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Environmental Risk Assessment of the GloFish® Electric Green®, Moonrise Pink®, and Sunburst Orange® Bettas: Three Lines of Transgenic Ornamental Fish

Colin McGowan, Melissa Gagné, and Rosalind Leggatt

Fisheries and Oceans Canada
Aquaculture, Biotechnology and Aquatic Animal Health Science
200 Kent Street,
Ottawa, ON K1A 0E6

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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

CEPA: *Canadian Environmental Protection Act*

CFIA: Canadian Food Inspection Agency

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

DNA: Deoxyribonucleic acid

dpf: days post fertilization

eGFP: Enhanced green fluorescent protein

GE: Genetically engineered

GxE: Genotype by environment interaction

HGT: Horizontal gene transfer

kb: Kilobase – 1000 base pairs of DNA

LD_{50}: Lethal dose that kills 50% of a population

LD_{100}: Lethal dose that kills 100% of a population

MOU: Memorandum of Understanding

mRNA: Messenger RNA

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

RFP: Red fluorescent protein

RNA: Ribonucleic acid

SEM: Standard error of the mean

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 three genetically engineered *Betta splendens* called the GloFish® Electric Green® Betta (GBS2019), Moonrise Pink® Betta (PiBS2019) and Sunburst Orange® Betta (OBS2019) for commercial sales 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 GBS2019, PiBS2019 and OBS2019 in the Canadian environment, outside of aquaria, is expected to be rare, isolated, and ephemeral due to its 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 line and relevant comparators, and lack of establishment of non-transgenic *B. splendens* 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 ratings range from negligible to moderate due to data limitations for the notified and surrogate organisms, and some reliance on expert opinion and anecdotal evidence. The use of guide RNA and Cas9 during line creation may have resulted in off-target mutations in the GloFish® Betta 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 GBS2019, PiBS2019 and OBS2019 as ornamental aquarium fish, or other potential uses.

EXECUTIVE SUMMARY

BACKGROUND

On February 4, 2021, Spectrum Brands (a division of GloFish LLC) submitted three regulatory packages (notifications) to Environment and Climate Change Canada (ECCC) under the *New Substances Notification Regulations (Organisms)* [NSNR(O)] of the *Canadian Environmental Protection Act, 1999* (CEPA) for the GloFish® Electric Green® Betta, Moonrise Pink® Betta, and Sunburst Orange® Betta; herein referred to collectively as the GloFish® Bettas. These ornamental fish are domesticated *Betta splendens* (Siamese Fighting Fish) that have been genetically engineered to fluoresce different colours in home aquaria. Note that similar risk assessments have been conducted on three different colours of GloFish® Danios (DFO 2020a, 2020b), and six different colours of GloFish® Tetras (DFO 2018, 2019).

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, 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 as the basis to determine if the organism is CEPA-toxic or capable of becoming CEPA-toxic.

Under a memorandum of understanding with ECCC and 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 an 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 organism, and the potential for the notified organism 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 discussed.

THE NOTIFIED ORGANISMS

The three GloFish® Betta strains are independent lines of genetically engineered diploid, hemizygous or homozygous, transgenic colour morphs of domesticated *B. splendens*. Each line possesses a different transgene for which expression results in a unique colour under natural light, and becomes fluorescent under blue or UV light. The proteins are expressed in the skin, musculature, fins, eyes, and likely other organs of the organism.

For each line, all individuals are descendants of a single founding individual (F0), with the transgene construct microinjected at the single cell stage. Uniform insert location(s) of the transgene(s), copy number, and Mendelian segregation were examined at the F2 and F3 generations.

GloFish® Bettas have been sold in the United States since 2019 (GBS2019) and 2020 (PiBS2019 and OBS2019), without incident. The targeted phenotypic change is the presence of a unique fluorescent colouration as novel colour morphs for the ornamental aquarium trade. Other unanticipated phenotypic changes noted by the company include impaired cold tolerance and a reduction of reproductive success in competition with non-transgenic competitors.

ENVIRONMENTAL RISK ASSESSMENT

The environmental risk assessment was conducted under Spectrum Brands' proposed use scenario: the import of GloFish® Bettas to aquarium wholesale locations in Canada, with further distribution to aquarium retail stores across the country, to be purchased by Canadian consumers for home aquaria.

Exposure

The intended housing for the GloFish® Bettas are indoor, static, physically contained aquaria at wholesalers, retail stores, and in consumer's homes. Based on historical records of aquarium fish in natural ecosystems in Canada and worldwide, it is highly likely that the organisms will be introduced purposefully or accidentally into natural freshwater ecosystems in Canada. Based on the expected number of fish to be purchased by individual consumers, it is expected that release events will be very low in magnitude (e.g., five fish or less per release), though larger magnitude releases cannot be ruled out.

Based on temperature preferences and limitations of non-transgenic and notified *B. splendens,* and recorded water temperature throughout freshwater systems in Canada, GloFish® Bettas cannot survive over winter in Canadian ecosystems. Indeed, there are no reports of long-term established populations of non-transgenic *B. splendens* in either Canada or the United States, despite decades of sales and trading across North America, and the occasional report of transient occurrences in the wild.

The occurrence of GloFish® Bettas in the Canadian environment is expected to be rare, isolated and ephemeral. Consequently, the likelihood of **exposure** to the Canadian environment is ranked **low**. The **uncertainty** associated with this estimation is **low**, given the quality of temperature tolerance data available for each line and valid surrogate organisms, and data available on the environmental parameters of the receiving environment in Canada.

Hazard

The potential for GloFish® Bettas to be hazards to Canadian environments was examined in the context of environmental toxicity, horizontal gene transfer, interactions with other organisms including hybridization, as a vector of disease, and through impacts to biogeochemical cycling, habitat, and biodiversity. Domesticated *B. splendens* is a small fish with expected limited activity due to low temperatures in most seasons in Canada and no history of invasiveness in Canada, despite its widespread use in the aquarium trade. There are no reports of phenotypic effects of the transgene that may increase the hazard potential of GloFish® Bettas above that of non-transgenic, domesticated *B. splendens*, and no evidence that potential gene transfer will result in harm to the Canadian environment.

Rankings for the specific **hazards** examined ranged from **negligible to low**. Uncertainty ranged from **negligible to moderate**, due to limited data specific to the organisms, limited direct data on comparator species, variable data from surrogate models (RFP Zebrafish), and the reliance on expert opinion for the assessment of some hazards. The technology used during line creation (CRISPR-Cas9 gene editing) may have resulted in off target mutations, contributing to the uncertainty regarding genetic changes to the organism, but does not alter the overall conclusions on risk. GloFish® Bettas are not expected to pose additional hazards if used in applications other than the intended use of ornamental fish for home aquaria.

CONCLUSIONS ON RISK

The overall **risk** of GloFish® Bettas to the Canadian environment is ranked **low**, and the notified organisms are not expected to cause harmful effects to Canadian environments at the assessed exposure level. While the uncertainty associated with some hazard classifications is moderate due to limited or no direct data on the notified organisms or comparator species, no evidence was identified to suggest that the GloFish® Bettas, under the proposed or other potential uses, could cause harm as a result of exposure to Canadian environments. These conclusions concur with previous assessments of notified GloFish® Danio and Tetra lines (DFO 2018, 2019, 2020a, 2020b).

PART 1: PROBLEM FORMULATION

1.1. PURPOSE OF PART 1

Part 1 of this document elaborates the problem formulation for the environmental risk assessment that will be conducted under the *Canadian Environmental Protection Act* (CEPA), with respect to the GloFish® Bettas; three lines of genetically engineered (GE) variants of the domesticated *B. splendens*, also known as the Siamese Fighting Fish, notified by Spectrum Brands (a division of GloFish LLC) under the *New Substances Notification Regulations (Organisms)* [NSNR(O)] for use in the ornamental aquarium trade. The problem formulation provides a foundation for the risk assessment through identification of environmental protection objectives and the elaboration of scope. It identifies protection goals and assessment endpoints that are aligned with the legislative protection goals in CEPA. The Problem Formulation also provides a characterisation of the three GloFish® Betta strains, the comparator species, and the potential receiving environment in Canada. Notification of the GloFish® Bettas under CEPA follows previous similar GloFish® notifications for three lines of GloFish® Danio (DFO 2020a, 2020b) and six lines of GloFish® Tetra (DFO 2018, 2019).

Further information on CEPA and NSNR(O), including guidance on the regulations, detailed guidance for information requirements, use of waivers, significant new activities, risk assessment outcomes and risk management can be found on the Biotechnology page of the Environment and Climate Change Canada (ECCC) website.

1.2. LEGAL CONTEXT, RISK ASSESSMENT FRAMEWORK, AND REGULATORY DECISION MAKING

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).

1.3. CHARACTERISATION OF THE ORGANISMS

In its current notifications, Spectrum Brands is requesting the import of three new transgenic strains of *B. splendens* from the US, for the ornamental aquarium trade in Canada. Trade names for the transgenic organisms are the GloFish® Electric Green® Betta (GBS2019), Moonrise Pink® Betta (PiBS2019), and Sunburst Orange® Betta (OBS2019). Figure 1.1 demonstrates the physical appearance of the three notified GloFish® Betta strains, as well as a non-transgenic, low pigment morph of domesticated *B. splendens* (background genotype or recipient of transgene), and wild *B. splendens*.

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

1.3.1. Electric Green® Betta (GBS2019)

1.3.1.1. Molecular Characterisation

GBS2019 is a genetically engineered white morph strain of domesticated *B. splendens*, possessing multiple copies of a transgenic insert containing fish-origin promotors that drive the expression of exogenous proteins. The insertion results in green colouration of the organism under ambient light, and fluoresces under UV or blue light. The purpose of this modification is to create a new green colour phenotype of *B. splendens* for the ornamental aquarium trade.

1.3.1.1.1. Production of the notified organism

The purified transgene expression cassette was mixed into a solution of Cas9 protein and guide RNA, then injected into newly fertilized eggs of *B. splendens.* 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 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.

1.3.1.1.2. Characterization of the transgene integrant

The sequence of the transgene construct as it is inserted into GBS2019 has not been determined, and the specific location of the insert within the organism's genome is unknown. Though elements used in the production of GBS2019, such as Cas9 protein with guide RNA and Betta homologous regions at each end of the construct, were included to encourage site-directed insertion of the cassette into the Betta genome, subsequent analysis of the targeted region indicated the construct had inserted elsewhere. There are no data examining whether off-target Cas9 mutations exist in GBS2019.

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.

1.3.1.1.3. 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 GBS2019 commercially since 2019. In this time they have observed the green fluorescent phenotype to be durable and stable across generations.

1.3.1.1.4. Methods to detect GBS2019 fish

GBS2019 individuals are distinguished from non-transgenic domesticated *B. splendens* by their uniform green colouration under natural light and fluorescence under blue or UV light. GBS2019 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 on a gel that distinguish GBS2019 from other transgenic fluorescent green Bettas if they are carrying a different cassette.

1.3.1.2. Phenotypic Characterization

1.3.1.2.1. Targeted phenotypic effects of the modification

The targeted phenotypic effect of the genetic modification is that GBS2019 appears green under ambient light and fluorescent under UV or blue light. The novel colour phenotype is present in muscle as well as skin and eye. Spectrum Brands reports that GBS2019 individuals that are hemizygous and homozygous for the transgene insert are visually indistinguishable from each other and are both part of the commercially available population.

1.3.1.2.2. Non-targeted phenotypic effects of the modification

No formal studies have compared the potential disease susceptibility of GBS2019 with that of non-transgenic *B. splendens* and there are no formal studies on potential non-target effects of the genetic modification on life-history of GSB2019 , environmental tolerances and requirements, metabolism, physiology, endocrinology, or behaviour; however, there are no anecdotal or otherwise reports of any non-target effects. Confidential data, submitted by the company as part of its regulatory package, suggest that GBS2019 is no more likely to be invasive than non-transgenic, domesticated *Betta splendens*.

1.3.2. GloFish® Moonrise Pink® Betta (PiBS2019)

1.3.2.1. Molecular Characterisation

PiBS2019 is a genetically engineered white morph strain of domesticated *B. splendens*, possessing a single site of insertion that contains multiple copies of a transgenic insert containing fish-origin promotors that drive the expression of exogenous proteins. The insert results in pink colouration of the organism under ambient light and fluoresces under blue light. The purpose of this modification is to create a new pink colour phenotype of *B. splendens* for the ornamental aquarium trade.

1.3.2.1.1. Production of the notified organism

The purified transgene expression cassette was mixed into a solution of Cas9 protein and guide RNA, then injected into newly fertilized eggs of *B. splendens*. 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 PiBS2019 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.

1.3.2.1.2. Characterization of the transgene integrant

The sequence of the transgene construct as it is inserted into PiBS2019 has not been determined, and the specific location of the insert within the organism's genome is unknown. Though elements used in the production of PiBS2019, such as Cas9 protein with guide RNA and Betta homologous regions at each end of the construct, were included to encourage site-directed insertion of the cassette into the Betta genome, subsequent analysis of the targeted region indicated the construct had inserted elsewhere. There are no data examining whether off-target Cas9 mutations exist in PiBS2019.

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.

1.3.2.1.3. Inheritance and stability of the transgene

The specific insert location of the transgene has not been determined, and consequently, it cannot be determined whether the transgene is 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 pink colouration and would, therefore, be removed from the breeding population.

Spectrum Brands has maintained this breeding line for over four generations and have produced PiBS2019 commercially since 2020. In this time they have observed the pink fluorescent phenotype to be durable and stable across generations.

1.3.1.1.4. Methods to detect PiBS2019 fish

PiBS2019 individuals are easily distinguished from non-transgenic domesticated *B. splendens* by their phenotypic uniform pink colouration under natural light and fluorescence under blue or UV light. PiBS2019 can be distinguished genetically by PCR amplification and detection of unique fragments of the transgene insert, followed by a restriction digest to generate unique fragments that can be separated into a series of bands that distinguish PiBS2019 from other transgenic fluorescent pink Bettas if they are carrying a different cassette.

1.3.2.2. Phenotypic Characterization

1.3.2.2.1. Targeted phenotypic effects of the modification

The targeted phenotypic effect of the genetic modification is that PiBS2019 appears pink under ambient light and fluorescent under UV or blue light, to create a new, bright colour variant for the ornamental aquarium trade. The novel colour phenotype is present in the muscle as well as skin and eyes. Spectrum Brands reports that PiBS2019 individuals that are hemizygous and homozygous for the transgene insert are visually indistinguishable from each other and are both part of the commercially available population.

1.3.2.2.2. Non-targeted phenotypic effects of the modification

No formal studies have compared the potential disease susceptibility of PiBS2019 with that of non-transgenic *B. splendens*. There are also no formal studies on potential non-target effects of genetic modification on life-history of PiSB2019, environmental tolerances and requirements, metabolism, physiology, endocrinology, or behaviour; however, there are no anecdotal or otherwise reports of any non-target 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 *Betta splendens*.

1.3.3. GloFish® Sunburst Orange® Betta (OBS2019)

1.3.3.1. Molecular Characterization

OBS2019 is a genetically engineered white morph strain of domesticated *B. splendens*, possessing a single site of insertion that contains approximately one copy of a transgenic insert containing fish-origin promotors that drive the expression of exogenous proteins. This insert results in orange colouration of the organism under ambient light and fluorescent colouration under UV or blue light. The purpose of this modification is to create a new orange colour phenotype of *B. splendens* for the ornamental aquarium trade.

1.3.3.1.1. Production of the notified organism

The purified transgene expression cassette was mixed into a solution of Cas9 protein and guide RNA, then injected into newly fertilized eggs of *B. splendens*. 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 PiBS2019 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.

1.3.3.1.2. Characterization of the transgene integrant

The sequence of the transgene construct as it is inserted into OBS2019 has not been determined, and the specific location of the insert within the organism's genome is unknown. Though elements used in the production of OBS2019, such as Cas9 protein with guide RNA and Betta homologous regions at each end of the construct, were included to encourage site-directed insertion of the cassette into the Betta 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 OBS2019.

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.

1.3.3.1.3. Inheritance and stability of the transgene

The specific insert location of the transgene has not been determined, and consequently, it cannot be determined whether the transgene is 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 have produced OBS2019 commercially since 2020. During this time, they have observed the orange fluorescent phenotype to be durable and stable across generations.

1.3.3.1.4. Methods to detect OBS2019 fish

OBS2019 individuals are easily distinguished from non-transgenic domesticated *B. splendens* by their phenotypic uniform orange colouration under natural light and fluorescence under blue or UV light. OBS2019 can be distinguished genetically by PCR amplification of a unique section of the cassette, and detection of unique fragments following a restriction enzyme digest. When digested with a restriction enzyme, the PCR product can be separated into a series of bands that distinguish OBS2019 from other transgenic fluorescent orange Bettas carrying a different cassette.

1.3.3.2. Phenotypic Characterization

1.3.3.2.1. Targeted phenotypic effects of the modification

The targeted phenotypic effect of the genetic modification is that OBS2019 appears orange under ambient light and fluorescent under UV or blue light, to create a new, bright colour variant for the ornamental aquarium trade. The novel colour phenotype is present in the muscle as well as skin and eyes. The company reports that OB2019 individuals that are hemizygous and homozygous for the transgene insert are visually indistinguishable from each other, and are both part of the commercially available population.

1.3.3.2.2. Non-targeted phenotypic effects of the modification

No formal studies have compared the potential disease susceptibility of OBS2019 with that of non-transgenic *B. splendens*. There are also no formal studies on potential non-target effects of genetic modification on life-history of OSB2019, environmental tolerances and requirements, metabolism, physiology, endocrinology, or behaviour; however, there are no anecdotal or otherwise reports of any non-target effects.

Confidential data, submitted by the company as part of its regulatory package, suggest that OBS2019 is no more likely to be invasive than non-transgenic, domesticated *Betta splendens*.

1.3.4. Pleiotropic Effects of Fluorescent Protein 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, 2006b), 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 Zebrafish transgenic for eGFP, driven by the Fli-1 protein promoter, were less cold tolerant than the source non-transgenic strain; however, two other eGFP lines driven by other promoters did not have diminished cold tolerance. This indicates that different transgenic lines may have different responses to extreme environmental stressors.

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) indicating decreased viability of fluorescent gametes or larvae in some fluorescent models.

Reports describing the effects of RFP transgenesis on vulnerability to predation have varied. Cortemeglia and Beitinger (2006a) 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 wild-caught Zebrafish. In contrast, Jha (2010) found a domesticated RFP Zebrafish strain in India was less preyed upon by wild-caught Snakeheads than 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 used, 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 they were raised with. 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. 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 off-target 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).

1.3.5. Characterization Relative to Previously Notified and Assessed GloFish®

The GloFish® Bettas were produced using similar methodologies and testing protocols as for previously notified and assessed GloFish® Danio and GloFish® Tetra 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. One difference with the production of the Betta GloFish® was the use of Betta-specific homology arms incorporated in the construct, and the use of Cas9 with Betta-specific guide RNA included in the injection mixture when producing the founding individuals for each line. This was to direct site-specific insertion of the construct into the Betta genome, though the company reported site-specific sequence similarity analysis revealed the transgenes were inserted in an unknown location other than the targeted location (data not provided).

The use of guide RNA and Cas9 to direct site-specific transgene insertion may have resulted in un-intended off-target mutations within the resulting populations of Bettas. Sequence analysis found no places on the published Betta genome with exact matches to the guide RNA (other than the intended site with one additional base pair). 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 off-target Cas9 mutations exist in the GloFish® Betta populations. The potential for off-target Cas9 mutations to affect risk-relevant traits in higher organisms has not been examined to our knowledge, and may be case-specific.

Molecular and phenotypic characterization tests conducted by the company were equal among the current and previously notified GloFish® lines, and results from tests conducted on the GloFish® Bettas overlap with some or all of previously notified lines.

1.4. CHARACTERISATION OF COMPARATOR SPECIES

For the purpose of this risk assessment, the domesticated *B. splendens* (the Siamese Fighting Fish) was selected as a comparator. *B. splendens* is a popular ornamental species that is bred, produced, and traded worldwide. It is one of the most frequently occurring fish in the aquarium trade, e.g. occurring in 97% of surveyed aquarium stores in Northwest United States (Strecker et al. 2011). It was first domesticated about 170 years ago, in what is present day Thailand, where they were sought after for the propensity of males to fight with one another (Monvises et al. 2009), and current aquarium-trade *B. splendens* are considered to be strongly domesticated (Teletchea 2016). Subsequent selection has resulted in an impressive variety of colours, iridescence, and fin morphologies that made the species a favorite among aquarium enthusiasts. More recently, interest in the genetics behind aggressive behaviour have made *B. splendens* an important model species for scientific research (Fan et al. 2018; Ramos and

Goncalves 2019; Vu et al. 2020). Wild populations of *B. splendens* within their natural range are currently in decline as a result of human activities such as pollution, habitat destruction, and the introduction of domesticated strains (Chailertrit et al. 2014).

1.4.1. Taxonomic Status

Betta splendens is a freshwater fish of the subfamily Macropodusinae, of the family Osphronemidae (also known as Gouramis). They are one of approximately 55 species of *Betta* that have been described from southeast Asia, with *B. splendens* among the most widely distributed (Monvises et al. 2009). As members of the suborder Anabantoidei (order Perciformes), Bettas possess a 'labyrinth organ'; a highly folded structure held in a cavity above the gills that is covered with respiratory epithelium and can assist in accessory air-breathing (Ruber et al. 2006). The organ is used to generate bubbles when building a nest, and can aid in survival when dissolved oxygen is limited. The genus *Betta* can be further divided into species that are 'mouth-brooders', where the male protects the fertilized eggs by keeping them in its mouth, and 'bubble-nesters', where the male builds a nest of bubbles in which it can incubate and guard developing embryos. Though the majority of *Betta* species are mouth-brooders, *B. splendens* is described as a bubble-nester (Ruber et al. 2006).

There are no published reports of hybridization among *Betta* species in the wild, or under culture; however, according to the International Betta Congress, the domesticated strains of *B. splendens* we see today were selectively bred from the wild types of *Betta imbellis*, *Betta mahachaiensis*, *Betta samaragdina*, and the original wild type *B. splendens* (Goldstein 2015).

Since their domestication, *B. splendens* has been selectively bred for traits such as colour and iridescence, fin morphology, and aggression (Monvises et al. 2009). Today, there is a large variety of *B. splendens* strains that can be purchased or traded, including the Black Orchid Betta, the Bumblebee Betta, the Half-moon Double Tail Betta and the Rose Petal Betta, to name but a few (USFWS 2019).

1.4.2. Distribution

Betta splendens are native to the Mekong River basin of Cambodia, Laos, Thailand and Viet Nam. They are the predominant *Betta* species and widespread in the central plain of Thailand (Monvises et al. 2009). Introductions of *B. splendens*, both accidental and intentional, have occurred in several countries, with established populations reported in Singapore, the Dominican Republic, Brazil, Colombia (USFWS 2019) and Australia (Hammer et al. 2019). In the United States, *B. splendens* were believed to be established in Florida, in bodies of water adjacent to ornamental fish farms, but were eradicated by extreme cold weather in 1977 (USFWS 2019). As well, evidence of a reproducing population was found in Connecticut in 1997 but failed to establish (see Nico and Neilson 2018; USFWS 2019). Prior to 1968, introductions of B. splendens were made to thermal springs in Banff National Park, Alberta, Canada (Renaud and McAllister 1988), but there is no record of their occurrence since.

1.4.3. Physical, Chemical and Biological Requirements

Wild *B. splendens* are carnivorous, feeding mostly on mosquito larvae and other small aquatic insects (Pleeging and Moons 2017). Their preferred habitats are small bodies of fresh water such as those in rice paddies, ponds, lagoons and marshes, with plenty of vegetation to help protect it from avian predators (Jaroensutasinee and Jaroensutasinee 2001; Monvises et al. 2009). In the aquarium, *B. splendens* prefer a neutral pH, will tolerate dissolved oxygen levels below 2 ppm (though they prefer 5 to 7 ppm), and do best at temperatures between 24 and 30°C (Goldstein 2015). Goodrich and Taylor (1934) established an optimal breeding temperature of approximately 27°C, and observed that males stop building bubble nests when temperatures fall below 25°C. In the wild, the habitat of nesting *B. splendens* in Thailand has been described as low in pH (5.28 to 5.80) and dissolved oxygen (0.00 to 7.39 ppm), high in temperature (27.00 to 31.50°C), and with water depths ranging from 2.00 to 9.40 cm (Jaroensutasinee and Jaroensutasinee 2001). There are no published records describing the ability of Bettas to survive outside their preferred temperature range; however, data collected by the notifier indicate that Bettas cannot survive below 6°C when temperatures fall quickly (1°C/30 min from 25 to 15°C, with 2 h incubation periods at 17.5 and 15°C, then decrease by 0.25°C/30 min from 15°C on, see Section 1.3.1.2.2). Unpublished data collected by DFO indicate that when temperatures drop slowly (1°C/day starting at 20.5°C), Bettas slowed feeding and activity around 17°C, stopped feeding at about 12.5°C, ceased activity at 11.5°C, and lost equilibrium between 11.2 and 8.0°C (average 10.0 ± 1.2°C, see Figure 1.2).

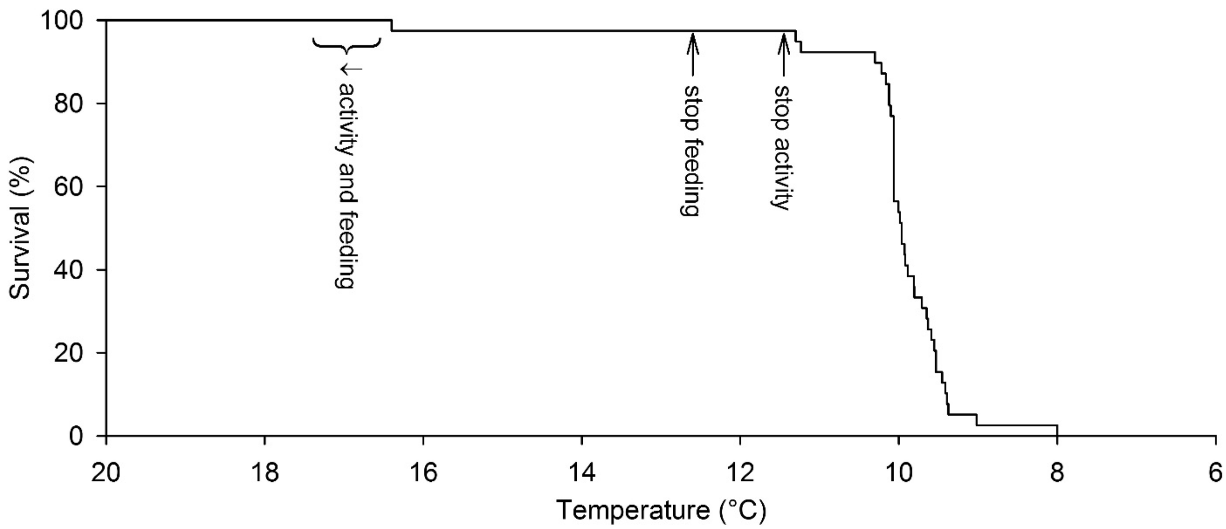

Figure 1.2. Activity and survival of Betta splendens during temperature drops of 1°C per day (unpublished data from DFO).

1.4.4. Life-history

The life-history of Betta bubble-nesters is described in detail by Goldstein (2015). When ready to reproduce, the male *B. splendens* builds a nest of bubbles at the water surface and begins to court a female. The bubbles are generated by the male using its labyrinth organ, and are coated with a mucus that helps to bind the nest together, and provides eggs and larvae with protection from microbes. Once a mate has been chosen, the male wraps its body around the female, inducing her to release eggs. Ten to 40 eggs are released with each embrace until the female is spent, a process that can take several hours and can result in approximately 100 to 1000 fertilized eggs. Fertilization is external, with the male catching eggs as they fall towards the substratum and lifting them into the nest where it can protect them from predators and ensure their development. When spawning is complete, the female is chased away and the male assumes responsibility for defending the nest. Eggs hatch after 24 to 36 hours of incubation, then remain in the nest for two or three days, as larvae, until their egg-sac is fully adsorbed. Fry leave the nest as free-swimming fish. Sexual dimorphism is apparent after three or four months and, though the growth rate of *B. splendens* is highly variable, fish can become sexual mature within five months and can reach a length of about 7.5 cm (Watson et al. 2019).

1.4.5. Background Genetics

The domesticated *B. splendens* is relatively well characterized genetically, though information on the genetic structure of wild populations is limited. Early studies of domesticated Bettas focused on the inheritance of colour, iridescence, and fin morphologies, as well as the mechanism of sex determination (Goodrich and Mercer 1934; Wallbrunn 1958; Lucas 1968). Though males are considered by many to be the heterogametic sex, realized sex ratios at adulthood are variable and can be significantly different from 50:50. Cytological studies of *B. splendens* established a diploid chromosome number of 42 (Furgala-Selezniow et al. 2008), but could not identify a morphologically distinct sex chromosome, suggesting that sex may be determined by several alleles that are spread among autosomes, or that environmental factors may be influencing the development of sex (Kipouros et al. 2011). More recently, Ramos and Goncalves (2019) found that selection for aggression in male *B. splendens* correlated with high female aggression, suggesting common genetic and physiological mechanisms to male and female aggression.

Molecular biology and genomics are also being used to further our understanding of *B. splendens* genetics. Khoo et al. (1997) used isoelectric focusing to investigate protein variation between four different colour strains of *B. splendens*, and found a high degree of genetic similarity among strains. Meejui et al. (2005) used allozyme electrophoresis to examine different hatchery populations and compare them with a wild population. They found that although there was less genetic variability in the hatcheries relative to the wild population, there was still enough variability to detect genetic structure among the hatchery populations.

Mitochondrial DNA sequence data have been used for phylogenetic analysis and to study the evolutionary relationship between mouth-brooding and bubble-nesting *Betta* species (Ruber et al. 2004; Ruber et al. 2006). These studies established Bettas as a monophyletic group, though the mouth brooding trait was found to be polyphyletic and may have evolved more than once within the genus. A complete sequence of the entire mitochondrial genome for *B. splendens* was published by Prakhongcheep et al. (2018) to help facilitate further genetic studies.

Fan et al. (2018) assembled the first reference genome for *B. splendens* as a foundation for the study of aggressive behaviour in the species. This reference map was used by Vu et al. (2020) when studying the brain-transcriptomics in pairs of fighting male *B. splendens*. They found synchronization of gene expression among fighting pairs, leading to pair-wise synchronization of genes associated with ion transport, synapse function, and learning and memory. Amparyup et al. (2020) also used transcriptomics to study *B. splendens* immune function.

1.4.6. History of Invasiveness

While there are examples of established *B. splendens* populations in various parts of the world (all tropical), it has only been reported as invasive in northern Australia, where a large and extensive population has persisted in the Adelaide river over consecutive years, with potential for further spread (Hammer et al. 2019). Here they are postulated to impact localized populations of native fish and tadpoles occupying similar niches through aggressive interactions and competition for space and food, although direct impacts of the introduced Betta have not been specifically examined (Hammer et al. 2019). Hill et al. (2017) estimated *B. splendens* invasion potential in conterminous United States to be low, using the Fish Invasiveness Screening Kit (FISK).

1.5. CHARACTERIZATION OF POTENTIAL RECEIVING ENVIRONMENT

Though the many lakes and rivers of Canada vary in their annual temperature profiles, as well as their average maximum and minimum temperatures, most reach 4°C or below at some point annually, and only a few isolated lakes in Southern Coastal British Columbia have minimum recorded temperatures above this. Of these latter lakes all but one has a minimum temperature recorded below 6°C, and the one lake with minimum temperature recording above 6°C (Cowichan Lake) had minimum temperature recorded below 6°C on occasion (see Leggatt et al. 2018b). It is worth noting that temperature recordings of these warmer lakes are often restricted to a single measurement per winter, and recorded temperatures may not represent the coldest or warmest temperature obtained during winter months.

Given the above, if an introduced fish cannot survive at 4°C or below, its occurrence in the Canadian environment will be seasonal at best, with possible localized overwintering pockets (e.g., industrial effluent, hot springs etc., isolated lakes if can survive between 4-6°C). It should be noted that many freshwater systems may 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 have more extreme temperatures than deep waters. Hot springs or warm water effluent may result in localized areas with year-round elevated temperatures. As well, mean freshwater surface temperatures in Canada are rising as a result of global climate change and are projected to increase by 1.5 to 4.0°C over the next 50 years (DFO 2013) and therefore, could increase the number of possible lakes in which organisms with moderate cold tolerance could survive.

A more detailed description of potential receiving environments in Canada relevant to the introduction of tropical freshwater fish is presented in Leggatt et al. (2018a).

PART 2: ENVIRONMENTAL RISK ASSESSMENT

2.1. PURPOSE OF PART 2

Part 2 of this document comprises the environmental risk assessment conducted under the *Canadian Environmental Protection Act* (CEPA) with respect to the three GloFish® Betta lines that are described in part one of this document, and have been notified by Spectrum Brands under the *New Substances Notification Regulations (Organisms)*. Given the common comparator species, and the physiological and ecological similarities between the three lines, the following section will consider all lines at the same time. The environmental risk assessment format follows that used for previously notified GloFish® Tetras (DFO 2018, 2019) and GloFish® Danios (DFO 2020a, 2020b). Results of the current assessment are equivalent to those from previous GloFish® assessments unless otherwise stated.

2.2. EXPOSURE ASSESSMENT

The exposure assessment for the three living organisms addresses both their potential to enter the environment (release) and fate once in the environment. The likelihood and magnitude of environmental exposure is determined through an extensive, cradle-to-grave assessment that details the potential for release, survival, persistence, reproduction, proliferation, and spread in the Canadian environment. Rankings for the likelihood of exposure to the Canadian environment are provided in Table 2.1.

Table 2.1. 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® Bettas 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 2.2 ranks uncertainty associated with the likelihood of occurrence and fate of the organism in the Canadian environment.

Table 2.2. 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 and at the point of entry. Demonstration of absence of Genotype by Environment Interaction (GxE) effects or complete understanding of GxE effects across relevant environmental conditions. Evidence of low variability.
Low	High-quality data on relatives of the organism or valid surrogate. Data on environmental parameters of the receiving environment. Understanding of potential GxE effects across relevant environmental conditions. Evidence of variability.
Moderate	Limited data on the organism, relatives of the organism or valid surrogate. Limited data on environmental parameters in the receiving environment. Knowledge gaps. Reliance on history of use or experience with populations in other geographical areas with similar or better environmental conditions than in Canada.
High	Significant knowledge gaps. Significant reliance on expert opinion.

All previous assessments of notified and assessed GloFish® Danio and Tetra lines concluded low rating for environmental exposure with low uncertainty (DFO 2018, 2019, 2020a, 2020b). There are no known molecular or phenotypic characteristics of GloFish® Bettas that suggest a different rating than previously assessed lines, and no new scientific literature has been published that would alter the previous ratings. Consequently, the environmental exposure assessments for GloFish® Bettas are low, with low uncertainty that is consistent with previously notified lines. Details supporting this conclusion follow.

2.2.1. Likelihood of Release

Though the stated purpose of the organism is for sale in the ornamental market, and hobbyists who purchase the product do, for the most part, follow the instructions for disposal that are recommended by the retailer or the company itself, there is still a high likelihood that GloFish® Bettas will be introduced into the Canadian environment. Once the organism has been sold into the retail market, it is 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 water suggest the practice of releasing aquarium fish into the environment is common and ongoing (Kerr et al. 2005; Rixon et al. 2005; Marson et al. 2009; Strecker et al. 2011). This concurs with a high likelihood of release for previously notified GloFish® Tetras and Danios. The extent to which GloFish® Bettas may be further exposed to the environment will therefore depend heavily on their ability to survive and reproduce in Canadian lakes and rivers.

2.2.2. Likelihood of Survival

As a tropical species, *B. splendens* is not expected to survive in a temperate region, where water temperatures are below optimal for survival. 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, *B. splendens* do best at temperatures between 24 and 30°C (Goldstein 2015, see Section 1.4.3).

In experiments at DFO (see Section 1.4.3), when water temperatures were dropped relatively slowly (decrease of 1°C/day from 20.5°C), non-transgenic domesticated *B. splendens* stopped feeding around 12.5°C, and 100% of fish had lost equilibrium by 8°C, though the majority of fish lost equilibrium between 11.2 and 9.0°C, with an LD_{50} of 9.91±0.13°C.

As discussed in the Problem Formulation (see Section 1.5) 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® Bettas to survive are possible for several Canadian lakes during the summer, it is extremely unlikely that GloFish® Bettas can survive the Canadian winter. At best, its occurrence in the environment would be seasonal or ephemeral. This is further supported by lack of establishment of *B. splendens* after noted occurrences in much warmer climates (e.g., Florida, USFWS 2019).

Though 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), 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), but continuation of any winter ice coverage would result in temperatures at or below 4°C at some point during the winter, preventing year-round survival of GloFish® Bettas.

Cold-tolerance data combined with the lack of establishment of *B. splendens* in regions warmer than Canada (e.g., Florida, USFWS 2019), suggest negligible potential for survival in Canadian waters, even with the increased water temperatures associated with climate change.

2.2.3. Likelihood of Reproduction

Though water temperatures in Canada will limit the persistence of any GloFish® Bettas that are introduced into the environment (see Section 2.2.2), 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 2013). While this may be a tolerable temperature range for GloFish® Bettas survival, warmer temperatures (25-30°C) are more ideal for reproduction (Goodrich and Taylor 1934, Goldstein 2015). Bettas could potentially reproduce in isolated areas of warm water (e.g., hot springs); however, they did not become established when released to a hot spring in Banff, AB (see USFWS, 2019).

2.2.4. Likelihood of Proliferation and Spread

The capacity for GloFish® Bettas to proliferate and spread in the Canadian environment is prevented by the fact that *B. splendens* cannot survive the winter. It should be noted that any released GloFish® Bettas are expected to occupy areas near the shoreline, based on what is known of wild-type habitat preferences (see Section 1.4.3). 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® Bettas, though fish may move to follow warmer water as temperatures drop. Warmer summer temperatures in these areas may increase potential for single-generation spawning.

2.2.5. Conclusions

Given the above analysis, the occurrence of GloFish® Bettas in the Canadian environment is expected to be rare, isolated and ephemeral. Consequently, the likelihood of exposure of GloFish® Bettas to the Canadian environment is ranked **low** according to Table 2.1. The uncertainty associated with this estimate is **low** (Table 2.2), given the quality of data (temperature tolerance) available for GloFish® Bettas and valid surrogate organisms, evidence of low variability, and data available on the environmental parameters of the receiving environment in Canada. This rating is consistent with the low exposure rating with low uncertainty concluded on for three lines of GloFish® Danio (DFO 2020a, 2020b), and six lines of GloFish® Tetra (DFO 2018, 2019).

The notifying company identifies the sole intended use for the notified organism as an ornamental fish for interior, static home aquaria. However, once purchased by consumers, other unintended uses cannot be discounted (e.g., rearing in outdoor ponds, as bait fish, etc.). While some unintended uses may lead to the release of GloFish® Bettas, they would not be expected to 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 have the potential to increase uncertainty when determining the ability of the notified organism to survive, reproduce, proliferate and spread in Canadian freshwater ecosystems.

2.3. HAZARD ASSESSMENT

The hazard assessment examines potential impacts to the environment that could result from exposure to GloFish® Bettas. The hazard identification process considers potential pathways to harm including through environmental toxicity (i.e., potential to be poisonous), gene transfer, trophic interactions, and as a vector for pathogens, as well as capacity to impact ecosystem components (e.g., habitat, nutrient cycling, biodiversity). Table 2.3 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 2.3. 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 may be significant due to clear knowledge gaps and lack of empirical data around the behaviour and effects of GloFish® Bettas 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 organism in the environment is provided in Table 2.4.

Table 2.4. 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. 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® Bettas 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® Bettas to Canadian environments. The majority of potential hazards posed by GloFish® Bettas (e.g., through interactions with other organisms, impacts to biogeochemical cycling, habitat and biodiversity) would be through direct release of GloFish® Bettas 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).

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

2.3.1. 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 ingestion by predators. Exposure of the fluorescent proteins to the environment is expected to be lower than exposure of the proteins to GloFish® Betta lines; 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 other species of GloFish® containing transgenes coding the same proteins as those in the GloFish® Betta lines. Consequently, the potential hazard to the environment due to environmental toxicity of GloFish® Bettas is ranked **negligible**. The uncertainty associated with this ranking is **moderate** due to limited direct data from the notified organisms or surrogate organisms, and reliance on anecdotal evidence and indirect evidence from other organisms. This concurs with assessment rankings for previously notified GloFish® Danios and Tetras (DFO 2018, 2019, 2020a, 2020b), and no new relevant data have become available since analyses of previous GloFish® lines.

2.3.2. 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 free transgenic DNA to novel organisms (most likely prokaryotes) 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 transposable elements that may 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 constructs of the current 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 genetically engineered fruit flies (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, although cautioned 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 is not 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 (e.g., reproduction, cold tolerance) advantage conferred by the fluorescent protein transgenes used suggest 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 limited knowledge of the location of the transgenes within the *B. splendens* genome, and lack of studies examining HGT of the transgenes and resulting consequences, results in **moderate** uncertainty. This concurs with the previous assessments for the GloFish® Danios, and Tetras, though in the latter group uncertainty was assessed as low (DFO 2018, 2019, 2020a, 2020b). Here, and for the Danios, the uncertainty rating was increased to better reflect the lack of or limited number of relevant studies of HGT and resulting consequences.

2.3.3. Potential Hazards Through Interactions with Other Organisms

Should GloFish® Bettas be released to the environment, they have the potential to interact with other organisms in Canadian freshwater aquatic ecosystems, including potential prey, competitors, and predators. The trophic interactions of wild *B. splendens* in its native range are not well documented (see Section 1.4.3), nor is there documentation of trophic interactions of escaped domesticated non-transgenic *B. splendens* in other areas. Limited data described below indicate non-transgenic *B. splendens* may have limited potential to impact Canadian species through trophic interactions, and GloFish® Bettas would likely have equal or less potential to impact through trophic interactions.

Wild *B. splendens* are carnivorous, feeding mostly on mosquito larvae and other small aquatic insects (Pleeging and Moons 2017). As such, they have the potential to impact localized populations of small prey organisms or competitors occupying similar niches at the location of release. Non-transgenic *B. splendens* are generally described as aggressive, and do not interact well with other ornamental fish species, and have been postulated to have localized impacts on organisms occupying similar niches when established outside native range, although this has not been directly examined (Hammer et al. 2019). Bettas were one of several tropical fish species introduced to a Banff, AB hot spring where introduced species are thought to have contributed to the extinction of the Banff Longnose Dace, although Bettas were not listed among species thought to have contributed to the dace's extinction (Lanteigne 1988; Renaud and

McAllister 1988). In typical water systems in Canada, activity and hence feeding levels of *B. splendens* are expected to be low during most seasons due to limited activity of Bettas at temperatures below 17°C (DFO unpublished data).

In other fluorescent protein transgenic models, GloFish® Electric Green® Tetras had similar aggressive behaviour and foraging success as non-transgenic siblings in paired feeding trials (Leggatt and Devlin 2019), while RFP Zebrafish had lower male mating aggression and success than non-transgenic siblings (Howard et al. 2015), suggesting fluorescent protein transgenesis may decrease or not affect competitive success in tropical fish - although both of these studies are on typically non-aggressive fish. Anecdotal information from the notifying company indicates there are no detected differences in behaviour between GloFish® Bettas and non-transgenic bettas in the pet industry.

Given the low temperatures expected for 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® Bettas to impact native aquatic species through prey acquisition and competition is expected to be negligible through most of the year, and is expected to be no greater than for non-transgenic *B. splendens*.

Released GloFish® Bettas also have potential to impact native predator populations by acting as a new prey source. 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® Bettas causes deleterious effects to the predator populations. The latter is not expected as GloFish® Bettas are not expected to be environmentally toxic (see Section 2.3.1 above). While the predation pressure on GloFish® Bettas relative to non-transgenic *B. splendens* has not been reported, non-transgenic populations that establish in the wild are reported to be more drab in colour (Hammer et al. 2019), suggesting more brightly coloured bettas such as the GloFish® Betta lines may be more subject to selective pressures such as predation. This effect has also been observed in other ornamental tropical fish species bred for colour (Tuckett et al. 2021). The effect of fluorescent transgenesis in another transgenic model (RFP Zebrafish) is conflicting, with RFP-expressing Zebrafish having higher (Hill et al. 2011), equal (Cortemeglia and Beitinger 2006a), or lower (Jha 2010) predation susceptibility relative to non-related non-transgenic fish. These variable findings may be due to differences in rearing history, genetic background, experimental conditions among studies, or GxE interactions. Whether any of the above studies could be applied to the GloFish® Bettas predation vulnerability in Canadian environments is not known and, consequently, the predation vulnerability of GloFish® Bettas relative to non-transgenic counterparts cannot be estimated with reasonable certainty. However, due to the lack of expected toxicity from ingesting GloFish® Bettas, the notified lines introduced at anticipated scales are not expected to pose a hazard as prey to native predators, regardless of potential predation sensitivity.

Based on low activity of *B. splendens* in cooler waters, and lack of noted alterations in trophic-related behaviour of the notified lines, GloFish® Bettas are not expected to influence trophic interactions of native organisms beyond natural fluctuations, with associated **negligible** hazard relative to non-transgenic counterparts. The lack of studies directly examining the hazards of GloFish® Bettas, limited available data on a surrogate (RFP Zebrafish) and poor understanding of GxE interactions in aggression and predation susceptibility in surrogate fluorescent transgenic models, result in a **moderate** level of uncertainty. This concurs with assessment rankings for previously notified GloFish® Danios and Tetras (DFO 2018, 2019, 2020a, 2020b).

2.3.4. Potential Hazards Through Hybridization with Native Species

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

2.3.5. 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 (e.g., Evans and Lester 2001; Řehulka et al. 2006; Whittington and Chong 2007; Hongslo and Jansson 2009; Rose et al. 2013).

Any disease agents GloFish® Bettas 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® Bettas once released to cooler Canadian freshwater environments.

B. splendens 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). Two other species of Gourami, *Trichogaster pectoralis* and *Trichogaster trichopterus* (also of the family Osphronemidae) are listed for Epizootic ulcerative syndrome (*Aphanomyces invadans*); however, *Betta* species are not on the CFIA list of species known to carry this pathogen (Species Susceptible to Epizootic Ulcerative Syndrome). Since the principal mode of entry into Canada of GloFish® Bettas will be through importation from the US, the CFIA will play a critical role in regulating disease agents of *B. splendens* that are imported into Canada. Anyone importing aquatic animals on the Susceptible Species list requires an Aquatic Animal Health Import Permit, while all other imports require a 'declaration' at the border.

Whether GloFish® Bettas, or any other transgenic fluorescent organism, have altered ability to act as a vector of disease agents has not been directly examined. Increased susceptibility to disease may increase vector capabilities through heightened ability to act as a reservoir and increased shedding of disease agents, or decrease vector capabilities by succumbing to disease quickly. 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 accredited 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® Bettas to have altered capacity as a vector for disease relative to non-transgenic *B. splendens*. As this has not been directly examined in GloFish® Bettas, there are limited data on a surrogate, and reliance on expert opinion, the uncertainty level for this rating is **moderate.** This concurs with assessment rankings for previously notified GloFish® Tetras and Zebrafish (DFO 2018, 2019, 2020a, 2020b).

2.3.6. Potential to Impact Biogeochemical Cycling

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

2.3.7. Potential to Affect Habitat

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

2.3.8. 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 as models for research, there have been no reports of *B. splendens* becoming invasive in the temperate regions of North America or Europe. Hill et al. (2017) estimated the invasiveness risk of *B. splendens* within the continental USA to be low, using the Fish Invasiveness Screening Kit (FISK). In tropical areas that support their establishment, their occurrences have not been reported as invasive; while they have been postulated to be invasive in northern Australia, the biological impacts of their establishment remain to be determined (Hammer et al. 2019). As well, there is no evidence that GloFish® Betta lines have increased fitness that may increase invasiveness relative to non-transgenic Bettas.

As elaborated above, GloFish® Bettas are not expected to negatively impact native species through trophic or hybrid interactions, act as a vector for disease agents of concern in Canada, impact biogeochemical cycling, or impact habitat. Addition of the transgenic construct and fluorescent protein in GloFish® Bettas 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® Bettas affecting biodiversity of Canadian ecosystems. Reliance on data from the comparator species for invasiveness and biodiversity effects results in a **low** degree of uncertainty with this ranking. This concurs with assessment rankings for previously notified GloFish® Danios and GloFish® Tetras (DFO 2018, 2019, 2020a, 2020b).

2.3.9. Conclusions

GloFish® Bettas are not expected to be hazardous to Canadian environments. Non-transgenic Bettas have no history of invasiveness in 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 effects to the environment via transfer of the transgene to native Canadian species through hybridization, or HGT. GloFish® Bettas and other fluorescent fish models have no reported differences in survival, disease susceptibility, behaviour, or husbandry care, 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 2.5), while uncertainty ranged from negligible to moderate, due to limited data specific to GloFish® Bettas, limited direct data on comparator species, variable data from surrogate models (e.g., RFP Zebrafish), and the reliance on expert opinion for the assessment of some hazards. Outside of its intended use as an ornamental fish in static aquaria, GloFish® Bettas are not expected to pose unique hazards beyond those of the intended use. Hazard ranking concurred with that previously assessed for GloFish® Danios and GloFish® Tetras, although uncertainty differed from that assessed in GloFish® Tetras in two hazard categories due to increased acknowledgement of data limitations (through HGT), or differences in family distributions (through hybridization, see Table 2.5).

Table 2.5. Summary of all ranks and uncertainty rating for environmental risk assessments of currently notified GloFish® Betta lines, as well as previously notified GloFish® Danios and GloFish® Tetras (DFO 2018, 2019, 2020a, 2020b). Underlines indicate where previous assessments differ from the current assessment.

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

2.4. 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 2.3. 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 rating is not estimated, rather uncertainty in the hazard and exposure assessments are discussed in the context of a final conclusion on risk.

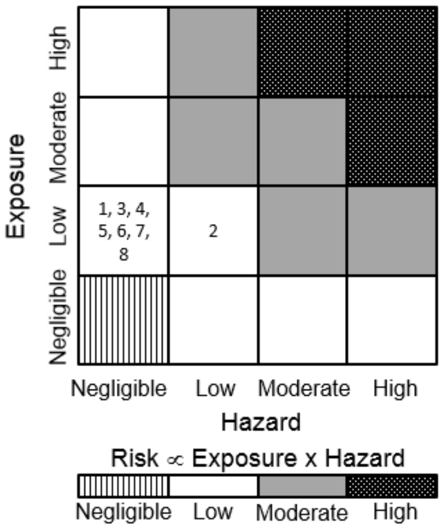

Figure 2.1. Risk matrix and pattern scale to illustrate how exposure and hazard are integrated to establish a level of risk in the environmental risk assessment. Risk assessments associated with assessed hazard components at the assessed exposure are identified by number: 1) through environmental toxicity; 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.

2.4.1. Risk Assessment of the GloFish® Bettas

The exposure assessment concluded that GloFish® Bettas used in the ornamental aquarium trade or other unintended uses would have a low likelihood of occurrence in the Canadian environment. This is due to the high likelihood of release of small numbers from home aquaria, but negligible likelihood for GloFish® Bettas to overwinter in Canadian aquatic ecosystems. As such, any exposure to Canadian freshwater ecosystems to GloFish® Bettas is expected to be isolated, rare, and ephemeral. The quality of data demonstrating lack of cold tolerance in GloFish® Bettas and domesticated *B. splendens*, relevant to Canadian freshwater temperatures result in low uncertainty associated with this ranking.

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

Using the risk matrix seen in Figure 2.3, GloFish® Bettas used in the ornamental aquarium trade or other uses in Canada pose **low risk** to Canadian environments. Individual hazards are expected to result in no harmful effects beyond natural fluctuations to Canadian environments under the assessed level of exposure. Sources of uncertainty in the environmental exposure and hazard assessments that may influence uncertainty in environmental risk assessment include a lack of data directly addressing hazards of the notified organism and comparator species, variability in data taken from surrogate organisms, and in some cases reliance on expert opinion.

Despite moderate uncertainty in some of the individual assessment components, there is no current evidence to suggest that overall risk ratings of GloFish® Bettas may be higher than the assessed low ranking for risk to Canadian environments. This concurs with low risk assessment rankings for previously notified GloFish® Danios and GloFish® Tetras (DFO 2018, 2019, 2020a, 2020b, see Table 2.5).

2.5. SUMMARY AND CONCLUSIONS

Use of GloFish® Bettas in home aquaria in Canada, or in other unintended uses, is expected to result in frequent, very small magnitude releases of GloFish® Bettas into the Canadian environment, although the potential for occasional high magnitude releases cannot be ruled out. Available high-quality data indicates that GloFish® Bettas do not have the capacity to overwinter in most Canadian freshwater ecosystems. This results in an exposure ranking of low, with associated uncertainty being low. 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® Bettas relative to non-transgenic domesticated *B. splendens*, 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® Bettas, uncertainty with hazard assessments ranged from negligible to moderate. Taken together, the overall risk of GloFish® Bettas to the Canadian environment is ranked low, and the notified organism is 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 organism or comparator species, no evidence was identified to suggest GloFish® Bettas, under the proposed or other potential uses, could cause harm as a result of exposure to the Canadian environment.

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