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Canadian Environmental Protection Act - Indirect Human Health Assessment Report on *Danio rerio* BZ2019 and PZ2019

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Foreword

This series documents the scientific basis for the evaluation of aquatic resources and ecosystems in Canada. As such, it addresses the issues of the day in the time frames required and the documents it contains are not intended as definitive statements on the subjects addressed but rather as progress reports on ongoing investigations.

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ABSTRACT

An indirect human health risk assessment was conducted on two lines of genetically modified *Danio rerio* (BZ2019 and PZ2019) that were notified under the *Canadian Environmental Protection Act* (CEPA). This risk assessment examined the potential for BZ2019 and PZ2019 to cause harmful effects to humans in Canada relative to wild-type *D. rerio* as a consequence of environmental exposure, including exposure in natural environments and environments under its intended use (i.e., home aquaria). BZ2019 and PZ2019 are modified lines of diploid, hemizygous or homozygous, Zebrafish, containing genes encoding for fluorescent blue and purple proteins, respectively. BZ2019 and PZ2019, which also appear blue and purple, respectively, under ambient light (including sunlight) will be imported from the United States for use as ornamental fish in home aquaria. The notified lines have been commercially marketed as aquarium fish throughout the United States except California since 2010 (BZ2019) and 2011 (PZ2019), and in California since 2015 without any reported incidents. The parental strain, *D. rerio*, has been available as a home aquarium fish since the early 1900s. There is no evidence to suggest a risk of adverse human health effects at the exposure levels predicted for the general Canadian population from use of BZ2019 and PZ2019 as ornamental aquarium fish as well as other identified potential uses. As such, there is no expectation that BZ2019 and PZ2019 pose any more risks to human health than wild-type *D. rerio.*

INTRODUCTION

The following indirect human health risk assessment was conducted on *Danio rerio* BZ2019 and PZ2019, two genetically modified lines of diploid, hemizygous or homozygous, Zebrafish, containing genes encoding for fluorescent blue or purple proteins, respectively. The risk assessment examines the potential for BZ2019 and PZ2019 to cause harmful effects to humans in Canada, relative to wild-type *D. rerio*, as a consequence of environmental exposure, including exposure in natural environments and environments under its intended use (i.e., home aquaria). BZ2019 and PZ2019 are blue and purple in colour, respectively, when displayed in ambient light, including sunlight, and will be imported from the United States for use as ornamental fish in home aquaria. The risk assessment was conducted under the *Canadian Environmental Protect Act* (CEPA) and *New Substances Notification Regulations (Organisms)* (NSNR[O]).

HAZARD ASSESSMENT

IDENTIFICATION AND CHARACTERIZATION OF *DANIO RERIO* BZ2019 AND PZ2019

Binomial name

Danio rerio BZ2019 and PZ2019

Taxonomy

Kingdom	Animalia
Phylum	Chordata
Subphylum	Vertebrata
Superclass	Actinopterygii
Class	Teleostei
Order	Cypriniformes
Genus	*Danio*
Species	*rerio*
Strains	BZ2019 and PZ2019

Synonyms, Common and superseded names

Synonyms: *Brachydanio rerio* (Hamilton, 1822); *Cyprinus rerio* (Hamilton, 1822)

Common names: Zebra Danio, Zebrafish

Trade names: BZ2019 - GloFish® Cosmic Blue® Danio

PZ2019 - GloFish® Galactic Purple® Danio

Characterization and substantiation of the taxonomic identification

Danio rerio BZ2019 and PZ2019 are genetically modified lines of diploid, hemizygous or homozygous, Zebrafish containing genetic constructs which makes them appear blue (BZ2019) and purple (PZ2019) under ambient light, including sunlight, and fluorescent under ultraviolet light. The two lines were derived from a line of stripe-free, Golden Zebrafish, a naturally occurring colour mutation of the pigmented wild-type Zebrafish (Clark and Ekker 2015). *D. rerio* distinguishing features include an incomplete lateral line extending to the base of the pelvic fin, two pairs of barbels, and five to seven dark blue longitudinal stripes extending from behind the operculum into the caudal fin (Barman 1991; Spence et al. 2008). However, the Golden Danio lacks the dark blue stripes due to the production of lighter pigment intensity compared to normal striped Zebrafish (Clark and Ekker 2015).

STRAIN HISTORY

BZ2019 and PZ2019 were developed from a population of Golden Zebrafish that was provided by 5-D Tropical Inc. (Plant City, Florida) in 2007. Golden Zebrafish is a naturally occurring colour mutation of the pigmented wild-type Zebrafish (Clark and Ekker 2015). The notified lines are each derived from a single DNA-injected egg. Greater detail regarding strain development and history of the notified lines has been provided by the company for the expressed purpose of the current risk assessment and review, but is identified as confidential business information and is not included in this report.

GENETIC MODIFICATIONS: PURPOSE, METHOD, GENETIC AND PHENOTYPIC CHANGES

The notified lines (BZ2019 and PZ2019), which have been modified to appear blue (BZ2019) and purple (PZ2019), are intended for use by the general public for home aquarium display purposes only. Just like the wild-type *D. rerio*, which is a non-food species that has been used safely in aquaria worldwide for more than a century, BZ2019 and PZ2019 are not intended for food use.

According to the information provided by the notifier, in addition to BZ2019 and PZ2019 appearing blue and purple, respectively, under ambient light, both lines have a lower reproductive success rate compared with the non-transgenic Golden Zebrafish siblings. The notifier also provided results from a temperature tolerance test that showed decreased tolerance to low temperatures for BZ2019 compared to non-transgenic Zebrafish. However, there was no significant difference ($P>0.05$) between the low temperature tolerance of PZ2019 and the non-transgenic Golden Zebrafish.

The approach used to produce, grow and prepare for sale the notified lines is considered adequate to ensure genetic stability of the broodstock because:

- The notified lines are each derived from a single injected egg with the resulting fry screened for fluorescence and used to select individuals to be used for mating with non-transgenic Golden Zebrafish to produce F_1 fish which are later mated again with the parental wild-type Golden Zebrafish to produce F_2 fluorescent fish. F_2 fluorescent fish were selected on the basis of phenotype and a confirmative Southern blot analysis to validate the presence of a single copy of the genetic modification. The selected fluorescent F_2 fish became the lines identified as either BZ2019 or PZ2019; and
- Approximate copy numbers of the genetic expression cassettes in the two notified lines were confirmed by quantitative real-time PCR. Breeding data for each line shows that the added genetic material is segregating during breeding as a single locus. Phenotypic markers, largely based on the colour of the fish, are used to ensure uniform genetic composition of the brood stock. Hemizygous and homozygous fish are visually indistinguishable from each other, and they are consequently both used in breeding stock. A loss or inactivation of the expression cassette would produce fish that are phenotypically indistinguishable from the unmodified Golden Zebrafish, which would simply be segregated from the fluorescent offspring. These Zebrafish are not used as broodstock. Breeding lines

of both colours of the fluorescent Zebrafish have been maintained for more than five generations and commercial production has continued for more than five years.

BIOLOGICAL AND ECOLOGICAL PROPERTIES

The wild-type *D. rerio* is a small shoaling cyprinid fish that is one of approximately 44 related species native to the flood-plains of the Indian sub-continent. The species is most commonly found in shallow ponds and slow-flowing waters that are often adjacent to rice fields, but it may also be found in rivers and hill streams. Although *D. rerio* rarely exceeds 40 mm Standard Length (SL), from the tip of the snout to the origin of the caudal fin (Spence et al. 2008), there is wide size variability with reports (Plaut 2000) of some strains reaching up to 61.5 mm Total Length (TL) between the most anterior part of the head to the posterior most tip of the caudal fin. Among the domesticated wild-type lines of *D. rerio*, there are a number of mutations including the “Leopard” Danio with spotted colour pattern instead of stripes and longfin aquarium variant, which is a dominant mutation resulting in elongated fins (Plaut 2000; Spence et al. 2008; Meyers 2018). Growth rates are considerably higher in domesticated populations while native fish are reported to have slower growth and achieve a smaller adult size (Spence et al. 2007). Zebrafish are omnivores with a diet consisting primarily of zooplankton and insects but may also include phytoplankton, filamentous algae, vascular plant material, spores, invertebrate eggs, fish scales, arachnids, and detritus (Spence et al. 2008).

D. rerio only spawn seasonally in nature, but will spawn throughout the year under captive breeding conditions. The fish are scatter breeders, providing no parental care after directly depositing their eggs onto the substrate (Hill and Yanong 2002; Spence et al. 2008). Hatching of eggs occurs between 48 and 72 hours post-fertilization at 28.5°C with the larvae immediately attaching to hard surfaces. Swimming, feeding, and active avoidance behaviours commence approximately 72 hours post-fertilization (Spence et al. 2008). Sexual maturity in domesticated strains is reported to be reached after approximately 75 days at 25.5°C. In captivity, Zebrafish have a mean lifespan of 42 months with a maximum reported lifespan of 66 months (Gerhard et al. 2002; Spence et al. 2008).

HUMAN HEALTH EFFECTS

Zoonotic Potential

In-house literature searches found no reports of zoonoses or other adverse effects attributed to the notified lines or to the wild-type *D. rerio* resulting from home aquarium exposure. However, while uncommon, there are reported cases of zoonotic infections from contact with tropical ornamental fish and indirect zoonoses due to ingestion of food or drinking water that has been contaminated with pathogens and parasites associated with ornamental or aquarium fish. Bacterial disease is extremely common in ornamental fish and is most frequently associated with bacteria that are ubiquitous in the aquatic environment acting as opportunistic pathogens secondary to stress (Roberts et al. 2009). Contact is the main route of transmission leading to bacterial infections in humans that develop from handling of aquatic organisms (Lowry and Smith 2007). Young children, pregnant women, and immunocompromised individuals are at higher risk for these infections (Dinç et al. 2015). Children are also more susceptible to severe disease outcomes as compared with adults and often have less stringent hygienic practices (Dunn et al. 2015). The most common bacterial species associated with tropical fish capable of causing human illness belong to the genera *Aeromonas* and *Salmonella* along with the species *Mycobacterium marinum* and *Streptococcus iniae* (CDC 2015) with the most commonly reported infections being associated with *M. marinum* (Weir et al. 2012).

In humans, *M. marinum* is the causative agent for the disease “fish tank granuloma” which results in ulcerative skin lesions or raised granulomatous nodules. These lesions are typically limited to the distal extremities such as the hands, legs, and feet as *M. marinum* has an optimum growth temperature range of 26°C to 32°C (Mutoji and Ennis 2012; Gauthier 2015). However, these nodular cutaneous lesions can progress to tenosynovitis, arthritis and osteomyelitis (Hashish et al. 2018). In addition, rare cases of systemic mycobacteriosis have been reported in immunocompromised individuals (Lowry and Smith 2007). Infections are generally contracted from exposure of wounds and skin abrasions to contaminated water (Gauthier 2015). Phan and Relic (2010) described the case of a 24-year old woman who owned multiple fish tanks and suffered a facial infection with sporothrichoid presentation three weeks after suffering a cat scratch to her left lower eyelid. The patient’s cats had a habit of standing on the tanks and immersing their paws in the water and the woman did not use gloves when performing monthly tank maintenance. Infection is also possible from contact with aquarium equipment as Doedens et al. (2008) reported on a case of an 18-month old girl with *M. marinum* abscesses on her right arm following contact with a contaminated bucket used for fish during aquarium cleanings. The child (born with Tetrology of Fallot, corrected at 1-month of age) never had direct contact with the fish as the aquarium was placed high in a bookcase out of the child’s reach. In addition, the father, who had eczema, had developed abscess-like lesions on his hands after cleaning the tank. The infections described in Phan and Relic (2010) and Doedens et al. (2008) were successfully treated with antibiotics.

While risk of infection is significantly increased for immunocompromised individuals (Koushk-Jalali et al. 2019), infections are also reported in immunocompetent individuals (Krooks et al. 2018; Bouceiro-Mendes et al. 2019). Lesions typically present as less than 2 cm in diameter with the size, tenderness and number of swellings increasing slowly over weeks to months (Boylan 2011). *M. marinum* infections are difficult to diagnose in humans and, therefore, history of exposure to aquarium water and/or fish is important to ensure proper diagnosis and antibiotic treatment (Beran et al. 2006). Monotherapy including clarithromycin, trimethoprim or ciprofloxacin has been reported as an effective treatment for skin and soft tissue infections, while a combination therapy of two drugs may be more effective in cases of deeper infections (Hashish et al. 2018).

Examples of reported cases of *M. marinum* infection from aquarium exposure in the literature include Huminer et al. (1986), Aubry et al. (2002), Lahey (2003), Wu et al. (2012), Slany et al. (2012; 2013), Riera et al. (2016), Veraldi et al. (2018), Bouceiro-Mendes et al. (2019), and Koushk-Jalali et al. (2019). Mason et al. (2016) reported on an outbreak of *M. marinum* in 2010 at a Zebrafish research facility at the University of Oregon that also affected facility personnel. Although three persons working in the facility reported reddish bumps on one hand, only one case was confirmed by PCR testing as an *M. marinum* infection. At the time of the outbreak, personal protective equipment (PPE) was not required in the facility and most staff did not use gloves for work involving direct contact with the Zebrafish. Changes implemented in the facility, including mandatory use of PPE by staff that have contact with the Zebrafish, resulted in zero cases of infection in the years following. While *M. marinum* has been associated with disease in Zebrafish (Ramsay et al. 2009), there are no reported zoonotic cases attributed to home aquarium exposure from either the notified lines or to wild-type *D. rerio*.

Other species of *Mycobacterium* that are capable of causing infections in Zebrafish include *M. abscessus*, *M. chelonae*, *M. fortuitum*, *M. haemophilum*, and *M. peregrinum* (Rowe et al. 2014). While most mycobacterial species are opportunistic pathogens, Zebrafish are reported to be particularly vulnerable to *M. haemophilum* (Whipps et al. 2007; Rowe et al. 2014). In immunosuppressed adults and children, *M. haemophilum* has been reported to be associated with subcutaneous infections, lymphadenitis, septic arthritis, osteomyelitis, pneumonitis and disseminated disease (Emmerich et al. 2019; Franco-Paredes et al. 2019). Cameselle-Martínez et al. (2007) reported on a cutaneous infection by *M. haemophilum* in a severely immunosuppressed AIDS patient following a bite from an aquarium fish. The infection was successfully treated following a combined therapy of six antibiotics. *M. abscessus*, *M. chelonae*, *M. fortuitum* and *M. peregrinum* are also associated with cutaneous infections in humans (Kamijo et al. 2012; Franco-Paredes et al. 2019). Li et al. (2014) reported on a successful treatment with antibiotics of a cutaneous *M. chelonae* infection on the left arm of an 82-year old woman with a hobby of rearing tropical fish. While cutaneous mycobacterial infections may be successfully resolved with antibiotics, the choice of antibacterial combinations and length of therapy is species-specific (Franco-Paredes et al. 2018).

Zoonotic infections from *S. iniae* have most often been associated with the handling and preparation of infected fish by persons with underlying medical conditions such as diabetes mellitus, chronic rheumatic heart disease, or cirrhosis (Baiano and Barnes 2009). Handling of live or recently killed infected fish can result in cellulitis of the hand or endocarditis, meningitis, and arthritis in severe systemic infections (Lowry and Smith 2007; Boylan 2011; Gauthier 2015). People with weakened immune systems or open skin wounds could get infected by *S. iniae* while handling fish or cleaning aquaria (CDC 2015). *Streptococcus*-like bacteria have been isolated in Zebrafish imported into Canada as aquarium fish (Ferguson et al. 1994) and Zebrafish have been used as a model organism for *S. iniae* infection (Rowe et al. 2014; Harvie and Huttonlocher 2015). However, there are no reports in the scientific literature of human streptococcal infections attributed to Zebrafish from home aquarium exposure.

Aeromonas spp. are opportunistic pathogens that are associated with a number of diseases in ornamental fish (Hossain et al. 2018). *Aeromonas hydrophila* is the most commonly reported Aeromonad that possesses zoonotic potential with *A. sobria* and *A. caviae* also having been reported (Boylan 2011). Water with high nutrient levels can cause bacterial blooms capable of being infectious to humans through wounds or ingestion; however, infections are rare and typically involve immune suppression (Boylan 2011). *A. hydrophila* was one of the species of bacteria isolated from cough swabs of an 11-month old boy with cystic fibrosis (Cremonesini and Thomson 2008). The authors believe the infection was the result of aerosol spread of the bacterium due to the aeration process of fish tanks in the home as isolations of *A. hydrophila* only ceased following removal of the tanks. Among the pathogenic *Aeromonas* spp., *A. veronii* appears to exhibit the broadest host range as species ranging from invertebrates to mammals, including humans, have shown susceptibility to this pathogen (Lazado and Zilberg 2018). Zebrafish have been shown to be more susceptible to *A. veronii* than the Guppy (*Poecilia reticulata*) (Lazado and Zilberg 2018). However, an in-house literature search found no reported cases of zoonotic infections of *A. veronii* from ornamental fish exposure.

Salmonella infection can occur through contact with an animal's habitat such as an aquarium (CDC 2015). While *Salmonella* is not a known pathogen for tropical fish, they may act as bacterial reservoirs and excrete *Salmonella* in their feces during periods of stress (Gaulin et al. 2005). Musto et al. (2006) reported on 78 cases of *Salmonella* Paratyphi B biovar Java infections in people having aquaria containing tropical fish in Australia. Infections were mostly seen in children (median age of cases was 3-years old) following exposure to aquarium water and resulted in diarrhoea, fever, abdominal cramps, vomiting, bloody stool, headaches, and myalgia. Similarly, out of 53 reported cases of *S.* Paratyphi B, var. Java infections reported in the province of Quebec from January 2000 to June 2003, 33 infected individuals owned an aquarium with 21 of the aquaria testing positive for *Salmonella* (Gaulin et al. 2005).

Zoonotic infections primarily occur through puncture, cuts, scrapes, abrasions or sores in the skin (Boylan 2011). Infections may be prevented through wearing gloves when handling fish or cleaning fish tanks and avoiding contact with any potentially contaminated water if any open skin wounds are present. Washing hands with soap and water after contact with aquarium water is also highly recommended. As well, people with compromised immune systems or underlying medical conditions as well as children should avoid cleaning tanks or handling fish (Haenen et al. 2013).

There are no reports specifically associating the notified organisms with any parasites of human health significance. Routine health evaluations (necropsy, microbiology) were conducted on limited sample sizes of six blue and six purple Zebrafish and histology was conducted on an additional six fish of each colour at a fish disease diagnostic laboratory at the University of Florida in 2010. For BZ2019, the health evaluation reported that all findings were normal except for the presence of low numbers of unidentified species of nematodes in two fish while all findings were reported to be normal for PZ2019. The reports did not examine wild-type fish but did state that the findings were unrelated to the genetic modification since parasites may commonly be found in ornamental fish (Florindo et al. 2017; Trujillo-González et al. 2018). As well for both lines, no bacterial growth was observed after 48 hours (at 28°C) in brain and posterior kidney samples plated onto blood agar plates. The histological examinations reported no significant pathologic lesions in any of the fish for both lines.

Allergenicity/Toxigenicity

In-house amino acid sequence analyses of all the expressed proteins were done using the AllergenOnline Database (v19; 10 February, 2019). No matches with greater than 35% identity nor exact matches for 80 and 8 sliding window amino acid segments, respectively were found for the expressed proteins. The 35% identity for 80 amino acid segments is a suggested guideline proposed by the Codex Alimentarius Commission for evaluating newly expressed proteins produced by recombinant-DNA plants (WHO/FAO 2009). Analyses conducted for all the other reading frames found a positive result using the 80mer sliding window for a putative ORF in the 3'5'Frame1 direction for one of the proteins. The ORF was found to have 35.03% identity with a putative serine protease from the fungal species *Aspergillus niger*. However, the full length alignment resulted in only 33% identity and there was a high E-value (expectation value) of 1.3e+3. Cross-reactivity typically requires the matches to be 40% identical over 80 amino acids with an E-value score of 1e-15 or less (Dr. Richard Goodman, University of Nebraska-Lincoln, personal communication, May 27, 2019). Thus, allergic cross reactivity is not likely for the putative ORF. Results provided by the notifier from analyses using the Allermatch website found no matches performing an 80 amino acid sliding window alignment using the 35% cutoff or exact matches using 7 or 8 amino acid lengths.

Analyses of the inserted sequences do not indicate any homologies to sequences of potential toxins or allergens. Furthermore, there is no evidence indicating any potential for BZ2019, PZ2019 or *D. rerio* to produce toxic or other hazardous materials that may accumulate in the environment or be consumed by humans or other organisms in the environment.

HISTORY OF USE

The notified lines have been commercially marketed as home aquarium fish throughout the United States except California since 2010 (BZ2019) and 2011 (PZ2019) and in California since 2015 with no reported incidents of adverse health effects in humans. The parental strain, *D. rerio* was first imported to Europe as a home aquarium fish in the early 1900s and has also been used as a model research organism since the 1930s (Clark and Ekker 2015).

HAZARD CHARACTERIZATION

The human health hazard potential of BZ2019 and PZ2019 is assessed to be low (Table 1) because:

1. BZ2019 and PZ2019 are genetically modified tropical fish containing copies of transgene constructs at a single site of insertion (although alternate insert patterns may exist in the population) that were confirmed to be stably integrated through multiple crossings;
2. The methods used to produce BZ2019 and PZ2019 do not raise any indirect human health concerns. Although some of the source organisms from which the inserted genetic material was derived produce toxins, there is no indication that any of the inserted genetic material or expressed proteins in these lines are associated with any toxicity or pathogenicity in humans;
3. While there are reported cases of zoonotic infections associated with tropical aquarium fish, particularly for immunocompromised individuals and children, there are no reported cases attributed to either the notified organisms or the wild-type *D. rerio*;
4. Sequence identities of the inserted transgenes do not match any known allergens or toxins. While analyses conducted on the other potential reading frames found one potential match for the purple fluorescent protein, the results suggest there is little evidence for cross-reactivity; and
5. There is a history of safe use for the BZ2019 and PZ2019 in the United States and globally for the wild-type species as an ornamental aquarium fish and model research organism.

Table 1: Considerations for hazard severity (human health).

HAZARD	CONSIDERATIONS
High	• Effects in healthy humans are severe, of longer duration and/or sequelae in healthy individuals or may be lethal. • Prophylactic treatments are not available or are of limited benefit. • High potential for community level effects.
Medium	• Effects on human health are expected to be moderate but rapidly self-resolving in healthy individuals and/or effective prophylactic treatments are available. • Some potential for community level effects
Low	• No effects on human health or effects are expected to be mild, asymptomatic, or benign in healthy individuals. • Effective prophylactic treatments are available. • No potential for community level effects.

UNCERTAINTY RELATED TO INDIRECT HUMAN HEALTH HAZARD ASSESSMENT

The ranking of uncertainty associated with the indirect human health hazard assessment is presented in Table 2. Adequate information was either provided by the notifier or retrieved from other sources that confirmed the identification of the notified organisms. Adequate information was also provided describing in good detail the methods used to genetically modify the wild-type *D. rerio* including the sources of the inserted genetic materials and the stability of the resulting genotypes and phenotypes. Sequence analyses of the inserted genetic material for both notified lines did not match any toxins or allergens and no reports were found of adverse effects attributed to the inserted proteins in humans.

While there were no reports of adverse human health effects directly associated with the notified organisms, surrogate information from the literature on other ornamental fish appear to indicate the potential for transmission of human pathogens. However, such cases of infections are common to all ornamental aquarium fish and are not unique to Zebrafish. However, data related to aquarium-associated zoonoses and infections is often incomplete, and sometimes it is unknown which of the various species present in the home may have caused the infection. Despite more than five years of commercially producing both BZ2019 and PZ2019 in the United States, there are no reports of adverse human health effects. Consequently, combining both empirical data on the notified organisms, surrogate information from the literature on other ornamental aquarium fish and the lack of adverse effects supported by the history of safe use in the United States, the indirect human health hazard assessment of BZ2019 and PZ2019 is considered to be **low** with **low uncertainty.** The uncertainty is considered low because much of the information on human health effects are based on reports from other ornamental aquarium fish and the fact that there are no particular studies that have investigated human health effects associated with fluorescent transgenic ornamental fish.

Table 2: Categorization of uncertainty related indirect human health hazard.

Description	Uncertainty Ranking
There are many reports of human health effects related to the hazard, and the nature and severity of the reported effects are consistent (i.e., low variability); OR The potential for human health effects in individuals exposed to the organism has been monitored and there are no reports of effects.	Negligible
There are some reports of human health effects related to the hazard, and the nature and severity of the effects are fairly consistent; OR There are no reports of human health effects and there are no effects related to the hazard reported for other mammals.	Low
There are some reports of human health effects that may be related to the hazard, but the nature and severity of the effects are inconsistent; OR There are reports of effects related to the hazard in other mammals but not in humans.	Moderate
Significant knowledge gaps (e.g., there have been a few reports of effects in individuals exposed to the organism but the effects have not been attributed to the organism).	High

EXPOSURE ASSESSMENT

IMPORT

Imported fish will enter Canada through various points of entry that have not been specifically identified. Broodstock, which are the descendants of the original F_2 fish for the two lines, are maintained on two farms (5-D Tropical, Inc. and Segrest Farms, Inc.) in Florida. Both farms use the same breeding protocol to produce fish that become the lines identified as BZ2019 and PZ2019. For each line, the broodstock contains Zebrafish with hemizygous and homozygous genotypes, which are visually indistinguishable from each other. In the United States, production of the notified lines is regulated by the Florida Department of Agriculture and Consumer Services' Division of Aquaculture to ensure the use of best management practices and help protect the environment. According to the notifier, adult fish will be shipped to distributors for eventual distribution to pet stores for purchase by the general public. The notified lines will be delivered to retailers in the quantity ordered where they will be held until sold.

INTRODUCTION OF THE ORGANISM

BZ2019 and PZ2019 will be marketed at retail outlets where ornamental aquarium fish are sold. The exact number and locations where the notified organisms will be available are not currently known. According to the notifier, approximately 750 retail outlets could be used to sell BZ2019 and PZ2019 for use as ornamental fish to be confined inside aquaria in homes and retail outlets. Human exposure pathways for BZ2019 and PZ2019 are presented in Figure 1.

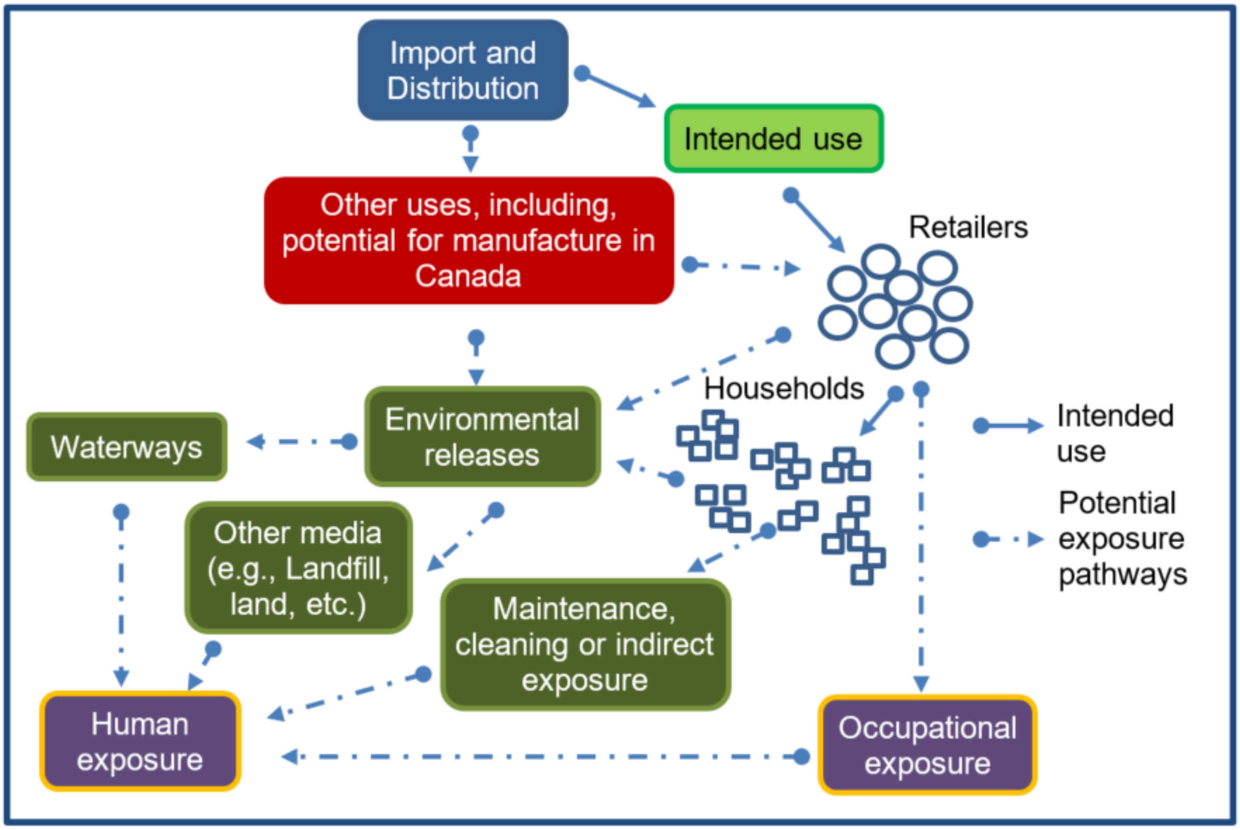

Figure 1: Human exposure pathways for BZ2019 and PZ2019.

With this introduction, human exposure during importation and distribution to retailers is expected to be largely occupational although accidental, deliberate or unintended environmental releases cannot be completely ruled out.

The most likely route of human exposure is expected to be through dermal contact with BZ2019 and PZ2019. Home aquarists that purchase the notified lines will most likely experience such contact during maintenance activities such as water changes and tank cleanings. It is not known what percentage of home aquarists may purchase BZ2019 or PZ2019; however, a 2009 survey estimated 12% of Canadian households owned fish (Perrin 2009; Whitfield and Smith 2014) and another survey (Marson et al. 2009) reported about 20% of respondents having danios in their aquaria. In the U.S., where about 8% of pet owners keep fish (AVMA, 2007), Glofish® hold an approximately 15% market share in aquarium fish sales according to a company estimate (Anderson 2017). Based on the notifier’s experience with the U.S. market, typical stocking of this species in home aquaria is one to two individual fish per gallon. No information is available on the health status of people that may be exposed but are likely to include immunocompromised individuals, children, those with underlying medical conditions, or other vulnerable individuals where caution is advised in handling pet-shop fish due to, for example, the risk of infection with non-tuberculosis mycobacteria (Kušar et al. 2017). One case of indirect exposure to immunocompromised individuals was reported by Vandepitte et al. (1983) where a 2-month-old Belgian infant contracted diarrhea that was associated with *Edwardsiella tarda* as the only potential pathogen considering that the same organism was isolated from a tropical aquarium fish in the home of the patient.

According to the information provided by the notifier, home aquaria established for *D. rerio* and similar types of tropical aquarium fish are generally maintained at temperatures of between 20 and 30°C, which are generally on the higher end of temperatures in their natural habitat but also the same temperatures preferred by pathogens like *M. marinum* (Kent et al. 2006; Mutoji and Ennis 2012; Gauthier 2015). Temperatures in the natural range of Zebrafish vary from as low as 6°C in winter to over 38°C in summer (Spence et al. 2008). The notifier has indicated that no specific procedures or treatments are required for disposal of the notified organisms (BZ2019 and PZ2019) compared to the wild-type species as the only difference (for each line) is the addition of a fluorescent protein derived from species of sea anenomes. Sale of the lines can be halted at any time if it is determined necessary to terminate their introduction in Canada. BZ2019 and PZ2019 are not for introduction into the wider environment and the methods of introduction do not favour their dispersal. However, *D. rerio* are known to spawn throughout the year under captive breeding conditions with females able to spawn a single clutch containing several hundred eggs every 2-3 days (Spence et al. 2008). Being scatter breeders, providing no parental care after directly depositing their eggs onto the substrate (Hill and Yanong 2002; Spence et al. 2008), it is possible that the population of BZ2019 and PZ2019 will likely increase in Canada beyond the import numbers through occasional breeding in home aquaria and thereby increase the likelihood of human exposure.

ENVIRONMENTAL FATE

According to the notifier, BZ2019 and PZ2019 are not intended for environmental release and the intended use is limited to aquaria in homes and retail outlets. However, it is understood that a proportion of fish kept in home aquaria may be released into the environment (Duggan et al. 2006). Should release occur into the environment, the fate of BZ2019 and PZ2019 in Canada is largely a function of the environmental conditions where factors relating to survival, growth and reproduction success are the main determinants of the potential to establish self-sustaining populations (Duggan et al. 2006; Leggatt 2018). Among the environmental factors, temperature tolerance is a key criterion for determining the ability of aquarium fish to survive, establish and overwinter in the Great Lakes and in Canadian waters as a whole (Rixon et al. 2005; DFO 2018; Leggatt et al. 2018).

The notifier supplied temperature tolerance data demonstrating LD50s of 5.66°C for BZ2019 and 5.79°C for PZ2019 compared to 5.5°C for non-transgenic Golden Zebrafish. These values all fall within the lethal water temperature ranges for Zebrafish. According to a study by Leggatt et al. (2018), functional minimal temperature tolerance of transgenic Zebrafish is between 6 and 8°C and that water temperatures between 5.4 and 5.9°C are lethal to several strains of transgenic Zebrafish leading to high mortalities. Unlike in the Tampa Bay region of Florida, United States, where Zebrafish accounted for about 0.2% of captured fish in the 2016 survey of non-native ornamental fish (Tuckett et al. 2017), the chances of BZ2019 and PZ2019 establishing self-sustaining populations are low in Canada due to their inability to survive when water temperatures are lower than 6°C.

In addition to survival and establishment in the Canadian environment, dispersal of BZ2019 and PZ2019 may have a bearing on the potential for human exposure through the environment.

Considering the inability of BZ2019 and PZ2019 to survive temperatures below 6°C, dispersal into the environment is less likely to happen. If live or dead BZ2019 and PZ2019 are released into the environment, it is expected that both fish and the inserted fluorescent protein would biodegrade normally, and not bioaccumulate or be involved in biogeochemical cycling in a manner different from other living organisms. Therefore, the likelihood of human exposure to the notified organism in the environment is low.

OTHER POTENTIAL USES

The sole intended use for BZ2019 and PZ2019 is as ornamental fish for interior home aquaria. According to the notifier, BZ2019 and PZ2019 are not suitable for use in outdoor ponds, as bait fish, for human consumption, or as environmental sentinels. However, Zebrafish (*D. rerio*) is an important vertebrate research animal model for understanding human development, disease, and toxicology (Spitsbergen and Kent 2003; Keller and Keller 2018). Characteristics of Zebrafish such as high fecundity, small size, rapid generation time, and optical transparency during early embryogenesis, have resulted in investigations in numerous other disciplines, including animal behavior, fish physiology, and aquatic toxicology (Lawrence 2007; Dai et al. 2014; Meyers 2018). The notifier has identified a potential use of BZ2019 and PZ2019 as scientific research organisms. Both wild-type and transgenic Zebrafish have been recommended as model systems for toxicology to monitor toxic heavy metals, endocrine disruptors, and organic pollutants (Dai et al. 2014). It has also been suggested that Zebrafish may have some value in mosquito control as studies involving their gut content analysis often identified aquatic larval forms of terrestrial insect species (Spence et al. 2008).

Manufacture of the notified organisms is not anticipated to occur in Canada as BZ2019 and PZ2019 are only produced in Florida. However, should manufacture occur, no additional risks are foreseen that are different from any other typical aquarium fish. The notifier recommends that individuals that no longer wish to maintain the organisms after purchase either return them to the retailer, give them to another aquarium hobbyist, or humanely euthanize them.

EXPOSURE CHARACTERIZATION

Risks from workplace exposure to the notified strain are not considered in this assessment[1]

The human exposure potential of both BZ2019 and PZ2019 is assessed to be low to medium (Table 3) because:

1. The primary sources of human exposures would stem from the proposed import of adult fish of the two lines (BZ2019 and PZ2019), through unidentified points of entry in Canada;
2. The intent is to have adult BZ2019 and PZ2019 fish be available for purchase by the public in up to 750 retail outlets throughout Canada where tropical aquarium fish are sold, and not for introduction into the Canadian environment;
3. The sole intended use of BZ2010 and PZ2019 is as ornamental aquarium fish, thus limiting potential exposure to the general public primarily to those that possess a home aquarium which may include immunosuppressed individuals, children, those with underlying medical

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

conditions or other vulnerable individuals. Recommended stocking rate for the notified lines in a home aquarium is one to two fish per gallon;

4. Typical human exposure to live or dead fish in the home is most often related to maintenance activities such as tank cleanings and water changes. Human exposure through the environment as a result of accidental or deliberate environmental releases cannot be ruled out;
5. No significant increase in human exposure is expected from other potential uses of BZ2019 and PZ2019, such as for bait fish, presence in outdoor ponds and use in mosquito control; and
6. Zebrafish, being a popular research model, leaves open the possibility for diverse potential uses ranging from study of human diseases to pollution diagnostics that may result in human exposure. However, use of BZ2019 and PZ2019 for scientific research would be expected to take place under containment with appropriate personal protective equipment and would thus result in a low likelihood of exposure to the general population.

Table 3: Exposure considerations (human health).

Exposure	Considerations
High	• The release quantity, duration and/or frequency are high. • The organism is likely to survive, persist, disperse proliferate and become established in the environment. • Dispersal or transport to other environmental compartments is likely. • The nature of release makes it likely that susceptible populations or ecosystems will be exposed and/or that releases will extend beyond a region or single ecosystem. • In relation to exposed humans, routes of exposure are permissive of toxic, zoonotic or other adverse effects in susceptible organisms.
Medium	• The organism is released into the environment, but quantity, duration and/or frequency of release is moderate. • The organism may persist in the environment, but in low numbers. • The potential for dispersal/transport is limited. • The nature of release is such that some susceptible populations may be exposed. • In relation to exposed humans, routes of exposure are not expected to favour toxic, zoonotic or other adverse effects.
Low	• The organism is used in containment (no intentional release). • The nature of release and/or the biology of the organism are expected to contain the organism such that susceptible populations or ecosystems are not exposed. • Low quantity, duration and frequency of release of organisms that are not expected to survive, persist, disperse or proliferate in the environment where released.

UNCERTAINTY RELATED TO INDIRECT HUMAN HEALTH EXPOSURE ASSESSMENT

The ranking of uncertainty associated with the indirect human health exposure assessment is presented in Table 4. Information was provided by the notifier on the sources of exposure and factors influencing human exposure including its import, retail distribution, and survival in the environment. It was indicated that the notified organism will not be manufactured in Canada and the source of exposure will be restricted to the import of BZ2019 and PZ2019. The survival of these fish is expected to be limited by their poor tolerance to temperatures below 6°C. This is based on empirical data comparing cold temperature tolerances between the notified lines and the wild-type *D. rerio*. Human exposure (general public and vulnerable individuals [i.e., immunocompromised, children, medical conditions, etc.]) in Canada is expected to occur through home aquaria mainly from maintenance and cleaning activities. The actual number of notified organisms to be imported in the following years is not known at this point and hence it is difficult to gauge public uptake and popularity beyond the first year of import. For this assessment, household surveys looking into aquarium fish ownership in Canada are based on reports dating back to 2009 (Perrin 2009; Marson et al. 2009). These reports are not specific to BZ2019 and PZ2019 and, apart from ownership statistics; they do not investigate factors influencing human exposure to aquarium fish. Therefore, because of limited information on exposure scenarios in the Canadian market, the human exposure to the notified organisms is considered low to medium with moderate uncertainty.

Table 4: Uncertainty ranking associated with the indirect human health exposure.

Available Information	**Uncertainty Ranking**
High quality data on the organism, the sources of human exposure and the factors influencing human exposure to the organism. Evidence of low variability.	Negligible
High quality data on relatives of the organism or valid surrogate, the sources of human exposure and the factors influencing human exposure to the organism or valid surrogate. Evidence of variability.	Low
Limited data on the organism, relatives of the organism or valid surrogate, the sources of human exposure and the factors influencing human exposure to the organism.	Moderate
Significant knowledge gaps. Significant reliance on expert opinion.	High

RISK CHARACTERIZATION

NOTIFIED USE

In this assessment, risk is characterized according to a paradigm: Risk ∝ Hazard x Exposure. The two components (“hazard” and “exposure”) are considered embedded in the definition of “toxic” under section 64 of CEPA 1999. Hence, a hazard and exposure to that hazard are both required for there to be a risk. The risk assessment conclusion is based on the hazard, and on what we can predict about exposure from the notified use.

BZ2019 and PZ2019 are genetically modified lines of fluorescent Zebrafish derived from a line of stripe-free, Golden Zebrafish. The blue and purple colours are the result of the introduction of expression cassettes containing either a fluorescent protein gene or a chromoprotein gene derived from species of sea anemones. The notified organisms will be marketed throughout Canada for use as ornamental fish in home aquaria.

Although there are reported cases of zoonotic infections from exposure to aquarium fish, Zebrafish are popular in home aquaria with a long history of safe use. Similarly, BZ2019 and

PZ2019 have been maintained as breeding lines for more than five generations and commercially produced for over five years in the U.S. with no reported adverse human health effects. The inserted fluorescent protein genes and the methods used to modify the notified lines do not present any pathogenic or toxic potential towards humans.

Owing to the low potential hazard and the low to medium potential exposure, the human health risk associated with the use of *D. rerio* BZ2019 and PZ2019 as ornamental aquarium fish is assessed to be low.

OTHER POTENTIAL USES

Other uses that have been identified include the use of the notified organisms in outdoor ponds, as bait fish, and in scientific research. While the notifier is discounting the possibility of some of these uses, the characteristics of the notified organisms do not support this claim. It is possible that the notified organisms may be used as bait fish and, when temperatures are favourable, also grown in outdoor ponds as in Florida where the fish are produced. Zebrafish are a commonly used research model, thus their use in research is possible; however, this would likely be done under contained conditions thereby limiting exposure to the general public. There are no reported cases in the literature of the notified organisms being used as an environmental sentinel, but regardless of the use, the available information does not indicate a potential human health implication from any of these uses. No additional risks to human health are foreseen that are different from those of any other typical aquarium fish.

RISK ASSESSMENT CONCLUSION

There is no evidence to suggest a risk of adverse human health effects at the exposure levels predicted for the general Canadian population from the use of *D. rerio* BZ2019 and PZ2019 as ornamental aquarium fish or any other potential uses. This risk to human health associated with *D. rerio* BZ2019 and PZ2019 is not suspected to meet criteria in paragraph 64(c) of CEPA 1999. No further action is recommended.

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