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ENVIRONMENTAL AND INDIRECT HUMAN HEALTH RISK ASSESSMENT OF THE GLOFISH® BETTAS: THREE LINES OF TRANSGENIC ORNAMENTAL FISH

Figure 1. Some variants of Betta splendens*. White morph of domesticated* B. splendens *male (A), Wild female* B. splendens *(B),Male and female Electric Green® Betta (C,D), Male and female Sunburst Orange® Betta (E,F), Male and female Moonrise Pink® Betta (G,H). All images provided by Spectrum Brands.*

Context:

The biotechnology provisions of the Canadian Environmental Protection Act (CEPA), 1999 take a preventative approach to environmental protection by requiring all new living organism products of biotechnology, including genetically engineered fish, to be notified and assessed prior to their import into Canada or manufacture in Canada, to determine whether they are "toxic"[1] or capable of becoming "toxic". Environment and Climate Change Canada (ECCC) and Health Canada (HC) are mandated to conduct all risk assessments under CEPA.

*On February 4, 2021, three notifications under the New Substances Notification Regulations (Organisms) [NSNR(O)] were submitted by Spectrum Brands to ECCC for evaluation of the GloFish® Electric Green® Betta (GBS2019, NSN 20660), the GloFish® Sunburst Orange® Betta (OBS2019, NSN 20659), and the GloFish® Moonrise Pink® Betta (PiBS2019, NSN 20658), which are, respectively, lines of fluorescent green, orange and pink genetically engineered Bettas (*Betta splendens*), intended for use as ornamental fish in home aquaria.*

Under a Memorandum of Understanding (MOU) between Fisheries and Oceans Canada (DFO), ECCC and HC, DFO conducts an environmental risk assessment as science advice, provides this advice t, and collaborates with HC to conduct an indirect human health risk assessment for any new living

[1] Under CEPA, a substance or living organism is "toxic" if it is entering or may enter the environment in a quantity or concentration or under conditions that (a) have or may have an immediate or long-term harmful effect on the environment; (b) constitute or may constitute a danger to the environment on which life depends; or (c) constitute or may constitute a danger in Canada to human life or health.

organism that is a fish product of biotechnology notified under CEPA and the NSNR(O). The advice will be conveyed to ECCC and HC in the form of this Science Advisory Report to inform the risk assessment they will conduct under CEPA.

This Science Advisory Report is from the April 22-23, 2021 Canadian Science Advisory Secretariat (CSAS) national advisory meeting on Environmental and Indirect Human Health Risk Assessment of GloFish® Moonrise Pink®, Sunburst Orange®, and Electric Green® Bettas: Transgenic Ornamental Fishes. Additional publications from this meeting will be posted on the Fisheries and Oceans Canada (DFO) Science Advisory Schedule as they become available.

*Related to this risk assessment, notifications for fluorescent lines of genetically engineered ornamental fish have been submitted for three lines of GloFish® Danio (*Danio rerio*) (DFO 2020a, 2020b), and six lines of GloFish® Tetra* (Gymnocorymbus ternetzi) *(DFO 2018, 2019).*

SUMMARY

- Pursuant to the *Canadian Environmental Protection Act* (CEPA), three notifications under the *New Substances Notification Regulations (Organisms*) (NSNR(O)) were submitted by Spectrum Brands to Environment and Climate Change Canada (ECCC) for genetically engineered *Betta splendens* (GloFish® Electric Green® Betta (GBS2019), GloFish® Moonrise Pink® Betta (PiBS2019) and GloFish® Sunburst Orange® Betta (OBS2019).
- Environmental and indirect human health risk assessments were conducted that included an analysis of potential hazards, likelihoods of exposure, and associated uncertainties to reach conclusions on risk and to provide science advice to ECCC and Health Canada (HC) to inform their CEPA risk assessment. Assessments were compared with the assessments of previously notified GloFish® Danio and Tetra lines.

Environmental Risk Assessment

- The environmental exposure assessment concluded that the occurrence of GBS2019, PiBS2019 and OBS2019 in the Canadian environment, outside of aquaria, is expected to be rare, isolated, and ephemeral due to their inability to survive typical low winter temperatures in Canada's freshwater environments. Consequently, the likelihood of exposure of GBS2019, PiBS2019 and OBS2019 to the Canadian environment is ranked low.
- The uncertainty associated with this environmental exposure estimation is low, given the available data for temperature tolerance of the notified lines and relevant comparators and the lack of establishment through the long history of use of non-transgenic *Betta splendens* in North America.
- The environmental hazard assessment concluded that the hazards of GBS2019, PiBS2019 and OBS2019 associated with environmental toxicity, trophic interactions, hybridization, vector for disease, biodiversity, biogeochemical cycling, and habitat are negligible. There is low hazard (i.e., no anticipated harmful effects) associated with horizontal gene transfer.
- The uncertainty levels, associated with the environmental hazard ratings, range from negligible to moderate due to data limitations and quality for the notified and surrogate organisms, or some reliance on expert opinion and anecdotal evidence.
- There is low risk of adverse environmental effects at the exposure levels predicted for the Canadian environment from the use of GBS2019, PiBS2019 and OBS2019 as an ornamental aquarium fish or other potential uses.

Indirect Human Health Risk Assessment

- The indirect human health (IHH) exposure assessment concluded that human exposure potential of GBS2019, PiBS2019 and OBS2019 is low to medium as their intended use is as an ornamental aquarium fish, thus largely limiting public exposure to those individuals who possess them for use in home aquaria, primarily through tank maintenance, and would include potentially vulnerable individuals (e.g., immunocompromised, children, those with medical conditions).
- Uncertainty associated with the IHH exposure assessment is moderate due to limited information on future import quantities and market uptake, and regarding exposure scenarios in Canada.
- The IHH hazard assessment concluded that the indirect human hazard potential of GBS2019, PiBS2019 and OBS2019 is low as there are no reported cases of zoonotic infections associated with the other commercially available GloFish® lines and only one report for the non-transgenic *Betta splendens* arising from aquarium use. Although some of the source organisms from which the inserted genetic material were derived produce toxins, there is no indication that the inserted genetic material is associated with any toxicity, allergenicity, or pathogenicity in humans.
- Uncertainty associated with the IHH hazard assessment is low, based on available data on the organisms, information from the literature on the non-transgenic *B. splendens* and other ornamental aquarium fishes, and the lack of adverse effects supported by the history of safe use of all commercially available GloFish® lines including GBS2019 and the non-transgenic *B. splendens* in Canada and other countries.
- There is a low risk of adverse indirect human health effects at the exposure levels predicted for the Canadian population from the use of GBS2019, PiBS2019 and OBS2019 as ornamental aquarium fish or other potential uses.

Conclusion and Summary

- The overall assessment of the use of GBS2019, PiBS2019 and OBS2019 in the ornamental aquarium trade or other potential uses in Canada is a low risk to the indirect human health of Canadians and to the Canadian environment. Although there was moderate uncertainty associated with a few of the assessment components, these do not affect confidence in the overall risk ratings. Assessment conclusions were consistent with those for the GloFish® Danios and GloFish® Tetras.
- The use of guide RNA and Cas9 during line creation may have resulted in off-target mutations in the GloFish® Betta populations, and this adds to the uncertainty in both hazard assessments, but there is no information to suggest that this would alter overall conclusions on risk.

BACKGROUND

On February 4, 2021, Spectrum Brands (a division of GloFish LLC) submitted three regulatory packages (notifications) to Environment and Climate Change Canada (ECCC) under the *New Substances Notification Regulations (Organisms)* [NSNR(O)] of the *Canadian Environmental Protection Act, 1999* (CEPA) for the GloFish® Electric Green® Betta, Moonrise Pink® Betta, and Sunburst Orange® Betta; herein referred to collectively as the GloFish® Bettas (Figure 1). These ornamental fish are domesticated *Betta splendens* (Siamese Fighting Fish) that have been genetically engineered to fluoresce different colours in home aquaria. They have been sold in

the USA since 2019 (GBS2019) and 2020 (PiBS2019 and OBS2019). Note that similar risk assessments have been conducted on three different colours of GloFish® Danios (DFO 2020a, 2020b), and six different colours of GloFish® Tetras (DFO 2018, 2019).

Production of notified lines

GBS2019, PiBS2019 and OBS2019 were produced using similar methodologies and testing protocols as the previously notified and approved GloFish® Danio and Tetra lines, except for the use of the CRISPR/Cas9 system, which hasn't been used previously. In general, transgene expression cassettes containing different colour fluorescent protein genes were incorporated into the genome of the notified lines. This results in ubiquitous targeted colouration of the organisms under ambient light, including sunlight. All previous and current notified GloFish® Danio and Tetra lines have used similar transgene expression cassettes and elements (promoters, terminator sequences), although the fluorescent protein genes vary in colours. One difference with the production of the Betta GloFish® was the use of Betta-specific homology arms incorporated in the transgene, and Cas9 with Betta-specific guide RNA included in the injection mixture when producing the founding individuals for each line. Though greater detail regarding the initial production of the transgenic lines has been provided by the company for review, it is considered confidential business information and is not included in this report.

Propagation of each line has been through batch breeding in populations that contain a mix of individuals hemizygous and homozygous for the transgene, with non-fluorescent *Betta splendens* removed from the population as they occur. The purpose of the modifications is to create new colour phenotypes of *B. splendens* for the ornamental aquarium trade.

Characterization of the notified organisms

Though greater detail regarding the development, structure, and function of the transgene construct has been provided by the company for review, it is considered confidential business information and is not included in this report. In addition, details regarding the design of experiments conducted by the company to characterize both genetic and phenotypic changes have been redacted.

Electric Green® Betta (GBS2019)

GBS2019 is a genetically engineered white morph of domesticated *B. splendens*, possessing multiple copies of a transgene construct. This insert results in green colouration of the organism under ambient light (Figure 1). Confirmation that F1 and F2 fish contain homogenous insert sites and constitute a single homogenous line was made via enzyme cleavage of total genomic DNA and Southern blot analysis, using a probe targeting the transgene. Confirmation that the vector backbone was not incorporated into the organism was performed on five F3 generation fish via PCR. A study conducted by the company indicated either a single insert location, or multiple insertion sites that are closely located and segregate together. Transgene copy number was estimated using quantitative real-time PCR (qPCR) on five F2 generation hemizygous GBS2019. Results indicate that multiple copies of the transgene cassettes were incorporated into the genome.

The targeted phenotypic effect of the genetic modification is that GBS2019 appears green under ambient light. Two other changes identified by the company are a decrease in reproductive success (in competition for mates with non-transgenic domesticated *B. splendens*), and diminished cold tolerance.

Moonrise Pink® Betta (PiBS2019)

PiBS2019 is a genetically engineered white morph of domesticated *B. splendens*, possessing a single site of insertion that contains multiple copies of a transgene construct. This insert results in pink colouration of the organism under ambient light (Figure 1). Confirmation that all F1 and F2 fish contain homogenous insert sites and constitute a single homogenous line was made via enzyme cleavage and Southern blot analysis, using a restriction enzyme and probe targeting the transgene. Lack of vector backbone was confirmed in five F2 hemizygous fish via PCR. Single-pair mating trials conducted by the company indicate a single insert location, or multiple insertion sites that are closely located and segregate together. Transgene copy number was estimated using quantitative real-time PCR (qPCR) on five F2 generation hemizygous PiBS2019. Results indicate that multiple copies of the transgene cassettes were incorporated into the genome.

The targeted phenotypic effect of the genetic modification is that PiBS2019 appears pink under ambient light. Two other changes identified by the company are a decrease in reproductive success (in competition for mates with non-transgenic domesticated *B. splendens*), and diminished cold tolerance.

Sunburst Orange® Betta (OBS2019)

OBS2019 is a genetically engineered white morph of domesticated *B. splendens*, possessing a single site of insertion that contains approximately one copy of a genetic construct. This insert results in orange colouration of the organism under ambient light (Figure 1). Confirmation that all F1 and F2 fish contain homogenous insert sites and constitute a single homogenous line was made via enzyme cleavage and Southern blot analysis using a probe targeting the transgene. Lack of vector backbone was confirmed in five F2 hemizygous fish via PCR, using primers for four different sections of the vector backbone. Single-pair mating trials conducted by the company indicated a single insert location. Transgene copy number was estimated using quantitative real-time PCR (qPCR) on five F2 generation hemizygous OBS2019. Results indicate a single copy of the transgene cassette was incorporated into the genome.

The targeted phenotypic effect of the genetic modification is that OBS2019 appears orange under ambient light. In reproduction competition trials, the observed proportion of transgenic (orange fluorescent) offspring did not indicate a reproductive disadvantage. In low-temperature tolerance trials conducted by the company, transgenic fish had similar cold tolerance as non-transgenic fish.

Comparator species

For the purpose of this risk assessment, the domesticated *B. splendens* (the Siamese Fighting Fish) was selected as a comparator. *B. splendens* is a popular ornamental species that is bred, produced, and traded worldwide. A freshwater fish of the family Osphronemidae (also known as Gouramis), they are one of approximately 55 species of *Betta* that have been described from southeast Asia, with *B. splendens* among the most widely distributed (Monvises et al. 2009). Since their domestication over 100 years ago, *B. splendens* has been selectively bred for traits such as colour and iridescence, fin morphology, and aggression (Monvises et al. 2009).

Wild *B. splendens* are carnivorous, feeding mostly on mosquito larvae and other small aquatic insects (Pleeging and Moons 2017). Their preferred habitats are small bodies of fresh water such as those in rice paddies, ponds, lagoons and marshes, with plenty of vegetation to help protect it from avian predators (Jaroensutasinee and Jaroensutasinee 2001; Monvises et al. 2009). In the aquarium, *B. splendens* prefer a neutral pH, will tolerate dissolved oxygen levels

below 2 ppm (though they prefer 5 to 7 ppm), and do best at temperatures between 24 and 30°C (Goldstein 2015). The habitat of nesting *B. splendens* in Thailand has been described as low in pH (5.28 to 5.80) and dissolved oxygen (0.00 to 7.39 ppm), high in temperature (27.00 to 31.50°C), and with water depths ranging from 2.00 to 9.40 cm (Jaroensutasinee and Jaroensutasinee 2001).

There are no published records describing the ability of Bettas to survive outside their preferred temperature range, however, data collected by the notifier indicate that Bettas cannot survive below 6°C when temperatures fall quickly (e.g., 1-2°C/hr). Unpublished data collected by DFO indicate that when temperatures drop slowly (e.g., 1°C/day), Bettas slowed feeding and activity around 17°C, stop feeding at about 12.5°C, ceased activity at 11.5°C, and lost equilibrium between 16.0 and 7.9°C (average 10.0 ± 1.2°C).

While there are examples of established *B. splendens* populations in various parts of the world (all tropical), it has only been postulated to be invasive in northern Australia, where a large and extensive population has persisted in the Adelaide river over consecutive years, with potential for further spread (Hammer et al. 2019). Hill et al. (2017) estimated *B. splendens* invasion potential in conterminous United States to be low, using the Fish Invasiveness Screening Kit (FISK).

Receiving environment

Though the many lakes and rivers of Canada vary in their annual temperature profiles, as well as their average maximum and minimum temperatures, most reach 4°C or below at some point annually, and only a few isolated lakes in Southern Coastal British Columbia have minimum recorded temperatures at or below 6°C. If an introduced fish cannot survive at 4°C or below, its occurrence in the Canadian environment will be seasonal at best, though possible localized overwintering pockets can occur (e.g., industrial effluent, hot springs etc., isolated lakes if can survive between 4-6°C). Also, it should be noted that mean freshwater surface temperatures in Canada are rising as a result of global climate change and are projected to increase by 1.5 to 4.0°C over the next 50 years (DFO 2013) and therefore, could increase the number of possible lakes in which organisms with moderate cold tolerance could survive. A more detailed description of potential receiving environments in Canada relevant to the introduction of tropical freshwater fish is presented in Leggatt et al. (2018).

RISK ASSESSMENT – ENVIRONMENTAL

Environmental exposure, hazard, and risk assessment conclusions for PiBS2019, OBS2019, or GBS2019 are, despite being conducted independently, consistent with previous risk assessments on GloFish® Danio lines, and rankings and most uncertainty ratings are equivalent to those for the previously notified and assessed GloFish® Tetra lines (Table 1). New relevant evidence in the scientific literature and differences in the current GloFish® notifications have not altered risk conclusions. Detailed environmental risk assessments can be found at (DFO 2018, 2019, 2020a, 2020b). An abbreviated summary of previous and current assessments follows.

Environmental exposure assessment

The exposure assessment for the three living organisms being assessed addresses both their potential to enter the environment (release) and fate once in the environment. The likelihood and magnitude of environmental exposure is determined through an extensive, cradle-to-grave assessment that details the potential for release, survival, persistence, reproduction, proliferation, and spread in the Canadian environment.

Though the stated purpose of the organism is for sale in the ornamental market, and hobbyists who purchase the product do, for the most part, follow the instructions for disposal that are recommended by the retailer or the notifier company itself, there is still a high likelihood that GloFish® Bettas will be introduced into the Canadian environment. Once the notified organisms have been sold into the retail market, they are no longer under the direct control of the importer, and there can be no guarantee of appropriate containment and disposal. The extent to which the organisms are further exposed to the environment will, therefore, depend heavily on their ability to survive and reproduce in Canadian lakes and rivers.

Table 1. Summary of all ranks and uncertainty rating for environmental risk assessments of currently notified GloFish® Betta lines, as well as previously notified GloFish® Danios (DFO 2020a, 2020b) and GloFish® Tetras (DFO 2018, 2019). Italicized text indicates where previous assessments differ from the current assessment.

Assessment	Rank/Uncertainty		
	GloFish® Bettas	**GloFish® Danios**	**GloFish® Tetras**
Exposure	**Low/Low**	**Low/Low**	**Low/Low**
Hazards:			
1. Environmental toxicity	Negligible/Moderate	Negligible/Moderate	Negligible/Moderate
2. HGT	Low/Moderate	Low/Moderate	Low/*Low*
3. Trophic interactions.	Negligible/Moderate	Negligible/Moderate	Negligible/Moderate
4. Hybridization	Negligible/Negligible	Negligible/*Moderate*	Negligible/Negligible
5. Vector for disease	Negligible/Moderate	Negligible/Moderate	Negligible/Moderate
6. Biogeochemical	Negligible/Moderate	Negligible/Moderate	Negligible/Moderate
7. Habitat	Negligible/Low	Negligible/Low	Negligible/Low
8. Biodiversity	Negligible/Low	Negligible/Low	Negligible/Low
Environmental Risk	**Low**	**Low**	**Low**

As a tropical species, *B. splendens* is not expected to survive in a temperate region, where water temperatures are below optimal for survival. In the aquarium, *B. splendens* do best at temperatures between 24 and 30°C (Goldstein 2015, see Section 1.4.3). Data provided by the notifier indicate that when the water temperature drops relatively quickly, approximately 50% to 100% of fish are dead by the time temperatures reach 7°C to 5.5°C. In experiments at DFO, when water temperatures were dropped relatively slowly (decrease of 1°C/day from 20.5°C), non-transgenic domesticated *B. splendens* stopped feeding around 12.5°C, and 100% of fish had lost equilibrium by 7.9°C, though the majority of fish lost equilibrium between 11.2 and 9.0°C, with an LD_{50} of 9.91±0.13°C.

There are no lakes in Canada that consistently remain above 7°C throughout the entire course of a year, or above 6°C across multiple years and almost all do not remain above 4°C throughout the year (Leggatt et al. 2018). Consequently, while the temperatures needed for the

notified lines to survive may be possible for several Canadian lakes during the summer, there is a very low likelihood that GBS2019, PiBS2019 and OBS2019 can survive the Canadian winter. At best, the occurrence of GloFish® Bettas in the Canadian freshwater environment would be seasonal or ephemeral.

Though water temperatures in Canada will limit the persistence of any GloFish® Bettas that are introduced into the environment, there may still be time to reproduce, if introduced at the start of a warm season. For example, Osoyoos Lake in the BC interior is one of Canada's warmest lakes in the summer, with an average temperature between 20 and 25°C for about two months of the year (mid-July to mid-September), with higher temperatures (e.g., 25°C) restricted to an even shorter window (e.g., end of July – beginning of August, (BCLSS 2013). While this may be a tolerable temperature range for GloFish® Bettas survival, warmer temperatures (25-30°C) are more ideal for reproduction (Goodrich and Taylor 1934; Goldstein 2015), although such temperatures may temporarily exist in isolated areas such as shorelines.

Given the above analysis, the occurrence of GloFish® Bettas in the Canadian environment is expected to be rare, isolated, and ephemeral. Consequently, the likelihood of exposure of GloFish® Bettas to the Canadian environment is ranked **low**. The uncertainty associated with this estimate is **low**, given the quality of data (temperature tolerance) available for GloFish® Bettas and valid surrogate organisms, evidence of low variability, and data available on the environmental parameters of the receiving environment in Canada (see Appendix Tables A1 and A2). The potential for off-target mutations from use of Cas9 and guide RNA does not alter the exposure ranking or associated uncertainty due to available data on cold tolerances of the GloFish® Betta lines. This rating is consistent with the low exposure rating with low uncertainty concluded on for three lines of GloFish® Danio (DFO 2020a, 2020b), and six lines of GloFish® Tetra (DFO 2018, 2019).

Environmental hazard assessment

The hazard assessment examines potential impacts to the environment that could result from exposure to GloFish® Bettas. The hazard identification process considers potential pathways to harm including through environmental toxicity (i.e., potential to be poisonous), gene transfer, trophic interactions, and as a vector for pathogens, as well as capacity to impact ecosystem components (e.g., habitat, nutrient cycling, biodiversity). The following assesses the hazards and uncertainty associated with the fluorescent protein transgenic modification in the notified lines, followed by an overall discussion of potential effects to hazard ratings and uncertainty associated with possible off-target mutations as a result of Cas9 and guide RNA use.

The notifications include a report screening the amino acid sequence of the fluorescent protein for allergenicity on Allermatch that found no functional matches to known human allergen amino acid sequences. After several years of commercial production in the US, there have been no reported toxic effects resulting from exposure to other species of GloFish® containing transgenes coding the same proteins as those in the GloFish® Betta lines, and no such reports during one year of commercial use of GBS2019. Consequently, the potential hazard to the environment due to environmental toxicity of GloFish® Bettas is ranked **negligible**. The uncertainty associated with this ranking is **moderate** due to limited direct data from the notified organisms or surrogate organisms, and reliance on anecdotal evidence and indirect evidence from other organisms. This concurs with assessment rankings for previously notified GloFish® Danios and Tetras (DFO 2018, 2019, 2020a, 2020b), and no new relevant data have become available since analyses of previous GloFish® lines.

Genes encoding fluorescence have been introduced to a wide range of organisms with few reports of harmful effects from the introduced transgenes. This suggests that the introduction of the transgene through horizontal gene transfer (HGT) to a novel host is not expected to result in harmful effects, should it occur. Though the introduction of a fluorescent transgene to a novel organism in Canadian environments through HGT cannot be excluded, the absence of expected harmful effects from such an introduction result in a hazard ranking of **low**. While the transgenes are well defined, the limited knowledge of the location of the transgenes within the *B. splendens* genome, and lack of studies examining HGT of the transgenes and resulting consequences, results in **moderate** uncertainty. This concurs with the previous assessments for the GloFish® Danios, and Tetras, though in the latter group uncertainty was assessed as low (DFO 2018, 2019, 2020a, 2020b). Here, and for the Danios, the uncertainty rating was increased to better reflect the lack of or limited number of relevant studies of HGT and resulting consequences.

Should GloFish® Bettas be released to the environment, they have the potential to interact with other organisms in Canadian freshwater aquatic ecosystems, including potential prey, competitors, and predators. Wild *B. splendens* are carnivorous and aggressive, feeding mostly on mosquito larvae and other small aquatic insects (Pleeging and Moons 2017). As such, they have the potential to impact localized populations of small prey organisms or competitors occupying similar niches at the location of release. Based on low activity of *B. splendens* in cooler waters, and lack of noted alterations in trophic-level related behaviour of the notified lines, GloFish® Bettas are not expected to influence trophic interactions of native organisms beyond natural fluctuations, with associated **negligible** hazard relative to non-transgenic counterparts. The lack of studies directly examining the hazards of GloFish® Bettas, limited available data on a surrogate (RFP Zebrafish) and poor understanding of genotype by environment (GxE) interactions in aggression and predation susceptibility in surrogate fluorescent transgenic models, result in a **moderate** level of uncertainty. This concurs with assessment rankings for previously notified GloFish® Danios and Tetras (DFO 2018, 2019, 2020a, 2020b).

Betta splendens is a freshwater fish of the family Osphronemidae (also known as Gouramis) that are native to Asia and do not occur in North America. Though *B. splendens* has many congeneric species within its natural range (Monvises et al. 2009), none are established in Canada and there are no other genera of the Osphronemidae family native to Canada. Consequently, there is **negligible** potential for GloFish® Bettas to cause hazard through natural hybridization with native fish in Canada. High quality data on the distribution of *Betta* species and related genera result in **negligible** uncertainty. This is concurrent with hazard conclusions in GloFish® Tetra lines, although uncertainty level in Danio® lines was higher due to the presence of native species sharing the same Family as *D. rerio* in Canada.

Whether GloFish® Bettas, or any other transgenic fluorescent organism, have altered ability to act as a vector of disease agents, has not been directly examined. Increased susceptibility to disease may increase vector capabilities through heightened ability to act as a reservoir and increased shedding of disease agents, or decrease vector capabilities by succumbing to disease quickly. Numerous other transgenic fluorescent aquarium species and lines have been grown on a commercial scale in the US starting in 2003. Spectrum Brands have provided statements from veterinarians claiming they had not seen increases in susceptibility to, or the transmission of, pathogens in any GloFish® line, though no empirical evidence was provided and the scale of the assessment was small. Fluorescent transgenic Zebrafish have been used extensively in laboratory conditions for research for years with no known reported effects on disease susceptibility. Consequently, there is **negligible** potential for GloFish® Bettas to have

altered capacity as a vector for disease relative to non-transgenic *B. splendens*. As this has not been directly examined in GloFish® Bettas, there are limited data on a surrogate, and reliance on expert opinion, the uncertainty level for this rating is **moderate.** This concurs with assessment rankings for previously notified GloFish® Tetras and Zebrafish (DFO 2018, 2019, 2020a, 2020b).

GloFish® Bettas are expected to contribute to nutrient cycles within habitats through ingestion of prey and other food items and release of waste (ammonia and feces). The potential effects of fluorescent protein in GloFish® Bettas on metabolism, and hence nutrient cycling, have not been examined. In a different model organism, eGFP transgenic mice were found to have alterations in the urea cycle, nucleic acid and amino acid metabolism, and energy utilization (Li et al. 2013). What impacts these changes may have on biogeochemical cycling should GloFish® Bettas have similar influences from fluorescent transgenic gene expression are not known, but the small size of *B. splendens* and potential low numbers of individuals in an ecosystem indicates a **negligible** potential for GloFish® Bettas to impact biogeochemical cycling in natural environments, even with altered metabolic pathways. Uncertainty is **moderate** due to a lack of studies directly examining this hazard. This concurs with assessment rankings for previously notified lines of GloFish® Danios and GloFish® Tetras (DFO 2018, 2019, 2020a, 2020b).

B. splendens are a small species and do not build structures that are expected to impact habitats of other species. Male Betta fish do build bubble nests for embryo incubation, but these are expected to be ephemeral in nature and there are no known reports of negative impacts of bubble nests to habitat in *B. splendens* or other species that build them. Goodrich and Taylor (1934) reported males stop building bubble nests when temperatures fall below 25°C which would greatly limit times and places released Bettas may build nests in Canadian waters. There have been no reports, anecdotal or otherwise, of GloFish® lines including Bettas having altered behaviour, relative to domesticated non-transgenic fish, that may influence effects on habitat structure. Consequently, GloFish® Bettas are expected to have **negligible** effects to habitat with **low** uncertainty associated with this rating. This concurs with assessment rankings for previously notified GloFish® Danios and GloFish® Tetras (DFO 2018, 2019, 2020a, 2020b).

The GloFish® Bettas are not expected to negatively impact native species through trophic or hybrid interactions, act as a vector for disease agents of concern in Canada, impact biogeochemical cycling, or impact habitat. The transgenic constructs and fluorescent proteins in GBS2019, PiBS2019 and OBS2019 are not expected to result in environmental toxicity, or cause hazards through HGT of the transgene, are not expected to increase potential hazards through interactions with native species, and there is no history of invasiveness of *B. splendens* in temperate climates despite extensive use. Taken together, there is a **negligible** hazard of GloFish® Bettas affecting biodiversity of Canadian ecosystems. Reliance on data from the comparator species for invasiveness and biodiversity effects results in a **low** degree of uncertainty with this ranking.

The examined hazards have negligible to low rankings (Table 2), while uncertainty ranged from negligible to moderate, due to limited data specific to GloFish® Bettas, limited direct data on comparator species, variable data from surrogate models, and the reliance on expert opinion for the assessment of some hazards (see Appendix Tables A3 and A4). Outside of its intended use as an ornamental fish in static aquaria, GloFish® Bettas are not expected to pose unique hazards beyond those of the intended use. Hazard ranking concurred with that previously assessed for GloFish® Danios and GloFish® Tetras, although uncertainty differed from that assessed in GloFish® Tetras or Danios in two hazard categories due to increased acknowledgement of data limitations (through HGT), or differences in family distributions (through hybridization).

Use of guide RNA and Cas9 in the creation of the GloFish® Betta lines adds additional uncertainty to the overall hazard assessment from potential off-target mutations in the Betta populations. There exists a theoretical risk that an off-target mutation could result in altered protein structure or expression that alters the phenotype of Bettas and may have downstream consequences to the environment (e.g., alteration of a protein to become allergenic or toxic to other organisms). In other models, the potential for off-target mutations from guide RNA and Cas9 is usually discussed in the context of potential harm or toxicity to the organism itself, and phenotypes of off-target mutations, when examined, are generally neutral or negative. Discussions on harmful effects of potential off-target mutations to the environment are theoretical at this point, and there are no known reports of off-target mutations resulting in phenotype that may result in environmental harm in other models, nor are there anecdotal reports of individuals in the GloFish® Betta populations have altered phenotype that may result in environmental harm. While this does not alter any hazard ratings for the GloFish® Bettas, it does add to uncertainty in the overall hazard assessment.

Environmental risk assessment

Consistent with similar risk assessments, an overall conclusion on Risk is based on the classic paradigm where: Risk ∝ Hazard x Exposure. Overall Risk is estimated by plotting overall Hazard against Exposure using a risk matrix or heat map, as illustrated in Figure 2. The matrix can be used as a tool for facilitating communication and discussion on risk. The uncertainty associated with risk is discussed in the context of uncertainty in the hazard and exposure assessments. Hazard and exposure uncertainty ratings are associated with quality of data used in assessments, and whether uncertainty may increase the range of possible ratings is context specific.

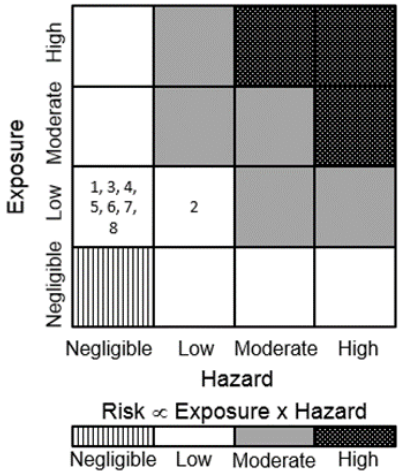

Figure 2. Risk matrix and pattern scale to illustrate how exposure and hazard are integrated to establish a level of risk in the environmental risk assessment. Risk assessments associated with assessed hazard components at the assessed exposure are identified by number: 1) through environmental toxicity; 2) through horizontal gene transfer; 3) through interactions with other organisms; 4) through hybridization; 5) as a vector of disease; 6) to biogeochemical cycling; 7) to habitat; and 8) to biodiversity.

The exposure assessment concluded that GloFish® Bettas used in the ornamental aquarium trade or other unintended uses would have a low likelihood of occurrence in the Canadian environment. This is due to the high likelihood of release of small numbers from home aquaria, but negligible likelihood for GloFish® Bettas to overwinter in Canadian aquatic ecosystems. As such, any exposure to Canadian freshwater ecosystems to GloFish® Bettas is expected to be

isolated, rare, and ephemeral. The quality of data demonstrating lack of cold tolerance in GloFish® Bettas and domesticated *B. splendens*, relevant to Canadian freshwater temperatures result in low uncertainty associated with this ranking.

The hazard assessment concluded that GloFish® Bettas poses negligible to low hazard to the Canadian environment, due to the lack of hazard associated with domesticated *B. splendens*, and no direct evidence that the expressed fluorescent protein would increase hazard, relative to domesticated *B. splendens*. Uncertainty ranking associated with individual hazard components ranged from negligible to moderate, due to limited data specific to GloFish® Bettas, limited direct data on comparator species, and the reliance on expert opinion for the assessment of some hazards.

Using the risk matrix seen in Figure 2, GloFish® Bettas used in the ornamental aquarium trade or other uses in Canada pose **low risk** to Canadian environments. Individual hazards are expected to result in no harmful effects beyond natural fluctuations to Canadian environments under the assessed level of exposure. Sources of uncertainty in the environmental exposure and hazard assessments that may influence uncertainty in environmental risk assessment include a lack of data directly addressing hazards of the notified organism and comparator species, variability in data taken from surrogate organisms, and in some cases reliance on expert opinion.

Despite moderate uncertainty in some of the individual assessment components, there is no current evidence to suggest that overall risk ratings of GloFish® Bettas may be higher than the assessed low ranking for risk to Canadian environments. This concurs with low risk assessment rankings for previously notified GloFish® Danios and GloFish® Tetras (DFO 2018, 2019, 2020a, 2020b).

RISK ASSESSMENT – INDIRECT HUMAN HEALTH

The following indirect human health risk assessment was conducted on *B. splendens* PiBS2019, OBS2019, and GBSZ2019, three genetically modified lines of diploid, hemizygous or homozygous, Bettas, containing genes encoding for modified versions of fluorescent red, yellow or green proteins, respectively. 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).

Indirect human health exposure, hazard, and risk assessment conclusions for GBS2019, PiBS2019 and OBS2019 are consistent with previous risk assessments on similar notified GloFish® Danio and Tetra lines (see Table 2). No new relevant evidence has been reported in the scientific literature, and no differences have been noted in the GloFish® Betta notifications relative to previously notified GloFish® lines that would alter indirect human health risk conclusions. As with the Environmental risk assessment, the use of Cas9 and guide RNA during line creation may have produced off-target mutations with unknown effects, however, these are not expected to alter the overall risk assessment conclusions for human health. While this adds to overall uncertainty regarding the hazards of the notified lines to indirect human health, it does not raise the uncertainty ranking.

Table 2. Summary of all ranks and uncertainty ratings for indirect human health (IHH) risk assessments of currently notified Betta lines, three previously notified Zebrafish lines, and six previously notified lines of GloFish® Tetras (DFO 2018, 2019, in review).

Indirect Human Health	GloFish® Bettas	GloFish® Danios	GloFish® Tetras
	Rank/Uncertainty	Rank/Uncertainty	Rank/Uncertainty
Exposure	Low to Medium/ Moderate	Low to Medium/ Moderate	Low to Medium/ Moderate
Hazard	Low/Low	Low/Low	Low/Low
IHH Risk	**Low**	**Low**	**Low**

Indirect human health exposure assessment

Risks from workplace exposure to the notified strains are not considered in this assessment[2]. The human exposure potential of PiBS2019, OBS2019, and GBS2019 is assessed to be low to medium because:

1. The primary sources of human exposures would stem from the proposed import through unidentified points of entry in Canada;
2. The intent is to have adult PiBS2019, OBS2019, and GBS2019 fish available for purchase by the public 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 which 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.

[2] 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.

Uncertainty related to indirect human health exposure assessment

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 fish. 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 of the 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 actual numbers of notified organisms to be imported in the following 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; Perrin 2009; Marson et al. 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 (see Appendix Tables A5 and A6).

Indirect human health hazard assessment

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

1. PiBS2019, OBS2019, and GBS2019 are genetically modified tropical fish containing copies of transgene constructs at a single site of insertion 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. Although 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 despite relatively widespread use. The zoonotic potential of PiBS2019, OBS2019, and GBS2019 is not expected to be any different than the 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 the PiBS2019, 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®.

Uncertainty related to indirect human health hazard assessment

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 transgenic genotypes and phenotypes. Sequence analyses of the inserted genetic material 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. As with the Environmental Risk assessment, there is a theoretical possibility that off-target mutations from the use of Cas9 and guide RNA could produce altered proteins with increased allergenicity, although this has not been identified in other models. Consequently, this is not expected to alter hazard ratings, but increases hazard uncertainty, though not sufficiently to raise uncertainty ranking above low. The uncertainty is considered low because much of the information on human health effects are 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 specific studies that have investigated human health effects associated with fluorescent transgenic ornamental fish (see Appendix Tables A7 and A8).

Indirect human health risk assessment

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.

Notified use

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 1950; 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 green and yellow fluorescent proteins used in GBS2019 and OBS2019, and PiBS2019 have been used in other Glofish® lines approved in Canada. There are no known reports of adverse human health effects specifically associated with the wild type Bettas. In general, the inserted fluorescent protein genes and the methods used to modify the notified lines leading to a conclusion that 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 *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, illegal bait fish use, 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 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.

The indirect human health low risk conclusion (including rankings for exposure, hazard, and relevant uncertainties) concurs with conclusions of low risk to indirect human health for the previously notified three lines of GloFish® Danio (DFO 2020a, 2020b) and six lines of GloFish® Tetra (DFO 2018, 2019).

Sources of Uncertainty

Sources of uncertainty in the indirect human health exposure and hazard assessments that may influence uncertainty in the risk assessment include limited information on exposure scenarios in the Canadian market, reliance on reports from surrogate models, and lack of direct data addressing hazards of GBS2019, PiBS2019, and OBS2019 specifically.

Sources of uncertainty in the environmental exposure and hazard assessments that may influence uncertainty in the risk assessment include lack of data directly addressing hazards of the notified organisms, variability in data taken from surrogate organisms, and a reliance on expert opinion for some of the hazard assessments (e.g., impacts through vector of disease agents).

In both assessments, the potential off-target mutations from use of Cas9 and guide RNA at line creation increases uncertainty in hazard assessments without altering overall ratings and does not affect uncertainty in exposure assessments. Currently there are no data from studies examining off-target effects in other CRISPR Cas9 models that indicate final risk rankings would be higher than low. Overall, though sources and levels of uncertainty may vary among hazard and exposure rankings, the reported levels of uncertainty are not currently expected to affect the overall risk assessment conclusions.

CONCLUSIONS AND ADVICE

Use of GloFish® Bettas for home aquaria is expected with moderate uncertainty to result in low to medium exposure to humans, primarily through tank maintenance by those who purchase or care for the fish. The hazard of GloFish® Bettas to indirect human health is ranked low (with low uncertainty), due to lack of pathogenicity, allergenicity or toxicity associated with the genetic modification, and history of safe use of commercially available GloFish® lines and non-transgenic comparator species. Taken together, available evidence does not suggest a risk of

adverse indirect human health effects at the exposure levels predicted for the general Canadian population from use of GloFish® Bettas as ornamental aquarium fish or in other identified potential uses.

Use of GloFish® Bettas in home aquaria is expected to result in potential repeated, but very small magnitude, releases to the Canadian environment. However, data available indicate GloFish® Bettas do not have the capacity to overwinter in most Canadian freshwater ecosystems, resulting in low environmental exposure with low uncertainty. The lack of evidence of hazards from the non-transgenic *B. splendens* despite long-term extensive use, as well as lack of evidence for increased hazards of GloFish® Bettas relative to non-transgenic fish, indicates ratings of negligible to low hazard of GloFish® Bettas to Canadian environments with negligible to moderate uncertainty. Taken together, the overall risk of PiBS2019, OBS2019, or GBS2019 to the Canadian environment is low, and the notified organisms are not expected to cause harmful effects to Canadian environments at the assessed exposure level.

The import of GloFish® Bettas into Canada, for use in the ornamental aquarium trade and home aquaria, is expected to pose low risk to indirect human health and the Canadian environment. While uncertainty associated with some exposure and hazard classifications is moderate due to limited or absent direct data on the notified organisms or comparator species, evidence was not identified that suggests GloFish® Bettas under the proposed use, or other potential uses, could cause harm as a result of exposure to Canadian populations or environments. While current limited data suggests the potential for off-target mutations from use of guide RNA and Cas9, this is not anticipated to alter risk ratings; though conclusions should be reassessed as the literature on this issue progresses. The conclusions of low risk to indirect human health and the environment from the notified organisms are consistent with conclusions for all previously GloFish® lines notified under CEPA.

OTHER CONSIDERATIONS

The impact of climate change on risk assessment conclusions was considered, but not fully assessed. Climate change is projected to increase average water temperatures 1.5 to 4.0°C over the next 50 years (DFO 2013), but is unlikely to impact the potential for GloFish® Betta to overwinter in Canada. Increased winter water temperatures in the few isolated lakes with infrequent ice coverage in Southwestern BC could increase the potential for overwinter survival in these isolated lakes. However, for the majority of freshwater systems experiencing ice coverage, temperatures would be expected to be at or below 4°C at some point during the winter, preventing year-round survival of GloFish® Bettas.

The current assessment highlighted some of the unknowns regarding risk-relevant effects of off-target mutations from use of gene editing (e.g., CRISPR-Cas9) to produce genetically engineered fish. Research is required in this area, particularly for CRISPR-Cas9 edited temperate or native species, to better address the risks to the Canadian environment and indirect human health from organisms produced using this technology.

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SOURCES OF INFORMATION

This Science Advisory Report is from the April 22-23, 2021 Canadian Science Advisory Secretariat (CSAS) national advisory meeting on Environmental and Indirect Human Health Risk Assessment of GloFish® Moonrise Pink®, Sunburst Orange®, and Electric Green® Bettas: Transgenic Ornamental Fishes. Additional publications from this meeting will be posted on the Fisheries and Oceans Canada (DFO) Science Advisory Schedule as they become available.

BCLSS. 2013. Osoyoos Lake 2005-2011. British Columbia Lake Stewardship Society, Kelowna, BC, Kelowna, BC. p. 4.

DFO. 2013. Risk-based assessment of climate change impacts and risks on the biological systems and infrastructure within Fisheries and Oceans Canada's mandate - Freshwater Large Aquatic Basin. DFO Can. Sci. Advis. Sec. Sci. Resp. 2013/011.

DFO. 2018. Environmental and indirect human health risk assessment of the Glofish® Electric Green® Tetra and the Glofish® Long-Fin Electric Green® Tetra (*Gymnocorymbus ternetzi*): a transgenic ornamental fish. DFO Can. Sci. Advis. Sec. Sci. Advis. Rep. 2018/027.

DFO. 2019. Environmental and indirect human health risk assessment of the GloFish® Tetras (*Gymnocorymbus ternetzi*): five lines of transgenic ornamental fish. DFO Can. Sci. Advis. Sec. Sci. Advis. Rep. 2019/002.

DFO. 2020a. Environmental and indirect human health risk assessment of the GloFish® Sunburst Orange® Danio (*Danio rerio*): A transgenic ornamental fish. DFO Can. Sci. Advis. Sec. Sci. Advis. Rep. 2020/015.

DFO. 2020b. Environmental and Indirect Human Health Risk Assessment of GloFish® Cosmic Blue® and Galactic Purple® Danios (*Danio rerio*): Transgenic Ornamental Fish. DFO Can. Sci. Advis. Sec. Sci. Advis. Rep. 2020/016.

Duggan, I.C., Rixon, C.A., and MacIsaac, H.J. 2006. Popularity and propagule pressure: determinants of introduction and establishment of aquarium fish. Biol. Invasions 8(2):377-382.

Gertzen, E., Familiar, O., and Leung, B. 2008. Quantifying invasion pathways: fish introductions Gertzen, E., Familiar, O., and Leung, B. 2008. Quantifying invasion pathways: fish introductions from the aquarium trade. Can. J. Fish. Aqua. Sci. 65(7):1265-1273.

Goldstein, R.J. 2015. The Betta Handbook. Barron's, New York.

Goodrich, H.B., and Taylor, H.C. 1934. Breeding reactions in *Betta splendens*. Copia 1934(4):165-166.

Hammer, M.P., Simoes, M.N.S., Needham, E.W., Wilson, D.N., Barton, M.A., and Lonza, D. 2019. Establishment of Siamese Fighting Fish on the Adelaide River floodplain: the first serious invasive fish in the Northern Territory, Australia. Biol. Invasions 21(7):2269-2279.

Hill, J.E., Tuckett, Q.M., Hardin, S., Lawson, L.L., Lawson, K.M., Ritch, J.L., and Partridge, L. 2017. Risk screen of freshwater tropical ornamental fishes for the conterminous United States. Trans. Am. Fish. Soc. 146(5):927-938.

Innes, W.T. 1950. Exotic Aquarium Fishes: A work of general reference. Philadelphia: Innes Publishing Company.

Jaroensutasinee, M., and Jaroensutasinee, K. 2001. Bubble nest habitat characteristics of wild Siamese fighting fish. J. Fish Biol. 58(5):1311-1319.

Leggatt, R.A., Dhillion, R.S., Mimeault, C., Johnson, N., Richards, J.G., and Devlin, R.H. 2018. Low-temperature tolerances of tropical fish with potential transgenic applications in relation to winter water temperatures in Canada. Can. J. Zool. 96:253-260.

Li, H., Wei, H., Wang, Y., Tang, H., and Wang, Y. 2013. Enhanced green fluorescent protein transgenic expression *in vivo* is not biologically inert. J. Proteome Res. 12(8):3801-3808.

Marson, D., Cudmore, B., Drake, D.A.R., and Mandrak, N.E. 2009. Summary of a survey of aquarium owners in Canada. Can. Manuscr. Rep. Fish. Aquat. Sci. 2905: iv + 20 p.

Monvises, A., Nuangsaeng, B., Sriwattanarothai, N., and Panijpan, B. 2009. The Siamese fighting fish: Well-known generally but little-known scientifically. ScienceAsia 35(1):8-16.

Perrin, T. 2009. The business of urban animals survey: the facts and statistics on companion animals in Canada. Canadian Vet. J. 50(1):48.

Pleeging, C.C.F., and Moons, C.P.H. 2017. Potential welfare issues of the Siamese fighting fish (*Betta splendens*) at the retailer and in the hobbyist aquarium. Vlaams Diergen. Tijds. 86(4):213-223.

Wallbrunn, H.M. 1958. Genetics of the Siamese fighting fish, *Betta splendens*. Genetics. 43(3):289-298.

APPENDIX: EXPOSURE AND HAZARD RANKING CONSIDERATIONS

Table A1: Rankings for likelihood of exposure of genetically engineered fish to the Canadian environment.

Exposure Ranking	Assessment
Negligible	No occurrence; Not observed in Canadian environment[1]
Low	Rare, isolated occurrence; Ephemeral presence
Moderate	Often occurs, but only at certain times of the year or in isolated areas
High	Often occurs at all times of the year and/or in diffuse areas

[1]extremely unlikely or unforeseeable

Table A2: Ranking of uncertainty associated with the likelihood of occurrence and fate of the organism in the Canadian environment (environmental exposure).

Uncertainty Ranking	Available Information
Negligible	High-quality data on the organism (e.g., sterility, temperature tolerance, fitness). Data on environmental parameters of the receiving environment and at the point of entry. Demonstration of absence of Genotype by Environment (GxE) interactions or complete understanding of GxE effects across relevant environmental conditions. Evidence of low variability.
Low	High-quality data on relatives of the organism or valid surrogate. Data on environmental parameters of the receiving environment. Understanding of potential GxE effects across relevant environmental conditions. Evidence of variability.
Moderate	Limited data on the organism, relatives of the organism or valid surrogate. Limited data on environmental parameters in the receiving environment. Knowledge gaps. Reliance on history of use or experience with populations in other geographical areas with similar or better environmental conditions than in Canada.
High	Significant knowledge gaps. Significant reliance on expert opinion.

Table A3: Ranking of hazard to the environment resulting from exposure to the organism.

Hazard Ranking	Assessment
Negligible	No effects[1]
Low	No harmful effects[2]
Moderate	Reversible harmful effects
High	Irreversible harmful effects

[1]No biological response expected beyond natural fluctuations
[2]Harmful effect: an immediate or long-term detrimental impact on the structure or function of the ecosystem including biological diversity beyond natural fluctuations

Table A4: Ranking of uncertainty associated with the environmental hazard.

Uncertainty Ranking	Available Information
Negligible	High-quality data on notified organism. Demonstration of absence of GxE effects or complete understanding of GxE effects across relevant environmental conditions. Evidence of low variability.
Low	High-quality data on relatives of notified organism or valid surrogate. Understanding of GxE effects across relevant environmental conditions. Some variability.
Moderate	Limited data on notified organism, relatives of organism or valid surrogate. Limited understanding of GxE effects across relevant environmental conditions. Knowledge gaps. Reliance on expert opinion.
High	Significant knowledge gaps. Significant reliance on expert opinion.

Table A5: Exposure considerations (indirect 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.
Medium	• It is released into the environment, but quantity, duration and/or frequency of release is moderate. • It 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	• It 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.

Table A6: Uncertainty ranking associated with the indirect human health exposure.

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

Table A7: Considerations for hazard severity (indirect human health).

Hazard Ranking	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 indirect 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 indirect 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.

Table A8: Ranking of uncertainty associated with the indirect human health hazard.

Uncertainty Ranking	Description
Negligible	There are many reports of indirect 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 indirect human health effects in individuals exposed to the organism has been monitored and there are no reports of effects.
Low	There are some reports of indirect human health effects related to the hazard, and the nature and severity of the effects are fairly consistent; OR There are no reports of indirect human health effects and there are no effects related to the hazard reported for other mammals.
Moderate	There are some reports of indirect 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.
High	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).

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