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Environmental Risk Assessment of the GloFish® Starfire Red®, Electric Green®, Sunburst Orange®, and Galactic Purple® Barbs (*Puntigrus tetrazona*): Transgenic Ornamental Fishes

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Foreword

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

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LIST OF ACRONYMS

bp: Base pair

Cas9: CRISPR-associated protein 9

CBA: Carp β-actin

CEPA: *Canadian Environmental Protection Act*

CFIA: Canadian Food Inspection Agency

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

DNA: Deoxyribonucleic acid

DFO: Fisheries and Oceans Canada

ECCC: Environment and Climate Change Canada

eGFP: Enhanced green fluorescent protein

GE: Genetically engineered

GxE: Genotype by environment interaction

gRNA: Guide RNA

HC: Health Canada

HGT: Horizontal gene transfer

kb: Kilobase – 1000 base pairs of DNA

LD_{50}: Lethal dose (temperature) that causes 50% population mortality

LD_{100}: Lethal dose (temperature) that causes 100% population mortality

MOU*:* Memorandum of Understanding

mRNA: Messenger RNA

µS/cm: Microsiemens per centimeter

NSNR(O): *New Substances Notification Regulations (Organisms)*

RFP: Red fluorescent protein

RNA: Ribonucleic acid

SEM: Standard error of the mean

zMLC: Zebrafish myosin light chain

ABSTRACT

Pursuant to the *Canadian Environmental Protection Act* (CEPA), a notification under the *New Substances Notification Regulations (Organisms)* (NSNR(O)) was submitted by Spectrum Brands to Environment and Climate Change Canada (ECCC) for the import of four genetically engineered lines of Tiger Barb (*Puntigrus tetrazona*) called the GloFish® Electric Green® Barb (GB2011), Starfire Red® Barb (RB2015), Sunburst Orange® Barb (OB2019), and Galactic Purple® Barb (PB2019), for commercial sale in Canada. The environmental risk assessment analyzed potential hazards, likelihood of exposure, and associated uncertainties to reach a conclusion on risk. 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 to the Canadian environment is ranked low. Uncertainty associated with the exposure assessment is low, given the available data for temperature tolerance of the notified lines and relevant comparators, and lack of establishment of non-transgenic *P. tetrazona* in North America despite a long history of use. The environmental hazard assessment concluded that potential hazards linked with environmental toxicity, trophic interactions, hybridization, disease, biodiversity, biogeochemical cycling, and habitat are negligible. There is low hazard (i.e., no anticipated harmful effects) related with horizontal gene transfer. Uncertainty associated with the environmental hazard rankings range from low to moderate due to data limitations for the notified and surrogate organisms, and some reliance on expert opinion and anecdotal evidence. The use of CRISPR during the creation of OB2019 and PB2019 may have resulted in unintended mutations in the GloFish® Barb populations, adding to uncertainty in the hazard assessment, but without altering the overall conclusions on risk. 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.

1. INTRODUCTION

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, Starfire Red® Barb, Sunburst Orange® Barb, and Galactic Purple® Barb; herein referred to collectively as the GloFish® Barbs. These ornamental fish are domesticated *Puntigrus tetrazona* (Tiger Barb, formerly *Puntius tetrazona*) that have been genetically engineered to fluoresce different colours in home aquaria. Similar risk assessments have been conducted on 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).

The biotechnology provisions of CEPA take a preventative approach to pollution by requiring all new living products of biotechnology, including genetically engineered fish, to be notified and assessed prior to import or manufacture in Canada, to ultimately determine whether they are "toxic" or capable of becoming "toxic". Under CEPA (Section 64), an organism is considered "toxic" if it can 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 or its biological diversity; (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. Anyone proposing to import or manufacture a living animal product of biotechnology in Canada, including genetically engineered fish, is required to provide ECCC with the information prescribed in NSNR(O) at least 120 days prior to the commencement of import or manufacture of the organism. This information is used to conduct an environmental risk assessment and an assessment of indirect human health (risk to human health from environmental exposure to the living organism), which are then used to determine whether the organism is CEPA-toxic or capable of becoming CEPA-toxic.

Under a memorandum of understanding with ECCC and Health Canada (HC), DFO provides science advice in the form of an environmental risk assessment for fish products of biotechnology under the NSNR(O). This advice is used to inform the CEPA risk assessment conducted by ECCC and HC. Under this arrangement, the Minister of Environment and Climate Change receives scientific advice from DFO and retains ultimate responsibility for regulatory decision making on the use of notified fish.

It is in this context that DFO conducted this environmental risk assessment of the notified organisms under the proposed use. Here, Risk is defined as a function of the potential for Canadian environments to be exposed to the notified organisms and the potential for the notified organisms to pose hazards to the Canadian environment. Exposure and Hazard assessments are conducted separately and then integrated into an assessment of Risk. Uncertainty in Exposure and Hazard assessments are determined, and uncertainty associated with the final risk assessment is discussed.

2. CHARACTERIZATION OF THE NOTIFIED ORGANISMS

In its current notifications, Spectrum Brands is requesting the import of four new transgenic strains of *P. tetrazona* from the US, for the ornamental aquarium trade in Canada. Trade names for the transgenic organisms are the GloFish® Electric Green® Barb (GB2011), Starfire Red® Barb (RB2015), Sunburst Orange® Barb (OB2019), and Galactic Purple® Barb (PB2019). Figure 1 demonstrates the physical appearance of the four notified GloFish® Barb strains, as well as a

non-transgenic, albino domesticated Tiger Barb (*P. tetrazona*) (background genotype or recipient of transgene) and common domesticated *P. tetrazona*.

Though greater detail regarding the structure, development, and function of the transgene constructs used to create the GloFish® Bettas has been provided by the company for review, it is considered confidential business information and is not included in this report.

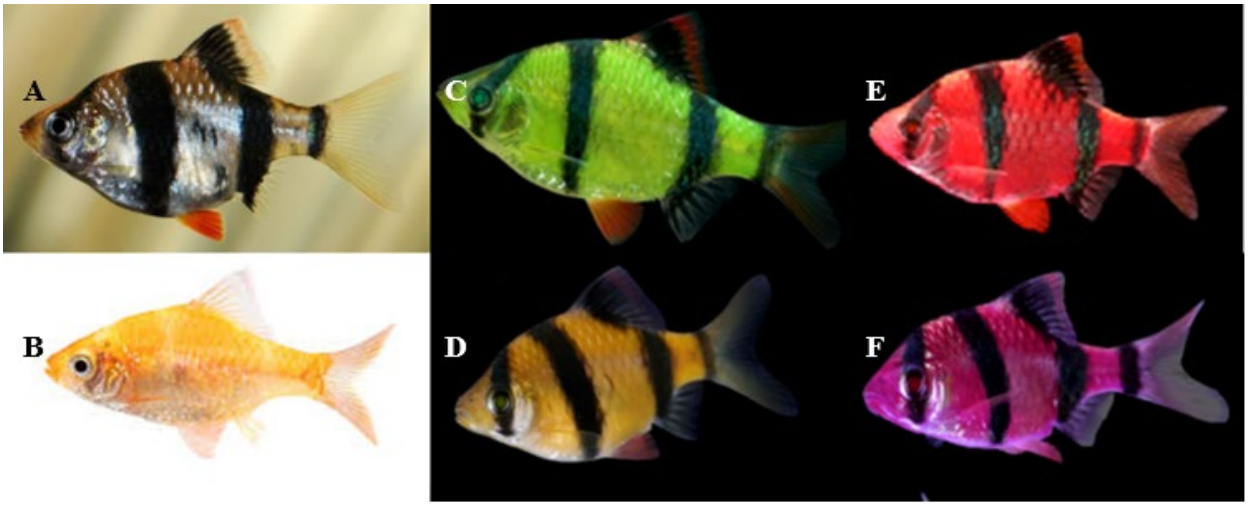

Figure 1. Some variants of Puntigrus tetrazona. Common domesticated P. tetrazona (A), albino domesticated P. tetrazona (B), Electric Green® Barb (C), Sunburst Orange® Barb (D), Starfire Red® Barb (E), and Galactic Purple® Barb (F). All images provided by Spectrum Brands except for B which is taken from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

2.1. ELECTRIC GREEN® BARB (GB2011)

2.1.1. Molecular Characterization

GB2011 is a genetically engineered Tiger Barb (*P. tetrazona*) possessing multiple copies of a transgene insert. The genetic modification results in ubiquitous green colouration of the organism under ambient white light and green fluorescence under ultraviolet light (Figure 1). The purpose of the modification is to create a new colour phenotype of *P. tetrazona* for the ornamental aquarium trade.

2.1.1.1. Production of the notified organism

The purified transgene expression cassette was injected into newly fertilized eggs of albino P. tetrazona.

Further details provided by the company that describe line development and analysis to confirm that GBS2019 constitutes a single homogeneous line and that the vector backbone was not incorporated along with the transgenes are considered confidential business information and are not reported here.

2.1.1.2. Characterization of the transgene integrant

The sequence of the cassette as it is inserted into the genome of GB2011 has not been determined, and the specific location of the insert within the GB2011 genome is unknown.

Details regarding the analysis to confirm that multiple copies of the transgene cassette were incorporated at a single insert location are considered confidential business information and are not reported here.

2.1.1.3. Inheritance and stability of the transgene

The specific insert location of the transgene has not been determined and it is unknown whether it has inserted into a stable genome location or in an area prone to silencing. Should transgene expression be silenced in an individual, it would not display the green colouration and would consequently be removed from the breeding population.

The company has maintained this breeding line for over four generations and have produced GB2011 commercially since 2012. Over this time, they have observed the green fluorescent phenotype to be durable and stable across generations.

2.1.1.4. Methods to detect the transgene

GB2011 individuals are distinguished from non-transgenic domesticated *P. tetrazona* by their green colouration under natural light and fluorescence under blue or UV light. GB2011 can be further identified genetically by PCR amplification of a unique section of the cassette followed by a restriction digest to generate unique fragments that can be separated into a series of bands that distinguish GB2011 from other transgenic fluorescent green Barbs if they are carrying a different cassette.

2.1.2. Phenotypic Characterization

2.1.2.1. Targeted phenotypic effects of the modification

The targeted phenotypic effect of the genetic modification is that GB2011 appears green under ambient light and fluorescent under UV or blue light. The novel colour phenotype is present in muscle as well as the skin and eyes. 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.

2.1.2.2. Additional phenotypic effects of modification

No formal studies have compared potential disease susceptibility of GB2011 and non-transgenic strains. There are also no formal studies on potential unintended phenotypic effects of genetic modification on life history (other than reproductive success and juvenile survival), environmental tolerances and requirements (other than low-temperature tolerance), metabolism, physiology, endocrinology, or behaviour; however, there are no anecdotal or otherwise reports of any unintended phenotypic effects other than those listed above.

2.2. STARFIRE RED® BARB (RB2015)

2.2.1. Molecular Characterization

RB2015 is a genetically engineered Tiger Barb (*Puntigrus tetrazona*) that contains multiple copies of a transgene insert. The genetic modification results in ubiquitous red colouration of the organism under ambient white light and red fluorescence under ultraviolet light (Figure 1). The purpose of the modification is to create a new colour phenotype of *P. tetrazona* for the ornamental aquarium trade.

2.2.1.1. Production of the notified organism

The purified transgene expression cassette was injected into newly fertilized eggs of albino *P. tetrazona*.

Further details provided by the company that describe line development and analysis to confirm that RB2015 constitutes a single homogeneous line and that the vector backbone was not incorporated along with the transgenes are considered confidential business information and are not reported here.

2.2.1.2. Characterization of the transgene integrant

The sequence of the cassette as it is inserted into the genome of RB2015 has not been determined, and the specific location of the insert within the RB2015 genome is unknown.

Details regarding the analysis to confirm that multiple copies of the transgene cassette were incorporated at a single insert location are considered confidential business information and are not reported here.

Transgene copy number was estimated using quantitative real-time PCR (qPCR). Results indicate that there are multiple copies of the transgene construct were incorporated into the genome of RB2015 fish.

2.2.1.3. Inheritance and stability of the transgene

The specific insert location of the transgene has not been determined and it is unknown whether it has inserted into a stable genome location or in an area prone to silencing. Should transgene expression be silenced in an individual, it would not display the red colouration and would consequently be removed from the breeding population.

The company has maintained this breeding line for over four generations and has produced RB2015 commercially since 2016. Over this time, they have observed the red fluorescent phenotype to be durable and stable across generations.

2.2.1.4. Methods to detect the transgene

RB2015 individuals are distinguished from non-transgenic domesticated *P. tetrazona* by their red colouration under natural light and fluorescence under blue or UV light. RB2015 can be further identified genetically by PCR amplification of a unique fragments of the transgene insert, followed by a restriction enzyme digest to generate unique fragments that can be separated into a series of bands that distinguish RB2015 from other transgenic fluorescent red Barbs if they are carrying a different cassette.

2.2.2. Phenotypic Characterization

2.2.2.1. Targeted phenotypic effects of the modification

The targeted phenotypic effect of the genetic modification is that RB2015 appears red under ambient light and fluorescent under UV or blue light. The novel colour phenotype is present in muscle as well as the skin and eyes. Spectrum Brands 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.

2.2.2.2. Additional phenotypic effects of modification

The influence of the genetic modification on any other phenotypes, including survival, has not been formally examined.

No formal studies have compared potential disease susceptibility of RB2015 and non-transgenic strains. There are also no formal studies on potential unintended phenotypic effects of genetic modification on life history (other than reproductive success), environmental tolerances and requirements (other than low-temperature tolerance), metabolism, physiology, endocrinology, or behaviour; however, there are no anecdotal or otherwise reports of any unintended phenotypic effects.

Confidential data, submitted by the company as part of its regulatory package, suggest that PiBS2019 is no more likely to be invasive than non-transgenic, domesticated *P. tetrazona.*

2.3. SUNBURST ORANGE® BARB (OB2019)

2.3.1. Molecular Characterization

OB2019 is a genetically engineered Tiger Barb (*Puntigrus tetrazona*) possessing a single site of insertion that contains multiple copies of a tandem transgene fish-origin construct. The genetic modification results in ubiquitous orange colouration of the organism under ambient white light and orange fluorescence under ultraviolet light (Figure 1). The purpose of the modification is to create a new colour phenotype of *P. tetrazona* for the ornamental aquarium trade.

2.3.1.1. Production of the notified organism

The purified transgene expression cassette was injected into newly fertilized eggs of albino *P. tetrazona*, along with Cas9 protein and a guide RNA.

Further details provided by the company that describe line development and analysis to confirm that OB2019 constitutes a single homogeneous line, and that the vector backbone was not incorporated along with the transgenes, are considered confidential business information and are not reported here.

2.3.1.2. Characterization of the transgene integrant

The sequence of the cassette as it is inserted into the genome of OB2019 has not been determined, and the specific location of the insert within the OB2019 genome is unknown.

There are no data examining whether on- or off-target mutations exist in OB2019 as a result of the attempted CRISPR gene editing.

Details regarding the analysis to confirm that multiple copies of the transgene cassette were incorporated at a single insert location are considered confidential business information and are not reported here.

2.3.1.3. Inheritance and stability of the transgene

The specific insert location of the transgene has not been determined and it is unknown whether it has inserted into a stable genome location or in an area prone to silencing. Should transgene expression be silenced in an individual, it would not display the orange colouration and would consequently be removed from the breeding population.

The company has maintained this breeding line for over four generations and has produced OB2019 commercially since 2020. Over this time, they have observed the orange fluorescent phenotype to be durable and stable across generations.

2.3.1.4. Methods to detect the transgene

OB2019 individuals are distinguished from non-transgenic domesticated *P. tetrazona* by their orange colouration under natural light and fluorescence under blue or UV light. OB2019 can be further identified genetically by PCR amplification of a unique section of the cassette and detection of unique fragments following a restriction enzyme digest. When digested with the restriction enzyme fragments can be separated into a series of bands that distinguish OB2019 from other transgenic fluorescent orange Barbs if they are carrying a different cassette.

2.3.2. Phenotypic Characterization

2.3.2.1. Targeted phenotypic effects of the modification

The targeted phenotypic effect of the genetic modification is that OB2019 appears orange under ambient light and fluorescent under UV or blue light. The novel colour phenotype is present in muscle as well as the skin and eyes 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.

2.3.2.2. Additional phenotypic effects of modification

The influence of the genetic modification on any other phenotypes, including survival, has not been formally examined.

No formal studies have compared potential disease susceptibility of OB2019 and non-transgenic strains. There are also no formal studies on potential unintended phenotypic effects of genetic modification on life history (other than reproductive success), environmental tolerances and requirements (other than low-temperature tolerance), metabolism, physiology, endocrinology, or behaviour; however, there are no anecdotal or otherwise reports of any unintended phenotypic effects other than those listed above.

Confidential data, submitted by the company as part of its regulatory package, suggest that OB2019 is no more likely to be invasive than non-transgenic, domesticated *P. tetrazona*.

2.4. GALACTIC PURPLE® BARB (PB2019)

2.4.1. Molecular Characterization

PB2019 is a genetically engineered Tiger Barb (*P. tetrazona*) possessing a single site of insertion that contains multiple copies of a transgene construct. The genetic modification results in ubiquitous purple colouration of the organism under ambient white light and purple fluorescence under ultraviolet light (Figure 1). The purpose of the modification is to create a new colour phenotype of *P. tetrazona* for the ornamental aquarium trade.

2.4.1.1. Production of the notified organism

The purified transgene expression cassette was mixed into a solution of Cas9 protein and a guide RNA, then injected into newly fertilized eggs of *P. tetrazona*. The Cas9 protein, directed by the guide RNA, was expected to cleave both strands of DNA at a site upstream of a gene that is most similar to the β-actin 2 gene. The gene construct was expected to be inserted at this location as a result of the homology arms included at the ends of the gene construct and the organism's own homology-directed DNA repair mechanism.

Further details provided by the company that describe line development and analysis to confirm that PB2019 constitutes a single homogeneous line, and that the vector backbone

was not incorporated along with the transgenes, are considered confidential business information and are not reported here.

2.4.1.2. Characterization of the transgene integrant

The sequence of the cassette as it is inserted into the genome of PB2019 has not been determined, and the specific location of the insert within the PB2019 genome is unknown.

Though elements used in the production of OB2019, such as Cas9 protein and guide RNA and homologues regions were included to encourage site-directed insertion of the cassette into the Tiger Barb genome, subsequent analysis (sequencing) of the targeted region indicated the construct had inserted elsewhere. There are no data examining whether off-target Cas9 mutations exist in OB2019.

Details regarding the analysis to confirm that multiple copies of the transgene cassette were incorporated at a single insert location are considered confidential business information and are not reported here.

2.4.1.3. Inheritance and stability of the transgene

The specific insert location of the transgene has not been determined and it is unknown whether it has inserted into a stable genome location or in an area prone to silencing. Should transgene expression be silenced in an individual, it would not display the purple colouration and would consequently be removed from the breeding population.

The company has maintained this breeding line for over four generations and has produced PB2019 commercially since 2020. Over this time, they have observed the purple fluorescent phenotype to be durable and stable across generations.

2.4.1.4. Methods to detect the transgene

PB2019 individuals are distinguished from non-transgenic domesticated *P. tetrazona* by their purple colouration under natural light and fluorescence under blue or UV light. PB2019 can be further identified genetically by PCR amplification of a unique section of the cassette, and detection of unique fragments following a restriction enzyme digest. When digested with the restriction enzyme the PCR product can be separated into a series of bands that distinguish PB2019 from other transgenic fluorescent purple Tiger Barbs if they are carrying a different cassette.

2.4.2. Phenotypic Characterization

2.4.2.1. Targeted phenotypic effects of the modification

The targeted phenotypic effect of the genetic modification is that PB2019 appears purple under ambient light and fluorescent under UV or blue light. The novel colour phenotype is present in muscle as well as the skin and eyes. The company states 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.

2.4.2.2. Additional phenotypic effects of modification

The influence of the genetic modification on any other phenotypes, including survival, has not been formally examined. No formal studies have compared potential disease susceptibility of PB2019 and non-transgenic strains. There are also no formal studies on potential unintended phenotypic effects of genetic modification on life history (other than reproductive success),

environmental tolerances and requirements (other than low-temperature tolerance), metabolism, physiology, endocrinology, or behaviour; however, there are no anecdotal or otherwise reports of any unintended phenotypic effects other than those listed above.

2.5. PLEIOTROPIC EFFECTS OF FLUORESCENT TRANSGENES IN OTHER FISH

Many fluorescent proteins, most commonly enhanced green fluorescent protein (eGFP), have widespread use for research in a variety of organisms, and some risk assessment relevant information is available on Zebrafish transgenic for red fluorescent protein (RFP) and other fluorescent proteins.

Zebrafish containing a RFP transgene were observed to be less cold tolerant than unrelated non-transgenic Zebrafish, when examined under different acclimation temperatures (Cortemeglia and Beitinger 2005, 2006a), though differences in strain background and rearing conditions (Schaefer and Ryan 2006) prior to experimentation may have impacted relative extreme temperature tolerance. Similarly, Leggatt et al. (2018b) reported that Zebrafish transgenic for eGFP, driven by the Fli-1 protein promoter, were less cold tolerant than the source non-transgenic strain. Leggatt et al. (2018b) also reported on two other eGFP lines, driven by different promoters, that did not exhibit diminished cold tolerance. This indicates that different transgenic lines may have different responses to extreme environmental stressors. Five of six previously notified GloFish® Tetras, three previously notified lines of GloFish® Danios, and two of three GloFish® Betta lines were also reported to have diminished cold tolerance (DFO 2018, 2019, 2020a, 2020b, 2021).

No effect of fluorescence protein transgenesis was observed on survival of RFP Zebrafish relative to related non-transgenic fish under laboratory conditions (Howard et al. 2015). In a population of eGFP, RFP, eGFP-RFP, and non-transgenic Zebrafish, eGFP fish had lower survival, but there was no effect of RFP or the double transgene on survival (Gong et al. 2003), indicating different transgenes or transgenic lines may also have different influences on survival. Paired crosses with non-transgenic siblings resulted in fewer fluorescent offspring than expected in two of six lines of GloFish® Tetras (DFO 2019), and two of three lines of GloFish® Danios (DFO 2020a,b, Table 1) indicating decreased viability of fluorescent gametes or larvae in some fluorescent models.

Reports describing the effects of RFP transgenesis on vulnerability to predation have shown varied outcomes. Cortemeglia and Beitinger (2006b) found that RFP and unrelated non-transgenic Zebrafish were equally preyed upon. Hill et al. (2011) found that GloFish® RFP Zebrafish were two times more vulnerable to predation than unrelated non-transgenic Zebrafish. In contrast, Jha (2010) found a domesticated RFP Zebrafish strain in India was less preyed upon by wild-caught Snakeheads than were wild-type wild-caught Zebrafish. Factors influencing the difference in relative vulnerability of RFP Zebrafish to predation are not known, but could include differences in genetic background or rearing history of transgenic and non-transgenic Zebrafish, innate preference or life history of predators, and/or experimental conditions (e.g., presence of shelter for prey species). Jha (2010) found RFP were more aggressive than wild-caught unrelated Zebrafish, although this may have been due to differences in domestication and/or rearing. GloFish® Electric Green® Tetra did not differ from non-transgenic Tetras in foraging success or aggression levels in paired foraging competition trials (Leggatt and Devlin 2019).

The reported influences of RFP and other fluorescent transgenes on reproductive success or preferences in Zebrafish are likewise inconsistent. RFP and non-transgenic Zebrafish had similar age at maturity for related females, as well as similar male and female fecundity (Howard et al. 2015). In a population containing equal numbers of eGFP and non-transgenic Zebrafish

eGFP offspring had no reproductive advantage or disadvantage (Gong et al. 2003). In contrast, Owen et al. (2012) found both non-transgenic and RFP Zebrafish females (related) preferred to associate with RFP rather than non-transgenic males, regardless of the proportion of non-transgenic to RFP fish with which they were raised. In another study, Howard et al. (2015) reported lower mating success in RFP males and less aggression towards both male and female fish compared to related non-transgenic males.

Snekser et al. (2006) found the RFP transgene did not influence social partner preferences for either shoaling or in a potential reproductive context in presumably unrelated populations of RFP and non-transgenic Zebrafish. Jiang et al. (2011) reported sex-specific differences in non-transgenic Cloud Mountain Minnow (*Tanichthys albonubes*) preference for non-transgenic or RFP transgenic conspecifics, where non-transgenic males tended to prefer transgenic shoals and females, while non-transgenic females preferred non-transgenic males and had no preference for shoal type. Howard et al. (2015) examined the fate of the RFP transgene over 15 generations in a serial competitive breeding experiment in 18 populations of GloFish® Zebrafish. In all populations, the frequency of the RFP transgene declined rapidly, and was eliminated in all populations except one, indicating a strong bias against the RFP transgene in reproduction. Overall, there are inconsistent reports of pleiotropic effects in other fluorescent protein transgenic models, and for the most part these effects would be considered detrimental to the organism (e.g., diminished cold tolerance, diminished reproductive success).

2.6. CHARACTERIZATION RELATIVE TO PREVIOUSLY ASSESSED GLOFISH®

The GloFish® Tiger Barbs were produced using similar methodologies and testing protocols as previously notified and assessed GloFish® Danio, Tetra, and Betta lines. All previously notified GloFish® lines have used similar transgene expression cassette production and elements (promoters, terminator sequences), though the pigment genes vary between colours of fish, and only the Betta lines have included species-specific homology arms in the construct to promote site directed insertion using Cas9 and guide RNA.

The use of guide RNA and Cas9 to direct site-specific transgene insertion may have resulted in unintended mutations within the resulting populations of Barbs. In other models, guide RNA and Cas9 has been demonstrated to bind and cut genomic DNA even when there are up to 3-5 base pair mismatches between the guide RNA and genomic DNA (Zhang et al. 2015). There are no data examining whether on- or off-target Cas9 mutations exist in the GloFish® Tiger Barb populations. The potential theoretical mutagenic effects of the utilized guide RNA sequence were examined using CRISPOR (Concordet and Haeussler 2018), and utilizing *P. tetrazona* genome assembly GCA_018831695.1/ASM1883169v1 (pers. comm. K.W. Wellband, Fisheries and Oceans Canada, West Vancouver BC). The guide RNA utilized was predicted to have low on-target affinity due to a number of features (e.g., low %GC content, high number of T bases near the 3’ end, additional G bases at the 5’ end from the T7 promotor), which may explain why it was not successful in integrating the transgene at the target site. There were some off-target sites with moderate to weak affinity to the guide RNA calculated (see Appendix 1), although the phenotypic consequences of mutations in these sites, should they occur, are not known.

Similar molecular and phenotypic characterization tests have been conducted by the company for the current and previously notified GloFish® lines, and results from tests conducted on the GloFish® Barbs overlap with some or all of previously notified lines (see Table 1).

Table 1. Characterization of GloFish® lines notified under CEPA for sale in Canada in the ornamental pet trade.

Characterization	GB20	RB2015	OB2019	PB2019	GBS2019	PiBS2019	OBS2019	BT2018	OT2018	PiT2018	PuT2018	RT2018	CGT2016	YZ2018	BZ2019	PZ2019
																
Commercial name	Electric Green® Barb	Starfire Red® Barb	Sunburst Orange® Barb	Galactic Purple® Barb	Electric Green® Betta	Moonrise Pink® Betta	Sunburst Orange® Betta	Cosmic Blue® Tetra	Sunburst Orange® Tetra	Moonrise Pink® Tetra	Galactic Purple® Tetra	Starfire Red® Tetra	Electric Green® Tetra	SunBurst Orange® Danio	Cosmic Blue® Danio	Galactic Purple® Danio
Long-fin variant present	yes	yes	yes	no	Yes[2]	Yes[2]	Yes[2]	No	yes	Yes	yes	Yes	yes	no	no	No
Homozygous fish present	yes	yes	yes	yes	yes	yes	yes	No	yes	No	no	Yes	yes	yes	yes	yes
Allowed for use date - USA/Canada	2012/in review	2016/in review	2020/in review	2020/in review	2019/2021	2020/2021	2020/2021	2014/2018	2013/2018	2013/2018	2013/2018	2014/2018	2012/2017	2012/2019	2010/2019	2011/2019

3. CHARACTERIZATION OF THE COMPARATOR

For the purpose of this risk assessment, the domesticated *Puntigrus tetrazona* (Tiger Barb) was selected as a comparator. *P. tetrazona* is a popular ornamental species that is bred, produced, and traded worldwide.

3.1. TAXONOMIC STATUS

Early efforts to combine redundant species descriptions and more recent attempts to resolve the large, diverse *Puntigrus* genus into smaller clades have led to many Tiger Barb taxonomic reassignments, with no current consensus (Kottelat 2013; Ren 2015). Tiger Barbs are part of the family Cyprinidae and were first described as *Capoeta tetrazona* in 1855 by Dutch ichthyologist Pieter Bleeker while exploring the Palembang region of Sumatra (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 taxonomical sources now recommend *Puntigrus tetrazona* (Kortmulder pers. comm.; Kortmulder and Robbers 2017; Kottelat 2013). Other taxonomists recommend resurrecting the genus name *Systomus* (Pethiyagoda et al. 2012; Rainboth 1996). Common names used for the Tiger Barb include Sumatra Barb and Partbelt Barb (Nico et al. 2019).

3.2. DISTRIBUTION

Tiger Barbs are likely native to Sumatra and Borneo (Froese and Pauly 2019; Kortmulder 1972; Kottelat 1992; Sakurai et al. 1993; Tan 2012; Welcomme 1988). Although species occurrences have been reported in other parts of Asia, including Thailand, Malaysia, and Cambodia (Frankel 1998; Naiman and Pister 1974; Tamaru et al. 1998), it is likely that at least some of these records refer to morphologically similar congenerics, particularly former subspecies *P. partipentazona*. Some of these records may also represent non-native Tiger Barb populations or new releases; for more details on the non-native distribution of Tiger Barbs, see Section 3.7.

3.3. HABITAT

Tiger Barbs are commonly found in shallow waters (approximately 2 feet) and along the banks of moderately flowing forest streams and tributaries with substrates of sand or rocks/pebbles of various sizes (Kortmulder 1972; Tamaru et al. 1998). Their temperature tolerances restrict them to tropical climates, where they prefer densely vegetated habitats, likely related to their reproductive strategy of depositing eggs on submerged vegetation (Innes 1979). They have been reported in both clear and turbid waters, and in swampy lakes subject to severe water level and quality fluctuations, indicating that wild Tiger Barbs may be tolerant of changing water quality (Tamaru et al. 1998; Vajargah and Rezaei 2015).

3.4. PHYSIOLOGICAL TOLERANCES

3.4.1. Oxygen

There are few experimental studies of the dissolved oxygen requirements of Tiger Barbs in captivity, and no such studies in wild populations, however, the lakes where Tiger Barbs are found often contain swamps, and oxygen levels are generally low (Kortmulder 1982). In a hypoxia experiment, juvenile Tiger Barbs exposed to 24 h of 4 mg/L dissolved oxygen had survival ranging from approximately 40% to greater than 90% depending on the level of

supplemental dietary linseed oil (Abolhasani et al. 2014). Experimental studies unrelated to oxygen requirements and hypoxia have reported maintaining oxygen concentrations in Tiger Barb tanks at greater than 5.5 mg/L (Chapman 1997), 6.0 to 7.8 mg/L (Ling et al. 1991), 6.4 mg/L (Abolhasani et al. 2014), and 7-9 mg/L (Abdallah et al. 2015).

3.4.2. Temperature

Tiger Barbs are considered stenotherms, capable of surviving in only a narrow range of environmental temperatures (Yanar et al. 2019). While Innes (1979) recommended maintaining ornamental Tiger Barbs at temperatures between 70 and 80°F (21.1-26.7°C), Tamaru et al. (1998) recommended a slightly narrower range of temperatures (22-25°C). Tiger Barbs appear to prefer slightly higher temperatures (23-28°C) during breeding (Tamaru et al. 1998). In its patent applications for “Green Transgenic Fluorescent Ornamental Fish” (Blake et al. 2016a) and “Red Transgenic Fluorescent Ornamental Fish” (Blake et al. 2019), GloFish LLC recommends water temperatures of 75 to 85°F (~23.9-29.4°C) during non-transgenic Tiger Barb spawning.

Recent controlled studies have elucidated previously unknown temperature tolerances for Tiger Barbs. Leggatt et al. (2018b) found that the temperature at which 50% of individuals lost equilibrium (LD_{50}) was 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 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 when acclimated at 20°C may be due to different experimental procedures (i.e., rate of temperature decline), as well as potential differences in rearing history or background genetics (Saillant et al. 2008; Schaefer and Ryan 2006; Tuckett et al. 2016). Recovery from the tests also differed between studies, with 100% recovery from the CT_{min} trial (Yanar et al. 2019) and 0% recovery from the CL_{min} trial (Leggatt et al. 2018b). 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. 2018b). As well, Liu et al. (2020) reported extensive tissue damage in the brain, gills, liver, and muscle of Tiger Barbs when the temperature was dropped to 13°C.

3.4.3. pH and water hardness

Tiger Barbs are tolerant of a pH range of 6.5-7.5 (Tamaru et al. 1998; Vajargah and Rezaei 2015), but appear to prefer slightly acidic water (Sakurai et al. 1993), particularly when breeding. In its patent applications for “Green Transgenic Fluorescent Ornamental Fish” (Blake et al. 2016a) and “Red Transgenic Fluorescent Ornamental Fish” (Blake et al. 2019), In controlled (aquarium) settings, Tiger Barbs thrive in water with a hardness of 100 to 250 ppm $CaCO_3$ (Tamaru et al. 1998).

3.4.4. Salinity

Although no data are available for natural environments, experimental studies provide some insight on Tiger Barb salinity preferences and tolerances. Abolhasani et al. (2014) found juvenile Tiger Barb survival was greater than 80% after 24 h at 6 ppt salinity under a controlled diet, and after 24 h at 10 ppt salinity if fed a linseed oil-enriched diet, while Tamaru et al. (1998) suggested salinity up to 9 ppt can be beneficial to reduce stress for commercially reared Tiger Barbs. In its patent applications for “Green Transgenic Fluorescent Ornamental Fish” (Blake et al. 2016a), and “Red Transgenic Fluorescent Ornamental Fish” (Blake et al. 2019), GloFish LLC

states that low salinity (conductivity 100-200 µS/cm) promotes spawning in non-transgenic Tiger Barbs.

3.5. MORPHOLOGY, LIFE HISTORY AND GROWTH

The Tiger Barb is so named for the four black bars that contrast strongly with its background colour. *P. tetrazona* is a small species with a standard length of 2.4 to 4.7 cm (Kortmulder 1972; Taki et al. 1978). It has a rhomboidal body and the background colour in the wild varies from silvery to brownish-yellow (Kortmulder 1972; Tan 2012). Other colour variations have been created by artificial selection, including the green and albino variants (Tamaru et al. 1998).

Male and female Tiger Barbs sometimes can be distinguished morphologically based on colour and body shape (Kortmulder 1972; Tamaru et al. 1998). The females generally have a fuller outline and a transparent tail fin, while the males have two distinct red streaks from the base to the tips of this fin (Innes 1979; Kortmulder 1972). Males are on average redder than females, although the intensity of other red patterns may overlap between the sexes (Takahashi and Shimizu 1983). During reproduction, the red markings become more intense in males and less intense in females (Kortmulder 1972; Takahashi and Shimizu 1983).

Tiger Barbs usually attain sexual maturity at a body length of 2-3 cm, around six to seven weeks of age (Tamaru et al. 1998). *P. tetrazona* gonads initially develop as ovaries followed, in males, by degeneration of the ovarian tissue and normal testis development by 50 days after hatching (Devlin and Nagahama 2002; Takahashi and Shimizu 1983).

Male Tiger Barbs are intermittently territorial, only guarding their territory during spawning and often schooling during the same day (Kortmulder 1972). Leading up to mating, males perform courtship displays, chase, and aggressively nip at the fins of females (Innes 1979; Kortmulder 1972). During courtship, males have been known to sometimes nip at female anal fins to such an extent that they kill them (Innes 1979). Following courtship, the male clasps the female who deposits 1-3 eggs at a time on broad-leaved plants, where the male will immediately fertilize them (Tamaru et al. 1998). Females can produce from 200-500 eggs per spawning event and have been reported to spawn at approximately two-week intervals (Tamaru et al. 1998). Both male and female Tiger Barbs are keen spawn-eaters (Innes 1979). Eggs hatch 2-3 days post fertilization and larvae become free-swimming once the yolk sac has been consumed, approximately 3 or 4 days post hatching (Kortmulder 1972; Tamaru et al. 1998). Tiger Barbs are reported to have a lifespan of six years in an aquarium setting (Vajargah and Rezaei 2015).

3.6. BACKGROUND GENETICS

Several studies have investigated the inheritance of various pigmentation characteristics in Tiger Barbs through phenotypic analysis of controlled crosses. Though Frankel (1998) found that band length is controlled by two additive gene loci, and band number is under simple monogenic Mendelian inheritance with five bands dominant over four bands, the study based its conclusions on crosses between apparent sub-species that have since been distinguished as separate species (Frankel 1998). Sheriff (1999) showed that in crosses of “normal”, “green”, and “yellow” Tiger Barbs, the ‘normal’ colour phenotype is dominant and the ‘green’ phenotype exhibits evidence of epistatic interactions. Albino Tiger Barb lines have been created through selective breeding of naturally occurring mutant colour variations (Kortmulder 1972). Aquarium-sourced Tiger Barbs are considered fully domesticated (i.e., selectively bred for specific goals) (Teletchea 2016).

Although functional (active) transposable elements have been identified in related cyprinids including other *Puntigrus spp.* (Ishiyama et al. 2017), this has not been specifically examined in Tiger Barbs. No studies were found regarding the population genetics of Tiger Barbs.

3.7. HISTORY OF INVASIVENESS

There have been no reports of established Tiger Barb populations in continental North America, most likely due to the temperature tolerance limitations of the species (Tamaru et al. 1998); however, there has reportedly been an established population of Tiger Barbs in eastern Puerto Rico since 1995 (Nico et al. 2019). Although there are established populations of related Barb species (*Puntigrus*) in Puerto Rico and Australia, no ecological impacts have been identified (Hill et al. 2014).

Though occurrences of Tiger Barbs in the continental U.S. (Florida, Texas, California and Wyoming) have been reported since the 1970s (Howells 2001; Naiman and Pister 1974; Nico et al. 2019; Welcomme 1988), there are no reports of establishment or reproduction, even with the discovery of a sexually mature male and female near a warm spring with ideal spawning temperatures (Dill and Cordone 1997; Naiman and Pister 1974). Tuckett et al. (2017) captured 163 individuals of 2 varieties within 500m of outdoor aquaculture ponds in Florida, though no fish were captured more than 500m away from source facilities. Despite the large number of captured fish, the authors attributed these individuals to recent releases, not establishment, presumably due to an absence of juveniles in the sample (Shafland et al. 2008).

Several recent studies have used the Fish Invasiveness Screening Kit (FISK) (Copp et al. 2005) risk screening tool to assess Tiger Barb invasiveness. Although FISK scores in Mediterranean climates range from 5 to 11.3 (medium risk) (Perdikaris et al. 2016; Range 2013), and FISK analyses have assessed the invasion potential of Tiger Barbs to be low to medium (scores: -2.5 to 1.8) in subtropical Florida (Hill et al. 2014; Hill et al. 2017; Lawson 2014), Tiger Barb invasion risk would likely be much lower in temperate climates.

Marcot et al. (2019) proposed a decision support system for evaluating freshwater fish invasiveness based, in part, on a modified Freshwater Fish Injurious Species Risk Assessment Model (FISRAM); the dominant probability outcome of their FISRAM model identified Tiger Barbs as not invasive in the United States.

3.8. TROPHIC INTERACTIONS (DIET, PREY, COMPETITORS, PREDATORS)

Tiger Barbs 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 aggressive, and often exhibit agonistic behaviours towards conspecifics and larger fishes (Innes 1979; Kortmulder 1972; Sakurai et al. 1993). Kortmulder (1972) reports Tiger Barbs “mobbing” a cichlid (*Cichlasoma severum*) that was introduced into the same tank, including biting and tearing at fins and following the cichlid when it attempted to escape. Similar, though less severe, behaviour has been observed directed towards a larger species of Barb (*Barbus schwanenfeldi*) (Kortmulder 1972). Innes (1979) identified similar behaviour exhibited by Tiger Barbs towards slow-moving fish species that have flowing fins, including Bettas, Angelfish, and Veiltail Guppies. Sakurai et al. (1993) also noted similar behaviour.

4. ENVIRONMENTAL RISK ASSESSMENT

This risk assessment is conducted within the legislative context of CEPA and the information requirements of the NSNR(O), Schedule 5. Potential risks to the Canadian environment that may be associated with the import or manufacture of GE fish is determined in accordance with the classical risk assessment paradigm, where risk is directly related to the exposure and hazard of the organism. The exposure assessment is based on the likelihood and magnitude of release into the environment, and the likelihood and magnitude of survival, reproduction,

establishment, and spread of the organism and potential descendants of the organism in the Canadian environment. The hazard assessment is focused on the potential for the organism to impact: (1) potential prey, predators, and competitors of the organism; (2) biological diversity; and, (3) habitat. The level of uncertainty for both exposure and hazard determinations is evaluated and communicated in terms of impact to the final risk assessment. DFO provides science advice in the form of peer-reviewed risk assessments to ECCC for regulatory decision-making under CEPA, based on risk to the environment and the uncertainty associated with the conclusion. A detailed overview of the legal context for the risk assessment process, the risk assessment framework, and regulatory decision making process under CEPA is provided in Leggatt et al. (2018a).

4.1. EXPOSURE ASSESSMENT

The exposure assessment for the four living organisms addresses both their potential to enter the environment (release) and their 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. Rankings for the likelihood of exposure to the Canadian environment are provided in Table 2.

Table 2. Rankings for likelihood of exposure of genetically engineered fish to the Canadian environment.

Likelihood of Exposure	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

Given the regulatory status of any GE fish undergoing environmental risk assessment under CEPA, a lack of empirical data regarding the survival, fitness, and ability of GloFish® Barbs to reproduce in the natural environment will contribute uncertainty to the exposure assessment. Uncertainty associated with the environmental fate of an organism or the failure of biological and geographical containment may depend on the availability and robustness of the scientific information related to the biological and ecological parameters of the organism, valid surrogates, and the receiving environment. Table 3 ranks uncertainty associated with the likelihood of occurrence and fate of the organisms in the Canadian environment.

Table 3. Ranking of uncertainty associated with the likelihood of occurrence and fate of the organism in the Canadian environment (environmental exposure).

Uncertainty	Available Information
Negligible	High-quality data on the organism (e.g., sterility, temperature tolerance, fitness). Data on environmental parameters of the receiving environment Demonstration of absence of Genotype by Environment Interaction (GxE) effects or complete understanding of GxE effects across relevant environmental conditions. Evidence of low variability.

Uncertainty	Available Information
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.

All previous assessments of notified and assessed GloFish® Danio, Tetra, and Betta lines concluded low ranking for environmental exposure with low uncertainty (DFO 2018, 2019, in review). There are no known molecular or phenotypic characteristics of GloFish® Barbs that suggest a different ranking than previously assessed lines, and no new scientific literature has been published that would alter the previous rankings. Consequently, the environmental exposure assessment for GloFish® Barbs is low, with low uncertainty that is consistent with previously notified lines. Details supporting this conclusion follow.

4.1.1. Characterization of the Receiving Environment

A detailed description of potential receiving environments in Canada relevant to the introduction of tropical freshwater fish is presented in Leggatt et al. (2018a). Emphasis is placed on water temperature as a key abiotic factor that affects both the survival and production of most freshwater fish populations, and is a pervasive determinant of habitat suitability (Amiro 2006; Elliott and Elliott 2010; Jobling 1981; Magnuson et al. 1979).

Briefly, the many lakes and rivers of Canada vary in their annual temperature profiles, as well as their average maximum and minimum temperatures, however, almost all reach 4°C or below at some point annually, and only a few isolated lakes in Southern Coastal British Columbia have minimum recorded temperatures above this. Of these latter lakes, all but one has a minimum temperature recorded below 6°C (see Leggatt et al. 2018b), though temperature recordings of these warmer lakes are often restricted to a single measurement per winter, and recorded temperatures may not represent the coldest temperature obtained during winter months.

During the summer, many Canadian lakes can reach surface temperatures above 20°C, however, only a few systems have been observed exceeding 25°C. For example, of the 83 lakes monitored under the British Columbia Lake Stewardship and Monitoring Program, there are 67 lakes where maximum surface water temperatures have been measured above 20°C during the summer, but only six where water surface temperatures have been measured above 25°C (BCLSS, accessed March 17, 2022). Exceptions to the above are highly localized and isolated pockets of warm water generated by hot springs or industrial effluent that can provide refuge to fish species with limited cold tolerance (Peterson et al. 2005; Renaud and McAllister 1988).

It should be noted that many freshwater systems have heterogeneity in temperature profiles; for example, groundwater contributions may increase or decrease temperatures in localized areas of a water body, and shorelines are expected to experience more extreme temperatures relative to deeper waters. Also, 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). This change could increase the number of possible lakes in which organisms with moderate cold tolerance can survive.

4.1.2. Likelihood of Release

Though the stated purpose of the organisms is for sale in the ornamental market, and hobbyists generally follow the instructions for disposal recommended by the retailer or the company itself, there is still a high likelihood that GloFish® Barbs will be introduced into the Canadian environment. Once the 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. Numerous aquarium fish have established themselves in natural waters in North America, and reoccurring, though isolated, reports of aquarium fish in Canadian waters suggest the practice of releasing aquarium fish into the environment is common and ongoing (Kerr et al. 2005; Marson et al. 2009; Rixon et al. 2005; Strecker et al. 2011). This concurs with a high likelihood of release for previously notified GloFish® Tetras, Danios, and Bettas. The extent to which GloFish® Barbs may be further exposed to the environment will therefore depend heavily on their ability to survive and reproduce in Canadian lakes and rivers.

4.1.3. Likelihood of Survival

As a tropical species, *P. tetrazona* is not expected to survive in a temperate region where water temperatures are below optimal. Indeed, water temperature is a key abiotic factor that affects both the survival and production of most freshwater fish populations, and is a pervasive determinant of habitat suitability (Jobling 1981; Magnuson et al. 1979).

In the aquarium, Tiger Barbs are typically kept at temperatures between 21 and 27°C (see Section 3.4.2). In a recent experiment, when *P. tetrazona* was acclimated at 20°C and temperatures lowered by 0.3°C per hour, the observed CT_{min} was 11.66±0.86°C (Yanar et al. 2019). Leggatt et al. (2018b) found that when domesticated Tiger Barb were acclimated at 20°C and temperatures lowered by 1°C per day, 50% of individuals lost equilibrium (LD_{50}) by 13.20°C, and the average CL_{min} of *P. tetrazona* was 13.36±0.02°C.

As discussed in Section 4.1.1 there are no known 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 (with the exception of hot springs and industrial effluent).

While the temperatures needed for GloFish® Barbs to survive may be possible for several Canadian lakes during short periods of the summer, it is extremely unlikely that GloFish® Barbs could survive the Canadian winter and their occurrence in the environment would be seasonal or ephemeral. This is further supported by lack of establishment of *P. tetrazona* despite noted occurrences in much warmer climates (e.g., Florida, Tuckett et al. 2017, see Section 3.7).

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). While the majority of freshwater systems experiencing significant ice coverage in the winter are expected to see a decrease in the number of ice-days in these systems (DFO 2013), any continuation of winter ice coverage would result in temperatures at or below 4°C at some point during the winter, preventing year-round survival of GloFish® Barbs.

Cold-tolerance data combined with the lack of establishment of *P. tetrazona* in North America suggest negligible potential for survival in Canadian waters, even with the increased water temperatures associated with climate change.

4.1.4. Likelihood of Reproduction

Though water temperatures in Canada will limit the persistence of any GloFish® Barbs that are introduced into the environment (see Section 5.1.3), 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 2 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, accessed March 17, 2022). While this may be a tolerable temperature range for GloFish® Barb survival, warmer temperatures (23 to 28°C) are more ideal for reproduction (Tamaru et al. 1998). Also, Tiger Barb have complex reproductive behaviour, with courtship displays and chase (See Section 3.5). If suitable mate and habitat for reproduction were to occur, the resulting offspring would perish in the cold of winter before reaching maturity.

Barbs could potentially reproduce in isolated areas of warm water (e.g., hot springs), however, there have been no observations of any Barb occurrences in hot springs, and there is no evidence of reproduction where Tiger Barbs persist in the effluent of ornamental fish farms (Tuckett et al 2017).

4.1.5. Likelihood of Proliferation and Spread

The capacity for GloFish® Barbs to proliferate and spread in the Canadian environment is precluded by the fact that *P. tetrazona* cannot survive the winter (see Section 4.1.3). It should be noted that any released GloFish® Barbs are expected to occupy areas near the shoreline, based on what is known of wild-type habitat preferences (see Section 3.3). These areas are expected to have more extreme temperature ranges than deep water or mid-lake areas that are often the source of water temperature measurements (Trumpikas et al. 2015). Consequently, periodic winter temperatures may be colder than indicated by recorded data, which may further reduce the potential for overwintering of GloFish® Barbs, though fish may move to follow warmer water as temperatures drop. Warmer summer temperatures in these habitats may increase the potential for single-generation spawning.

4.1.6. Conclusions

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 according to ranking criteria in Table 10. The uncertainty associated with this estimate is low (see Table 3 for ranking criteria), 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 ranking is consistent with the low exposure ranking with low uncertainty concluded on 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).

The notifying company identifies the sole intended use for the notified organisms as an ornamental fish for interior, static, home aquaria; once purchased by consumers, however, the possibility of other unintended uses cannot be excluded (e.g., rearing in outdoor ponds, use as bait fish, etc.). While some unintended uses may lead to the release of GloFish® Barbs, they would not alter the organism's ability to overwinter in Canadian environments, or otherwise alter the low environmental exposure ranking for the organism.

Changing water temperature patterns associated with global climate change do not increase uncertainty when determining the ability of the notified organisms to survive, reproduce, proliferate, and spread in Canadian freshwater ecosystems.

4.2. 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). Table 4 categorizes the severity of the biological consequences based on the severity and reversibility of effects to the structure and function of the ecosystem. Any difference in measurement endpoint is evaluated relative to 'normal' variation, based on published studies and expert opinion.

Table 4. 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.

Uncertainty around the hazard assessment is significant due to clear knowledge gaps and lack of empirical data around the behaviour and effects of GloFish® Barbs in the natural environment. Criteria for the assessment of uncertainty address potential effects to the environment, which may rely heavily on information and data found in published and peer-reviewed scientific literature. A description of rankings for uncertainty regarding the potential hazards of the organisms in the environment is provided in Table 5.

Table 5. Ranking of uncertainty associated with the environmental hazard.

Uncertainty Ranking	Available Information
Negligible	High quality data on the organisms. 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 the organisms or valid surrogate. Understanding of GxE effects across relevant environmental conditions. Evidence of some variability.
Moderate	Limited data on the organisms, relatives of the organisms 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.

For uncertainty, the quality of data refers to the data or information available for each parameter being examined, the integration of this information and breadth of experimental conditions

examined, sample size, appropriateness of controls, statistical analysis, as well as the experimental design and interpretations of the results. Variability refers to both the range of phenotypic differences among individuals or strains within the same environment as well as the range of physical, chemical, and biological conditions that may be experienced by a GE fish in the receiving environment. Broad principles influencing uncertainty in hazard assessments of GE fish (e.g., GxE, effects of background genetics, off-target/pleiotropic effects) are detailed in Leggatt et al. (2018a) and Devlin et al. (2015).

The proposed use of GloFish® Barbs in Canada (i.e., importation and transport in static containers, holding in static tanks in commercial wholesalers and retailers, rearing in static tanks in home aquaria) provide minimal pathways of effects of GloFish® Barbs to Canadian environments. The majority of potential hazards posed by GloFish® Barbs (e.g., through interactions with other organisms, impacts to biogeochemical cycling, habitat, and biodiversity) would be through direct release of GloFish® Barbs into natural aquatic ecosystems, although some potential hazards could act indirectly through the release of waste water and carcasses into the environment (e.g., environmental toxicity, horizontal gene transfer, as a vector for disease).

All assessments of previously notified and assessed GloFish® Tetra, Danio, and Betta lines concluded with negligible ranking for most environmental hazard pathways and low hazard ranking through horizontal gene transfer (HGT), with uncertainty ranging from negligible to moderate (DFO 2018, 2019, 2020a, 2020b, 2021). While *P. tetrazona* differ from previously notified species *G. ternetzi* and *D. rerio* in some phenotypes (i.e., aggression, reproductive behaviour; they are similar to *B. splendens* in these attributes), there are no known molecular or phenotypic characteristics of GloFish® Barbs derived from the genetic modifications that suggest a different ranking than previously assessed lines, and no new scientific literature has been published that would alter the previous rankings. Consequently, the environmental hazard assessments for GloFish® Barbs follow those of the previously notified GloFish® Tetras, GloFish® Danios, and GloFish® Bettas. Details supporting these conclusions follow, and greater detail for each hazard assessment can be found in Leggatt et al. (2018a).

4.2.1. Potential Hazards Through Environmental Toxicity

Potential routes of environmental toxicity include exposure of aquatic ecosystems to the whole animal and its waste, as well as through ingestion by predators. Exposure of the environment to the fluorescent proteins is expected to be lower than exposure of GloFish® Barb lines to the proteins; though different routes of exposure are not necessarily comparable. Fluorescent proteins are commonly used as neutral markers in research in a wide range of organisms with almost no reports of toxicity (Stewart 2006). The few reports of negative effects are generally specific to transgenic organisms with especially high expression of fluorescent transgenes (Huang et al. 2000; Devgan et al. 2004; Guo et al. 2007). Any toxic effects to host organisms are likely due to production of the protein within the host cell, and are not expected to have equal effects from contact or ingestion exposure.

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 GloFish® Barbs, or any other species of GloFish® containing transgenes coding the same proteins as those in the GloFish® Barb lines in both Canada and 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.

This concurs with assessment rankings for previously notified GloFish® Tetras, Danios, and Bettas (DFO 2018, 2019, 2020a, 2020b, 2021). No new relevant data has become available since the analyses of previous GloFish® lines.

4.2.2. Potential Hazards Through Horizontal Gene Transfer

Horizontal gene transfer is the non-sexual exchange of genetic material between organisms of the same or different species (DFO 2006). Pathways of exposure of novel organisms (most likely prokaryotes) to free transgenic DNA include exposure within the gut, or through feces, mucus, and other waste sloughed off by the fish into the water. The transgene construct does not contain known transposable elements that would increase the potential for DNA uptake/mobility to a new organism. In order for the transgene to be expressed and result in phenotypic change, it requires co-transfer of regulatory elements. The close proximity of the promoters to the pigment transgenes could increase the likelihood of them being co-transferred and expressed, though vertebrate promoters generally have poor activity in prokaryotes. As well, the identified presence of the bacteriophage T3 promoter in the transgene construct of one current (GB2011) and some previously notified lines may increase the potential for functional HGT to occur, and the T3 promoter has been shown to result in expression of cnidarian fluorescent protein transgenes in *Escherichia coli* (Wu et al. 2015). One recent study examined the potential for HGT of fluorescent protein transgenes using a genetically engineered fruit fly (transgenic for DsRed) and its parasitoid (Ramirez-Santos et al. 2018). The authors did not find any evidence of HGT of the fluorescent protein transgene over 16 generations of experimental rearing, though they cautioned that their experimental design may not have detected rare events of HGT or transfer of mutated transgenes.

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 HGT to a novel host would not be expected to result in harmful effects, should it occur. Graham and Davis (2021) recently demonstrated HGT of an environmentally advantageous gene (antifreeze protein) between two fish species at an evolutionary scale. While this demonstrates HGT can occur between higher organisms, the lack of fitness advantage (e.g., reproduction, cold tolerance) conferred by the present fluorescent protein transgenes suggests that if HGT transfer occurred it would likely be on an individual organism level. 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 lack of knowledge of the location of the transgenes within the *P. tetrazona* 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® Tetras, Danios, and Bettas, though in the Tetra lines uncertainty was assessed as low (DFO 2018, 2019, 2020a, 2020b, 2021). Here, as with the Danios and Bettas, the uncertainty ranking was increased to better reflect the limited number of relevant studies of HGT and resulting consequences.

4.2.3. Potential Hazards Through Interactions with Other Organisms

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. In typical Canadian

freshwater systems, however, activity and feeding levels of *P. tetrazona* are expected to be low due to water temperatures that are below ideal for the species (see Section 3.4.2 and 5.1.1), and anecdotal information from the company indicates there have been no detected differences in behaviour between GloFish® Barbs and non-transgenic domesticated Tiger Barbs in several years of development and commercial use. Given the low temperatures of Canadian freshwater systems for most of the year, and lack of evidence of altered behaviour from genetic modifications, the potential for anticipated numbers of released GloFish® Barbs to impact native aquatic species through prey acquisition, competition, and aggression is expected to be negligible through most of the year, and is expected to be no greater than for non-transgenic *P. tetrazona*.

Released GloFish® Barbs may also have potential to impact native predator populations as a new source of prey. This could have a positive effect on predator populations by providing a new food source, or a negative effect on predator populations if consuming GloFish® Barbs causes deleterious effects to the predator populations. The latter is not expected as GloFish® Barbs are not expected to be environmentally toxic (see Section 5.2.1 above). Consequently, the notified lines introduced at anticipated scales are not expected to pose a hazard to native predators.

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 GxE interactions in aggression and predation susceptibility, result in a **moderate** level of uncertainty. This concurs with assessment rankings for previously notified GloFish® Tetras, Danios, and Bettas (DFO 2018, 2019, 2020a, 2020b, 2021).

4.2.4. Potential Hazards Through Hybridization with Native Species

There is little potential for Tiger Barb to hybridize with native species in Canada. *P. tetrazona* is a member of the taxonomic family Cyprinidae, with 53 species occurring in Canada and over 1500 species worldwide (Coad 2015). There are several cyprinid genera in Canada, and intergeneric hybrids have been noted for two cyprinid genera in Europe (Hayden et al. 2010), suggesting hybrids between *P. tetrazona* and Canadian cyprinids could be possible. As well, in the piscine family Mormyridae survival of intergeneric hybrids was related to the phylogenetic distance of the parent species (i.e., greater phylogenetic distance resulted in decreased viability, and increased occurrence of malformations, Kirschbaum et al. 2016). Given Canadian cyprinid genera are expected to be of further phylogenetic difference than the above genera that did not produce viable hybrids, it is unlikely that Canadian cyprinids would form viable hybrids with *P. tetrazona*.

Tiger Barbs are also not broadcast spawners, but directly fertilize eggs that have been laid on substrate (see Section 3.5), minimizing potential for cross-species fertilization. In addition, Tiger Barbs prefer warm water temperatures (23°C-28°C) during breeding (Tamaru et al. 1998), conditions that are hard to find in Canada (see Section 4.1.1) where native cyprinids are more likely to breed at cooler temperatures. Consequently, there is **negligible** potential for the GloFish® Barbs to cause hazards through viable hybridizations with native fish in Canada. High quality information on the distribution of cyprinids and breeding requirements of *P. tetrazona*, and some data on intergeneric hybridization result in **low** uncertainty associated with this ranking. The negligible conclusion ranking is in line with the assessment rankings for previously assessed GloFish® Tetras, Danios and Bettas, although uncertainty ranking differ from the negligible uncertainty ranking in Tetras and Bettas that do not share families with Canadian fish

species, and the moderate uncertainty in Danios (family Cyprinidae) that do not have data available on intergeneric hybridization (DFO 2018, 2019, 2020a, 2020b, 2021, see Table 6).

4.2.5. Potential to Act as a Vector of Disease Agents

Commercial ornamental aquarium fish are commonly reported to carry numerous disease agents including viruses, bacteria, fungi, and parasites (Evans and Lester 2001; Hongslo and Jansson 2009; Řehulka et al. 2006; Rose et al. 2013; Whittington and Chong 2007).

Any disease agents GloFish® Barbs would be harbouring are expected to be tropical in origin, and/or persist in warm waters normally found in home aquarium (e.g., 25-28°C), and, therefore, may have limited ability to persist within or outside GloFish® Barbs once released to cooler Canadian freshwater environments. As well, *P. tetrazona* is not listed among the few tropical species susceptible to diseases of significant importance to aquatic animal health and the Canadian economy by the Canadian Food Inspection Agency (CFIA) (Susceptible Species of Aquatic Animals).

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. 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. Some studies of fluorescent cultured cell models used in research have reported potential alterations in 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 signalling pathways (Baens et al. 2006), and alter expression of genes involved in immune function (Coumans et al. 2014) and response to stress (Badrian and Bogoyevitch 2007). As well, Chou et al. (2015) reported mice transgenic for DsRed had alterations in some white blood cell numbers (lymphocytes and monocytes) but not others.

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. Fluorescent 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® 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 ranking is **moderate.** This concurs with assessment rankings for previously notified GloFish® Tetras, Danios, and Bettas (DFO 2018, 2019, 2020a, 2020b, 2021).

4.2.6. Potential to Impact Biogeochemical Cycling

GloFish® Barbs 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® Barbs 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® Barbs have similar influences from fluorescent transgenic gene expression are not known, but the small size of *P. tetrazona* and potential low numbers of individuals anticipated to enter an ecosystem indicate a **negligible** potential for GloFish® Barbs 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® Tetras, Danios, and Bettas (DFO 2018, 2019, 2020a, 2020b, 2021).

4.2.7. Potential to Affect Habitat

P. tetrazona are a small species and 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 ranking. This concurs with assessment rankings for previously notified GloFish® Tetras, Danios, and Bettas (DFO 2018, 2019, 2020a, 2020b, 2021).

4.2.8. Potential to Affect Biodiversity

Biodiversity can be negatively impacted by numerous drivers, including invasive species and the introduction of disease. Despite their long-standing use in the ornamental aquarium trade, and numerous introductions (see Section 3.7) there have been no reports of *P. tetrazona* becoming invasive in the temperate regions of North America. As well, there is no evidence that GloFish® Barb lines have increased fitness that may increase invasiveness relative to non-transgenic Tiger Barbs.

As elaborated above, GloFish® Barbs 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. 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. This concurs with assessment rankings for previously notified GloFish® Tetras, Danios, and Bettas (DFO 2018, 2019, 2020a, 2020b, 2021).

4.2.9. Conclusions

GloFish® Barbs are not expected to be hazardous to Canadian environments. Non-transgenic Tiger Barbs have no history of invasiveness in temperate regions including North America, despite widespread use. There is no evidence of environmental toxicity associated with the constructs, and the majority of other fluorescent models do not report toxicity associated with fluorescent transgenes. There is also no indication of potential hazardous effects to the environment via transfer of the transgene to native Canadian species through hybridization, or HGT. GloFish® Barbs and other fluorescent fish models have no reported differences in survival, disease susceptibility, behaviour, or husbandry care, have no advantageous changes in temperature tolerance or reproduction, and are not expected to have an altered ability to act as a vector for disease or impact biogeochemical cycling.

The examined hazards have negligible to low rankings (Table 6), 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 (e.g., RFP Zebrafish), and the reliance on expert opinion for the assessment of some hazards. Use of guide RNA and Cas9 in the creation of OB2019 or PB2019 lines adds additional uncertainty to the overall hazard assessment from potential off-target mutations in the Barb populations. Off-target 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 off-target

mutations from guide RNA and Cas9 has been discussed for other models 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. Possible harmful effects of off-target 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 does not alter any hazard ratings for the GloFish® Barbs, it does increase uncertainty in the overall hazard assessment.

Outside of its intended use as an ornamental fish in static aquaria, GloFish® Barbs are not expected to pose unique hazards beyond those of the intended use. Hazard ranking concurred with those previously assessed for GloFish® Tetras, Danios, and Bettas, although uncertainty in hazard via hybridization differed from other models due to data availability and presence of Canadian confamilials, and from GloFish® Tetras in uncertainty of hazard via HGT due to increased acknowledgement of data limitations.

Table 6. Summary of all ranks and uncertainty rankings 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.

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/<u>Low</u>
3. Trophic interactions.	Neg./Mod.	Neg./Mod.	Neg./Mod.	Neg./Mod.
4. Hybridization	Neg./<u>Low</u>.	Neg./Neg.	Neg./<u>Mod.</u>	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**

4.3. ASSESSMENT OF RISK

Risk is the likelihood that a harmful effect is realized as a result of exposure to a hazard. The risk assessment incorporates the nature and severity of the harmful effect, the likelihood that the harmful effect is realized, and the uncertainty associated with each conclusion. DFO's science advice to ECCC and HC for a regulatory decision is based on the overall risk of the organism, carried out in the context of the applicant's proposed use scenario, and all other potential use scenarios. An overall conclusion on Risk is based on the classic paradigm where Risk is proportional to Hazard and Exposure: Risk $\propto$ Exposure $\times$ Hazard.

For each endpoint, hazard and exposure are ranked as: negligible, low, moderate, or high, and include an analysis of uncertainty for each. Overall Risk is estimated by plotting Hazard against Exposure, using a matrix or heat map, as illustrated in Figure 2. Though the matrix cannot be used as a tool for establishing a discreet conclusion or decision on risk, it can be used to facilitate communication and discussion. The uncertainty associated with overall Risk ranking is not estimated, rather uncertainty in the hazard and exposure assessments are discussed in the context of a final conclusion on risk.

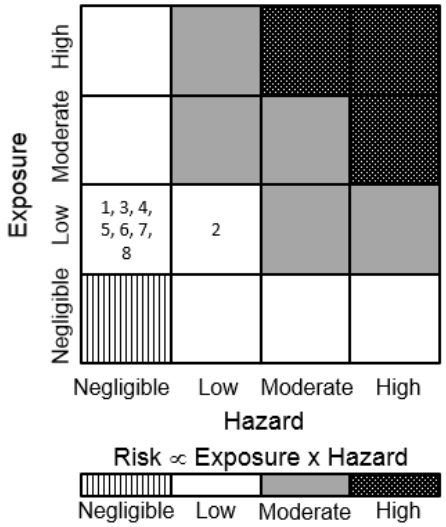

Figure 2. Risk matrix and pattern scale to illustrate how exposure and hazard are integrated to establish a level of risk in the environmental risk assessment. Risk assessments associated with assessed hazard components at the assessed exposure are identified by number: 1) through environmental toxicity; 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.

4.3.1. Risk Assessment of the GloFish® Barbs

The exposure assessment concluded 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 for GloFish® Barbs to persist or overwinter in Canadian aquatic ecosystems. As such, any exposure to Canadian freshwater ecosystems to GloFish® Barbs is expected to be isolated, rare, and ephemeral. The quality of data demonstrating lack of cold tolerance in GloFish® Barbs and domesticated *P. tetrazona*, relevant 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 ranking associated with individual hazard components 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.

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 organisms 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 rankings 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® Tetras, Danios, and Bettas (DFO 2018, 2019, 2020a, 2020b, 2021, see Table 6).

4.3.2. Summary and Conclusions

Use of GloFish® Barbs in home aquaria in Canada, or for other unintended uses, is expected to result in frequent, very small magnitude releases of GloFish® Barbs into the Canadian environment, though the potential for occasional high magnitude releases cannot be excluded. Available high-quality data indicates that GloFish® Barbs do not have the capacity to overwinter in Canadian freshwater ecosystems. This results in an exposure ranking of low, with low associated uncertainty. The lack of evidence of hazards from non-transgenic comparator species despite long-term extensive use, and a lack of evidence for increased hazards of GloFish® Barbs relative to non-transgenic domesticated *P. tetrazona*, indicates negligible to low hazard ranking to Canadian ecosystems. Due to a lack of, or limited direct information on, the hazards of base models or GloFish® Barbs, uncertainty with hazard assessments ranged from low to moderate. Taken together, the overall risk of GloFish® Barbs to the Canadian environment is ranked low, and the notified organisms are not expected to cause harmful effects to the Canadian environment at the assessed exposure level. Though uncertainty with some of the hazard estimates is moderate due to limited and or no direct data on the notified organisms or comparator species, no evidence was identified to suggest GloFish® Barbs, under the proposed or other potential uses, could cause harm as a result of exposure to the Canadian environment.

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