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Indirect Human Health Risk Assessment of the GloFish® Moonrise Pink® Betta (PiBS2019), GloFish® Sunburst Orange® Betta (OBS2019) and the GloFish® Electric Green® Betta (GBS2019) for use as ornamental aquarium fish in Canada

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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 three lines of genetically modified Bettas (*Betta splendens*) known as the GloFish® Moonrise Pink® Betta (PiBS2019), GloFish® Sunburst Orange® Betta (OBS2019), and GloFish® Electric Green® Betta (GBS2019) that were notified under the *Canadian Environmental Protection Act* (CEPA). PiBS2019, OBS2019, and GBS2019 are modified lines of diploid, hemizygous or homozygous, Bettas, containing genes encoding for different fluorescent proteins. PiBS2019, OBS2019, and GBS2019, which under ambient light (including sunlight) appear pink, orange, or green, respectively, will be imported from the United States for use as ornamental fish in home aquaria. This risk assessment examined the potential for the three lines to cause harmful effects to humans in Canada relative to wild-type Bettas as a consequence of environmental exposure including from their intended use in home aquaria. The parental strain, *B. splendens*, has been available as a home aquarium fish since the 1930s. 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 PiBS2019, OBS2019, and GBS2019 as ornamental aquarium fish as well as other identified potential uses. As such, there is no expectation that PiBS2019, OBS2019, and GBS2019 pose any more risks to human health than wild-type *B. splendens.*

INTRODUCTION

The following indirect human health risk assessment was conducted on *Betta splendens* PiBS2019, OBS2019, and GBS2019, three genetically modified lines of diploid, hemizygous or homozygous, Bettas, containing genes encoding for modified versions of fluorescent red, yellow or green proteins, respectively. Bettas, also known as Siamese Fighting Fish, are a popular tropical ornamental fish in Canada and other parts of the world. The risk assessment examines the potential for PiBS2019, OBS2019, and GBS2019 to cause harmful effects to humans in Canada, relative to wild-type *B. splendens*, as a consequence of environmental exposure, including exposure in natural environments and from environments under their intended use (i.e., home aquaria). PiBS2019, OBS2019, and GBS2019 are pink, orange, and green 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 *BETTA SPLENDENS* PiBS2019, OBS2019, AND GBS2019

Binomial name

Betta splendens PiBS2019, OBS2019, and GBS2019

Taxonomy

Kingdom	Animalia
Phylum	Chordata
Subphylum	Vertebrata
Superclass	Actinopterygii
Class	Teleostei
Order	Perciformes
Family	Osphronemidae
Genus	*Betta*
Species	*Splendens*
Strains	PiBS2019, OBS2019, and GBS2019

Synonyms, common and superseded names

Synonym: *Betta splendens* (Regan 1910)

Common names: Siamese fighting fish, Betta, fighting fish

Trade names: PiBS2019 - GloFish® Moonrise Pink® Betta

OBS2019 - GloFish® Sunburst Orange® Betta

GBS2019 - GloFish® Electric Green® Betta

Characterization and substantiation of the taxonomic identification

Betta splendens PiBS2019, OBS2019, and GBS2019 are genetically modified lines of diploid, hemizygous or homozygous, Bettas containing genetic constructs which makes them appear pink (PiBS2019), orange (OBS2019), or green (GBS2019) under ambient light, including sunlight. All three lines were derived from a line of light-coloured Betta, a pigment variant created during domestication of the species.

Betta species can be classified into two categories: bubble-nest building and mouth brooders, based on mode of paternal care for fertilized eggs and hatchlings (Monvises et al. 2009; Chailertrit et al. 2014; Panijpan et al. 2014). Panijpan et al. (2014) compiled a list of fighting fish species, which include the bubble nest Bettas, *B. splendens, B. imbellis, B. smaragdina, B. mahachaiensis, B. coccina* and *B. livida* and the mouth brooders, *B. apollon, B. chloropharynx, B. pi, B. prima, B. pugnax, B. simplex*, and *B. stigmosa*. Some distinguishing external features of the bubble-nesters include iridescence and colour of the operculum and body scales, body colouration, and the bars and patterns on the fins (Kowasupat et al. 2012). Other morphological distinguishing features used to identify Bettas include body shape, colour patterns, fin size, scale iridescence and the various attributes of dorsal and caudal fins (Monvises et al. 2009; Kowasupat et al. 2012; Panijpan et al. 2014; U.S. Fish and Wildlife Service 2019). In *B. splendens,* pelvic fins are situated below or slightly in advance of the pectorals, scales are large, regularly arranged with the lateral line being vestigial or absent (U.S. Fish and Wildlife Service 2019).

Unlike fish bred for fighting, which are selected for a large and strong body with hard scales (hard targets) but smaller fins (flimsy targets) as protection against bites of the opponent, the features favoured in ornamental Bettas are variations of colour intensity, colour patterns, scale iridescence, body shape, and fin size (Monvises et al. 2009; Valentin et al. 2013).

Strain history

The notified lines PiBS2019, OBS2019, and GBS2019 were produced by microinjection of the expression cassettes containing the corresponding transgenes into eggs of light-coloured *B. splendens*. Upon hatching, fry batches were tested for fluorescence of the inserted gene colour. 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.

Broodstocks for PiBS2019, OBS2019, and GBS2019 are maintained separately with the same breeding protocol used for all three lines.

Genetic modifications: purpose, method, genetic and phenotypic changes

The notified lines which have been modified to appear pink (PiBS2019), orange (OBS2019), or green (GBS2019) under ambient light, including sunlight, are intended for use by the general

public for home aquarium display purposes only. As for wild-type *B. splendens*, which is a non-food species that has been used safely in aquaria worldwide for approximately 90 years, PiBS2019, OBS2019, and GBS2019 are not intended for food use.

According to the information provided by the notifier, in addition to PiBS2019, OBS2019, and GBS2019 appearing pink, orange, and green, respectively, under ambient light, the three lines have a lower reproductive success rate compared with the non-transgenic Betta siblings. The notifier also provided results from a temperature tolerance test that showed slight increased sensitivity to low temperatures for the three lines compared to non-transgenic Bettas. These data together with the breeding trials suggest viability does not differ between transgenic and non-transgenic lines. The approach used to produce, grow, and prepare for sale the notified lines is considered adequate to ensure genetic stability of the broodstock. However, it should be noted that there is the potential for off-target mutations with unknown effects in the Betta genome that may arise from the use of Cas9 and guide RNA in the development of the notified lines.

Biological and ecological properties

Bettas are native to Southeast Asia with *B. splendens* being the most famous of the 91 species (Srikulnath et al. 2021). Their natural habitats are small bodies of water, such as paddy fields, ponds, lagoons and marshes with plenty of vegetation that helps to provide cover against fish-eating birds (Jaroensutasinee and Jaroensutasinee 2001; Monvises et al. 2009; Pleeging and Moons 2017). According to Jaroensutasinee and Jaroensutasinee (2001), the shallow bodies of water and marginal areas of the rice paddy field is characterized by high temperature (27 to 31.5 °C), low dissolved oxygen (0 to 7.39 mg/L), high free CO_2 (0.45 to 1.91 mg/L), low salinity and was acidic (pH 5.28 to 5.80). Bettas possess a labyrinth organ that enables consumption of oxygen from the air and are capable of surviving in water with levels of oxygen as low as 0 – 2 ppm (Pleeging and Moons 2017).

Wild fish have a brown-green colour and are 5 – 5.5 cm in length while domesticated fish are bred in a wide variety of colours and can reach 6 – 6.5 cm in length (Pleeging and Moons 2017). Bettas are carnivorous and prefer live and moving prey such as tiny infusorium organisms, water fleas, blood worms and especially mosquito larvae (Monvises et al. 2009, Pleeging and Moons 2017).

Bettas, especially the males, are considered to be territorial fish and will aggressively defend a territory (Castro et al. 2006, Plegging and Moons 2017). In the wild, males will establish a territory of approximately 1.7 males per square metre (Dzieweczynski et al. 2017). Female Bettas prefer to shoal with other females and, with the exception of 24 hours before mating, will choose solitude over being in the presence of a male (Dzieweczynski et al. 2017; Plegging and Moons 2017).

Reproductive style is bubble nesting where eggs and larvae are guarded and defended by the male (Srikulnath et al. 2021). Creation of a bubble nest is considered a sign of well-being in males and it has been observed that nests were not built in water temperatures below 24.4°C or above 27.7°C with an optimal reported temperature of 26.6°C (Pleeging and Moons 2017). Nests are built by the male by blowing bubbles around a floating base. Females will spawn around 400 to 500 eggs under the nest while the male waits to fertilize. Reproductive success will depend on the size of the nest as larger nests may support more fertilized eggs and facilitate efficient oxygenation (Srikrishnan et al. 2017). *B. splendens* have a relatively short embryonic development period as eggs hatch approximately 36-hours post-fertilization (Murcia-Ordoňez et al. 2016). Yolk sacs are totally absorbed at 73 hours post-hatching with metamorphosis completed 768 hours post-hatching as the larvae become juveniles (Valentin et

al. 2015). Sexual maturity may be reached at approximately 75 days with nest building by males starting as soon as 53 days (Rainwater 1967). Males are morphologically distinguishable from females at approximately two months post-hatching and need to be separated from other males at four months to prevent fighting (Monvises et al. 2009).

HUMAN HEALTH EFFECTS

Zoonotic potential

While rare, 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. There are few reports of zoonoses by parasitic, fungal, and viral pathogens from aquatic organisms with bacteria being reported as the main etiological agents of zoonotic infection (Iqbal et al. 2018). 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 as they often have less stringent hygienic practices (Dunn et al. 2015). While most infections are self-limiting, more serious cases are associated with immune deficiency, infection with highly virulent strains, contact with a large inoculum, depth of skin penetration, or a combination of these factors (Haenen et al. 2020). The most common bacterial species associated with tropical fish capable of causing human illness are *Aeromonas* sp.*, Mycobacterium marinum, Salmonella* sp.*,* and *Streptococcus iniae* (CDC 2015) with the most commonly reported infections being associated with *M. marinum* (Weir et al. 2012).

In-house literature searches found no reports of zoonoses or other adverse effects attributed to the notified lines or to other commercially available GloFish® lines. However, there is a reported zoonotic case in the scientific literature involving wild-type *B. splendens*. Cassetty and Sanchez (2004) reported on a case of a *Mycobacterium marinum* infection in a 49-year old man in New York state who possessed an aquarium with a pair of Bettas. The case involved nodules on the dorsum of the man's right hand that reappeared three months following a successful treatment with multiple antibiotics. Cultures grown found *Mycobacterium marinum* and were negative for other bacteria and fungi. With the reappearance of the nodules, a new regimen of different multiple antibiotics was started. It should be noted that the man reported wearing gloves when cleaning the tank; however, he was also on medications for a medical history that included diabetes mellitus and coronary artery disease.

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, or 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). In humans, mycobacteriosis is classified into four types (I – IV). Type I is found in patients who are immunocompetent with clinical signs including superficial lesions with crusted and ulcerated nodules or verrucuous plaques. The lesions are small, painless, bluish-

red papules approximately 1 to 2 cm in diameter. Signs develop over the course of weeks to months. Type II occurs in immunosuppressed individuals and involves lesions with abscesses, inflammatory nodules, and granulomas. The lesions may be single or multiple subcutaneous granulomas, with or without ulceration. In Type III, infections occur in deep tissues with or without skin lesions with clinical signs including arthritis, tenosynovitis, osteomyelitis, and bursitis. Type IV is very rare, but can occur in patients with lung disease (Delghandi et al. 2020).

It is probable that almost all species of fish are susceptible to *Mycobacterium* sp. with levels of mortality ranging from 10% to 100% of infected fish (Delghandi et al. 2020). Other examples of species of *Mycobacterium* known to cause infections in fish include *M. abscessus*, *M. chelonae*, *M. flavescens*, *M. fortuitum*, *M. gordonae, M. haemophilum*, *M. kansasii*, and *M. peregrinum* (Cardoso et al. 2019; Pate et al. 2019; Puk and Guz 2020). While *M. fortuitum* and *M. marinum* are most commonly isolated in Bettas (Pleeging and Moons 2017), *M. chelonae*, *M. gordonae,* and *M. kansasii* have also been reported to be isolated from *B. splendens* (Řehulka et al. 2006). A study by Sirimalaisuwan et al. (2017) found 5.5% of clinically healthy *B. splendens* carried *M. marinum* and could therefore play a role as a source for mycobacterial infections in ornamental fish hobbyists.

Although most cases of fish-related infections in humans are caused by *M. marinum*, home aquarists should also be aware of the zoonotic potential of other species of *Mycobacterium* (Puk and Guz 2020). In immunosuppressed humans 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 cutaneaous 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* are opportunistic and have most often been associated with puncture wounds from 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; Haenen et al. 2020). From the handling of live or recently killed infected fish, *S. iniae* may cause severe disease including septicaemia, endocarditis, arthritis, meningitis, fever, abdominal distension, and pneumonia (Lowry and Smith 2007; Boylan 2011; Gauthier 2015; Haenen et al. 2020). People with weakened immune systems or open skin wounds could get infected by *S. iniae* while handling fish or cleaning aquaria (CDC 2015). However, there are no reports in the scientific literature of human streptococcal infections attributed to Bettas 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). Kanchan et al. (2019) reported on the virulence potential of *A. hydrophila* in healthy *B. splendens*. Following intraperitoneal injections of either $7.5x10^7$ or $7.5x10^5$ cfu per 0.05 mL or saline control, the authors reported a cumulative mortality over two-weeks post-injection of 98.33%, 20%, and 0%, respectively, for the groups. 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). In humans, *A. hydrophila* exposure may result in local skin infections and occasionally, diarrheal disease (Haenen et al. 2020). *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. While the report by Cremonesini and Thomson (2008) didn't identify the species of fish, there are no reported cases of *A. hydrophila* zoonotic infections attributed to *B. splendens* exposure. 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). 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 three years old) following exposure to aquarium water and resulted in diarrhea, fever, abdominal cramps, vomiting, bloody stool, headaches, and myalgia. Types of tropical fish reported in this study included Tetras, Guppies and Angel Fish. Similarly, out of 53 reported cases of *S. paratyphi* B biovar. 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). While the authors did not identify tropical fish species, the report did state that Thailand was one of the sources for tropical fish found in Quebec. An in-house literature search found no reports of *Salmonella* zoonotic infections attributed to *B. splendens* exposure.

Zoonotic infections primarily occur through punctures, 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 and skin with soap and water after contact with aquarium water and fish is also highly recommended. In addition, people with compromised immune systems or underlying medical conditions as well as children should avoid cleaning tanks or handling fish (Haenen et al. 2013; 2020).

There are no reports specifically associating either the notified organisms or wild-type *B. splendens* with any parasites of human health significance. Routine health evaluations (necropsy, microbiology) were conducted on limited sample sizes of six fish of each colour as well as six non-transgenic Bettas and histology was conducted on an additional six fish of each colour plus six non-transgenics at a fish disease diagnostic laboratory at the University of Florida in 2018 (GBS2019), 2019 (PiBS2019 and OBS2019), and 2020 (albino, non-transgenic).

The notifier provided a letter following the study of GBS2019 from the veterinarian involved that stated the fluorescent fish exhibited no gross morphologies that differ from those seen in their wild-type counterparts. In addition, the veterinarian stated that he observed no evidence for a difference in susceptibility to, or transmission of pathogens. The monogean and ciliate parasites reported in these studies have previously been isolated from *B. splendens* and as well, parasites may commonly be found in ornamental fish (Florindo et al. 2017a,b; Iqbal et al. 2018; Trujillo-González et al. 2018). In addition, no bacterial growth was observed after 48 hours (at 28°C) in brain and posterior kidney samples plated onto blood agar plates for all four groups. The notifier also supplied a quality control (QC) SOP specifying that fish leaving the producers are inspected for behavioural and physical signs of disease before being shipped to sellers and

distributors. If a batch does not meet the QC standards, shipment of fish from that batch is cancelled.

Allergenicity/Toxigenicity

In-house amino acid sequence analyses of all the expressed fluorescent proteins were done using the AllergenOnline Database (v21; 14 February, 2021). Similar to previous analyses on these fluorescent proteins done on previously notified GloFish® lines, no matches with greater than 35% identity nor exact matches for 80 and 8 sliding window amino acid segments, respectively, were found for any of the fluorescent proteins. Analyses conducted for all the other reading frames found the same positive result using the 80mer sliding window for a putative ORF in the 5'→3' (Frame1) direction in the expression cassette sequences for both GBS2019 and OBS2019. This ORF was found to have 35.03% identity with a predicted collagen alpha-1(I) chain-like isoform X1 from the Barramundi (*Lates calcarifer*). However, the full-length alignment resulted in only 35.4% identity and there was a high E-value (expectation value) of 99. 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 Lincoln-Nebraska, personal communication). Thus, allergic cross reactivity is not likely for the putative ORF. In addition, Basic Local Alignment Search Tool (BLAST) analysis on the amino acid sequence with BLASTP found no significant similarity to a known protein. Analyses on the inserted nucleotide sequences for prediction of translation initiation sites using an online program only found sites associated for the expected fluorescent proteins. Therefore, this putative ORF would most likely not result in an expressed protein in either OBS2019 or GBS2019.

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). Similarly, results provided by the notifier from analyses using the Allermatch website found no matches for 80 amino acid sliding window alignments using the 35% cutoff or exact matches using 8 amino acid lengths.

BLAST analyses of the inserted fluorescent protein sequences do not indicate any homologies to sequences of potential toxins or allergens. No adverse effects were observed in male rats fed pure green fluorescent protein (GFP) or canola expressing GFP for 26 days (Richards et al. 2003). Furthermore, there is no evidence indicating any potential for PiBS2019, OBS2019, GBS2019 or *B. splendens* 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

GBS2019 received an Enforcement Discretion decision by the U.S. Food and Drug Administration (USFDA) in 2019 and has been commercially available in the United States since early 2020. PiBS2019 and OBS2019 received their Enforcement Discretion from the USFDA in November 2020. The fluorescent proteins used in GBS2019 and OBS2019 have been used in other GloFish® lines since as early as 2006 and there has been more than seven years of use for the fluorescent protein found in PiBS2019. Wild-type Bettas have been sold as aquarium fish since the 1930s (Innes 1935; Wallbrunn 1958).

HAZARD CHARACTERIZATION

The human health hazard potential of PiBS2019, OBS2019, and GBS2019 is assessed to be low (Table 1) because:

1. PiBS2019, OBS2019, and GBS2019 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) and that were confirmed to be stably integrated through multiple crossings;

2. The methods used to produce PiBS2019, OBS2019, and GBS2019 do not raise any indirect human health concerns. However, the potential for off-target effects from use of the Cas9 and guide RNA remains unknown. While some of the source organisms from which the inserted genetic material was derived appear to 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 any of the commercially available lines of GloFish® including GBS2019. The zoonotic potential of PiBS2019, OBS2019, and GBS2019 is not expected to be any different than for wild-type Bettas currently commercially available;
4. Sequence identities of the inserted transgenes do not match any known allergens or toxins. Amino acid sequences of the three fluorescent proteins are identical to those used in previously assessed GloFish® lines. While analyses conducted on the other potential reading frames found the same potential match in both OBS2019 and GBS2019, the results suggest there is little evidence for cross-reactivity; and
5. While there is no history of safe use for PiBS2019 and OBS2019, and limited history of safe use for GBS2019 in the United States due to their recent introduction, the wild-type species has been safely used as an ornamental aquarium fish since the 1930s. In addition, there is a history of safe use for the other commercially available lines of GloFish®.

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 *B. splendens* including the sources of the genetic materials and the stability of the resulting genotypes and phenotypes. Sequence analyses of the inserted transgene constructs for the three 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 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 Bettas. While there is only a short history of commercial production for these three lines, the inserted fluorescent proteins have been used in other lines of GloFish® for several years and 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 for other lines of GloFish®, the indirect human health hazard assessment of PiBS2019, OBS2019, and GBS2019 is considered to be **low** with **low uncertainty.** However, there is a theoretical possibility that off-target mutations from the use of Cas9 and guide RNA could produce unknown effects such as altered proteins with increased allergenicity, although this has not been identified in other models. Consequently, this is not expected to alter the hazard rating, but increases uncertainty, although not sufficiently to raise the ranking above low. The uncertainty is considered low because much of the information on human health effects is based on reports from other ornamental aquarium fish, there is no history of safe use for these notified lines, 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

OVERVIEW

As illustrated in the generalized human exposure pathways in Figure 1, it is assumed that the main pathways for human exposure of PiBS2019, OBS2019, and GBS2019 are:

a. During import from the United States and distribution to retailers in Canada;

b. Introduction in Canada through the intended use as ornamental fish in home aquaria;

c. Exposure through the environment and environmental fate following accidental, deliberate or unintended environmental releases; and

d. Other potential uses.

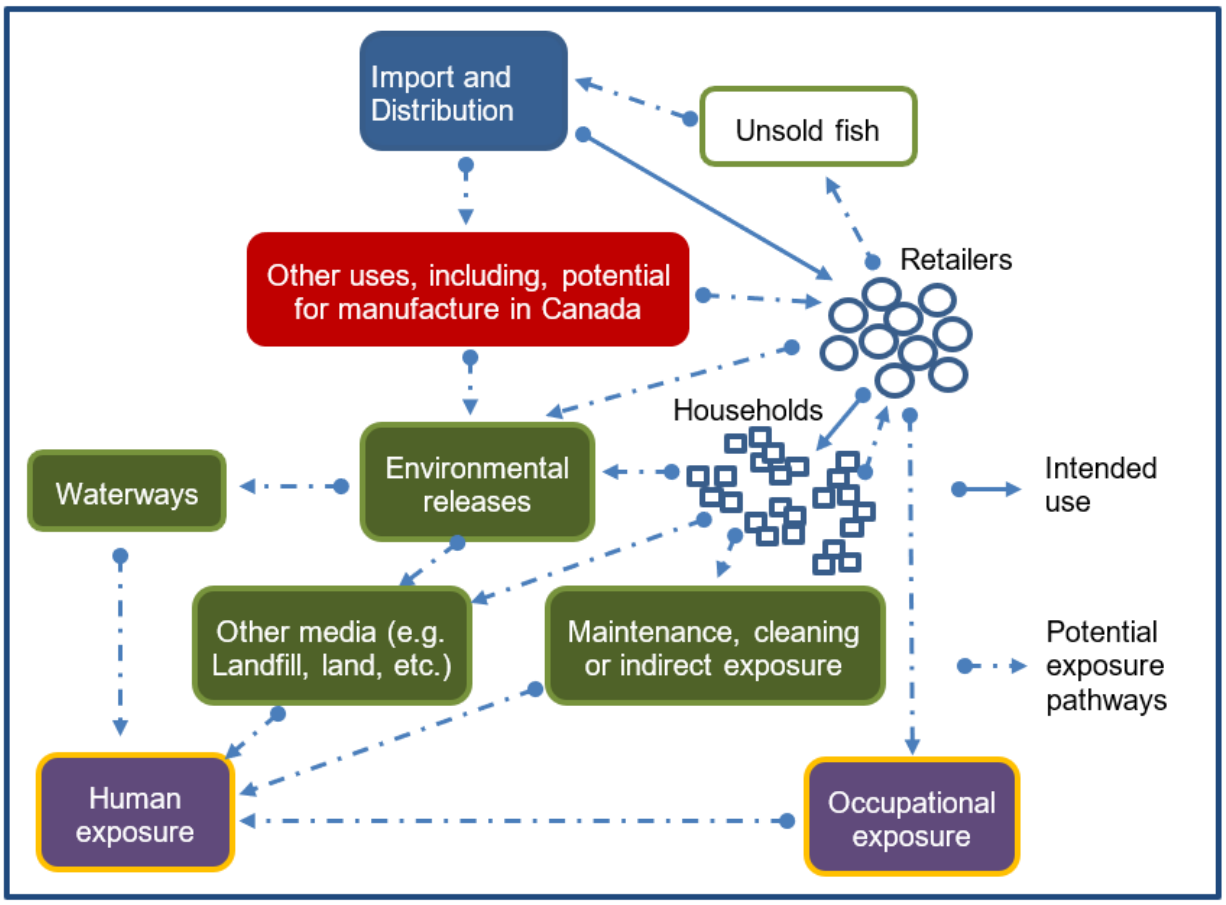

Figure 1: Generalized human exposure pathways for PiBS2019, OBS2019, and GBS2019.

IMPORT

Imported adult fish from the United States will enter Canada through various points of entry that have not been specifically identified. Broodstock are maintained separately in Florida, USA with the same breeding protocol used for all types of F_2 fish that become the lines identified as PiBS2019, OBS2019, and GBS2019. 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 Canadian 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.

The notifier plans to market PiBS2019, OBS2019, and GBS2019 fish in Canada using approximately 500 retail outlets where ornamental aquarium fish are sold. While the exact number and locations where the notified organisms will be available for sale are not currently

known, fish will be primarily sold for use as ornamental fish to be confined inside aquaria in homes and retail outlets. For the intended use, human exposure could happen during distribution involving the transportation of fish by the importer as well as during storage, handling and sale by the retailer. Based on a survey of store owners in Montréal, Quebec, fish are either kept and put on sale by retailers until sold or returned to the distributor and are less likely to be released into the environment by retailers (Gertzen et al. 2008). Since retailers are not expected to be the final users of PiBS2019, OBS2019, and GBS2019 fish, human exposure during importation and distribution to retailers is expected to be largely occupational.

INTRODUCTION OF THE ORGANISM

Home aquarists that purchase the notified lines directly from retailers or receive them from other aquarists will most likely experience dermal exposure through contact with the notified fish during maintenance activities such as water changes and tank cleanings. Estimating the extent of human exposure through the intended use, as ornamental fish in home aquaria, requires the knowledge of stocking rate per household and the number of households planning to purchase the notified lines. According to the information provided by the notifier, the typical stocking of wild type Bettas in home aquaria is typically one male or female per aquarium or several females. Experience in the U.S. market shared by the notifier point to consumers with more than one Betta aquarium. While the proportion of home aquarists planning to purchase PiBS2019, OBS2019, and GBS2019 is not known, 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 28% of respondents having Bettas in their aquaria. In another survey done in Montréal, Quebec (Gertzen et al. 2008), about 2.4% of the fish sold by the pet stores were the Siamese fighting fish (*Betta splendens*). According to the 2016 census, Canada has 14 million households (Statistics Canada 2017). Putting these numbers together, about half a million households in Canada have Bettas (i.e., 28% having Bettas of 12% having fish as pets of the 14 million households). Considering a stocking rate of at least one male and one female, there would be about a million Bettas in Canada.

According to the information provided by the notifier, home aquaria established for *B. splendens* and similar types of tropical aquarium fish are generally maintained at temperatures of between 20 and 30°C, which mirrors the temperatures of tropical waters associated with rice paddies of between 26 and 28°C (79 - 82°F) where *B. splendens* inhabits in nature. These are the same temperatures preferred by opportunistic pathogens like *M. marinum* (Kent et al. 2006; Mutoji and Ennis 2012; Gauthier 2015). In the households expected to purchase the notified lines, we have no knowledge of 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. Caution is advised in handling pet-shop fish due to, for example, the risk of infection with non-tuberculosis mycobacteria (Kušar et al. 2017). *M. marinum* is considered to be the most important fish pathogen but it is also an opportunistic pathogen of humans (Cassetty and Sanchez 2004; Kušar et al. 2017; Hashish et al. 2018). Presence of *M. marinum* zoonotic bacterial pathogens in healthy Siamese fighting fish underlines the infection risk to humans of not only exposure to infected fish, but also when they manipulate clinically asymptomatic fish (Sirimalaisuwan et al. 2017). In addition to *M. marinum,* indirect exposure to pathogens through tropical aquarium fish was suspected as the cause of diarrhea associated with *Edwardsiella tarda* in the case a two-month-old Belgian infant where the same organism was isolated from a tropical aquarium fish in the home of the patient (Vandepitte et al. 1983).

ENVIRONMENTAL FATE

According to the notifier, PiBS2019, OBS2019, and GBS2019 are not intended for environmental release. The intended use is limited to aquaria in homes and retail outlets. However, it is likely that a proportion of fish kept in home aquaria may be released into the environment (Duggan et al. 2006; Gertzen et al. 2008). According to Gertzen et al. (2008), aquarists could potentially release unwanted aquarium fish into the environment when they become bored with the fish or when fish become aggressive, sick, large in size, or reproduce rapidly. Should there be releases of live fish into the environment, the fate of PiBS2019, OBS2019, and GBS2019 in Canada would be largely a function of the environmental conditions at the point of release and the ability of the released fish to survive, grow, reproduce, disperse and establish self-sustaining populations (Duggan et al. 2006; Strecker et al. 2011; Leggatt et al. 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 results from three studies demonstrating LD_{50}s of 7.44°C for PiBS2019, 6.93°C for OBS2019 and 7.49°C for GBS2019. In these studies, the corresponding LD_{50}s for the non-transgenic comparators were 7.2°C when compared with PiBS2019, 6.83°C when compared to OBS2019 and 7.15°C when compared with GBS2019. In general, transgenic genotypes (PiBS2019, OBS2019, and GBS2019) showed higher (not significant for OBS2019/non-transgenic comparison) sensitivity to cold water temperatures compared to their non-transgenic siblings but still within the lethal water temperature ranges for *B. splendens*. In a recent unpublished study by Leggatt (2021), *B. splendens* lost equilibrium at a mean temperature of 10.0 ± 1.2°C (range 16 - 7.9°C). Even with the most cold-tolerant *B. splendens,* fish lost equilibrium several degrees above typical winter water temperatures in Canada (4°C or less) and above temperatures in the warmest recorded lakes (6°C or less in winter). In addition to low potential for survival and establishment in the Canadian environment, PiBS2019, OBS2019, and GBS2019 are expected to have limited dispersal capabilities in the environment due mainly to the low tolerance to cold temperatures. Furthermore, if live or dead PiBS2019, OBS2019, and GBS2019 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 PiBS2019, OBS2019, and GBS2019 establishing self-sustaining populations in Canada is very low due to their inability to survive water temperatures lower than 8°C. Therefore, the likelihood of human exposure to the notified organisms in the environment is low.

Although the intended use is not for introduction into the wider environment, the potential for environmental release and ultimately human exposure to the notified lines may be exacerbated by the shear increases in the populations of PiBS2019, OBS2019, and GBS2019 in Canada due to occasional breeding in home aquaria. Important factors are survival, growth and reproduction which are ensured and supported by home aquarists through the intended use. In nature, Bettas, especially the males, are considered to be territorial fish where males tend to aggressively defend a territory and females preferring to shoal with other females but tend to isolate in the period of 24 hours before mating (Castro et al. 2006, Dzieweczynski et al. 2017; Plegging and Moons 2017). Reproductive success will depend on the size of the nest as larger nests may support more fertilized eggs and facilitate efficient oxygenation (Srikrishnan et al. 2017). *B. splendens* have a relatively short embryonic development period as eggs hatch approximately 36-hours post-fertilization (Murcia-Ordoňez et al. 2016). Yolk sacs are totally absorbed at 73 hours post-hatching with metamorphosis is complete 768 hours post-hatching as the larvae become juveniles (Valentin et al. 2015).

In the event a fish dies before sale to or while in the care of a home aquarist, the notifier suggests a disposal procedure similar to all other domestic waste and there are no special handling or disposal procedures required. The notifier has indicated that no specific procedures or treatments are required for disposal of the notified organisms (PiBS2019, OBS2019, and GBS2019) compared to the wild-type species as the only difference (for each line) is the addition of a fluorescent protein derived from a species of coral. Additionally, sale of these lines can be halted at any time if it is determined necessary to terminate the introduction of PiBS2019, OBS2019, and GBS2019 in Canada.

OTHER POTENTIAL USES

The sole intended use for PiBS2019, OBS2019, and GBS2019 is as ornamental fish for interior home aquaria. According to the notifier, the three notified lines are not suitable for use in outdoor ponds, as bait fish, for human consumption, or as environmental sentinels. However, *B. splendens* has been studied for potential use as a bio-indicator of freshwater pollution. Alyan (2007) examined the influence of two metal pollutants (mercuric chloride and sodium azide) on specific aggressive male behaviours. Less aggressive behaviour was observed in males under treatment conditions and the author concluded that male aggressive behaviour in *B. splendens* could potentially be used as a bio-indicator of freshwater pollutants. In addition, *B. splendens* is known to favour mosquito larvae (Monvises et al. 2009) and the species has been investigated for potential use in mosquito population control (de Oliveira Lima et al. 2010; Miraldo and Pecora 2017; Mah et al. 2018). As a research organism, Bettas are considered to be an exciting vertebrate model in such fields as evolution and phylogenetics, functional morphology, sex determination, hybridization, physiology, developmental biology, functional trait diversity, biological diversity, aggressive behavior, and population genetics (Srikulnath et al. 2021).

Manufacture of the notified organisms is not anticipated to occur in Canada as PiBS2019, OBS2019, and GBS2019 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 PiBS2019, OBS2019, and GBS2019 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 for the three lines (PiBS2019, OBS2019, and GBS2019) through unidentified points of entry in Canada;

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

2. The intent is to have adult PiBS2019, OBS2019, and GBS2019 fish available for purchase by the public in up to 500 retail outlets throughout Canada where tropical aquarium fish are sold, and not for introduction into the Canadian environment;
3. The sole intended use of PiBS2019, OBS2019, and GBS2019 is as ornamental aquarium fish, thus limiting potential exposure to the general public primarily to those that possess a home aquarium. These households may include immunosuppressed individuals, children, those with underlying medical conditions or other vulnerable individuals. Recommended stocking rate for the notified lines in a home aquarium is one male and up to four females per household;
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 is remotely possible because of poor cold temperature tolerance leading to limited survival, reproduction and dispersal in the environment; and
5. No significant increase in human exposure is expected from other potential uses of PiBS2019, OBS2019, and GBS2019, as bio-indicators of environmental pollution, for mosquito control and for research purposes.

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

Uncertainty ranking associated with the information used to assess indirect human health exposure for PiBS2019, OBS2019, and GBS2019 is presented in Table 4. This exposure assessment is based on information provided by the notifier on the sources of exposure and factors influencing human exposure including importation, retail distribution, and survival in the environment. As indicated, the notified organisms will not be manufactured in Canada and the source of exposure will be restricted to the import of adult fish for the three lines. In the environment, empirical data supports the conclusion that the survival of these fish is expected to be limited by their poor tolerance to temperatures below 10°C. However, this does not preclude the potential for human exposure (general public and vulnerable individuals [i.e., immunocompromised, children, medical conditions, etc.]) in Canada through home aquaria mainly from maintenance and cleaning activities. This exposure assessment is also limited by the lack of information on actual number of notified organisms to be imported in subsequent years making it difficult to gauge public uptake and popularity beyond the first year of import. Furthermore, household surveys looking into aquarium fish ownership in Canada are based on reports from more than 10 years ago (Duggan et al. 2006; Gertzen et al. 2008; Marson et al. 2009; Perrin 2009). These reports are not specific to PiBS2019, OBS2019, or GBS2019 and do not investigate factors influencing human exposure to aquarium fish. Therefore, because of limited information on the specific 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 and hence, there is no risk in absence of either. The risk assessment conclusion is based on the hazard, and on what we can predict about exposure from the notified use.

PiBS2019, OBS2019, or GBS2019 are genetically modified lines of diploid, hemizygous or homozygous, Bettas containing fluorescent protein genetic constructs derived from species of sea anemones or soft corals which makes them appear pink (PiBS2019), orange (OBS2019), and green (GBS2019) under ambient light, including sunlight. All three lines were derived from a line of light-coloured Betta, a pigment variant created during domestication of the species.

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, wild type Bettas are popular in home aquaria with a long history of safe use having been sold as aquarium fish since the 1930s (Innes 1935; Wallbrunn 1958). The three notified lines (PiBS2019, OBS2019, and GBS2019) received Enforcement Discretion decisions by the U.S. Food and Drug Administration (USFDA) in 2019 and 2020, and GBS2019 has been commercially available in the United States since early 2020. The fluorescent proteins used in GBS2019, OBS2019, and PiBS2019 have been used in other GloFish® lines approved in Canada. Although there is one reported case of infection associated with the wild-type Bettas, the inserted fluorescent protein genes and the methods used to modify PiBS2019, OBS2019, and GBS2019 lead to a conclusion that the notified lines do not present any pathogenic or toxic potential towards humans higher than wild-type *B. splendens*.

Owing to the low potential hazard and the low to medium potential exposure, the human health risk associated with the use of *B. splendens* PiBS2019, OBS2019, or GBS2019 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 as bio-indicators of environmental pollution, for mosquito control and for research purposes. 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 wild-type *B. splendens* or 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 PiBS2019, OBS2019, or GBS2019 as ornamental aquarium fish or any other potential uses. This risk to human health associated with PiBS2019, OBS2019, or GBS2019 is not suspected to meet criteria in paragraph 64(c) of CEPA 1999. No further action is recommended.

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