard to morphological or biochemical characteristics. This creates ambiguity in attributing research-findings or cases of infection to a particular species of *Aspergillus* section *Nigri*. Also the same isolate has been preserved in culture collections under different species names (Abarca et al., 2004) creating multiple synonyms. This can create an ambiguous-identification (Alastruey-Izquierdo et al., 2012; Raper and Fennell, 1965; Samson et al., 2006).

Strains of *A. awamori* are often called *A. niger* in the literature and it is not clear if *A. awamori* is a synonym or a variety of *A. niger* or a species on its own. *A. niger* and *A. awamori* differ in their occurrence on various substrates and in certain physiological characteristics such as elastase activity and the ability to use 2-deoxy-D-glucose as the sole carbon source (Varga et al., 2011). *A. awamori* was revalidated as a cryptic species within *A. niger* (Perrone et al., 2011) meaning that the two species cannot reliably be distinguished by morphological characteristics or extrolite profiles. Varga et al. (2011) indicated that only molecular approaches, including sequence analyses of calmodulin or β-tubulin genes, amplified fragment length polymorphism (AFLP) analysis, universally primed polymerase chain reaction (UP-PCR) analysis or mitochondrial DNA restriction fragment length polymorphism (mtDNA RFLP) analysis, could really distinguish *A. awamori* from *A. niger*. Further complicating the taxonomy of *A. awamori* is the lack of type strain (Hong et al., 2013). Originally, *A. awamori* was identified as the koji fungus used in the fermentation of the beverage awamori; however the type strain of *A. awamori* (CBS 557.65) did not originate from awamori fermentation (Hong et al., 2013), and was shown, with the use of the β-tubulin sequence*,* to be identical to *A. welwitschiae.* Therefore, *A. awamori* has been reduced to a synonym of *A. welwitschiae.* β-tubulin sequence analysis also identified the DSL strain ATCC 22342 as *A. niger* (Hong et al., 2013) and for this reason, the DSL strain will be characterized using surrogate information from *A. niger* instead of *A. awamori* and will be referred as *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342*)* in this screening assessment since both names are still currently used to describe the same strain.

A. brasiliensis, a new *Aspergillus* section *Nigri* species, accepted in 2007, includes a number of strains previously identified as *A. niger*, including the DSL *A. brasiliensis* strain ATCC 9642, (Varga et al., 2007). Varga *et al.* (1994) observed that six out of

13 Brazilian *A. niger* isolates exhibited a different type of mtDNA and rDNA, and as a consequence, could not be accurately classified. Analyses of intergenic transcribed region, β-tubulin and calmodulin gene sequences, AFLP analysis and extrolite profiles contributed evidence for the establishment of a new species (Samson et al., 2007a; Varga et al., 2007). *A. brasiliensis* was also shown to be the only species of the black aspergilli to be able to grow on D-galactose (Meijer et al., 2011).

1.1.2 Phenotypic and Molecular Characteristics

Since species in the *Aspergillus* section *Nigri* are difficult to distinguish from one another based on morphology alone, identification of species within this group relies on the characterization of variable DNA sequences such as β-tubulin, calmodulin, actin and other intron-rich genes, physiological and ecological data and extrolite profiles (Samson et al., 2007a). Based on the review done by Samson et al. (2007b), all species in section *Nigri* can be distinguished with the use of calmodulin sequence data. Calmodulin sequences are also able to differentiate between *A. awamori/welwitschiae* and *A. niger.* However for sister species like *A. niger* and *A. awamori* or *A. lacticoffeatus*, a multilocus identification is better.

Fungi are often differentiated on the basis of morphology, with particular reliance on the structure of spore-forming bodies such a conidiophores and resting structures such as sclerotia. Conidiophore morphology and associated terminology are illustrated in Figure 1-1. Sclerotia (not included in Figure 1-1) which are only produced by certain strains are hardened, thick-walled spherical structures formed for survival under adverse conditions. *A. niger* is morphologically characterized by sterigmata in two series, conidial heads that appear carbon black to the naked eye, and conidia that are globose at maturity, mostly 4.0 to 5.0 µm in size, irregularly roughened with conspicuous ridges and echinulations not arranged as longitudinal striations (Raper and Fennell, 1965). These characteristics are similar to those of *A. brasiliensis*, as seen in Table 1-1.

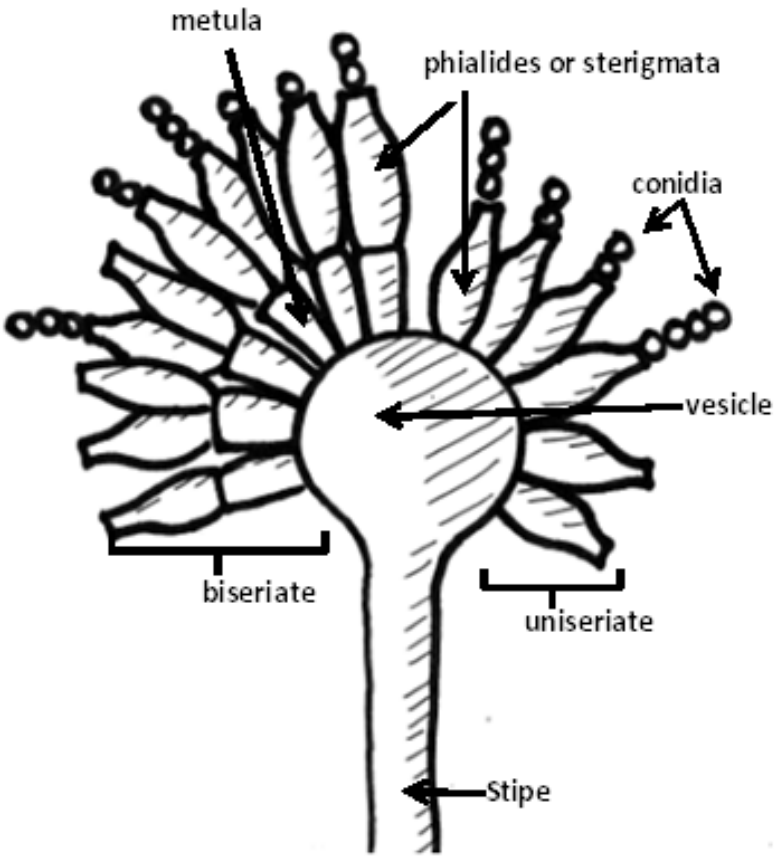

Figure 1-1 Terminology used to describe conidiophores morphology for the identification of *Aspergillus* species

Table 1-1 Characteristics of *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642

Chracteristics	*A. awamori* ATCC 22342 (= *A. niger* ATCC 22342)	*A. brasiliensis* ATCC 9642	Reference
Strain designations	NRRL 3112	SN 26; CBS 246.65; DSM 63263; IFO 6342; IMI 91855; NRRL 3536; NRRL A-5243; QM 386	(ATCC, 2013a; ATCC, 2013b)
Growth temperature range	28EC -37EC[a] no result for temperature below 28EC	28EC -37EC[a] no result for temperature below 28EC.Good growth and sporulation at 37EC.	(Varga et al., 2007)
Colony	Black colonies with a white leading edge CYA for 7 days at 25°C[a]	Colony first white then dark brown to black. CYA at 25 and 37 EC, 71–76 mm;	(Varga et al., 2007)
Conidial head	globose [a]	Globose at first and later radiate occasionally developing into several conidial columns	(Varga et al., 2007)
Conidial head size (µm)	50.9 ±17.2[a]	70.8 ±15.6[a]	N/A
Conidiophore / stipe	Smooth and colourless [a]	Walls thick, smooth, pale brown	(Varga et al., 2007)
Conidiophore (µm)	Not available	700–1700 x 8–13 mm	(Varga et al., 2007)
Vesicle size (µm)	Not available	30-45	(Varga et al., 2007)
Metulae	Not available	Metulae covering the entire surface of the vesicle	(Varga et al., 2007)
Metulae size (µm)	Not available	22-30 x 3-6	(Varga et al., 2007)
Sterigmata/ phialides	Not available	Biseriate, flask-shaped	(Varga et al., 2007)
Sterigmata size (µm)	Not available	7-9 x 3-4	(Varga et al., 2007)
Conidia	Forms chains, smooth, globose, indented center [a]	Subglobose, echinulate	(Varga et al., 2007)
Conidia diameter(µm)	4.2 ± 0.5 [a]	5.4± 1.0 [a]	N/A
Colour and size of sclerotia (µm)	Not available	None	(Varga et al., 2007)
Catalase activity	weak [a]	Inconclusive [a]	N/A
Extrolites produced	Ochratoxin A, fumonisin B,unalenone (kotanins), naphtho-γ-pyrones, pyranonigrin A, pyrophen, tensidol A and B	Aurasperone B and other naphtho-γ-pyrones; tensidol A and B; DERH; pyophen; dihydrocarolic acid; aflavine	(Frisvad et al., 2011; Varga et al., 2007)

[a] Data generated by Health Canada's Environmental Health Science and Research Bureau

Health Canada's Environmental Health Science and Research Bureau used growth kinetics at different temperatures (Appendix A), growth on different media at 28°C and 37°C (Appendix A), and sequence analyses of the consensus fungal D2 region, ITS region, or calmodulin gene to independently characterize *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642. Calmodulin sequence analysis and dendogram construction with randomly selected *Aspergillus* entries showed that *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 featured distinct genes (Appendix B). In the dendogram, these genes were rooted near other calmodulin genes from the respective species; this was similar to the observation that sequence analysis of calmodulin or β-tubulin genes could distinguish *A. awamori* and *A. niger*. (Varga et al, 2011; Perrone et al., 2011). None of these techniques could differentiate the DSL-listed strains from other *A. niger* or *A. brasiliensis* strains.

The whole genome sequences of three strains of *A. niger*, ATCC 1015, NRRL 3 and CBS 513.88, have been published (Andersen et al., 2011; Baker, 2006; Pel et al., 2007), and intensively examined using transcriptomics and metabolomics to explore and understand growth, differentiation, chemistry and physiology of the species (Andersen et al., 2008a; Andersen et al., 2008b; Jorgensen et al., 2009; Nielsen et al., 2009; Pel et al., 2007; Sun et al., 2007). The genome of the type strain *A. brasiliensis* CBS 101740 is available on the Department of Energy Joint Genome Institute website (http://genome.jgi-psf.org/Aspbr1/Aspbr1.info.html), but no analyses have yet been published.

1.1.3 Biological & Ecological Properties of the Organism

1.1.3.1 Growth Conditions

Aspergillus, Section *Nigri* is generally considered ubiquitous in nature (Baker, 2006). *A. niger* has been found globally, both in marine and terrestrial environments (Andersen et al., 2011). It is one of the most commonly encountered fungi contaminating food, feed and occurring in soil and indoor environments (Frisvad et al., 2011; Schuster et al., 2002).

Aspergillus section *Nigri* are among the fungi most frequently isolated from soils and vineyards and also dried fruits, coffee and cocoa, perhaps because of their rapid growth rate and tolerance of high temperatures and low water activity. They rapidly colonize and easily degrade available organic matter. *A. niger* is cosmopolitan and has been isolated from locations around the globe, indicating that it is able to propagate efficiently in a wide range of environments (Meijer et al., 2011). Populations of *Aspergillus* are isolated equally readily from forests, wetlands, grasslands and cultivated soil (Klich, 2002). Vegetative growth occurs over a range of temperatures between 6°C and 47°C (Schuster et al., 2002), but is favoured by higher temperatures (35°C) and water activities (0.95) (Belli et al., 2004)). *Aspergillus* hyphae are extremely tolerant to freezing injury. They survive storage at many different sub-zero temperatures, from -20 to -196 °C, and the majority of the

hyphae in the mycelium remain intact during freezing and thawing (Kozakiewicz and Smith 1994).

Optimal water activity and pH are not known for *A. brasiliensis,* but *A. niger* is acidophilic (Person et al., 2010; Xavier et al., 2008) and has optimum water activity range between 0.95 and 0.99 (Astoreca et al., 2007; Astoreca et al., 2010; Belli et al., 2004; Esteban et al., 2006a; Esteban et al., 2006b; Leong et al., 2006b; Meijer et al., 2011). Optimal growth conditions for *Aspergillus* section *Nigri* is 30-37 °C (Belli et al., 2004). *A. brasiliensis* grows poorly at 15°C, and has an optimal growth temperature around 35°C (Meijer et al., 2011).

1.1.3.2 Ecological Properties

Section *Nigri* species are mainly saprophytic and are able to develop in a vast variety of substrates where they play an essential role in the recycling of carbon and nitrogen (Van Diepeningen et al., 2004; Gugnani, 2003). *A. niger* can grow aerobically on organic matter, in litter, in compost and on decaying plant material (Leong et al., 2006b; Schuster et al., 2002; Semova et al., 2006; Staples and Burchfield, 1960). *A. niger* and *A. brasiliensis* have been isolated from soil, grapes, cereal, coffee, corn and corn based food and animal feed (Dalcero et al., 2002; Magnoli et al., 2004; Magnoli et al., 2005; Magnoli et al., 2006; Serra et al., 2006; Varga et al., 2007). *A. niger* is considered to be ubiquitous in nature. Different strains of *A. brasiliensis* have been isolated from various geographical locations (Varga et al., 2007), however the species is relatively rarely occurring and not ubiquitous.

A. niger is known as a phosphate solubilizing microorganism (PSM), and hence is used as a biofertilizer (Reddy et al., 2002; Seshadri et al., 2004)). *A. niger* has biodegradation and biotransformation abilities (Kanaly et al., 2005). It can absorb lead, copper, nickel, cadmium and zinc from the environment by either adsorption to fungal cell wall components, or complexation with organic acids produced by the fungus (Kapoor et al., 1999; Kapoor and Viraraghavan, 1998 Naseem et al., 1995; Price et al., 2001). Its tolerance of zinc, lead, cadmium and nickel (Iram et al., 2009) allows it to function in environments that are heavily contaminated with metals. *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) is also able to degrade phenol and phenol derivatives through the production of extracellular enzymes, but the mechanism of degradation is not well understood (Stoilova et al.,2006).

1.1.3.3 Life Cycle

All black aspergilli are presumed to be asexual, and vegetative compatibility between natural isolates is very rare (Van Diepeningen et al., 1997). Like most filamentous fungi, the majority of aspergilli, including *A. niger,* reproduce asexually through the formation of conidia, a type of spore (Adams et al., 1998). The conidia of *Aspergillus* are composed of hydrophobic proteins which confer resistance to extreme atmospheric conditions (Guarro et al., 2010) and enable them to survive periods of environmental stress until conditions that favour vegetative growth are

restored (Krijgsheld et al., 2013). They can resist low water activity, low or high temperatures, and UV radiation (van Leeuwen et al., 2013), enabling the organism to survive in an inactive state. *Aspergillus* conidia survive temperatures up to 50°C for one hour (Ruijter et al., 2003).

Some *Aspergillus* species can produce sclerotia, which are compact masses of hyphae containing food reserves. These resting bodies are a survival mechanism for adverse environmental conditions (Dyer and O'Gorman, 2011). Sclerotia have been observed in some strains of *A. brasiliensis* (Varga et al., 2007), but rarely occur in *A. niger* (Samson et al., 2004).

1.1.3.4 Pathogenic and Toxigenic Characteristics

The ability of *Aspergillus* section *Nigri* to produce infections in humans and non-human species is attributed to a wide array of mechanisms, including adherence, invasion, evasion of host defences and damage to host cells. No reports in the literature investigated the potential pathogenicity of *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) or *A. brasiliensis* ATCC 9642 to plants, animals or humans; however, taking into consideration the recent taxonomic reclassification of *A. brasiliensis* and the lack of distinction between species of black aspergilli in the literature, the following section also includes information on black aspergilli in general.

Infection caused by black aspergilli are more frequent in hot, humid, tropical and semitropical climates (Kredics et al., 2008), suggesting that the virulence and growth are affected by temperature and humidity. Most of the clinically important species can grow well at 37°C (Klich, 2008) and both *A. awamori ATCC 22342* (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 can grow at that temperature (Appendix A).

In cytotoxicity studies at Health Canada's Environmental Health Science and Research Bureau, *A. brasiliensis* caused cell detachment and reduced cellular metabolism, as measured by bioreduction activity. No significant change was reported in cells exposed to *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342*)*. Furthermore, in murine endotracheal exposures, *A. brasiliensis* ATCC 9642 persisted for at least one week post-exposure. Experiments of greater duration would need to be done to determine the time to complete clearance of the fungus.

Black aspergilli, including *A. niger,* have many mechanisms that may contribute to their pathogenicity, including secretion of secondary metabolites, such as mycotoxins; formation of calcium oxalate; formation of aspergillomas or fungal balls; sporulation; and tolerance of physiological temperature and pH.

A. niger has properties that allow it to act as a biocontrol agent due to its antagonistic effects on a variety of species. *In vitro*, culture filtrates of *A. niger* show biocontrol potential against root-knot nematode, *Meloidogyne incognita*, on tomato

(Hemlata and Gopal, 2001; Radwan, 2007), and *in vivo*, application of *A. niger* reduces *Meloidoyne javanica* infestation of sunflower and okra roots (Dawar et al., 2008). *A. niger* also inhibits white rot and brown rot wood decay fungi (Tiwari et al., 2011). *A. niger* culture filtrates have also shown enhanced lethal effects against larval and adult mosquitoes (*Anopheles stephensi*, *Culex quinquefasciatus*, and *Aedes aegypti*) (Singh and Prakash, 2012).

A) Enzymes

Members of the *Aspergillus* section *Nigri* are particularly efficient producers of extracellular enzymes (Samson et al., 2004; Serra et al., 2006).

As a soil saprobe, *A. niger* grows predominantly on dead plant material, which consists mainly of cell walls containing polymeric components, such as cellulose, hemicellulose, pectin, lignin and proteins, of which the polysaccharides make up about 80 % of the biomass (de Vries and Visser, 2001). *A. niger* produces a wide array of hydrolytic and oxidative enzymes which are responsible for the breakdown of plant lignocelluloses (Meijer et al., 2011; Pel et al., 2007). These features of *A. niger* enable it to decay various organic substances (Baker, 2006). *A. niger* has been reported to have high xylanase, beta xylosidase and polygalacturonase activity (Al-Hindi et al., 2011; Lemos et al., 2001). *A. niger* was found to have elastase activity. Elastase production is considered a virulence factor in the human pathogen *A. fumigatus* by having a role in the invasiveness of the fungus during infection (Varga et al., 2011). Elastase is secreted by the fungus in infected lungs to degrade elastin (Krijgsheld et al., 2013).

Limited information is available on enzyme production by *A. brasiliensis*. Like *A. niger,* some strains of *A. brasiliensis* are known to produce xylanase, polygalacturonases and thermostable beta xylosidases (Bussink et al., 1991; Gomes et al., 2011; Pedersen et al., 2007).

B) Fungus ball and formation of calcium oxalate crystals

Aspergillomas or fungal balls are vegetative masses that can form in a host. In humans, aspergillomas are predominantly found in the lungs (Kimmerling and Tenholder 1992; Ma et al., 2011; Severo, 1981) but can also form in other body cavities such as sinuses, brain and heart (Anandaraja et al., 2006; Goel et al., 1996; Naim-Ur-Rahman et al 1996).

A. niger fungal balls release oxalic acid which is able to complex with free Ca^{2+} in the infected tissues and blood to form calcium oxalate, which can be deposited as crystals (Denning, 2001; Roehrl et al., 2007). The formation of calcium oxalate crystals is characteristic of *A. niger* infection (Person et al., 2010; Vakil et al., 2010). Calcium oxalate crystals can be locally toxic, causing haemorrhage and tissue necrosis (Roehrl et al., 2007). Calcium oxalate crystals are found in tissue in 25% of pulmonary aspergillomas, 100% of sinus aspergillomas and 8% of disseminated

Aspergillus infections (Nime and Hutchins 1973: reviewed in Denning 2001). The exact mechanism of calcium oxalate crystal toxicity (oxalosis) has not yet been exactly determined, however, oxalate is known to function as a ligand for a variety of metal cations (Ghio et al., 1992).

C) Mycotoxins and secondary metabolites:

Species in the *Aspergillus* section *Nigri* are known to produce several highly specific secondary metabolites, including mycotoxins. Secondary metabolites are compounds produced by an organism that are not required for a physiological function (growth, development or reproduction of the organism), some of which are presented here because they have been reported to have negative effects on hosts. 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. niger* and *A. brasiliensis,* are listed in Appendix C. The LD 50 for *A. niger* and its toxins are also listed in Appendix C.

Aspergillus section *Nigri* is not known to produce the clinically significant mycotoxins gliotoxin, aflatoxin, cyclopiazonic acid, citrinin, sterigmatocystin, or patulin which can be produced by other *Aspergillus* species, and no orthologous genes have been identified in the genome sequence of *A. niger* strain CBS 513.88 (Pel et al. 2007).

A. niger produces the following secondary metabolites: nigragillin, aspergillin, asperrubrol, asperyellone, asperenones, orobols, tubingensins, yanuthones, tetra cyclic compounds pyranonigrin A, tensidol A, tensidol B, funalenone, naphtho-*γ*-pyrones, kotanins and mycotoxins such as malformins (sensu stricto), ochratoxin and fumonisins (Bouras et al., 2005; Curtis and Tanaka, 1967; Frisvad et al., 2007b; Leong et al., 2007a; Nielsen, 2003; Perrone et al., 2011; Samson et al., 2004 Storari et al., 2012). *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) was found to produce ochratoxin and fumonisins, which are mycotoxins of concern (Iriuchijima and Curtis, 1969; Frisvad et al., 2007b).

Ochratoxin produced by *A. niger* has been detected in a variety of foods and animal feeds (Frisvad et al. 2011; Nielsen et al. ,2009). Ochratoxin A contaminates a variety of agricultural products, including coffee, beer, wine, grape juice, and milk, in the field, and during storage or processing. It is most often associated with stored cereal grains, swine and poultry meat (Abarca et al., 2001; Varga et al., 2010a). Ochratoxin A can enter the human or animal food chain through these products (Holmberg et al., 1991; Kuiper-Goodman and Scott, 1989; Marquardt et al., 1988; Marquardt et al., 1990; Perrone et al., 2006). It is classified by the International Agency for Research on Cancer (IARC) as a potential human carcinogen, based on its nephrotoxic, hepatotoxic, immunotoxic, tetratogenic and carcinogenic effects (Storari et al.,

2010). *A. niger* aggregates normally produce ochratoxin A at 20-25°C, and water activity at 0.95/0.98 (Esteban et al., 2006a; Esteban et al., 2006b) and therefore are not produced during mammalian infection.

Fumonisins are a family of polyketide-derived mycotoxins that are suspected to enter the human food chain through the contamination of corn-based food and feeds (Mogensen et al., 2010; Nielsen et al., 2009), and fumonisins produced by *A. niger* have been detected in grapes, raisin, coffee beans and wine (Frisvad et al., 2007b; Logrieco et al., 2009; Mansson et al., 2010; . Fumonisin is associated with a number of animal and human diseases (Marasas, 2001). It is neurotoxic, hepatotoxic, and nephrotoxic in animals, and has been classified as a possible group 2B carcinogen to humans by IARC (Bondy et al., 2012; FAO/WHO 2012; Stockman et al., 2008). Fumonisin production in *A. niger* is favoured by low water activity and high temperatures (25-30^{o}C) (Mogensen et al., 2009a), which suggests that they are most likely to be produced during the drying process of harvest crops associated with decreasing water activity (Knudsen et al., 2011). Fumonisin production is induced by sporulation (K. Nielsen, personal communication).

Mycotoxin production and contamination may occur in the field and is largely dependent on environmental factors (Blumenthal, 2004; Bryden, 2012; Logrieco et al., 2009; Logrieco et al., 2010; Marquardt, 1996; Mogensen et al., 2010). Factors known to affect production of these mycotoxins in fruit include fruit type and cultivar, geographical location where the fruit is grown and harvested, climate, pre-harvest treatments, method of harvest, and presence of surface defects on the fruit, post-harvest treatments and storage conditions. Mycotoxin accumulation in fruits can occur in the field, during harvest, postharvest and during storage. Gentle and sanitary handling of the fruit during harvest and in storage and processing facilities is essential for reducing fungal decay and mycotoxin production in fruits (Jackson and Al-Taher, 2008).

A. brasiliensis ATCC 9642 produces secondary metabolites in common with *A. niger*, such as some naphtho-*γ*-pyrones, including aurasperones, malformins, tensidol A and B and pyrophen, and several other unique compounds (Nielsen et al., 2009; Samson et al., 2004; Samson et al., 2007a; Varga et al., 2007). However, *A. brasiliensis* does not produce mycotoxins with real hazard for human and animal health like *A. niger* (G. Perrone, personal communication). None have been found to produce ochratoxin A, fumonisin, kotanins, funalenone, antafumicins, asperzine or pyranonigrins, all of which are common in other species in the *A. niger* complex (Frisvad et al., 2011; Pedersen et al., 2007;Samson et al., 2007a; Varga et al., 2007).

1.1.4 Effects

In general, case reports of *Aspergillus* infection in the literature do not distinguish between species of *Aspergillus* section *Nigri*. To ensure that all cases of infection possibly involving the DSL listed strains would be identified, the following section

also includes information on cases of infection or intoxication with *A. niger* and *Aspergillus* section *Nigri* in general.

An in depth scientific literature search for information on the DSL-listed strains *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342), *A. brasiliensis* ATCC 9642 and their synonyms yielded no evidence of adverse effects towards plants, animals and humans.

1.1.4.1 Environment

A) Plants

Species of the genus *Aspergillus* are saprophytes, and are also considered weak plant pathogens. *A. niger* associated with *Aspergillus* rot has been isolated frequently from vineyards where it exists as a saprophyte in the top layer of the soil beneath vines (Leong et al., 2006a; Leong et al., 2007b). *A. niger* has been reported as a significant component of the fungal community on grapes while *A. brasiliensis* has been detected to a minor extent (Perrone et al., 2006; Perrone et al., 2007).

Although not considered to be a major cause of plant disease, *A. niger* has been reported to grow and damage a large number of crops and foods worldwide, including corn, peanuts, onions, mango and apples (Perrone et al., 2007; Pitt and Hocking, 1997; Sedaghati et al., 2012). *A. niger* has also been reported by Pawar et al. (2008) as a plant pathogen on *Zingiber officinale* (ginger). *A. niger* is responsible for many rot diseases in plants and a list of them can be found in Appendix D.

Aspergillus is known to enter berries through wounds caused by birds, insects, or other mechanical means such as cracks and fruit injuries during ripening (Pisani and Dubler, 2011). Pathogenicity tests indicate that *A. niger* can induce rot in healthy cassava tubers and induce high level of infection under the ear husks of maize after re-inoculation (Okigbo et al., 2009; Windham and Williams, 2012). Pathogenicity tests conducted with species from *Aspergillus* section *Nigri* isolated from various vineyards demonstrate that *A. niger* caused *Aspergillus* vine canker with no difference in virulence between different species (Vitale et al., 2012). Culture filtrates of *A. niger* exhibit phytotoxicity against onion and tomato by reducing seed germination and root elongation (Narayana et al., 2007). Natural seed contamination and artificial infestation of onion seeds with spore suspension of *A. niger* have been shown to reduce seed germination, emergence and distort seedling growth (El-Nagerabi and Ahmed, 2001).

Some black aspergilli have been isolated from the surface of maize, onion, garlic and peanuts, indicating that these species exist as symptomless endophytes. However, these were characterised as latent pathogens which have the capacity to produce secondary metabolites, some of which may be toxic under certain conditions. Symptomless infections pose problems from a food safety concern, as

commodities contaminated by such infections are not obvious, appear normal, but can contain toxic metabolites (Palencia et al., 2010).

In plant testing conducted at Environment Canada[1], red clover was grown for 42 days in clay loam soil inoculated with 10^3 µg dry culture of *A. brasiliensis* ATCC 9642 per gram of dry soil at days 0, 14 and 28. No significant adverse effects were observed for shoot length, root length or dry weight (Environment Canada, 2010). No data were generated for *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342).

B) Animals

A. niger has been found as part of the natural mycobiota and isolated from digestive tract of many species of triatomines (Moraes et al., 2001a; Moraes et al., 2001b). *A. niger* has been isolated from bird feathers and animal hair of several species (Moorthy et al., 2011). *Aspergillus* has the ability to colonize living and dead animal tissue. Its invasion of living tissue is responsible for many forms of disease in warm and cold blooded animals (Prelusky et al., 1994); however, the immune status of the host is pivotal (Baker and Bennett, 2007). Two types of disease are caused by *Aspergillus* section *Nigri* which are mycotoxicosis (from the ingestion of feed containing toxic metabolites) and infection (mycosis) (Austwick. 1965).

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 (livestock, poultry, fish) (Marasas and Nelson, 1987; Moss, 1996; Palencia et al., 2010). Grains, cereals or products made from such grains are common sources of mycotoxin exposure, but other sources of exposure exist (Binder, 2007; Richard, 2007; Sweeney and Dobson, 1998). Not all cases of feed contamination are reported, as the source of toxicity or the contaminating organisms are not clearly identified. The signs elicited by mycotoxin consumption range from reduced animal productivity (reduced body weight gain, reduced fertility) and immune suppression (Oswald and Comera, 1998), resulting in increased susceptibility to diseases and parasites to overt disease and death. Clinical signs of mycotoxin intoxication include diarrhea, liver and kidney damage, pulmonary edema, vomiting, haemorrhaging and tumours (Binder, 2007; Bryden, 2012). Under field conditions, mycotoxins usually occur in concentrations leading to reduced animal performance and/or immune suppression without causing any overt clinical signs (Marquardt, 1996). Toxicity outbreaks related to consumption of contaminated sorghum straw with *A. niger* have been observed in cattle (Nirmala et al., 2009).

[1] Tests conducted according to the "*Guidance Document for Testing the Pathogenicity and Toxicity of New Microbial Substances to Aquatic and Terrestrial Organisms* (EPS 1/RM/44, March 2004)"

Fumonisins are involved in leukoencephalomalacia in horses, pulmonary edema in pigs and cancer, disruption of sphingolipid metabolism, cardiovascular dysfunction and neural tube defects in experimental rodents (Gelderblom et al., 1988; Marasas, 2001; Stockmann-Juvala and Savolainen, 2008). Experimentally, fumonisins cause liver damage in farm animals and also kidney damage in rabbits, cattle and sheep or equivalent organs in fish (FAO/WHO, 2012). Ochratoxin is often reported to exhibit immunosuppressive and carcinogenic properties in animals (Kuiper-Goodman and Scott, 1989; Pfohl-Leszkowicz and Manderville, 2007).

To help prevent the formation and consumption of mycotoxins, the feed industry has established internal monitoring methods. Similarly, government regulatory agencies, including the Canadian Food Inspection Agency, 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, 2013).

A. niger, as an opportunistic animal pathogen, infects cavities such as the ear, nose, and paranasal sinuses where the infection can be invasive or non-invasive. It also tends to invade blood vessels and thus is easily disseminated to other organs (Landry and Parkins, 1993). Aspergillosis, a common term used to describe animal infections caused by *Aspergillus* species, is relatively uncommon in mammals, but dogs, horses, cows and dolphins are susceptible (Tell, 2005). With invasive aspergillosis, the immune system has collapsed and little or no defence can be mounted (Baker and Bennett, 2007). *A. niger* as one of the causative agents has been isolated from the milk of buffaloes having mastitis in India (Mahapatra et al., 1996). Outbreaks of Deg Nala disease were diagnosed in buffaloes and cattle. Clinical signs included ulcerative wounds and gangrene of the limbs, tail, ears, muzzle and tongue and younger animals were more susceptible. Examination of rice straw fed to the animals mentioned above revealed the presence of *A. niger* (Maqbool et al., 1997). Respiratory infection caused by *A. niger* was reported in a horse (Carrasco et al., 1997) and in a dog (Kim et al., 2003). Avian aspergillosis is usually seen as a respiratory infection where the fungi colonize the mucosal surfaces of the respiratory tract and also the serosal surfaces of the avian sacs, resulting in mycotic airsacculitis (Richard, 2007). *A. niger* was reported to cause respiratory infection in a broiler breeder flock (Akan et al., 2002), an Alpaca (Muntz, 1999), a one year old ostrich (Perez et al., 2003), and a great horned owl (Bubo virginianus) (Wobeser and Saunders, 1975). Aspergillosis is a major cause of mortality in birds (Tell, 2005), however most cases of avian aspergillosis are caused by *A. fumigatus* (Nardoni et al., 2006).

A. niger was identified from visceral lesions of tilapia cultured in Kenya and exhibiting Aspergillomycosis symptoms (Paperna, 1996). *A. niger* was also reported to infect two species of Asian freshwater catfishes which showed hemorrhagic ulcer like patches on the gills and skin. In an experiment to confirm the pathogenicity of the fungus, healthy fishes from these species exposed to a contaminated area

suffered from dermal ulcerations and died (Bhattacharya,1988). No other pathogenicity information on aquatic species was found in the literature.

Immunocompromised animals, those receiving corticosteroids, cytotoxic drugs, or prolonged antibiotic therapy, and those with concurrent debilitating disease have a significantly increased risk of developing the systemic form of otomycosis (Landry and Parkins, 1993). *A. niger* was found to be pathogenic to hydrocortisone-treated mice when infected intravenously with high doses of the fungus (Jacob et al., 1984). Addition of decadron, a steroid hormone, to the culture medium of *A. niger* induced more vigorous corneal ulceration in rabbit eyes infected with spores compared to animals inoculated with spores from medium without the steroid (Hasany et al., 1973).

Pathogenicity and toxicity testing was performed at Environment Canada[2] using *A. brasiliensis* ATCC 9642 on a soil arthropod, *Folsomia candida*. The invertebrate was grown for 28 days in clay loam soil inoculated with 10^3 µg dry culture of *A. brasiliensis* ATCC 9642 per gram of dry soil at days 0 and 14. No significant adverse effects were observed for adult survival and juvenile production (Environment Canada, 2010). No data were generated for *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342).

1.1.4.2 Human Health

Few cases of human disease are directly attributed to *A. brasiliensis*; however, *A. brasiliensis* was recently reported as the causative agent of keratitis in two healthy individuals, and was suggested to be responsible for a significant proportion of corneal infections formerly attributed to black aspergilli (Manikandan et al., 2010).

Because most clinical case reports do not distinguish between black *Aspergillus* species, all *A. niger, Aspergillus* section *Nigri* and black *Aspergillus* infections reported in the literature are considered in this assessment. In recent years, the number of aspergillosis has increased, possibly coinciding with a parallel rise in the number of patients whose immune function is compromised for prolonged periods as a result of modern diseases or therapies for a variety of conditions (e.g., AIDS, and therapies related to cancer, surgery and organ transplantation) (Anderson et al.,1996; Denning 1998; Denning et al., 2002; Fianchi et al., 2004; Gugnani, 2003; Hajjeh and Warnock, 2001; Misra et al., 2011; Abdul Salam et al., 2010; Xavier et al., 2008). Like other opportunistic human pathogens, members of *Aspergillus* section *Nigri* are capable of causing an array of infections in favourable circumstances. *A. niger* has been reported as having a lower level of virulence compared to other black aspergilli (Person et al., 2010; reviewed in Severo et al.,

[2] Tests conducted according to the "*Guidance Document for Testing the Pathogenicity and Toxicity of New Microbial Substances to Aquatic and Terrestrial Organisms* (EPS 1/RM/44, March 2004)"

1997), but it has nevertheless been reported to cause infections of the lungs, skin, ears, eyes and heart, as well as systemic infections.

Respiratory infections caused by *A. niger* occur mainly in individuals with compromised immune function, underlying disease, or a history of diseases (especially lung diseases such as tuberculosis), smoking and long-term steroid use (Person et al., 2010; Waraich et al., 2009; Roehrl et al., 2007; Muto et al., 2006; Fianchi et al., 2004; Denning, 1998; Severo et al., 1997; Anderson et al., 1996; Yamaguchi et al., 1992; Wiggins et al., 1989; Pervez et al., 1985; Kauffman et al., 1984; Gefter et al., 1981). Cavities in the lungs provide an ideal environment for fungal ball formation (Severo et al., 1997; Severo et al., 1981; Kimmerling and Tenholder, 1992; Gefter et al., 1981). Fungal ball formation is not necessarily indicative of tissue invasion (Roehrl et al., 2007; Procop, 1997) but necrotic tissue has been observed as the result of the formation of calcium oxalate crystals (Vakil et al., 2010; Roehrl et al., 2007; Yamaguchi et al., 1992). Pulmonary aspergillosis or oxalosis caused by *A. niger* has been reported in an immunocompetent individual (Rajalingham and Anshar, 2012). Treatment outcomes for pulmonary infections caused by *A. niger* are not always favourable and deaths have been reported (Xavier et al., 2008; Fianchi et al., 2004; Nakagawa et al., 1999; Kimmerling and Tenholder, 1992; Yamaguchi et al., 1992; Wiggins et al., 1989; Pervez et al., 1985; Gefter et al.,1981; Severo et al., 1981; Nime and Hutchins, 1973; Utz et al.,1959).

Skin, ear and eye infections caused by *A. niger* have been reported in both immunocompromised and immunocompetent individuals of all ages and gender (Shinohara et al., 2011; Amod et al, 2000; Aswani and Sukla, 2011; Aneja et al., 2010; Fasunla et al., 2008 Avino-Martínez et al., 2008; Ugurlu et al., 2001). Moisture is often implicated as a predisposing factor and consequently infections frequently occur in moist and humid areas such covered skin, the ear canals, and the eyes (Fasunla et al., 2008; Amod et al., 2000; Johnson et al., 1993). *A. niger* skin infections (dermatomycoses) persist as a rash or superficial lesion (Robinson et al., 2011; Shinohara et al., 2011; Amod et al., 2000; Loudon et al., 1996; Johnson et al., 1993; Cahill et al., 1967). Autoinoculation from infected skin or nails may be responsible for prolonged or chronic infections (Shinohara et al., 2011; Ozcan et al., 2003). Fungal ear infections (otomycoses) are common worldwide, especially in sub-tropical and tropical regions (Barati et al., 2011; Aneja et al., 2010; Fasnula et al., 2008; Kumar, 2005; Ozcan et al., 2003; Loh et al., 1998). They generally involve the external ear and ear canal (Aswani and ShuKla, 2011; Barati et al., 2011; Mishra et al., 2004; Ozcan et al., 2003; Vennewald et al., 2003) but may also affect the middle ear (Barati et al., 2011; Fasunla et al., 2008; Vennewald et al., 2003; Ozcan et al., 2003), as well as the mastoid cavity (Barati et al., 2011; Paulose et al., 1989). Left untreated, otomycoses can lead to various conductive hearing impairments (Fasunla et al, 2007). Eye infections may affect the cornea (Keratitis) and the orbits (Avino-Martínez et al., 2008; Paula et al., 2006; Brar et al., 2002; Ugurlu et al., 2001; Jager et al., 1994). Antifungal and antibacterial therapies along with better hygiene regimens and surgery, in some instances, have been applied to resolve infections (Aswani and ShuKla, 2011; Fasunla et al., 2008; Mishra et al., 2004; Noguchi, 2003;

Vennewald et al., 2003; Loh et al., 1998). If not treated rapidly, *Aspergillus* eye infections can cause loss of vision due to retinal necrosis and choroidal damage (reviewed in Chhablani, 2011).

Endocarditis caused by *A. niger* has been reported. Predisposing factors include open heart surgery or aortic/mitral valve replacement (Balajee et al., 2009; Anandaraja et al., 2006; Duygu et al., 2006; Kreiss et al., 2000; Vivas, 1998; Moore et al., 1984; Mahvi et al., 1968); however, infections have been reported in patients who have not undergone heart surgery (Parameswaran, 2008; McCracken et al., 2003; Atra et al., 1998). Antifungal drugs are often the prescribed treatment, but surgery may be required to remove vegetation.

Fungemia (Duthie and Denning, 1995), or multiple concurrent fungal infections affecting the lungs, skin, liver and gastrointestinal tract (Gercovich et al., 1975), is less common and is associated with predisposing factors such as indwelling devices or underlying illness (Duthie and Denning, 1995; Gercovich et al., 1975).

A. niger has occasionally been associated with other types of infection including bone infections (Shelton et al., 2002; Winslow et al., 2001); infection of silicone breast implants (Williams et al, 1983); and *A. niger* fungal granuloma of the pituitary gland (Wollschlaeger et al. 1970).

Antifungal drugs used in the treatment of black aspergilli infections include amphotericin B, caspofungin, fluconazole, itraconazole, metronidazole, micronazole, nystatin, and voriconazole. Other drugs, such as antibiotics and steroids, used during treatment of black *Aspergillus* infections include atropine, azithromycin, ceftazidime, cefazolin, cephalexin, ciprofloxacin, clindamycin, dexamethasone, diclofenac sodium, gentamycin, levofloxacin, mercurochrome, neomycin/polymyxin/hydrocortisone, penicillin, prednisone, triamcinlone, tobramycin, and vancomycin. *A. niger* is resistant to fluconazole and highly susceptible to terbinafine (Szigeti et al., 2012). Itraconazole resistance was common in *Aspergillus* section *Nigri* (Howard et al., 2011). Black aspergilli have high susceptibility to terbinafine and a low susceptibility to ketoconazole. The susceptibility of black aspergilli to itraconaxole and amphotericin B is less clear since results vary between studies (Szigeti et al., 2012). Table 1-2 represents an antibiogram generated by Health Canada for the characterization of *A. awamori ATCC* 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642.

Table 1-2 Minimal Inhibitory Concentration (MIC) for *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642[ab]

Antifungal MIC (µg/mL)	***A. awamori* ATCC 22342 (=*A. niger* ATCC 22342)**	***A. brasiliensis* *ATCC 9642***
Amphotericin B	>24	>24
5-Fluocystine and	6.8 ± 3.8	>24

Amphotericin B		
5-Fluocystine	4.1 ± 2.3	> 24
Clotrimazole	1.5 ± 0.0	1.5 ± 0.0
Griseofulvin	>24	>24
Itraconazole	9.0 ± 3.5	13.5 ± 7.5
Isoconazole	1.5 ± 0.0	8.6 ± 10.4
Micafungin	>24	>24
Nystatin	12 ± 0.0	18 ± 6.9
Terbinafine	0.4 ± 0.0	0.7 ± 0.6

[a] The reported values are based on a minimum of 3 independent experiments. Values correspond to the minimal inhibitory concentration (µg/mL) for select *A. awamori. and A. brasiliensis* (104 cfu/ 20 µL) grown in the presence of antifungal for 48 hrs at 37°C.

[b] Data generated by Health Canada's Environmental Health Science and Research Bureau

A. brasiliensis is more resistant to antifungals compared with *A. niger*. In the event of infection caused by the DSL-listed strains of *A. niger* or *A. brasiliensis,* there are clinically relevant antifungals that may be used. Nevertheless, the toxicity of antifungal drugs is a therapeutic obstacle. Fungal drug targets are structurally analogous to components of human cells (reviewed in Lamb et al., 2000). Antifungal treatments are known to interfere with sterol biosynthesis and drug and xenobiotic metabolism in the liver; to cause myelotoxicity, hepatotoxicity, and nephrotoxicity; to have targeted effects on genes involved with fatty acid catabolism and sterol metabolism; and disruption of pathways involved in androgen-estrogen metabolism, bile acid biosynthesis, P450 xenobiotic metabolism and signalling. Anaemia, neutropenia, thrombocytopenia, adverse haemolytic activity and changes in permeability of cell membranes have also been reported. For these reasons, the availability of effective antifungal treatments will not weigh heavily in the assessment of hazard for these micro-organisms.

Secondary metabolites produced by *Aspergillus* such as Ochratoxin A and fumonisins have been reported to have an impact on human health. Many reviews consider ochratoxin A as a possible etiologic agent of the Balkan Endemic Nephropathy (Hope and Hope 2012; Reddy and Bhoola, 2010). Ochratoxin A has been linked to kidney disease in humans, particularly in Northeastern European countries and Africa (Reddy and Bhoola, 2010). The effect of fumonisins on humans is not well-known but evidence suggests a role in human oesophageal cancer (Bennett and Klich, 2003; Pitt et al., 2000).

All micro-organisms are potential sensitizers; several instances of sensitisation to *A. niger* have been reported. IgE sensitisation to both xylanase and phytase produced by *A. niger* (Baur et al., 1998; Doekes et al., 1999) and bronchospasms as a result of repeated exposure to its spores have been described (Topping et al., 1985). Sensitization upon exposure to the DSL-listed strains is equally to be expected.

1.2 Hazard Severity

The complexity of *Aspergillus* section *Nigri* taxonomy creates uncertainties for assessing the hazard of *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642.

Limited information on the pathogenicity of *A. brasiliensis* is available in the literature. There have been no reports of animal or plant disease that are specifically attributed to *A. brasiliensis* ATCC 9642. Although *A. brasiliensis* ATCC 9642 does not produce fumonisin and ochratoxin, the species is closely related to the opportunistic pathogen *A. niger*. Based on their morphological characteristics, *A. niger* and *A. brasiliensis* cannot be distinguished from other black aspergilli, so most reports identify any of the black aspergilli as *A. niger*. For this reason, any cases of disease in animals or plants attributed to black aspergilli or *A. niger* have been considered as possibly being caused by *A. brasiliensis* for the purposes of this risk assessment. Therefore, because of that uncertainty, the **environmental hazard severity** for *A. brasiliensis* ATCC 9642 is estimated to be **low to medium**.

Even though *A. niger* is recognized as an important biotechnology organism, which is well characterized, has a demonstrated history of safe use in industrial fermentation and is considered non-toxic under industrial conditions (Schuster et al., 2002), certain *A. niger* strains produce moderately to highly toxic mycotoxins and secondary metabolites. *A. awamori* ATCC 22342(=*A. niger* ATCC 22342) is known to produce mycotoxins such as fumonisin and ochratoxin. Both fumonisin and ochratoxin are reported to cause adverse effects in animals. Although there have been no reports of animal or plant disease that are specifically attributed to *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) , some *A. niger* strains have been reported as pre harvest and post harvest plant pathogens and opportunistic animal pathogens causing mycoses, mastitis and aspergillosis. Therefore, the **environmental hazard severity** for *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) is estimated to be **medium**.

A. niger is reported as a human opportunistic pathogen that can cause a wide array of infections, including lung, skin, eye, heart and systemic infections, and that produces a wide variety of extracellular enzymes and toxins that are important factors for its pathogenicity in humans. The risk of *A. niger* infection increases with pre-disposing factors such as debilitating disease, surgery, the presence of indwelling medical devices and immune deficiency of the individual, but *A. niger* also has pathogenic potential in otherwise healthy humans, and recent research suggests the same potential in *A. brasiliensis*. The vast majority of *Aspergillus niger*-related diseases in healthy humans are mild, self-resolving and usually treatable, but there have been mortalities in immunocompromised individuals, and ear and eye infections in healthy individuals could result in irreversible damage to the ears or eyes, such as hearing or vision loss. *A. brasiliensis* and *A. niger* are both resistant to fluconazole, which could limit treatment options. Although no information was found to indicate that *A. brasiliensis* or *A. niger* has the ability to acquire or disseminate

antifungal resistance genes, and other effective treatments are available, because of the adverse effects associated with antifungal treatment, the availability of clinically relevant drugs is not strongly weighted as a mitigating factor in this hazard assessment. Based on evidence in the scientific literature implicating *A. niger* in adverse human health effects, the **human hazard severity** for *A. awamori ATCC 22342 (=A. niger* ATCC 22342*)* is estimated to be **medium**. Based on the taxonomic ambiguity between *A. brasiliensis* and *A. niger*, and some evidence in the scientific literature that *A. brasiliensis* can infect humans, the **human hazard severity** for *A. brasiliensis* ATCC 9642 is estimated to be **medium**.

2. Exposure Assessment

2.1 Sources of Exposure

This assessment focuses on exposure to *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 from their deliberate use in consumer or commercial products and industrial processes in Canada.

A. awamori ATCC 22342 (=A. niger ATCC 22342*)* and *A. brasiliensis* ATCC 9642*,* like most of the *Aspergillus* section *Nigri,* have properties that make them of commercial interest. *A. awamori* ATCC 22342 *(=A. niger* ATCC 22342) was nominated to the DSL for its past use in industrial processes and *A. brasiliensis* ATCC 9642 was nominated based on its past use in consumer and commercial products.

The basis of numerous uses of *A. niger* and *A. brasiliensis* is the production of enzymes which have a high value in food and pharmaceutical industries. These include hydrolytic enzymes, such as amylases or lipases, and organic acids, such as citric acid and gluconic acid (Baker, 2006; Howard et al., 2011; Pel et al., 2007; Varga et al., 2000; Ward et al., 2005). In other applications, these micro-organisms could be used alone or with other micro-organisms to colonize a particular environment and produce these enzymes *in situ* to perform a particular function.

A search of the public domain (internet, patent databases) suggests multiple potential uses, including food processing, production of fermentation extract, biochemical and enzyme production, bioremediation and biodegradation, bioleaching, textile processing, municipal and industrial wastewater treatment, and as a probiotic in broiler chickens.

In 2007, a voluntary questionnaire was sent to a subset of key biotechnology companies in Canada. The responses, combined with information obtained from other federal government regulatory and non-regulatory programs indicated that *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) was not used. However, survey responses indicated that 10,000 to 100,000 kg of products potentially containing *A. brasiliensis* ATCC 9642 (formulation and concentration unknown) were imported

into or manufactured in Canada in 2006-2007 for use in consumer and commercial products.

In 2009, the government conducted a mandatory information-gathering Notice (survey) under Section 71 of CEPA 1999 as published in the Canada Gazette Part 1 on October 3rd, 2009. The Notice applied to any persons who, during the 2008 calendar year, manufactured or imported *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) or *A. brasiliensis* ATCC 9642, whether alone, in a mixture, or in a product. Anyone meeting these requirements was legally obligated to respond. Respondents were required to submit information on the industrial sector, uses and any trade names associated with products containing these strains, as well as the quantity and concentration of the strain imported or manufactured in the 2008 calendar year. No commercial or consumer activity was reported for *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) or *A. brasiliensis* ATCC 9642 in response to Section 71 Notice. *A. brasiliensis* ATCC 9642 was reported to be used in very small quantities for academic research, teaching, and research and development activities. For *A. awamori ATCC 22342 (=A. niger* ATCC 22342*)*, the nominator has confirmed that they no longer use the DSL-listed strain.

2.2 Exposure Characterization

The exposure characterization is based on activities reported in the Notice (R&D and teaching). Measures to reduce human and environmental exposure to risk group 2 pathogens from their use in research and teaching laboratories are in place under the Canadian Biosafety Standards and Guidelines, 2013 (CBSG 2013). These include specific laboratory design, operational practices and physical requirements. For example, all material must be contained and is decontaminated prior to disposal or reuse in such a way as to prevent the release of an infectious agent, and equipment for emergency and decontamination response must be readily available and maintained for immediate and effective use. As *A. niger* and *A. brasiliensis* are Risk Group 2 for humans as defined by the Public Health Agency of Canada, the measures specified in the CBSG 2013 apply. Note that although R&D and teaching uses were only reported for *A. brasiliensis* ATCC 9642, these measures would also apply to future uses of *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342).

Arrangements for shipping of *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642, must also meet requirements under Canada's *Transportation of Dangerous Goods Act and Regulations*. These measures are designed to prevent any human or environmental exposure to the micro-organisms during transport. Human and environmental exposure to *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642, through R&D and teaching uses reported under the Notice is therefore expected to be low.

2.2.1 Environment

The **environmental exposure** to *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 is estimated to be **low** based on responses to the Notice, which suggest that these strains are 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. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 resume, have been considered along with persistence and survival properties of these micro-organisms. Due to the expanding commercialization of microbial-based products, some potentially containing *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642, there is a likelihood of an increase in the use and release of these micro-organisms in the environment (Chatzipavlidis et al, 2013).

The magnitude of plant and animal exposure to *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 will depend on their persistence and survival in the environment. The persistence and survival for the two DSL strains are unknown but data are available for *A. niger*. After inoculation of *A. niger* live cells into intact soil microcosm, there was a reduction in *A. niger* DNA by day 46, and by day 126, the concentration had declined approximately 14-fold relative to the measures on day 2. Both qualitative and quantitative PCR analyses indicated that *A. niger* declined in abundance initially but then survived for the full test period of 126 days (Hynes et al., 2006). *A. niger* can survive in soil, including under cold conditions, for several months, indicating that fungi used in industrial applications could survive in highly competitive soil environment and if released without proper attenuation, and are likely to persist for at least one season (Hynes et al., 2006). *A. niger* conidia survive both in shaded areas and under direct sunlight for 15 months under desert conditions (Dose et al., 2001). Conidia of *Aspergillus* survive periods of high temperature of 50°C for 1 hour (Ruijter et al., 2003). When conidia are released into the air, they have the potential to remain there for extended periods of time and will contaminate anything in direct contact with the air (VandenBergh et al., 1999; van Leeuwen et al., 2013). The ability of *A. niger* to survive wastewater treatment of industrial discharges, probably due to its resistant conidia, suggests that it is likely to persist after introduction into aquatic environments (USEPA, 1997). Although no specific data comparing the survivability of *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 were available in the literature, their survival is likely to be the same since they are morphologically similar and *A. brasiliensis* also produces conidia. Hence, the above mentioned information indicates that in most scenarios, *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 will be able to survive and persist in the environment.

No relevant reports concerning persistence in the environment of toxins produced by *A. brasiliensis* and *A. niger* have been found. In Ontario, Canada, fumonisins do not

occur regularly, but were present in 1993 in areas with above-normal temperatures and moisture stress (Miller, 2001). Other Canadian government regulatory agencies survey for the occurrence of mycotoxins in animal feeds and establish regulatory limits, so exposure of domestic animals through the consumption of contaminated feeds is not within the scope of this assessment.

Former and probable future uses are described in Section 2.1 *Sources of Exposure*. These are likely to introduce *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 into both aquatic and terrestrial ecosystems. For example, uses in bioremediation and biodegradation would involve direct application to soils, and subsequent rainfall events could introduce *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 into waterways. In addition, their potential use in waste water treatment facilities, and for the production of biofuels, organic acids (citric acid) or enzymes could lead to direct input into waterways. *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) has properties that allow it to act as a potential biocontrol and biopesticide agent and as livestock probiotics; however these uses are assessed by other Canadian government agencies and are not within the scope of this assessment.

In the event that consumer, commercial or industrial activities resume, environmental exposure to *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 could change based on the exposure scenarios described above.

2.2.2 Human

The **human exposure** to *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 is estimated to be **low** based on responses to the Notice, which indicate that these strains are no longer used in consumer or commercial products or for industrial processes in Canada.

Nevertheless, human exposure scenarios in the event that consumer, commercial or industrial activities with *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 resume have been considered. These are based on former and probable future uses as described in Section 2.1 *Sources of Exposure*. Hazards related to microorganisms used in the workplace should be classified accordingly under the Workplace Hazardous Materials Information System (WHMIS). [3].

[3] Note: A determination of whether one or more of the criteria of section 64 of CEPA 1999 are met is based upon 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 1999, on these substances, is not relevant to, nor does it preclude, an assessment against the hazard criteria for WHMIS that are specified in the Controlled Products Regulations for products intended for workplace use. Similarly, a conclusion based on the criteria contained in section 64 of CEPA 1999 does not preclude actions being taken under other sections of CEPA or other Acts.

Should potential uses of *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 be realized, human exposure would be expected primarily through direct contact with consumer, household and commercial products containing *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) or *A. brasiliensis* ATCC 9642. Skin and eye contact and inhalation of aerosolized droplets or particles are likely routes of direct user and bystander exposure.

For commercial products containing one of these micro-organisms, the general population could be exposed as bystanders during product application. The route and extent of exposure will depend on the application method, the concentration of *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) or *A. brasiliensis* ATCC 9642 in the product, the amount of product applied, and proximity to the site of application. The general population could also come into contact with residual *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 on treated surfaces. Industrial uses in fermentation facilities for enzyme production should not increase human exposure if the micro-organisms are not released into the environment.

Indirect exposure to *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 in the environment subsequent to its use in bioremediation and biodegradation, bioleaching, textile processing, municipal and industrial wastewater treatment, or disposal of waste from its use in the production of enzymes and fermentation extract is also likely to occur. Certain uses in waste and wastewater treatment or in industrial processes may introduce *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) or *A. brasiliensis* ATCC 9642 into bodies of water. Human exposure to these strains through recreational activities is expected to be low. Drinking water treatment processes might not eliminate these micro-organisms (Sisti et al., 2012); however ingestion of these microorganisms is not of concern. The microorganism could be inhaled from water droplets, but only in minimal quantities.

Because other government regulatory agencies survey for the occurrence of mycotoxins in foods and establish regulatory limits, human exposure to fumonisin and ochratoxin A, which is only expected from the consumption of contaminated foods, is not within the scope of this assessment.

In the event that consumer, commercial or industrial activities resume, the **human exposure** to *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) or *A. brasiliensis* ATCC 9642 could change based on the exposure scenarios described above.

3. Decisions from Other Juridictions

3.1 Domestic

In Canada, other acts and regulations regulate living organisms, some targeting their uses for product safety and others directly protect Canadians, animals and plants against diseases: the *Pest Control Products Act* and Regulations, the *Feeds Act* and Regulations, the *Fertilizers Act* and Regulations, the *Seeds Act* and Regulations and

the *Health of Animals Act* and Regulations. To our knowledge, none of these acts and regulations has approved *A. brasiliensis* to be used on the Canadian market at this time. Some enzymes produced by *A. niger* have been approved under the *Food and Drugs Act* but not the micro-organism itself.

A. brasiliensis is considered risk group level 1 animal pathogens and *A. niger* is considered risk group level 2 animal pathogen according to the Animal Pathogen Import Program, Office of Biohazard Containment & Safety at the Canadian Food Inspection Agency (CFIA).

A. brasiliensis and *A. niger* are considered as Risk Group 2 human pathogens according to the Public Health Agency of Canada. They are regulated under the *Human Pathogens and Toxins Act* and their use in research and teaching laboratories should be in compliance with the Canadian Biosafety Standards and Guidelines (CBSG, 2013), 1st edition.

3.2 International

To our knowledge, no other jurisdiction has published a decision on *A. brasiliensis* at this time. Therefore this section contains mainly information for *A. niger*.

A. niger has undergone a risk assessment by the biotechnology program of the *Toxic Substances Control Act* (TSCA) under the United States Environmental Protection Agency (USEPA) and is included as a recipient microorganism at õ 725.420 for the tiered exemption (USEPA, 1994). Many enzymes produced by *A. niger* are generally recognized as safe (GRAS) for use as Food Ingredient by the U.S. Food and Drug Administration. *A. niger* strains are considered as plant pests of quarantine importance in the Commonwealth of Dominica (Pest list of the Commonwealth of Dominica, 17 November 2005) and are considered as endemic (not regulated) pests of rice in Cambodia (Cambodian Endemic and quarantine pest of rice, 06 May 2005).

In Europe, *Aspergillus* species are treated as low-risk-class microorganisms, i.e., category 2 of the European Federation of Biotechnology (Frommer *et al.,* 1989). Category 1 of the European Federation of Biotechnology scale includes organisms deemed harmless, which can be grown under good industrial large scale practices (GILSP), while category 2 organisms like *Aspergillus* require more stringent containment.

The joint FAO/WHO committee on Food additives experts have repeatedly reviewed and accepted enzyme preparations from *A. niger* including the organism itself (FAO/WHO, 1972; 1978; 1981; 1987; 1990; 1992), listing them with an Acceptable Daily Intake of ‘not specified’.

4. Risk Characterisation

Human and environmental exposure to the DSL-listed strains *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 from their deliberate use in industrial processes or consumer or commercial products in Canada is not currently expected. Based on the current level of exposure, the risk is estimated to be **low** to both the environment and human health from the DSL-listed strains *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642.

However, given that *A. brasiliensis* is morphologically indistinguishable from *A. niger*, that the taxonomy in the *A. niger* group is still evolving, that *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) produces fumonisin and ochratoxin and finally, that recent publications report infection in healthy individuals for both *A. niger* and *A. brasiliensis*, we might assume that *A. awamori* and *A.brasiliensis* could have the same pathogenic properties as *A. niger* until proven otherwise.

Both *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 have properties that make them suitable for use in industrial activities and in commercial and consumer products, and it is likely that resumption of the import, manufacture or use of *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) or *A. brasiliensis* ATCC 9642 would result in an increased level of human and environmental exposure (as described in Section 2.2), which would increase the associated risk.

A. niger is an opportunistic animal pathogen, known to cause mycosis and mycotoxicosis. Cows and other farm animals could be exposed to elevated concentrations of *A. niger* and *A. brasiliensis* from their use in bioremediation, biotransformation or biodegradation in contaminated sites adjacent to farms or pastures. This is expected to be a rare occurrence, so the overall risk to cows and other farm animals is expected to be low. Cows and other farm animals could also be exposed to elevated concentrations of *A. niger* and *A. brasiliensis* through their use in water or waste water treatment should products containing the micro-organisms be applied to cattle watering troughs or irrigation ponds or should treated wastewater or biosolids be applied to agricultural land. The overall risk from these uses to Canadian dairy herd is nevertheless expected to be low, as there have been only two reported cases of bovine mycotic mastitis known to be caused by *A. niger*, and these cases were effectively treated with the use of antifungal drugs.

Aquatic animals could inadvertently be exposed to *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) or *A. brasiliensis* ATCC 9642 through their use in waste water treatment, through runoff from terrestrial applications or from industrial effluents. *A. niger* and *A. brasiliensis* would probably persist and survive in the aquatic environment due to their resistant conidia; however, the dilution of products containing *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) or *A. brasiliensis* ATCC 9642 is expected to be such that concentrations required to see adverse effects will not likely be reached. There have been only two reported cases of

A. niger causing aspergillomycosis disease in asian freshwater catfish in the scientific literature. No other pathogenicity information on aquatic species was found.

Therefore the use of *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 in bioremediation, biodegradation, or waste water treatments, is unlikely to have a long term impact on terrestrial and aquatic populations and trends over an entire ecosystem or an ecozone.

Based on the considerations outlined above, the risk to the environment from foreseeable future uses is expected to be low.

The risk to human health will depend on the route of exposure. Of all routes identified, inhalation and dermal exposures are the most likely to cause harm in humans. *A. niger* is reported as a human opportunistic pathogen leading to a wide array of infections including lung, skin, eyes, heart and systemic infections. Although effective antifungal treatments are available for *A. niger* and *A. brasiliensis,* in light of the toxicity of antifungal drugs, the availability of treatments is not heavily weighted as a mitigating factor in this risk assessment. The risk of *A. niger* infection increases with pre-existing factors such as debilitating disease, surgery, the presence of indwelling medical devices and immune deficiency of the individual, so that the use of products containing *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) or *A. brasiliensis* ATCC 9642 in hospitals or tertiary care centres could pose health risks to susceptible populations, including the elderly and neonates. In addition, *A. niger and A. brasiliensis* are known to cause ear and eye infections in healthy individuals. *A. niger* is also known to be the most common cause of fungal ear infection, which can occur in both immunocompromised and immunocompetent individuals. Although usually considered mild, these infections can result in irreversible damage to the ears and eyes such as hearing/vision loss.

Therefore, it is recommended that new activities with *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342*)* or *A. brasiliensis* ATCC 9642 involving their addition to products that could be used in hospitals or tertiary care settings, and new activities with *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342*)* or *A. brasiliensis* ATCC 9642 involving their addition to consumer products, such as cleaning products, be assessed to ensure that these activities do not present additional risks.

5. Conclusion

Based on responses to the 2009 Notice, it is concluded that both *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 are 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 proposed that those substances do not meet the criteria as set out in section 64 of the CEPA 1999.

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Appendix A: Growth of *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 in Various Media

Table A-1 Growth of *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 in liquid media at various temperature[a]

A) *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342)

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	–	–	–	–

B) *A. brasiliensis* ATCC 9642

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

Table A-2 Growth Characteristics of *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 on Solid Media at various temperature[a]

A) *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342)

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]	+	+
Yeast Mould Agar [h]	+	+

B) *A. brasiliensis* ATCC 9642

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]	+	+
Yeast Mould Agar [h]	+	+

[a] Data generated by Heath Canada's Environmental Health Science and Research Bureau
(+) Positive for growth
(-) Negative for growth
[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] Yeast Mould Agar. Cultivation of yeasts, molds and other aciduric microorganisms.

Appendix B: Phylogenetic Neighbour Joining Tree of Selected *Aspergillus* Species Inferred from Calmodulin Sequences

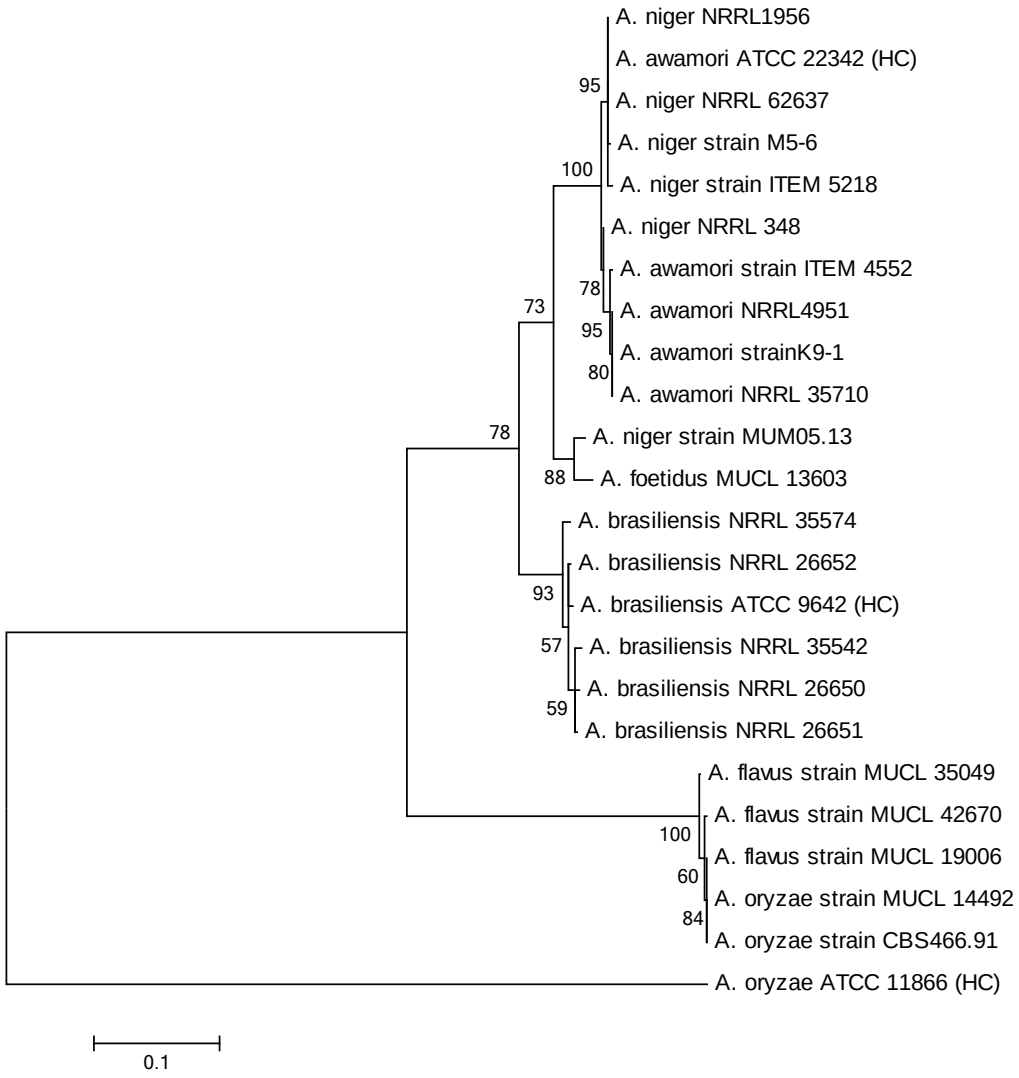

Figure B-1 Phylogenetic tree generated by the Environmental Health Science and Research Bureau using partial calmodulin gene sequences of *A. awamori* ATCC 22342 (=*A. niger* ATCC 22342) and *A. brasiliensis* ATCC 9642 alongside *Aspergillus* sp. calmodulin genes selected from Genbank. The alignment was generated by Muscle and analyzed using the Kimura 2-parameter distance model, which was then used to construct a phylogenetic tree using MEGA version 5.2 (Tamura et al., 2011)

Appendix C: Toxins and Secondary Metabolites Produced by *A. brasiliensis* and *A. niger*

Table C-1 List of toxin and secondary metabolites produced by *A. brasiliensis* and *A. niger*

Toxins	Description	Produced by	References
Fumonisin	• Most *A. niger* are able to produce fumonisin in media with high sugar content. • Fumonisin B_2 is a carcinogenic mycotoxin, less acutely toxic than aflatoxins, but found in greater quantity in corn. Known to cause fatal equine disease (leukencephalomalacia) and possibly oesophageal cancer in humans • Fumonisins are associated with a number of animal and human diseases. • Fumonisins have been shown to be involved in leukoencephalomalacia in horses, pulmonary edema in pigs and cancer and neural tube defects in experimental rodents	*A. awamori ATCC 22342* (=*A. niger* ATCC 22342) and *A. niger*	(Frisvad et al., 2007a; Frisvad et al., 2007b; Knudsen et al., 2011; Marasas, 2001; Miller, 2001; Mogensen et al., 2009a; Mogensen et al., 2009b; Mogensen et al., 2010; Nielsen et al., 2009; Noonim et al., 2009; Palumbo et al., 2011; Perrone et al., 2011; Somma et al., 2012; Stockmann-Juvala and Savolainen, 2008; Storari et al., 2012; Susca et al., 2010; Varga et al., 2010b; Varga et al., 2011)
Malformins •Malformin A1 •Malformin A2 •Malformin B1a •Malformin B1b •Malformin B2 •Malformin B3 •Malformin B5 •Malformin C	• Malformins are a group of cyclic pentapeptides • Toxicity of malformins may be attributed to the interaction of its disulfide group with essential thiol compounds. • Fungal production of malformins caused calcium depletion and other physiological abnormalities • They cause deformations, malformations and downward curvatures in bean plants and corn plants. • It stimulates root hair and lateral root formation, promotes radial expansion, inhibits elongation, wet and dry weight, cell division and cell wall synthesis in roots of Zea mays, but has no effect on protein synthesis • Malformin C shown antibacterial	*A. brasiliensis* and *A. niger*	(Al-Hindi et al., 2011; Anderegg et al., 1976; Blumenthal, 2004; Curtis et al., 1974; Ehrlich et al., 1984; Inokoshi et al., 1999; John and Curtis, 1974; Kim et al., 1993; Kobbe et al., 1977; Nielsen et al., 1999; Nielsen et al., 2009; Schuster et al., 2002; Steyn, 1977; Sugawara et al., 1990; Yoshizawa et al., 1975)

	activity against a variety of gram posit*ive and gram negative organisms (Bacillus subtilis, B. megaterium, Staphylococcus aureus, Atreptococcus faecalis, Proteus mirabilis* and *Sarcina lutea*) and cytostatic properties		
Naphtho-y-pyrones (NGPs)	• The NGP group of compounds comprises a series of aurasperones, fonsecinones, and nigerones, as well as monomers such as flavasperone and rubrofusarin B. • No data on the bioavailability of these compounds exist. Therefore these compounds cannot currently be considered mycotoxins *sensu stricto*, since this requires toxicity via a natural route of exposure. • The total naphtho- gamma -pyrones and one of its major components, aurasperone D, in doses of 50 mg/kg intraperitoneally, produced marked central nervous system depressant effects in albino mice and rats leading to death by respiratory failure.	*A. brasiliensis, A. brasiliensis ATCC 9642* and *A. niger*	(Blumenthal, 2004; Bouras et al., 2005; Ehrlich et al., 1984; Ghosal et al., 1979; Guang-Yi et al., 1989; Nielsen et al., 2009; Perrone et al., 2011; Samson et al., 2004; Varga et al., 2007; Varga et al., 2011)
Nigerazines	• Nigerazines were found to inhibit root growth of lettuce seedlings.	*A. niger*	(Iwamoto et al., 1985)
Nigragillin	• Nigragillin purified from cultures filtrates tested in animal studies demonstrated to be toxic to silkworm larvae	*A. niger*	(Caesar et al., 1969; Isogai et al., 1975);
Ochratoxin A (OTA)	• In general, ochratoxin A production is only present in 5-10 % of the *A. niger* strains • OTA production is positively associated with the presence of a putative polyketide synthase (PKS) gene (An15g07920) • OTA is a nephrotoxic mycotoxin in monograstric animals such as pigs and poultry, carcinogenic in kidney, teratogenic of the central nervous system and immunosuppressive in laboratory animals. It also has shown to possess genotoxic properties.	*A. awamori ATCC 22342* (=*A. niger* ATCC 22342) and *A. niger*	(Abarca et al., 2001; Dalcero et al., 2002; Esteban et al., 2006a; Esteban et al., 2006b; Ferracin et al., 2012; IARC, 1993; Kuiper-Goodman and Scott, 1989; Magnoli et al., 2004; Magnoli et al., 2006; Magnoli et al., 2007; Marquardt et al., 1988; Marquardt et al., 1990; Pfohl-Leszkowicz and Manderville, 2007; Somma et al., 2012;

	• OTA produced by *A. niger* has been detected from a variety of food and animal feed • Animals consuming OTA have decreased growth rates and may also be more susceptible to subclinical intoxications. • OTA is often cited as a possible causal agent in Balkan Endemic Nephropathy and associated with urinary tract tumours. • OTA is reported to exhibit immune suppressive and carcinogenic properties in humans and animals • The International Agency for Research on Cancer (IARC) has given OTA a Group 2B classification, a possible human carcinogen based on its nephrotoxic, hepatotoxic, immunotoxic, tetratogenic and carcinogenic effects • CFIA recommends a tolerance level of 2 mg/kg in pig and poultry feed.		Storari et al., 2010; Varga et al., 2010a) (Ferracin et al., 2012).
Tensidol A and B	• Both tensidols A and B are furopyrrols and have the common skeleton of 6-benzyl-6H-furo-[2,3-b]pyrrole. • Both are soluble in organic compounds such as methanol, trichloromethane, ethanoic acid, insoluble in water • Tensidols A and B potentiated miconazole activity against *Candida albicans*. Tensidols also show moderate antimicrobial activity against *Pyricularia oryzae*	*A. brasiliensis ATCC 9642, A. brasiliensis* and *A. niger*	(Frisvad et al., 2007b; Mogensen et al., 2010; Perrone et al., 2011; Somma et al., 2012; Storari et al., 2012; Varga et al., 2010b; Varga et al., 2011)

Table C-2 LD50 values for *A. niger* and its toxins

Substance	Organism	LD_{50} or LC_{50} LD50 (mg/kg)	Strain	Reference	Route

Ochratoxin A	Rat Mouse Rat Mouse Sheep Rat Mouse Chicken Duck Quail Turkey	12.6 mg/kg 22 mg/kg 12.8 mg/kg 25.7 mg/kg 1 mg/kg 20 mg/kg 46 mg/kg 3.3 mg/kg 0.5 mg/kg 16.5 mg/kg 5.9 mg/kg	*A. niger*	(Abarca et al., 1994)	IP IP IV IV IV O O O O O O
Ochratoxin A	Young rats Day old chicks	20 mg/kg 3.6 mg/kg	*A. niger*	(Pitt et al., 2000)	O
Aurasperone D	Mouse	47 mg/kg	*A. niger*	(Ehrlich et al., 1984)	IP
Malformins A and Malformin C	Mouse Rat	3.1 mg/kg 0.9 mg/kg	*A. niger*	(Anderegg et al., 1976; Curtis, 1958; Curtis et al., 1974; Iriuchijima and Curtis, 1969; Varoglu and Crews, 2000)	IP IP
Nigerazine B	Mouse	75 mg/kg	*A. niger*	(Iwamoto et al., 1985)	IP
Nigragillin	Cokerels*	150 mg/kg		(Caesar et al., 1969)	O

IP, intraperitoneal; IV, intravenous; O, oral; SC, subcutaneous

Appendix D: Plant Rots and Diseases Caused by *A. niger*

Table D-1 Plant rots and diseases caused by *A. niger*

Name of the disease	Host	Reference
Black rot of onions	*Allium cepa* . (Onion)	(Narayana et al., 2007)
Crown rot of peanuts	*Pisum sativum* (Peanut)	(Anderegg et al., 1976)
Stem rot of Dracaena	*Dracaena sanderiana* Mast.	(Abbasi and Aliabadi, 2008)
Black mold rot of cherry	*Prunus avium* (Cherry)	(Lewis et al 1963)
Kernel rot of maize	*Zea mays* (Corn)	(Palencia et al., 2010)
Fruit rot of grapes	*Vitis sp* (Grapes)	(Sharma and Dharam, 1986)
Fruit rot of banana	*Musa sp*. (Banana)	(Adebesin et al., 2009)
Rot of Tomatoes	*Solanum lycopersicum* (Tomato)	(Purnima and Saxena, 1987)
Mango rot	*Mangifera indica* (Mango)	(Om and Raoof, 1988)
Bunch rots, sour rot and vine canker on table grapes	Table grapes	(Latorre et al., 2002; (Michailides et al., 2002; Rooney-Latham et al., 2008; Vitale et al., 2008))
Leaf spot disease	*Zingiber officinale* (Ginger)	(Pawar et al., 2008).
Bole rot of Sisal	*Agave sisalana*	(Coutinho et al., 2006).
Stem end rot of mango	*Mangifera indica*	(Huang et al., 2012)
Fig smut	*Ficus carica*	(Bayman et al., 2002; Doster et al., 1996; Doster and Michailides, 2007).
Stem rot of dracaena	*Dracaena sanderiana*	(Abbasi and Aliabadi, 2008)
Rot of Bael fruits	*Aegle marmelos*	(Arya et al., 1986)