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ENVIRONMENTAL AND INDIRECT HUMAN HEALTH RISK ASSESSMENT OF GLOFISH® STARFIRE RED®, ELECTRIC GREEN®, SUNBURST ORANGE®, AND GALACTIC PURPLE® BARBS (*PUNTIGRUS TETRAZONA*): TRANSGENIC ORNAMENTAL FISHES

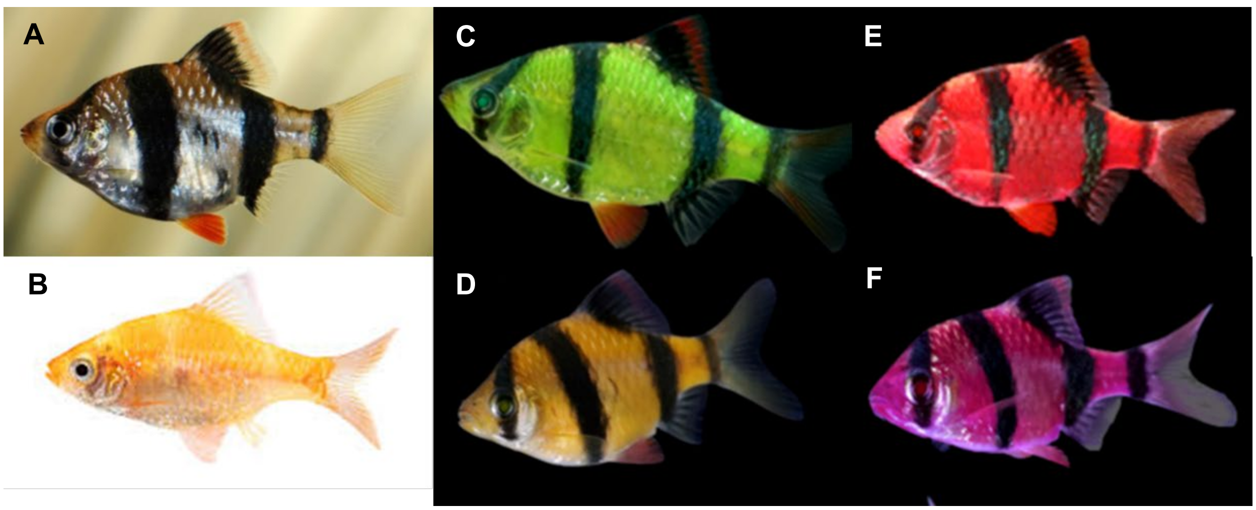

Figure 1. Some variants of Puntigrus tetrazona*. Common domesticated* P. tetrazona *(A), Albino domesticated* P. tetrazona *(B), Electric Green® Barb GB2011 (C), Sunburst Orange® Barb (D), Starfire Red® Barb (E), and Galactic Purple® Barb (F). All images provided by Spectrum Brands except for B which is taken from Petco.com.*

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 January 21, 2022, four notifications under the New Substances Notification Regulations (Organisms) (NSNR[O]) were submitted by Spectrum Brands to ECCC for evaluation of the GloFish® Electric Green® Barb (GB2011), the GloFish® Starfire Red® Barb (RB2015), the GloFish® Sunburst

[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.

November 2023

*Orange® Barb (OB2019), and the GloFish® Galactic Purple® Barb (PB2019), which are, respectively, lines of fluorescent green, red, orange, and purple genetically engineered Tiger Barbs (*Puntigrus tetrazona*), 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 to ECCC, and collaborates with HC to conduct an indirect human health risk assessment for any new living 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 13-14, 2022 Canadian Science Advisory Secretariat (CSAS) meeting: Environmental and Indirect Human Health Risk Assessment of the Glofish® Starfire Red®, Electric Green®, Sunburst Orange®, and Galactic Purple® Barbs: 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 six lines of GloFish® Tetra (*Gymnocorymbus ternetzi*) (DFO 2018, 2019), three lines of GloFish® Danio (*Danio rerio*) (DFO 2020a, 2020b), and three lines of GloFish® Bettas (*Betta splendens*) (DFO 2021).*

SUMMARY

- Pursuant to the *Canadian Environmental Protection Act* (CEPA), four notifications were submitted under the *New Substances Notification Regulations (Organisms)* (NSNR[O]) by Spectrum Brands to Environment and Climate Change Canada (ECCC) for genetically engineered *Puntigrus tetrazona*: GloFish® Electric Green® Barb (GB2011), GloFish® Starfire Red® Barb (RB2015), GloFish® Sunburst Orange® Barb (OB2019), and GloFish® Galactic Purple® Barb (PB2019).
- 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® Tetra, Danio, and Betta lines.

Environmental Risk Assessment

- The environmental exposure assessment concluded that the occurrence of GB2011, RB2015, OB2019, and PB2019 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 GB2011, RB2015, OB2019, and PB2019 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 *P. tetrazona* in North America.
- The environmental hazard assessment concluded that the hazards of GB2011, RB2015, OB2019, and PB2019 associated with environmental toxicity, trophic interactions,

hybridization, disease transmission, biodiversity, biogeochemical cycling, and habitat are negligible. There is low hazard associated with horizontal gene transfer.

- The uncertainty levels associated with the environmental hazard ratings range from low to moderate due to data limitations for the notified and surrogate organisms, and 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 GB2011, RB2015, OB2019, and PB2019 as 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 GB2011, RB2015, OB2019, and PB2019 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 (e.g., exposure through tank maintenance), potentially including vulnerable individuals (e.g., immunocompromised, children, those with medical conditions).
- Uncertainty associated with the IHH exposure assessment is moderate due to limited information regarding future import quantities, market uptake, and exposure scenarios in Canada.
- The IHH hazard assessment concluded that the indirect human hazard potential of GB2011, RB2015, OB2019, and PB2019 is low as there are no reported cases of zoonotic infections associated with the other commercially available GloFish® lines or wild-type *P. tetrazona* arising from aquarium use. Although the inserted genetic material was derived from some source organisms that 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 *P. tetrazona* and other ornamental aquarium fishes, and the lack of adverse effects supported by the history of safe use of all commercially available GloFish® lines and non-transgenic *P. tetrazona* 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 GB2011, RB2015, OB2019, and PB2019 as ornamental aquarium fish or other potential uses.

Conclusion and Summary

- Overall, GB2011, RB2015, OB2019, and PB2019 imported for aquarium use pose a low risk to the Canadian environment and the indirect human health of Canadians. Although there was moderate uncertainty associated with some of the assessment components, these do not affect confidence in the overall risk ratings. Assessment conclusions were consistent with those for the GloFish® Tetras, Danios, and Bettas.

BACKGROUND

On January 21, 2022, Spectrum Brands (a division of GloFish LLC) submitted four 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® Barb (GB2011), Starfire Red® Barb (RB2015), Sunburst Orange® Barb (OB2019), and Galactic Purple® Barb (PB2019); herein referred to collectively as the GloFish® Barbs (Figure 1). These ornamental fish are domesticated *Puntigrus tetrazona* (Tiger Barb) that have been genetically engineered to produce unique colours and fluorescence for use in home aquaria. They have been approved for use in the USA since 2012 (GB2011), 2016 (RB2015), and 2020 (OB2019 and PB2019). Note that similar risk assessments have been conducted in Canada for six different colours of GloFish® Tetras (DFO 2018, 2019), three different colours of GloFish® Danios (DFO 2020a, 2020b), and three different colours of GloFish® Bettas (DFO 2021).

Production of the notified lines

The GloFish® Barbs were produced using similar methodologies and testing protocols as the previously notified and approved GloFish® lines, except for the use of the CRISPR/Cas9 system in OB2019 and PB2019 lines, which previously had been used only with the Betta lines. Transgene expression cassettes containing genes for different coloured fluorescent proteins were incorporated into the genomes of the notified lines, resulting in ubiquitous colouration of the organisms under ambient light. All previous and current notified GloFish® lines have used similar transgene expression cassettes and elements (promoters, terminator sequences and fluorescent protein genes).

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.

Lines have been propagated through batch breeding in populations that contain both hemizygous and homozygous individuals, with non-fluorescent *P. tetrazona* removed from the population as they occur. The purpose of the modifications is to create new colour phenotypes of *P. tetrazona* for the ornamental aquarium trade.

Characterization of the notified organisms

Though greater detail regarding the development, structure, and function of the transgene constructs 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® Barb (GB2011)

GB2011 possesses a single site of insertion that contains multiple copies of a transgene construct. The genetic modification results in ubiquitous green colouration of the organism under ambient white light and fluorescent green under ultraviolet light (Figure 1). The notifier reports that GB2011 individuals that are hemizygous and homozygous for the transgene insert are indistinguishable from each other phenotypically and are both part of the commercially available population. Two other changes identified by the company are a decrease in reproductive success (in competition for mates with non-transgenic domesticated *P. tetrazona*), and diminished cold tolerance.

Starfire Red® Barb (RB2015)

RB2015 possesses a single site of insertion that contains multiple copies of a transgene construct. The genetic modification results in ubiquitous red colouration of the organism under ambient white light and fluorescent red under ultraviolet light (Figure 1). The notifier reports that RB2015 individuals that are hemizygous and homozygous for the transgene insert are indistinguishable from each other phenotypically and are both part of the commercially available population. One other change identified by the company is a decrease in reproductive success (in competition for mates with non-transgenic domesticated *P. tetrazona*).

Sunburst Orange® Barb (OB2019)

OB2019 possesses a single site of insertion that contains multiple copies of a tandem transgene construct. The genetic modification results in ubiquitous orange colouration of the organism under ambient white light and fluorescent orange under ultraviolet light (Figure 1). The notifier reports that OB2019 individuals that are hemizygous and homozygous for the transgene insert are indistinguishable from each other phenotypically and are both part of the commercially available population. Two other changes identified by the company are a decrease in reproductive success (in competition for mates with non-transgenic domesticated *P. tetrazona*), and diminished cold tolerance.

Galactic Purple® Barb (PB2019)

PB2019 possesses a single site of insertion that contains multiple copies of a tandem transgene construct, FP635. The genetic modification results in ubiquitous purple colouration of the organism under ambient white light and fluorescent purple under ultraviolet light (Figure 1). The notifier reports that PB2019 individuals that are hemizygous and homozygous for the transgene insert are indistinguishable from each other phenotypically and are both part of the commercially available population. One other change identified by the company is a decrease in reproductive success (in competition for mates with non-transgenic domesticated *P. tetrazona*).

Comparator species

For the purpose of this risk assessment, domesticated *Puntigrus tetrazona* (Tiger Barb) was selected as a comparator. *P. tetrazona* is a popular ornamental species that is produced and traded worldwide. Tiger Barbs are part of the family Cyprinidae and were first described from the Palembang region of Sumatra in 1855 as *Capoeta tetrazona* (Kottelat 2013). Since then, the scientific name has undergone numerous iterations, including: *Puntius tetrazona*, *Barbus tetrazona, Systomus tetrazona*, *Systomus sumatranus*, and *Systomus sumatrensis* (Froese and Pauly 2019). Of these, *Puntius tetrazona* is still in wide use within the scientific community, though taxonomic sources now recommend *Puntigrus tetrazona* (Kortmulder pers. comm., Kottelat 2013).

Tiger Barbs are likely native to Sumatra and Borneo (Froese and Pauly 2019), though species occurrences have been reported in other parts of Asia, including Thailand, Malaysia, and Cambodia (Tamaru et al. 1998). They are commonly found in shallow waters (approximately two feet) and along the banks of moderately flowing forest streams and tributaries with substrates of sand or rocks/pebbles of various sizes (Kortmulder 1972). Their temperature tolerance restricts them to tropical climates, where they prefer densely vegetated habitats, likely related to their reproductive strategy of depositing eggs on submerged vegetation (Innes 1979). Lakes where Tiger Barbs are found often contain swamps, and oxygen levels are generally low (Kortmulder 1982). Tiger Barbs are tolerant of a pH range of 6.0-8.0 (Vajargah and Rezaei 2015), but appear to prefer slightly acidic water (Sakurai et al. 1993), particularly when breeding. They are known to eat plants, crustaceans, and detritus (Mills and Vevers 1989). More recently, Tiger Barbs have been identified as effective predators of mosquito larvae under laboratory and semi-field conditions (Barik et al. 2018).

Tiger Barbs are considered stenotherms, capable of surviving in only a narrow range of environmental temperatures (Yanar et al. 2019). Recommended temperatures for Tiger Barb rearing range from 21.1°C to 26.7°C (Innes 1979) and 23°C to 28°C while breeding (Tamaru et al. 1998). Recent controlled studies have identified temperature tolerances for Tiger Barbs: Leggatt et al. (2018) found that 50% of individuals lost equilibrium (LD_{50}) at 13.20°C, and the average chronic lethal minimum temperature (CL_{min}) was 13.36°C, when acclimated initially at 20°C. Another study found when acclimated at temperatures between 20°C and 28°C, the critical thermal minimum (CT_{min}) of Tiger Barbs ranged from 11.66 to 13.94°C, and the critical thermal maximum (CT_{max}) ranged from 34.54 to 39.91°C (Yanar et al. 2019). Differences in reported cold tolerance between the studies may be due to different experimental procedures (i.e., rate of temperature decline), as well as potential differences in rearing history or background genetics (e.g., Tuckett et al. 2016). During slow temperature declines (i.e., 1°C per day), Tiger Barbs decreased activity at 19°C, decreased feeding below 17°C, and stopped feeding and activity below 14°C (Leggatt et al. 2018). As well, Liu et al. (2020) reported extensive tissue damage in the brain, gills, liver, and muscle of Tiger Barbs when temperature was dropped to 13°C.

Though occurrences of Tiger Barbs in the continental U.S. have been reported since the 1970s (Nico et al. 2019), there are no reports of establishment or reproduction, even with the discovery of a sexually mature male and female near a warm spring at ideal spawning temperatures (Dill and Cordone 1997). Recent studies in subtropical Florida using the Fish Invasiveness Screening Kit (FISK) (Copp et al. 2005) have assessed the invasion potential of Tiger Barbs as low to medium (Hill et al. 2014, Lawson 2015); invasion risk would likely be much lower in temperate climates.

Receiving environment

Though the many lakes and rivers of Canada vary in their annual temperature profiles, 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 above 6°C. If an introduced fish cannot survive at 4°C or below, its occurrence in the Canadian environment will be seasonal, though possible localized overwintering pockets can occur (e.g., in industrial effluent, hot springs, isolated lakes). During the summer, many Canadian lakes can reach surface temperatures above 20°C; however, only a few systems have been observed exceeding 25°C. It should also 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 GB2011, RB2015, OB2019 and PB2019 are consistent with previous risk assessments on GloFish® lines, and rankings and most uncertainty ratings are equivalent to those for the previously notified GloFish® lines (Table 1). New relevant evidence in the scientific literature and differences in the current GloFish® notifications have not altered previous risk conclusions. Detailed environmental risk assessments can be found at DFO 2018, 2019, 2020a, 2020b, 2021. An abbreviated summary of previous and current assessments follows.

Table 1. Summary of all ranks and uncertainty ratings for environmental risk assessments of currently notified GloFish® Barb lines, as well as previously notified GloFish® Tetras, Danios, and Bettas (DFO 2018, 2019, 2020a, 2020b, 2021). Underlines indicate where previous and current assessments differ. Neg refers to negligible rankings; Mod refers to moderate rankings.

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

Environmental exposure assessment

The exposure assessment for the four living organisms 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, once the notified organisms have been sold into the retail market, there can be no guarantee of appropriate containment and disposal. Given the high likelihood that GloFish® Barbs will be introduced into the Canadian environment, the extent to which the organisms are further exposed to the environment will depend heavily on their ability to survive and reproduce in Canadian lakes and rivers.

As a tropical species, *P. tetrazona* is not expected to survive across multiple seasons in a temperate region, where water temperatures trend below optimal. In the aquarium, Tiger Barbs are typically kept at temperatures between 21 and 27°C (see Comparator species). Data provided by the notifier indicate that when the water temperature drops relatively quickly, at least 50% of GloFish® Barbs are dead by the time temperatures reach 11.3°C, and 100% of fish are dead before temperatures reach 10°C. These results are consistent with temperature tolerance metrics for non-transgenic domesticated *P. tetrazona* from the primary literature (Leggatt et al. 2018; Yanar et al. 2019).

There are no lakes in Canada that consistently remain above 7°C throughout the year, or above 6°C across multiple years and almost none remain above 4°C throughout the year (Leggatt et al. 2018). Consequently, while the temperatures needed for the notified lines to survive may occur in several Canadian lakes during the summer, there is a very low likelihood that GB2011, RB2015, OB2019, and PB2019 can survive the Canadian winter. At best, the occurrence of GloFish® Barbs in the Canadian freshwater environment would be ephemeral.

Though water temperatures in Canada will limit the persistence of any GloFish® Barbs that are introduced into the environment, there may still be time to reproduce if introduced at the start of a warm season. Ideal temperatures for GloFish® Barb reproduction (23 to 28°C; Tamaru et al. 1998) exist seasonally in select lakes and isolated areas such as shorelines across Canada. For example, Osoyoos Lake in the BC interior is one of Canada's warmest lakes in the summer, with average temperatures above 20°C for two months of the year, and higher temperatures (e.g., 25°C) restricted to a period of several weeks (BCLSS 2021).

Given the above analysis, the occurrence of GloFish® Barbs in the Canadian environment is expected to be rare, isolated, and ephemeral. Consequently, the likelihood of exposure of GloFish® Barbs 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® Barbs and valid surrogate organisms, evidence of low variability, and data available on the environmental parameters of the receiving environment in Canada. This rating is consistent with the low exposure rating with low uncertainty for six lines of GloFish® Tetra (DFO 2018, 2019), three lines of GloFish® Danio (DFO 2020a, 2020b), and three lines of GloFish® Betta (DFO 2021).

Environmental hazard assessment

The hazard assessment examines potential impacts to the environment that could result from exposure to GloFish® Barbs. 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 unintended mutations as a result of CRISPR/Cas9 use.

A report screening the amino acid sequence of the fluorescent protein found no functional matches to known human allergens. There have been no reported toxic effects resulting from exposure to GloFish® Barbs or any other species of GloFish® containing transgenes coding the same proteins as those in the GloFish® Barb lines in either Canada or the USA. Consequently, the potential hazard to the environment due to environmental toxicity of GloFish® Barbs 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.

Graham and Davis (2021) recently demonstrated that HGT can occur between higher organisms; however, the present transgenes would not be expected to proliferate throughout a population due to a lack of fitness advantage. Based on the rare reports of harmful effects from fluorescence transgenes despite relatively wide usage, any possible introduction of the discussed transgenes to a novel host through HGT is not expected to result in harmful effects. The low predicted occurrence, permanence, and associated harm of an HGT event results in a hazard ranking of **low**. The unknown location of the transgenes within the *P. tetrazona* genome, and lack of studies directly examining HGT of the transgenes and resulting consequences, results in **moderate** uncertainty.

Should GloFish® Barbs 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. Tiger Barbs are known to eat plants, crustaceans, detritus and mosquito larvae (Mills and Vevers 1989; Barik et al. 2018), and often exhibit agonistic behaviours towards conspecifics and larger fishes (Innes 1979; Kortmulder 1972; Sakurai et al. 1993). 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 *P. tetrazona* in cooler waters, and lack of noted alterations in trophic-related behaviour of the notified lines, GloFish® Barbs 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® Barbs, and poor understanding of Genotype x Environment interactions in aggression and predation susceptibility, result in a **moderate** level of uncertainty.

P. tetrazona is a member of the taxonomic family Cyprinidae, with 53 species across several genera occurring in Canada. Intergeneric hybridization has been observed between two genera of Cyprinidae in Europe (Hayden et al. 2010), suggesting hybrids between *P. tetrazona* and Canadian cyprinids could be possible. Attempted *in vitro* crosses of *P. tetrazona* and closely related Cyprinidae genera [Tinfoil Barb (*Barbonymus schwanenfeldii*), Cherry Barb (*Puntius titteya*), and Rosy Barb (*Pethia conchonius*)] have not resulted in viable offspring, and greater phylogenetic distance is expected to result in decreased hybrid viability (Kirschbaum et al. 2016). Given that Canadian genera of Cyprinidae are more distantly related to *P. tetrazona* than the above genera, it is unlikely that Canadian cyprinids would form viable hybrids with *P. tetrazona*. Tiger Barbs are expected to experience further reproductive isolation from native cyprinids due to direct egg fertilization (not broadcast spawning) and incompatible breeding temperature preferences. Consequently, there is **negligible** potential for the GloFish® Barbs to cause hazards through hybridization with native fish in Canada. High quality information on the distribution of Cyprinidae and breeding requirements of *P. tetrazona*, and some data on intergeneric hybridization result in **low** uncertainty associated with this rating.

Whether GloFish® Barbs, or any other transgenic fluorescent organism, have altered ability to act as a vector of disease agents has not been directly examined; however, some studies of fluorescent cultured cell models have reported potential altered disease susceptibility. For example, GFP expression has been shown to decrease T-cell activation (Koelsch et al. 2013), induce cytokine IL-6 secretion (Mak et al. 2007), inhibit immune-related signaling pathways (Baens et al. 2006), and alter expression of genes involved in immune function (Coumans et al. 2014). As well, Chou et al. (2015) reported mice transgenic for DsRed had altered lymphocyte and monocyte counts. Consequently, there is **negligible** potential for GloFish® Barbs to have altered capacity as a vector for disease relative to non-transgenic *P. tetrazona*. As this has not been directly examined in GloFish® Barbs, there is limited data on a surrogate, and reliance on expert opinion, the uncertainty level for this rating is **moderate**.

GloFish® Barbs are expected to contribute to nutrient cycles within habitats through ingestion of prey and other food items and release of waste. While eGFP transgenic mice have altered urea cycling, nucleic acid and amino acid metabolism, and energy utilization (Li et al. 2013), similar alterations have not been noted or investigated in GloFish® Barbs. Given the small size of *P. tetrazona* and potential low numbers of individuals anticipated to enter an ecosystem, GloFish® Barbs have a **negligible** potential 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.

Tiger Barbs do not build structures that are expected to impact habitats of other species. There have been no reports, anecdotal or otherwise, of GloFish® Barbs having altered behaviour, relative to domesticated *P. tetrazona*, that may influence effects on habitat structure. Consequently, GloFish® Barbs are expected to have **negligible** effects to habitat with **low** uncertainty associated with this rating.

GloFish® Barbs are not expected to negatively impact native biodiversity through trophic or hybrid interactions, act as a vector for disease agents of concern in Canada, impact biogeochemical cycling, or impact habitat. Addition of the transgenic construct and fluorescent protein in GloFish® Barbs is not expected to result in environmental toxicity, or cause hazards through HGT of the transgene. Taken together, there is a **negligible** hazard of GloFish® Barbs 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 1), while uncertainty ranged from low to moderate, due to limited data specific to GloFish® Barbs, limited direct data on comparator species, variable data from surrogate models, and the reliance on expert opinion for the assessment of some hazards. All GloFish® Barb hazard and uncertainty rankings concur with rankings for previously notified GloFish® Tetras, Danios, and Bettas with the following exceptions: the uncertainty ranking associated with HGT was increased relative to the Tetras due to increased acknowledgement of data limitations, and the low uncertainty associated with hybridization differs from Tetras and Bettas (negligible) that lack Canadian confamiliars, and Danios (moderate) that broadcast spawn and lack data on intergeneric hybridization (DFO 2018, 2019, 2020a, 2020b, 2021). GloFish® Barbs are not expected to pose unique hazards beyond those of the intended use as ornamental fish in static aquaria.

Use of CRISPR/Cas9 in the creation of two of the GloFish® Barb lines adds additional uncertainty to the overall hazard assessment from potential unintended (on- and off-target) mutations in the Barb populations. Mutations could theoretically result in altered protein structure or expression that alters the phenotype of Barbs and may have downstream consequences to the environment. The potential for unintended mutations from CRISPR use has been discussed for other models in the context of potential harm or toxicity to the organism itself, and phenotypes of these mutations, when examined, are generally neutral or negative. Possible harmful effects of unintended mutations to the environment have not been examined experimentally or reported in other models, nor are there anecdotal reports of individuals in the GloFish® Barb populations having altered phenotypes that may result in environmental harm. While this additional consideration does not alter any hazard ratings for the GloFish® Barbs, it does increase 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 4. 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.

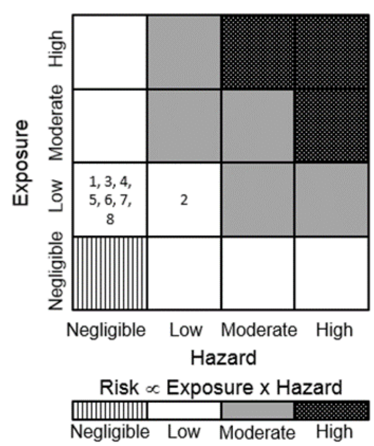

Figure 2. Risk matrix and pattern scale to illustrate how exposure and hazard are integrated to establish a level of risk in the environmental risk assessment. Risk assessments associated with assessed hazard components at the assessed exposure level are identified by number: 1) through environmental 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 concludes that GloFish® Barbs used in the ornamental aquarium trade or for 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 of GloFish® Barbs overwintering in Canadian aquatic ecosystems. As such, any exposure of Canadian freshwater ecosystems to GloFish® Barbs is expected to be isolated, rare, and ephemeral. The quality of data demonstrating a lack of cold tolerance in

GloFish® Barbs and domesticated *P. tetrazona*, relative to Canadian freshwater temperatures result in low uncertainty associated with this ranking.

The hazard assessment concluded that GloFish® Barbs pose negligible to low hazard to the Canadian environment, due to the lack of hazard associated with domesticated *P. tetrazona*, and no direct evidence that the expressed fluorescent protein would increase hazard, relative to domesticated *P. tetrazona*. Uncertainty rankings associated with individual hazard components ranged from negligible to moderate, due to limited data specific to GloFish® Barbs, 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® Barbs 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® Barbs 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, Tetras, and Bettas (DFO 2018, 2019, 2020a, 2020b, 2021).

RISK ASSESSMENT – INDIRECT HUMAN HEALTH

This risk assessment examines the potential for GB2011, RB2015, OB2019, and PB2019 to cause harmful effects to humans in Canada, relative to wild-type *P. tetrazona*, as a consequence of environmental exposure, including exposure in natural environments and environments related to its intended use (i.e., home aquaria).

Indirect human health exposure, hazard, and risk assessment conclusions for GB2011, RB2015, OB2019, and PB2019 are consistent with previous risk assessments on similar notified GloFish® Tetra and Danio lines (see Table 2). No new relevant evidence has been reported in the scientific literature, and no differences have been noted in the GloFish® Barb notifications relative to previously notified GloFish® lines that would alter indirect human health risk conclusions. As with the environmental risk assessment, the use of CRISPR/Cas9 during line creation may have produced unintended 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 Barb lines, six previously notified GloFish® Tetra lines, three previously notified GloFish® Danio lines, and three previously notified GloFish® Betta lines (DFO 2018, 2019, 2020a, 2020b, 2021).

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

Indirect Human Health Exposure Assessment

Risks from workplace exposure to the notified strain are not considered in this assessment[2].

The human exposure potential of GB2011, RB2015, OB2019, and PB2019 is assessed to be low to medium because:

1. The primary sources of human exposures would stem from the proposed import of fish through unidentified points of entry in Canada and distribution through retail outlets;
2. The sole intended use of GB2011, RB2015, OB2019, and PB2019 is as ornamental aquarium fish, thus limiting potential exposure primarily to those possessing a home aquarium;
3. Like other aquarium fish, human exposure may include immunosuppressed individuals, children, those with underlying medical conditions, or other vulnerable individuals. Due to *P. tetrazona's* aggressive behaviour when kept in small numbers, keeping groups of five or more fish is recommended;
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. Low winter water temperatures in Canadian waters and low cold tolerance of notified fish limits human exposure through the environment; and
5. No significant increase in human exposure is expected from other potential uses of GB2011, RB2015, OB2019, and PB2019, such as mosquito control or 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

Uncertainty ranking associated with the information used to assess indirect human health exposure for GB2011, RB2015, OB2019, and PB2019 is presented in Table 2. 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 (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 limited by a lack of information on the number of notified organisms to be imported in subsequent years and poor survey data on household ownership of ornamental fish. It is therefore difficult to gauge public uptake and popularity beyond the import number in the first year. Furthermore, data on 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 GB2011, RB2015, OB2019, and PB2019 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.

Indirect Human Health Hazard Assessment

The human health hazard potential of GB2011, RB2015, OB2019, and PB2019 is assessed to be low (Table 1) because:

1. GB2011, OB2019, PB2019, and RB2015 are genetically modified tropical fish containing copies of transgene constructs at a single site of insertion (although alternate insert patterns may exist in the population) that were confirmed to be stably integrated through multiple crossings;
2. The methods used to produce GB2011, RB2015, OB2019, and PB2019 do not raise any indirect human health concerns. However, the potential for off-target effects from use of CRISPR/Cas9 in OB2019 and PB2019 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® or to wild-type Tiger Barbs;
4. Sequence identities of the inserted transgenes do not match any known allergens or toxins. Amino acid sequences of the four fluorescent proteins are identical to those used in previously assessed GloFish® lines. While analyses conducted on the other potential reading frames found potential matches in both GB2011 and PB2019, the results suggest there is little evidence for cross-reactivity; and
5. There is a history of safe use for the notified lines (while limited for OB2019 and PB2019 due to their recent introduction, no additional safety concerns are anticipated compared to GB2011 and RB2015) in the United States and the wild-type species has been safely used globally as an ornamental aquarium fish since the 1950s.

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 also was provided describing in good detail the methods used to genetically modify the wild-type *P. tetrazona* including the sources of the genetic materials and the stability of the resulting genotypes and phenotypes. Sequence analysis of the inserted transgene constructs for the four notified lines did not match any toxins or allergens and no reports were found of adverse effects in humans attributed to the inserted proteins.

While there were no reports of adverse human health effects directly associated with the notified organisms, surrogate information from the literature on other ornamental fish appear to indicate the potential for transmission of human pathogens. However, such cases of infections are common to all ornamental aquarium fish and are not unique to Tiger Barbs. 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 GB2011, RB2015, OB2019, and PB2019 is considered to be **low** with **low uncertainty**. While there is a theoretical possibility that unintended mutations from the use of CRISPR/Cas9 could produce unknown effects, such as altered proteins with increased allergenicity, 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 are based on reports from other ornamental aquarium fish, there is a limited history of safe use for two of the notified lines (OB2019 and PB2019), and there are no particular studies that have investigated human health effects associated with fluorescent transgenic ornamental fish.

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 Tiger Barbs are popular in home aquaria with a long history of safe use having been sold as aquarium fish since the 1950s (Innes 1950). The four notified lines received Enforcement Discretion decisions by the U.S. Food and Drug Administration (USFDA) in 2011 (GB2011), 2016 (RB2015), and 2020 (OB2019, PB2019), and GB2011 has been commercially available in the United States since early 2012. The fluorescent proteins used in the four notified lines have been used in other GloFish® lines that are now commercially available in Canada. There are no reported adverse human health effects associated with wild type Tiger Barbs in general, the inserted fluorescent protein genes, or 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 GB2011, RB2015, OB2019, and PB2019 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 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 any other typical aquarium fish.

Risk Assessment Conclusions

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 GB2011, RB2015, OB2019, and PB2019 as ornamental aquarium fish or any other potential uses. This risk to human health associated with GB2011, RB2015, OB2019, and PB2019 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 lines of GloFish® Tetras (DFO 2018, 2019), Danios (DFO 2020a, 2020b), and Bettas (DFO 2021).

SOURCES OF UNCERTAINTY

Sources of uncertainty in the environmental exposure and hazard assessments that may influence uncertainty in the risk assessment include the 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 components (e.g., impacts through vector of disease agents).

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 GB2011, RB2015, OB2019, and PB2019 specifically.

In both assessments, the potential unintended effects from use of CRISPR/Cas9 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, or effects to the target region, in other CRISPR 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® Barbs in home aquaria is expected to result in potential repeated, but very small magnitude, releases to the Canadian environment. However, data available indicate GloFish® Barbs do not have 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 *P. tetrazona* despite long-term extensive use, as well as lack of evidence for increased hazards of GloFish® Barbs relative to non-transgenic fish, indicates ratings of negligible to low hazard of GloFish® Barbs to Canadian environments with negligible to moderate uncertainty. Taken together, the overall risk of GB2011, RB2015, OB2019, or PB2019 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.

Use of GloFish® Barbs for home aquaria is expected with moderate uncertainty to result in low to medium exposure to humans, primarily through tank maintenance by those who care for the fish. The hazard of GloFish® Barbs to indirect human health is ranked low (with low uncertainty), due to lack of pathogenicity, allergenicity or toxicity associated with the genetic modification, and the 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® Barbs as ornamental aquarium fish or in other identified potential uses.

The import of GloFish® Barbs into Canada, for use in the ornamental aquarium trade and home aquaria, is expected to pose low risk to the Canadian environment and indirect human health. 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 suggested GloFish® Barbs 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 unintended mutations from use of CRISPR/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 the environment and indirect human health 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® Barbs to overwinter in Canada. 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® Barbs. Increased winter water temperatures in the few isolated lakes with infrequent ice coverage in Southwestern BC is not expected to increase the potential for overwinter survival of GloFish® Barbs.

The use of certain elements in the constructs of all notified GloFish® lines may have Canadian Food Inspection Agency regulatory implications, but is not expected to influence environmental or indirect human health risk.

The current assessment highlights some of the unknowns regarding risk-relevant effects of off- or on-target mutations from use of gene editing (e.g., CRISPR) to produce genetically engineered fish. Research is required in this area, particularly for CRISPR-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 13-14, 2022 National Advisory Meeting on the Environmental and Indirect Human Health Risk Assessment of the Glofish® Starfire Red®, Electric Green®, Sunburst Orange®, and Galactic Purple® Barbs: 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.

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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 Ranking	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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