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Environmental Risk Assessment of the GloFish® Sunburst Orange®, Starfire Red®, Galactic Purple®, Moonrise Pink®, and Cosmic Blue® Tetras; Transgenic Ornamental Fish, Imported to Canada for Sale in the Pet Trade

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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, 1999

CT_{min}: Critical thermal minima

CL_{min}: Chronic lethal minimum temperature

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

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

RFP: Red fluorescent protein

RNA: Ribonucleic acid

SEM: Standard error of the mean

UAS: Upstream activating sequence

UV: Ultra violet

GLOSSARY

Assessment endpoint: ecological entities that are susceptible to harm upon exposure to a stressor and should be protected to achieve established protection goals

Biological diversity: As defined in CEPA, “biological diversity” means the variability among living organisms from all sources, including, without limiting the generality of the foregoing, terrestrial and marine and other aquatic ecosystems and the ecological complexes of which they form a part and includes the diversity within and between species and of ecosystems

Cassette: fragment of DNA carrying one or more genes of interest including required regulatory sequences for expression (e.g., promoter and terminator sequences)

CEPA toxic: a substance or an organism that may enter the environment in a quantity or concentration or under conditions that (a) have or may have an immediate or long-term harmful effect on the environment 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

Construct: Artificially constructed recombinant DNA sequence encoding one or more genes of interest including required regulatory sequences for expression, designed to be transplanted into a target cell

Diversity: the absolute number of species in an assemblage, community or sample; species richness; a measure of the number of species and their relative abundance in a community, assemblage or sample; the fact of being varied or different

Ecosystem: As defined in the CEPA, “ecosystem” means a dynamic complex of plant, animal and micro-organism communities and their non-living environment interacting as a functional unit

Entry: arrival of the living novel organism in the Canadian aquatic environment, through release in Canada, or immigration from other jurisdictions

Exposure: likelihood that the organism will come into contact with susceptible species and/or environmental components in Canada

Fate: the final outcome or expected result of normal development

Fluorescent: A substance that absorbs light of a short wavelength and emits light of a longer wavelength

Genetically engineered: the deliberate modification of the characteristics of an organism by manipulating its genetic material through artificial means

G0: the founding individual into which the transgene construct was first microinjected at the single cell stage

Genotype × Environment (GxE) interactions: how the genotype interacts with the environment to shape the observed phenotype; the differential morphological, physiological or behavioral responses of two or more genotypes to environmental fluctuations; plasticity

Habitat: the area or type of site where an individual or wildlife species naturally occurs and depends on directly or indirectly to carry out its life processes. It includes the biological, chemical, and physical attributes of the environment that living organisms require to complete their life process and life cycle

Harmful effect: an immediate or long-term detrimental impact on the structure or function of the ecosystem including biological diversity

Hazard: potential to cause a harmful effect

Horizontal gene transfer: the transfer of genes between organisms in a manner other than by conventional sexual or asexual reproduction

Invasiveness: property of an organism that arrived, established and spread in a new aquatic ecosystem and resulted in harmful consequences for the natural resources in the native aquatic ecosystem and/or the human use of the resource

Measurement endpoint: a measurable characteristic of the selected assessment endpoint

Mesocosm: experimental water enclosure designed to provide a limited body of water with close to natural conditions, in which environmental factors can be realistically manipulated

Persist: survives to the reproductive stage

Predation pressure: the effects of predation on the dynamics of a prey population

Productivity: the potential rate of incorporation or generation of energy or organic matter by an individual, population or trophic unit per unit time per unit area or volume; the organic fertility or capacity of a given area or habitat

Risk: the likelihood that a harmful effect will be realized as a result of exposure to a hazard. Risk incorporates the notion of the nature and severity of the harmful effect as well as the likelihood that the harmful effect will be realized

Transgenic: an organism that contains genetic material into which DNA from an unrelated organism has been artificially introduced

Uncertainty: the lack of knowledge regarding the true value of a parameter resulting from either randomness, incompleteness or both

The sources used for the definitions in this glossary include (Lincoln et al. 1988; Burgman 2005; Kapuscinski et al. 2007; Mair et al. 2007; Levin 2009; Moon et al. 2010).

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 five genetically engineered White Skirt Tetras (*Gymnocorymbus ternetzi*), called the GloFish® Sunburst Orange® Tetra (OT2018), Moonrise Pink® Tetra (PiT2018), Starfire Red® Tetra (RT2018), Galactic Purple® Tetra (PT2018), and Cosmic Blue® Tetra (BT2018), 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 OT2018, PiT2018, RT2018, PT2018 and BT2018 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 lines and relevant comparators, and lack of establishment of non-transgenic *G. ternetzi* 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 low to moderate due to data limitations for the notified and surrogate organisms, and some reliance on expert opinion and anecdotal evidence. There is low risk of adverse environmental effects at the exposure levels predicted for the Canadian environment from the use of OT2018, PiT2018, RT2018, PT2018 and BT2018 as ornamental aquarium fish, or other potential uses.

EXECUTIVE SUMMARY

BACKGROUND

On May 11, 2018, GloFish LLC submitted five 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 1999) for the GloFish® Sunburst Orange® Tetra, Moonrise Pink® Tetra, Starburst Red® Tetra, Galactic Purple® Tetra, and Cosmic Blue® Tetra. These ornamental fish are White Tetras (*Gymnocorymbus ternetzi*) that have been genetically engineered to fluoresce different colours for use in home aquaria. Note that a previous risk assessment was conducted on a related GloFish® Electric Green® Tetra in 2017 and has been published as a Science Advisory Report (DFO 2018), and as a Research Document (Leggatt et al. 2018b).

The biotechnology provisions of CEPA take a preventative approach to pollution by requiring all new living organism 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 paragraph 64 of CEPA, 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 is 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 Health Canada (HC), Fisheries and Oceans Canada (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 ECCC 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 five GloFish® Tetras are independent lines of genetically engineered diploid, hemizygous or homozygous, long- or regular-fin, transgenic colour morphs of the White Tetra (*G. ternetzi*), a white morph of the Black Tetra (also *G. ternetzi*). Each line possesses different transgenes for which expression results in a unique colour under natural light, and becomes fluorescent under

blue or UV light. The protein is 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 (G0) that had the transgene construct microinjected at the single cell stage. Insertion of the transgene at a single site for each line was confirmed at the F1 generation, and transgene copy number and Mendelian segregation were confirmed at the F2 generation.

The five notified GloFish® Tetras have been marketed in the USA ornamental aquarium trade since 2013 as the GloFish® Sunburst Orange® Tetra, the GloFish® Moonrise Pink® Tetra, the GloFish® Galactic Purple® Tetra, or since 2014 as the GloFish® Starburst Red® Tetra, and the GloFish® Cosmic Blue® Tetra. The targeted phenotypic change is the presence of a unique fluorescent colouration as novel colour morphs for the ornamental aquarium trade.

ENVIRONMENTAL RISK ASSESSMENT

The environmental risk assessment was conducted under GloFish LLC's proposed use scenario: the importation of GloFish® Tetras to four aquarium wholesale locations in Canada, for further distribution to aquarium retail stores across the country for purchase by Canadian consumers for home aquaria.

Exposure

The intended housing for GloFish® Tetras is in indoor, static, physically contained aquaria at wholesalers, retail stores, and in homes of customers. Based on historical records of aquarium fish in natural ecosystems in Canada and worldwide, it is highly likely that GloFish® Tetras will be introduced purposefully or accidentally into natural freshwater ecosystems in Canada. Based on the expected number of GloFish® Tetras to be purchased by individual consumers, it is expected that release events will be very low magnitude (e.g., five fish or less per release). Though larger magnitude releases cannot be ruled out, they are expected to occur at a low frequency. Based on temperature preferences and limitations of non-transgenic *G. ternetzi* and recorded water temperature throughout freshwater systems in Canada, GloFish® Tetras have potential to survive in many natural ecosystems in the summer in Canada, and some ecosystems in the autumn and spring. However, the lower temperature tolerance of the GloFish® Tetras and non-transgenic *G. ternetzi* preclude the fish from surviving over winter in most Canadian freshwater ecosystems. Indeed, there are no reports of established populations of *G. ternetzi* in either Canada or the United States, despite decades of sales and trading across North America, and occasional reports of transient occurrences.

There are some lakes in Canada that reach temperatures for a short time in the summer months that are adequate for reproduction of GloFish® Tetras. The minimum time to maturation for *G. ternetzi* is four months in ideal conditions, and, as such, the potential exists for only one reproductive cycle prior to termination over the winter. Given the above analysis, the occurrence of GloFish® Tetras in the Canadian environment is expected to be rare, isolated, ephemeral, and likely in low numbers. Consequently, the likelihood of **exposure** of GloFish® Tetras to the Canadian environment is ranked **low**. The **uncertainty** associated with this estimation is **low**, given the quality of temperature tolerance data available for GloFish® Tetras and valid surrogate organisms, and data available on the environmental parameters of the receiving environment in Canada.

Hazard

The potential for the GloFish® Tetras to cause a hazard to Canadian environments was examined in the context of environmental toxicity, through horizontal gene transfer, through interactions with other organisms including hybridization, as a vector of disease, and through impacts to biogeochemical cycling, habitat, and biodiversity. *G. ternetzi* is a small, non-aggressive fish with expected limited activity due to low temperatures in most seasons in Canada, there is no anecdotal evidence demonstrating susceptibility to diseases of concern in Canada (although this has not been directly examined), and it has no history of invasiveness reported in Canada or worldwide despite its wide use in the aquarium trade spanning greater than 65 years. There are no reports of phenotypic effects of the inserted transgenes that may increase hazard potential of GloFish® Tetras above that of non-transgenic domesticated *G. ternetzi*, no evidence of toxicity of the fluorescent proteins used (i.e., not poisonous to organisms or the environment), and no evidence that potential gene transfer will result in harm to Canadian environments. Some evidence suggests GloFish® Tetras may have lower potential to impact other species through trophic interactions relative to non-transgenic *G. ternetzi*, as lower cold tolerance of some strains may further limit activity in cooler water temperatures. Taken together, the five lines of GloFish® Tetra are expected to pose **negligible to low environmental hazard** if released to Canadian aquatic ecosystems. The uncertainty rankings associated with individual hazard assessments ranged from negligible to moderate due to limited data specific to GloFish® Tetras and transgenes used, limited direct data on the comparator species, variable data from a surrogate model (red fluorescent protein Zebrafish), and the reliance on expert opinion for the assessment of some hazards.

CONCLUSIONS ON RISK

The overall risk of GloFish® Tetras to the Canadian environment is **low**, and the notified organisms are not expected to cause harmful effects to Canadian environments at the assessed exposure level. 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 GloFish® Tetras under the proposed or other potential uses, could cause harm as a result of exposure to Canadian environments.

1. 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® Sunburst Orange® Tetra, Starfire Red® Tetra, Galactic Purple® Tetra, Moonrise Pink® Tetra, and Cosmic Blue® Tetra; genetically engineered variants of the Black Tetra (*Gymnocorymbus ternetzi*) notified by GloFish LLC under the *New Substances Notification Regulations (Organisms)* [NSNR(O)] for use in the ornamental aquarium trade. These notifications follow a previous notified and assessed fluorescent Tetra of the same species, specifically the GloFish® Electric Green® Tetra (DFO 2018; Leggatt et al. 2018b) and the current document refers to Leggatt et al. (2018b) where appropriate.

This 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 five GloFish® Tetra strains, the comparator species, and the potential receiving environment in Canada.

It is critical to accurately reflect the scope and focus of risk assessments conducted under CEPA at the outset, so that an appropriate and scientifically defensible risk assessment can be concluded within the legislated 120-day timeframe specified by the NSNR(O). 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 website.

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

A detailed overview of the legal context for the risk assessment, the risk assessment framework, and the regulatory decision making process is provided in Leggatt et al. 2018b.

1.3. CHARACTERISATION OF GLOFISH® TETRAS

GloFish LLC is requesting the importation of five new transgenic strains of Tetra from the US, for the ornamental aquarium trade in Canada. Trade names for the five transgenic organisms are the Sunburst Orange® Tetra, the Starfire Red® Tetra, the Galactic Purple® Tetra, the Moonrise Pink® Tetra, and the Cosmic Blue® Tetra. Long-finned naturally occurring variants of each strain except the Cosmic Blue® Tetra are included in the notification. Figure 1.1 demonstrates the physical appearance of the five notified GloFish® Tetra strains, as well as the non-transgenic Black Tetra, white morph of the Black Tetra (White Skirt Tetra), and an example of the long-finned variant.

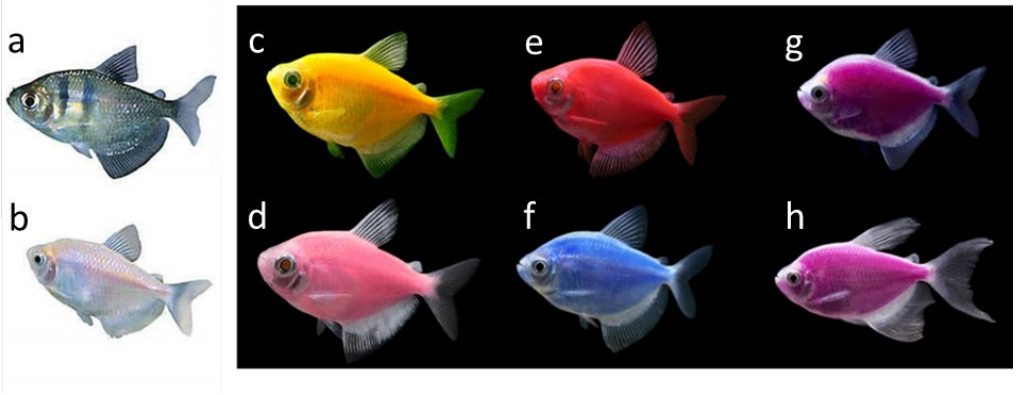

Figure 1.1: Some variants of Gymnocorymbus ternetzi available in the ornamental pet trade worldwide (a, b), and notified transgenic variants currently only available in the United States of America (c, d, e, f, g, h). Non-transgenic Black Tetra (a), White Tetra (b), Sunburst Orange® Tetra (c), Moonrise Pink® Tetra (d), Starfire Red® Tetra (e), Cosmic Blue® Tetra (f), and Galactic Purple® Tetra (g, h). Taken from www.petsmart.com (a, b), www.glofish.com (c, d, e, f, g, h). Galactic Purple® Tetra are shown in both regular (g) and long-fin varieties (h). All lines shown are available in long-fin variety except for Cosmic Blue® Tetra.

1.3.1. Cosmic Blue® Tetra (BT2018)

1.3.1.1. Molecular Characterisation

BT2018 is a genetically engineered white morph of the Black Tetra (*G. ternetzi*) possessing multiple copies of the transgenic insert containing fish-origin promotors that drive the expression of exogenous proteins. This insert results in blue colouration of the organism under white light, and fluorescent blue colouration under ultraviolet or blue light. The purpose of this modification is to create a new colour phenotype of *G. ternetzi* for the ornamental aquarium trade.

Though greater detail regarding the structure, development, and function of the transgene construct has been provided by the company for review, it is considered confidential business information and is not included in this report.

1.3.1.1.1. Production of the notified organism

The transgene expression construct was injected into newly fertilized eggs of non-transgenic white-variant of the Black Tetra (*G. ternetzi*). To date no homozygous BT2018 fish have been recovered: i.e., interbreeding of BT2018 individuals always produces some white offspring. Proportions of White Tetra produced in crosses have not been reported, and, therefore, it cannot be confirmed whether the White Tetras are due to a lack of homozygous fish or silenced genes. The proportion of fluorescent to non-fluorescent fry produced in hemizygous BT2018 x White Tetra crosses is close to 50:50, indicating the construct is likely inherited at a single locus.

1.3.1.1.2. Characterization of the transgene integrant

The specific sequence and location of the gene construct within the BT2018 genome has not been determined. Transgene copy number was estimated using quantitative real-time PCR (qPCR) against a standard curve. Results indicate that multiple copies of the transgene construct were incorporated into the genome of BT2018 fish.

While BT2018 may theoretically include individuals that are hemizygous (i.e., a construct copy on only one member of a chromosome pair) or homozygous (i.e., construct copies at that same locus on both members of a chromosome pair) for the transgenic sequence, GloFish Inc. reports no homozygous individuals have been identified. The cause of this is not reported, but indicates viability issues with homozygous transgene insert sites (i.e., that the insert may have disrupted a vital gene in the genome). Regardless, the large number of tandem copies of the insert provides an opportunity to facilitate rearrangement.

1.3.1.1.3. 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 (Uh et al. 2006). The apparent lack of homozygous individuals suggests that either homozygosity of the transgene is not viable, or that gene silencing can lead to white phenotypes in the presence of heterozygosity (e.g., as observed in a Her2/ErbB2 transgenic line of mice where the transgene was integrated into the *Pds5b* gene, Yong et al. 2015). In other transgenic organisms, high copy number of inserted fluorescent proteins resulted in gene silencing through epigenetic modification. For example, Zebrafish containing UAS-driven green fluorescent protein transgene were more likely to have transgenerational silencing of the transgene when copy number was relatively high (e.g., fourteen copies) than low (e.g., four copies, Akitake et al. 2011). As phenotype and genotype were not compared to assess the presence of silenced transgenes in non-blue fish, whether decreased blue offspring were due to silencing, transgene instability, or viability issues cannot be determined. Southern-blot banding patterns of F1 and F2 fish used for line propagation indicate inheritance of the inserted transgenes as a single unit, but available data does not discount the potential for separation of parts of the inserted copies through crossing-over events during meiosis. As well, inheritance and stability have not been examined in subsequent generations. Should transgene expression be silenced in an individual, this individual would not display phenotypic blue colouration.

1.3.1.1.4. Methods to detect BT2018 fish

BT2018 individuals are easily distinguished from non-transgenic White or Black Tetras by their phenotypic uniform blue colouration under natural/normal light. No known non-transgenic tetra species has similar uniform blue colouration, making BT2018 individuals easily phenotypically

distinguishable from other non-transgenic tetra species, unless the transgene has been silenced. There is some practice of dyeing white *G. ternetzi* different colours for the ornamental aquarium trade, including blue, but this practice is not common, does not result in permanent colouration, and dyed individuals can be distinguished from BT2018 through genetic tests.

1.3.1.2. Phenotypic Characterization

1.3.1.2.1. Strain history and production

White Tetras used in BT2018 production are a naturally occurring colour variant of the Black Tetra (Frankel 2004, see Figure 1.1), generally bred as a separate strain from the Black Tetra that was introduced to North America prior to 1950 (Innes 1950). The individual fish used to produce BT2018 were sourced from an ornamental aquarium fish producer (5-D Tropical) in 2007. All BT2018 fish are descended from a single G0 individual injected with the notified transgene construct at the single cell stage. This G0 individual was crossed to two non-transgenic White Tetras to produce two F1 hemizygous groups.

Based on line description of propagation of an earlier notified strain (GloFish® Electric Green® Tetra, CGT2016, Notification document 19261), further continuation of the BT2018 line is through batch breeding of BT2018 individuals and all white individuals are removed from the BT2018 population and transferred to separate non-transgenic White Tetra populations. While batch breeding is expected to produce both hemizygous and homozygous individuals, it is stated in BT2018 Notification documentation that no homozygous individuals have been identified. All atypical individuals are culled. BT2018 has been in commercial production for the ornamental aquarium trade in the US excluding California since 2014, and for California since 2015. BT2018 are produced by two aquarium fish producers in Florida, USA.

1.3.1.2.2. Targeted Phenotypic Effects of the Modification

The targeted phenotypic effect of the genetic modification is that BT2018 appears blue under ambient light, including sunlight, to create a new, bright colour variant for the ornamental aquarium trade. Under violet or UV light the protein in the notified organism fluoresces blue. The novel colour phenotype is present in the muscle as well as skin. Although the transgene could be expressed in internal organs, resulting in phenotypic blue colour, this has not been reported for BT2018. Available pictures of GloFish® Cosmic Blue® Tetra do not demonstrate visible blue colour in the eye, although GloFish® Electric Green® Tetra marketed in Canada demonstrate both green and not green eyes, suggesting this may be a variable trait in GloFish® transgenic tetras.

1.3.1.2.3. Non-targeted phenotypic effects of the modification

Two off-target phenotypic effects have been identified by GloFish LLC in BT2018: diminished tolerance to low temperature, and decreased fluorescent offspring from paired reproductive trials with non-transgenic siblings. As well, homozygous fish are not present in the population. Off-target phenotypes are not expected to impact the organism's fitness in home aquaria, but may impact on the organism's ability to survive and reproduce in the Canadian environment. The influence of the genetic modification on any other phenotypes, including survival, fecundity and behaviour, has not been formally examined.

GloFish LLC provided a summary of diagnostic examination by the Fish Disease Diagnostic Lab, University of Florida from tissues from twelve BT2018 individuals (six used for routine health evaluation, six used for histology).

Regarding necropsy results: "All findings were normal except for: Gills: light (1 of 6 fish) to moderate (1 of 6 fish) excess mucus, moderate hypertrophy (3 of 6 fish), and mild telangiectasia (3 of 6 fish). These findings are believed to be unrelated to the transgenic nature of these fish."

Regarding microbiology results from brain and posterior kidney: "*Growth on brain and kidney of 1 of 6 fish at 24 hours: Pseudomonas aeruginosa*" – the report stated this bacteria was sensitive to 6 tested antibiotics, and resistant to 7 tested antibiotics.

Regarding histological examination of major organs (all on females): "*Overall, these fish are basically healthy, and do not demonstrate evidence of infectious disease. However, based on their diminished degree of hepatocellular vacuolation (storage of starch and fat in the liver) and paucity of internal body fat, these reproductively active females appear to be utilizing a high proportion of their available energy for egg production. This negative energy balance may not be sustainable for the long term and it could potentially affect spawning success, as evidenced by varying degrees of oocyte atresia (egg degeneration) in the ovaries of some females. Stomachs that were present in the sections of sections of several fish were usually full of ingesta, which suggests that the fish are feeding adequately. Consequently, for optimal performance in reproducing females it may be advisable to increase the energy content of the feed relative to the current diet.*"

It should be noted that necropsy, microbiology and histology were not directly compared to non-transgenic fish, and whether any findings would be similar to equally-reared non-transgenic tetras is not known. In addition, GloFish LLC provided a statement from the veterinarian of one of the companies involved in production of all GloFish® lines that state no evidence for increased susceptibility to, or transmission of, water-borne pathogens, and that fluorescent transgenic tetra lines require the same husbandry care as non-transgenic counterparts, i.e.: "*Spectrum Brands' fluorescent ornamental fish require the same husbandry practices as their non-fluorescent counterparts, including veterinary care. I have seen no evidence of any increased susceptibility to, or transmission of, water-borne pathogens compared to their nonfluorescent counterparts*". As well, "*During my tenure with 5-D over the last four and a half years, I have observed multiple generations of Spectrum Brands' fluorescent fish in a variety of circumstances including their breeding, maturation, harvest, and eventual commercial distribution. The fluorescent tetras exhibit traditional Mendelian dominant inheritance with respect to their fluorescence, as do all of Spectrum Brands' fluorescent fish lines. The fluorescence traits are stably inherited from generation to generation with a durable phenotype that is readily distinguishable from their non-genetically engineered counterparts.*" (From Notification Document NSN-19575).

No formal studies have compared potential disease susceptibility of BT2018 and non-transgenic strains. There are also no formal studies on potential non-target effects of the genetic modification on life-history (other than reproductive success), environmental tolerances and requirements (other than low temperature tolerance), metabolism, physiology, endocrinology, or behaviour; however, there are no anecdotal or otherwise reports of non-target effects other than those listed above. A detailed overview of fluorescent protein transgenes in other fish and non-fish models is provided in Leggatt et al. (2018b).

1.3.1.3. History of Use

BT2018 has been marketed in the United States, except for California, since 2014, and in California since 2015. The history of fluorescent protein use, fluorescent fish, and the use of fluorescent fish in the ornamental aquarium trade is reviewed in Leggatt et al. (2018b).

1.3.2. Sunburst Orange® Tetra (OT2018)

1.3.2.1. Molecular Characterisation

OT2018 is a genetically engineered White Skirt Tetra possessing a single site of insertion that contains multiple copies of the construct. This genetic change results in orange colouration of

the organism under ambient white light, and fluorescent orange under ultraviolet light. The purpose of the modification is to create a new colour phenotype of *G. ternetzi* for the ornamental aquarium trade.

Though greater detail regarding the structure, development, and function of the transgene construct has been provided by the company for review, it is considered confidential business information and is not included in this report.

1.3.2.1.1. Production of the notified organism

The transgene expression construct was injected into newly fertilized eggs of White Skirt Tetras. Transgene copy number was estimated using quantitative real-time PCR (qPCR) using targeted primers. Results indicate that multiple copies of the transgene cassette were incorporated into the genome of OT2018 fish. Lack of vector backbone was confirmed by PCR amplification using targeted primers. The sequence of the cassette as it is inserted into the genome of OT2018 has not been determined, and the specific location of the insert within the OT2018 genome is unknown. The long-finned variant of OT2018 was produced by selective breeding of a natural variant within the OT2018 population that has longer fins. GloFish LLC reports that OT2018 individuals are either hemizygous or homozygous for the transgene insert.

1.3.2.1.2. Characterization of the transgene integrant

Estimates of transgene copy number made using qPCR analysis indicate multiple copies of the cassette have been inserted into the genome of OT2018. The sequence of the cassette as it is inserted into the genome of OT2018 has not been determined, and the specific location of the insert within the OT2018 genome is unknown.

1.3.2.1.3. Inheritance and stability of the transgene

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

1.3.2.1.4. Methods to detect OT2018 fish

OT2018 individuals are easily distinguished from non-transgenic White or Black Tetra by their uniform orange colouration under natural light. No known tetra species has similar colouration making OT2018 individuals easy to distinguish from non-transgenic tetras. There is some practice of dyeing white *G. ternetzi* different colours for the ornamental aquarium trade, including orange, but this practice is not common, does not result in permanent colouration, and dyed individuals can be distinguished from OT2018 through genetic tests.

1.3.2.2. Phenotypic Characterization

1.3.2.2.1. Strain history and production

White Tetras used in the production of OT2018 are a naturally occurring colour variant of the Black Tetra (Frankel 2004, see Figure 1.1) and are generally bred as a separate strain from Black Tetras. The individual fish used to produce OT2018 were sourced from an ornamental aquarium fish producer (5-D Tropical) in 2007. All OT2018 fish are descended from a single G0 individual injected with the notified transgene construct at the single cell stage. The G0 individual was crossed to a non-transgenic White Tetra to produce a F1 hemizygous group.

Based on the line propagation of an earlier notified strain (the GloFish® Electric Green® Tetra, CGT2016, notification 19261), further continuation of the OT2018 line is presumed to be through batch breeding of OT2018 individuals so that the OT2018 population contains a mix of

individuals hemizygous or homozygous for the transgene. All white individuals are removed from the population and transferred to separate non-transgenic White Tetra populations.

1.3.2.2.2. Targeted Phenotypic Effects of the Modification

The targeted phenotypic effect of the genetic modification is that OT2018 appears orange under ambient light, and fluorescent orange under ultraviolet light. The novel colour phenotype is present in muscle as well as skin and in some cases the eye. Although the transgene could be expressed in internal organs, resulting in phenotypic orange colour, this has not been reported for OT2018. GloFish LLC reports that OT2018 individuals that are hemizygous and homozygous for the transgene insert are indistinguishable from each other phenotypically, and both are part of the commercially available population.

1.3.2.2.3. Non-targeted phenotypic effects of the modification

Two off-target effects have been identified by GloFish LLC in OT2018: diminished tolerance to low temperature and a decrease in reproductive success. The results from a competitive mating trial suggest that OT2018 may have lower reproductive success than non-transgenic White Tetras or may suffer lower survival at the hatching and early fry stage. GloFish LLC also conducted a low-temperature tolerance test comparing the survival of OT2018 and sibling non-transgenic White Tetras during a rapid decrease in temperature.

The influence of the genetic modification on any other phenotypes, including survival, has not been formally examined. GloFish LLC provided a summary of diagnostic examination by the Fish Disease Diagnostic Lab, University of Florida, for 12 OT2018 individuals (six used for routine health evaluation and six used for histology). The document reports all findings normal, with the exception of several external gill parasites (Dactylogyrous, Trichodinids, *Ichthyophthirius multifiliis*), in low numbers, which were reported as unrelated to the transgenic nature of the fish. Histological examination of the major organs found no remarkable abnormalities, and no bacterial growth was observed in cultured brain and posterior kidney. In addition, GloFish LLC provided a statement from the veterinarian of one of the companies involved in production of all GloFish® lines that state no evidence for increased susceptibility to, or transmission of, water-borne pathogens, and that fluorescent transgenic tetra lines require the same husbandry care as non-transgenic counterparts.

No formal studies have compared potential disease susceptibility of OT2018 and non-transgenic strains. There are also no formal studies on potential non-target effects of genetic modification on life-history (other than reproductive success), environmental tolerances and requirements (other than low temperature tolerance), metabolism, physiology, endocrinology, or behaviour; However, there are no anecdotal or otherwise reports of any non-target effects other than those listed above.

1.3.2.3. History of Use

OT2018 has been marketed in the United States, except for California, since 2013, and in California since 2015.

1.3.3. Moonrise Pink® Tetra (PiT2018)

1.3.3.1. Molecular Characterisation

PiT2018 is a genetically engineered white morph of the Black Tetra (*G. ternetzi*) possessing multiple copies of an expression cassette. This insert results in pink colouration of the organism under ambient light and fluorescent pink colouration under blue light. The purpose of this modification is to create a new pink colour phenotype of *G. ternetzi* for the ornamental aquarium trade.

Though greater detail regarding the structure, development, and function of the transgene construct has been provided by the company for review, it is considered confidential business information and is not included in this report.

1.3.3.1.1. Production of the notified organism

The transgene expression construct was injected into newly fertilized eggs of White Skirt Tetras. Transgene copy number was estimated using quantitative real-time PCR (qPCR) using targeted primers. Results indicate that multiple copies of the transgene cassette were incorporated into the genome of PiT2018 fish. Lack of vector backbone was confirmed by PCR amplification using targeted primers. The sequence of the cassette as it is inserted into the genome of PiT2018 has not been determined, and the specific location of the insert within the PiT2018 genome is unknown. The proportion of fluorescent to non-fluorescent fry is functionally close to 50:50, indicating the construct is likely inherited at a single locus.

While PiT2018 may theoretically include individuals that are hemizygous (i.e., a single gene copy at a locus) or homozygous (i.e., two gene copies at that same locus) for the transgenic sequence, GloFish LLC reports no homozygous individuals have been identified. The cause of this is not reported, but indicates viability issues with homozygous transgene insert sites.

The long-fin variant of PiT2018 (market name GloFish® Long-Fin Moonrise Pink® Tetra) was produced by crossing pink tetras with long-fin white (presumably non-transgenic) progeny of Electric Green® (CGT2016) and/or Sunburst Orange® (OT2018) tetra lines. Progeny from these crosses were selected for fluorescent colour and long fins to establish pink long-fin breeding lines.

1.3.3.1.2. Characterization of the transgene integrant

The specific sequence and location of the gene construct within the PiT2018 genome has not been determined, although all F1 fish produced had the same insert location. A breeding trial indicated a single insert location of the transgene with Mendelian segregation. Results of a quantitative real-time PCR (qPCR) experiment indicate that approximately two copies of the transgene cassette were incorporated into the genome of PiT2018 fish.

While PiT2018 may theoretically include individuals that are hemizygous (i.e., a single copy at a locus) or homozygous (i.e., two copies at that same locus) for the transgenic sequence, GloFish LLC reports no homozygous individuals have been identified. The cause of this is not reported, but suggests viability issues with homozygous transgene insert sites.

1.3.3.1.3. Inheritance and stability of the transgene

The PiT2018 line has no evidence of homozygous individuals; see section 1.3.1.1.3 for possible causes of homozygous genotype absence.

1.3.3.1.4. Methods to detect PiT2018 fish

PiT2018 individuals are easily distinguished from non-transgenic White or Black Tetras by their phenotypic uniform pink colouration under natural/normal light. No known non-transgenic tetra species has similar uniform pink colouration, making PiT2018 individuals easily phenotypically distinguishable from other non-transgenic tetra species. There is some practice of dyeing white *G. ternetzi* different colours for the ornamental aquarium trade, including pink, but this practice is not common, does not result in permanent colouration, and dyed individuals can be distinguished from PiT2018 genetically by PCR amplification and detection of unique fragments of the transgene insert.

1.3.3.2. Phenotypic Characterization

1.3.3.2.1. Strain history and production

White Tetras used in PiT2018 production are a naturally occurring colour variant of the Black Tetra (Frankel 2004, see Figure 1.2), generally bred as a separate strain from the Black Tetra that was introduced to North America prior to 1950 (Innes 1950). The individual fish used to produce PiT2018 were sourced from an ornamental aquarium fish producer (5-D Tropical) in 2007. All PiT2018 fish are descended from a single G0 individual injected with the notified transgene expression cassette at the single cell stage. For details on line maintenance, see section 1.3.1.2.1.

1.3.3.2.2. Targeted Phenotypic Effects of the Modification

The targeted phenotypic effect of the genetic modification is that PiT2018 appears pink under ambient light, including sunlight, to create a new, bright colour variant for the ornamental aquarium trade (see Figure 1.2). PiT2018 will also fluoresce pink under blue light. The novel colour phenotype is present in the muscle as well as skin. Although the transgene could be expressed in internal organs, resulting in phenotypic pink colour, this has not been reported for PiT2018. Available pictures of GloFish® Moonrise Pink® Long-fin and regular Tetra indicate visible pink colour may be present or absent in the eye depending on the individual fish.

1.3.3.2.3. Non-targeted phenotypic effects of the modification

One off-target phenotypic effect has been identified by GloFish LLC in PiT2018, specifically decreased reproductive success in paired trials with non-transgenic siblings. As well, homozygous fish are not present in the population. PiT2018 did not significantly differ from non-transgenic siblings in measured cold tolerance. Results from a reproductive success trial indicate decreased viability of sperm, egg or embryos containing the notified transgenic construct. It could also indicate silencing of the transgene in some individuals.

The influence of the genetic modification on any other phenotypes, including survival, has not been formally examined. GloFish LLC provided a summary of diagnostic examination by the Fish Disease Diagnostic Lab, University of Florida. The document reported regarding necropsy results: "*All findings were normal except for presence of low numbers of the external parasite Gyrodactylus in 1 of 6 fish and a moderate number of granulomas around the pyloric cecae of 2 of 6 fish. These findings are unrelated to the transgenic nature of these fish*" Regarding microbiology results from brain and posterior kidney: "*No bacterial growth was observed*". Regarding histological examination of major organs, there was evidence of current or previous parasitic infection in 3 of 12 samples and 2 incidences of minor histological abnormalities. Overall conclusions on histology were: "*No significant pathologic lesions were noted in any of the sections examined*". It should be noted that necropsy, microbiology and histology were not directly compared to non-transgenic fish, and whether any findings would be similar to equally-reared non-transgenic tetras is not known. In addition, GloFish LLC provided a statement from the veterinarian of one of the companies involved in production of all GloFish® lines that state no evidence for increased susceptibility to, or transmission of, water-borne pathogens, and that fluorescent transgenic tetra lines require the same husbandry care as non-transgenic counterparts. No formal studies have compared potential disease susceptibility of PiT2018 and non-transgenic strains. There are also no formal studies on potential non-target effects of the genetic modification on life-history (other than reproductive success), environmental tolerances and requirements (other than low temperature tolerance), metabolism, physiology, endocrinology, or behaviour, however, there are no anecdotal or otherwise reports of non-target effects other than listed above.

1.3.3.3. History of Use

PiT2018 has been marketed in the United States, except for California, since 2013, and in California since 2015.

1.3.4. Galactic Purple® Tetra (PuT2018)

1.3.4.1. Molecular Characterisation

PuT2018 is a genetically engineered White Skirt Tetra possessing a single site of insertion that contains multiple copies of the transgenic construct. This genetic change results in purple colouration of the organism under ambient white light, and fluorescent purple under ultraviolet light. The purpose of the modification is to create a new colour phenotype of *G. ternetzi* for the ornamental aquarium trade.

Though greater detail regarding the structure, development, and function of the transgene construct has been provided by the company for review, it is considered confidential business information and is not included in this report.

1.3.4.1.1. Production of the notified organism

The transgene expression construct was injected into newly fertilized eggs of White Skirt Tetras. Transgene copy number was estimated using quantitative real-time PCR (qPCR) using targeted primers. Results indicate that multiple copies of the transgene cassette were incorporated into the genome of PuT2018 fish. The sequence of the cassette as it is inserted into the genome of PuT2018 has not been determined, and the specific location of the insert within the PuT2018 genome is unknown.

1.3.4.1.2. Characterization of the transgene integrant

The specific sequence and location of the gene construct within the PuT2018 genome has not been determined. Transgene copy number was estimated using quantitative real-time PCR (qPCR) against a standard curve. Results indicate that multiple copies of the transgene construct were incorporated into the genome of PuT2018 fish.

While PuT2018 may theoretically include individuals that are hemizygous (i.e., a single gene copy at a locus) or homozygous (i.e., two gene copies at that same locus) for the transgenic sequence, GloFish LLC reports no homozygous individuals have been identified. The cause of this is not reported, but suggests viability issues with homozygous transgene insert site.

1.3.4.1.3. Inheritance and stability of the transgene

The PuT2018 line has no evidence of homozygous individuals; see section 1.3.1.1.3 for possible causes of homozygous genotype absence.

1.3.4.1.4. Methods to detect PuT2018 fish

PuT2018 individuals are easily distinguished from non-transgenic White or Black Tetra by their uniform purple colouration under natural light. No known tetra species has similar colouration making PuT2018 individuals easy to distinguish from non-transgenic tetras. There is some practice of dyeing white *G. ternetzi* different colours for the ornamental aquarium trade, but this practice is not common, does not result in permanent colouration, and dyed individuals can be distinguished from PuT2018 through genetic tests.

1.3.4.2. Phenotypic Characterization

1.3.4.2.1. Strain history and production

White Tetras used in the production of PuT2018 are a naturally occurring colour variant of the Black Tetra (Frankel 2004) and are generally bred as a separate strain from Black Tetras. The individual fish used to produce PuT2018 were sourced from an ornamental aquarium fish producer (5-D Tropical) in 2007. All PuT2018 fish are descended from a single G0 individual injected with the notified transgene construct at the single cell stage.

Based on the line propagation of an earlier notified strain (the GloFish® Electric Green® Tetra, CGT2016, notification 19261), further continuation of the PuT2018 line is presumed to be through batch breeding of PuT2018 individuals so that the PuT2018 population contains a mix of individuals hemizygous or homozygous for the transgene. All white individuals are removed from the population and transferred to separate non-transgenic White Tetra populations. PuT2018 has been in commercial production in the United States excluding California since 2013, and in California since 2015. PuT2018 are produced at two aquarium fish farms in Florida.

1.3.4.2.2. Targeted phenotypic effects of the modification

The targeted phenotypic effect of the genetic modification is that PuT2018 appears purple under ambient light. PuT2018 will also fluoresce purple under blue light. The novel colour phenotype is present in muscle as well as skin. Although the transgene could be expressed in internal organs, resulting in phenotypic purple colour, this has not been reported for PuT2018. Available pictures of GloFish® Galactic Purple® Long-fin and regular Tetra indicate PuT2018 do not demonstrate visible purple colour in the eye, although GloFish® Electric Green® Tetra marketed in Canada demonstrate both green and not green eyes, suggesting this may be a variable trait in GloFish® transgenic tetras.

1.3.4.2.3. Non-targeted phenotypic effects of the modification

One off-target phenotypic effect has been identified in PuT2018; decreased cold tolerance. As well, the company has reported that no homozygous fish are present in the population. GloFish LLC provided information on PuT2018 multigenerational reproductive success in competition. There was no significant difference in the proportion of fluorescing to non-fluorescing fry. In a low-temperature tolerance test, all fish died between 8.5ºC and 6.0ºC; however, Galactic Purple® Tetras demonstrated higher cold sensitivity relative to non-transgenic White Tetras.

The influence of the genetic modification on any other phenotypes, including survival, has not been formally examined. GloFish LLC provided a summary of diagnostic examination by the Fish Disease Diagnostic Lab, University of Florida, for 12 PuT2018 individuals (six used for routine health evaluation and six used for histology). The document reports all findings normal, with the exception of several external gill and skin parasites, in low numbers, which were reported as unrelated to the transgenic nature of the fish. No bacterial growth was observed in brain or posterior kidney. Histological examination of the major organs found no remarkable abnormalities except that ovaries were filled with oocytes and primordial germ cells that appeared to be degenerative. It should be noted that necropsy, microbiology and histology were not directly compared to non-transgenic fish, and whether any findings would be similar to equally-reared non-transgenic tetras is not known. In addition, GloFish LLC provided a statement from the veterinarian of one of the companies involved in production of all GloFish® lines that state no evidence for increased susceptibility to, or transmission of, water-borne pathogens, and that fluorescent transgenic tetra lines require the same husbandry care as non-transgenic counterparts (see Section 1.5.2.3 Non-targeted phenotypic effects of the modification).

No formal studies have compared potential disease susceptibility of PuT2018 and non-transgenic strains. There are also no formal studies on potential non-target effects of genetic modification on life-history (other than reproductive success), environmental tolerances and requirements (other than low temperature tolerance), metabolism, physiology, endocrinology, or behaviour; however, there are no anecdotal or otherwise reports of any non-target effects other than those listed above.

1.3.4.3. History of Use

PuT2018 has been marketed in the United States, except for California, since 2013, and in California since 2015.

1.3.5. Starfire Red® Tetra (RT2018)

1.3.5.1. Molecular Characterisation

RT2018 is a genetically engineered White Skirt Tetra possessing a single site of insertion that contains multiple copies of the transgenic construct. This genetic change results in red colouration of the organism under ambient white light, and fluorescent red under ultraviolet light. The purpose of the modification is to create a new colour phenotype of *G. ternetzi* for the ornamental aquarium trade.

Though greater detail regarding the structure, development, and function of the transgene construct has been provided by the company for review, it is considered confidential business information and is not included in this report.

1.3.5.1.1. Production of the notified organism

The transgene expression cassette was injected into newly fertilized eggs of White Skirt Tetras. A single founding individual (G0) was identified by phenotype (red colour) and separately crossed to two non-transgenic White Tetras to produce two F1 groups.

1.3.5.1.2. Characterization of the transgene integrant

Estimates of transgene copy number made using qPCR analysis indicate that multiple copies of the cassette have been inserted into the genome of RT2018. The sequence of the cassette as it is inserted into the genome of RT2018 has not been determined, and the specific location of the insert within the RT2018 genome is unknown.

1.3.5.1.3. Inheritance and stability of the transgene

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

1.3.5.1.4. Methods to detect RT2018 fish

RT2018 individuals are easily distinguished from non-transgenic White or Black Tetra by their uniform red colouration under natural light. No known tetra species has similar colouration making RT2018 individuals easy to distinguish from non-transgenic tetras. There is some practice of dyeing white *G. ternetzi* different colours for the ornamental aquarium trade, but this practice is not common, does not result in permanent colouration, and dyed individuals can be distinguished from RT2018 through genetic tests.

1.3.5.2. Phenotypic Characterization

1.3.5.2.1. Strain history and production

White Tetras used in the production of RT2018 are a naturally occurring colour variant of the Black Tetra (Frankel 2004) and are generally bred as a separate strain from Black Tetras. The individual fish used to produce RT2018 were sourced from an ornamental aquarium fish producer (5-D Tropical) in 2007. All RT2018 fish are descended from a single G0 individual injected with the notified transgene construct at the single cell stage.

Based on the line propagation of an earlier notified strain (the GloFish® Electric Green® Tetra, CGT2016, notification 19261), further continuation of the RT2018 line is presumed to be through batch breeding of RT2018 individuals so that the RT2018 population contains a mix of individuals hemizygous or homozygous for the transgene. RT2018 has been in commercial production in the United States excluding California since 2014, and in California since 2015. RT2018 are produced at two aquarium fish farms in Florida.

1.3.5.2.2. Targeted Phenotypic Effects of the Modification

The targeted phenotypic effect of the genetic modification is that RT2018 appears red under ambient light. RT2018 will also fluoresce under blue light. The novel colour phenotype is present in muscle as well as skin. Although the transgene could be expressed in internal organs, resulting in phenotypic red colour, this has not been reported for RT2018. Available pictures of Starfire Red® Tetra suggest visible red colour may be present or absent in the eye.

1.3.5.2.3. Non-targeted phenotypic effects of the modification

Two off-target effects have been identified by GloFish LLC in RT2018: diminished tolerance to low temperature and a significant decrease in reproductive success. The results of a reproductive success trial suggest that Starfire Red® Tetras may have lower reproductive success than non-transgenic White Tetras or may suffer lower survival at the embryo, hatching and early fry stages. In a low-temperature tolerance test, all fish died between 8.5ºC and 6.6ºC; however, RT2018 demonstrated higher cold sensitivity relative to non-transgenic White Tetras.

The influence of the genetic modification on any other phenotypes, including survival, has not been formally examined. GloFish LLC provided a summary of diagnostic examination by the Fish Disease Diagnostic Lab, University of Florida, for 12 RT2018 individuals (six used for routine health evaluation and six used for histology). The document reports all findings normal, with the exception of several external skin and gill parasites (Gyrodactylids and Dactylogyrids respectively), in low numbers, which were reported as unrelated to the transgenic nature of the fish. Histological examination of the major organs found no remarkable abnormalities, and no bacterial growth was observed on cultured brain and posterior kidney tissue. In addition, GloFish LLC provided a statement from the veterinarian of one of the companies involved in production of all GloFish® lines that state no evidence for increased susceptibility to, or transmission of, water-borne pathogens, and that fluorescent transgenic tetra lines require the same husbandry care as non-transgenic counterparts.

No formal studies have compared potential disease susceptibility of RT2018 and non-transgenic strains. There are also no formal studies on potential non-target effects of genetic modification on life-history (other than reproductive success), environmental tolerances and requirements (other than low temperature tolerance), metabolism, physiology, endocrinology, or behaviour; however, there are no anecdotal or otherwise reports of any non-target effects other than those listed above.

1.3.5.3. History of Use

RT2018 has been marketed in the United States, except for California, since 2014, and in California since 2015.

1.3.6. Summary of Notified Line Characterizations

Table 1.1: Summary of characterization of notified lines (BT2018, OT2018, PiT2018, PuT2018, RT2018), as well as previously approved Electric Green® Tetra (CGT2016). Off-target phenotypes (e.g., altered cold tolerance) are not expected to impact the organism's fitness in home aquaria, but may impact on the organism's ability to survive and reproduce in the Canadian environment.

Characterization	**BT2018**	**OT2018**	**PiT2018**	**PuT2018**	**RT2018**	**CGT2016**
						
Commercial name	Cosmic Blue® Tetra	Sunburst Orange® Tetra	Moonrise Pink® Tetra	Galactic Purple® Tetra	Starfire Red® Tetra	Galactic Green® Tetra
Commercial production date - USA	2014	2013	2013	2013	2014	2012
Long-fin variant present	no	yes	yes	yes	yes	yes
% fluorescent offspring in paired crosses (=diff from expected 50%)*	48.4±0.6*	49.2±0.4	46.5±1.4*	48.0±1.6	50.0±1.2	50.2±1.9
% fluorescent offspring in reproductive competition with non-transgenics (=diff from expected 40 or 43.75%)*	38.6±3.2	35.9±3.2*	35.1±3.9	39.4±4.6	19.0±5.7*	24.9±5.1*
LD_{50} of notified vs non-transgenic tetra during rapid decrease in temperature	8.02 vs 7.64ºC*	9.07 vs 8.95ºC*	8.03 vs 7.95ºC	7.28 vs 7.08ºC*	7.78 vs 7.31ºC*	8.11 vs 7.94ºC*
Homozygous fish present	no	yes	no	no	yes	yes

1.4. CHARACTERISATION OF COMPARATOR SPECIES

For the purpose of this assessment, the Black Tetra (*G. ternetzi*) is used as a comparator for the notified organism (See Figure 1.1). A detailed overview of the Black Tetra is provided in Leggatt et al. (2018b).

1.5. CHARACTERIZATION OF POTENTIAL RECEIVING ENVIRONMENT

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

1.6. SUMMARY

Within the legislative context of CEPA and the information requirements of the *New Substances Notification Regulations (Organisms)* Schedule 5, this document elaborates the Framework for the assessment of potential risks to the Canadian environment that may be associated with the import or manufacture of GE fish. The environmental risk assessment is conducted 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 Environment and Climate Change Canada to advise the CEPA risk assessment and regulatory decision-making, based on risk to the environment and the uncertainty associated with the conclusion.

2. 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 five GloFish® Tetra lines that are described in part one of this document, and have been notified by GloFish LLC under the *New Substances Notification Regulations (Organisms)*. Given the common comparator species, and the physiological similarities among the five lines, the following section will consider all five lines at the same time.

2.2. EXPOSURE ASSESSMENT

The exposure assessment for living GloFish® Tetras 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. When considering the potential for GloFish® Tetras to reproduce and proliferate in the Canadian environment, the reproductive fitness of both the organism and potential descendants of the organism, the stability of the sex-determination systems, and the influence of propagule pressure on occurrence should be considered. Rankings for the likelihood of exposure to the Canadian environment are provided in Table 2.1.

Table 2.2: Rankings for exposure of GloFish® Tetras to the Canadian environment.

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

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® Tetras 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 2.3: Ranking of uncertainty associated with the likelihood of occurrence and fate of the organism in the Canadian environment (environmental exposure).

Uncertainty Ranking	Available Information
Negligible	High-quality data on the organism (e.g., sterility, temperature tolerance, fitness). Data on environmental parameters of the receiving environment and at the point of entry. Demonstration of absence of Genotype by Environment 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.

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® Tetras will be introduced into the Canadian environment. Numerous aquarium fish have established themselves in natural waters in North America, and reoccurring, though isolated reports of aquarium fish in Canadian water suggests the practice of releasing aquarium fish into the environment is common and ongoing (Dumont et al. 2002). Indeed Rixon et al. (2005), and Kerr et al. (2005) both cite the aquarium hobby industry as a significant source for introductions of exotic aquatic organisms into the Great Lakes Basin. In the Pacific Northwest, Strecker et al. (2011) estimated there are 2500 fish released annually into the Puget Sound region by aquarists, and a survey of aquarium fish owners in Ontario reported 2% of unwanted ornamental aquarium fish had been released to the environment (Marson et al. 2009). 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. Consequently, it is appropriate for GloFish® Tetras to be considered under a scenario of full release. The extent to which the organism is further exposed to the environment will, therefore, depend heavily on its ability to survive and reproduce in Canadian lakes and rivers. The magnitude of each release event is expected to be very small; however, the possibility of larger releases from larger purchases or breeding GloFish® Tetras in the home aquaria cannot be excluded.

2.2.2. Likelihood of Survival

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 (Magnuson et al. 1979; Jobling 1981; Amiro 2006; Elliott and Elliott 2010). As a tropical species, the Black Tetra is not expected to survive in a temperate region where water temperatures are below optimal for survival. Whereas the optimal temperature for Black Tetras may lie somewhere between 20 and 29°C (see Problem Formulation), data collected by DFO for White Tetras indicate a lower lethal temperature of approximately 9.8°C, when temperature is lowered slowly from the optimum, at a rate of 1°C per day (Leggatt et al. 2018a). Leggatt et al. (2018a) also reported changes in White Tetra activity and feeding level with decreasing temperature, where White Tetras decreased feeding level and activity at 17°C, stopped feeding when temperatures approached 12°C and stopped activity just above 10°C (see Figure 2.2). Data provided in the five notifications indicate that for all lines of GloFish® Tetra, low temperature tolerance is no greater than that of the comparator species, and all lines except PiT2018 were significantly less tolerant of cold than non-transgenic siblings.

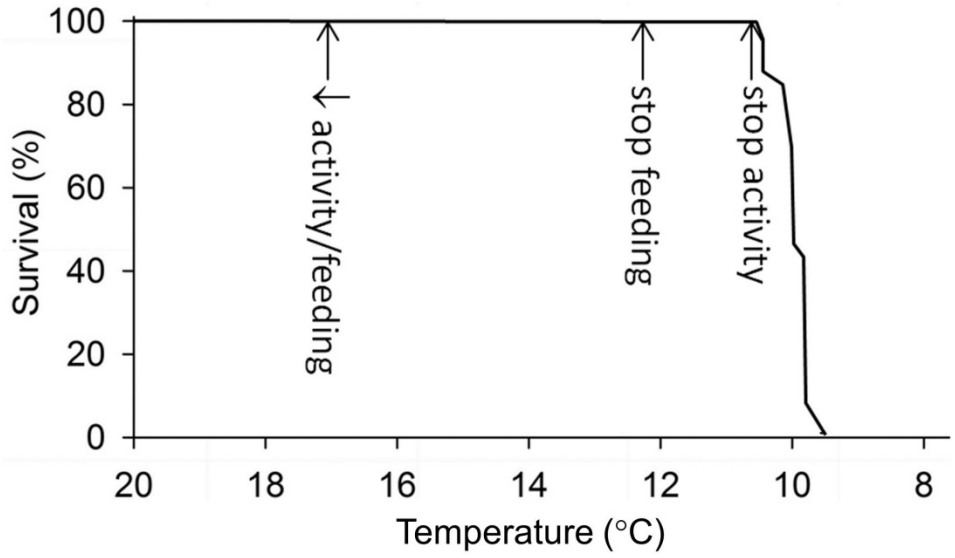

Figure 2.1: Survival and changes in activity and feeding level in White Tetra when temperatures are lowered gradually from 20°C at a rate of 1°C per day. Modified from Leggatt et al. (2018a).

As discussed in the Problem Formulation (see Characterization of potential receiving environment) there are no lakes in Canada that consistently remain above 6°C throughout the entire course of a year, and most do not remain above 4°C throughout the year. Based on what is known of non-transgenic habitat preferences, any released GloFish® Tetras are expected to occupy areas near the shoreline, avoiding areas of deep-water. These areas are expected to have more extreme temperature ranges than deep-water or mid-lake areas that are often the source for water temperature measurements (Trumpickas et al. 2015). Consequently, there may be more warm temperature areas suitable for breeding in summer months than indicated by recorded data. Conversely, winter temperatures may be colder than recorded, reducing the potential for overwintering.

Consequently, though temperatures needed for the GloFish® Tetras to survive are possible for several Canadian lakes during the spring, summer and autumn, it is highly unlikely that the GloFish® Tetras can survive the Canadian winter. At best, its occurrence in the environment would be seasonal or ephemeral. One possible exception to this would be in a minimal number

of isolated pockets of warm water (e.g., hot springs or in warm water industrial effluent). For example, establishment of other tropical fish species have been reported in Banff hot springs, Alberta, where local aquarists purposefully introduced numerous tropical freshwater aquarium species in 1960 (Mayhood 1995).

2.2.3. Likelihood of Reproduction

Though water temperatures in Canada will limit the occurrence of any GloFish® Tetras 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. As noted in the Problem Formulation (see section on Comparator Species), Tetras can be prolific breeders under ideal conditions, though in nature, fish larval production is strongly correlated with zooplankton abundance and limits population growth. Consequently, any reproduction would be limited to a short window of opportunity during the summer, regardless of its age at the time of introduction. Data provided in the five notifications indicate that for all lines of GloFish® Tetra, reproductive success is no greater than that of the comparator species and may be less in competition with non-transgenic tetras for all lines except PuT2018. Consequently, though isolated opportunities for reproduction in the Canadian environment could occur, it would never result in more than a single generation present in the environment.

2.2.4. Likelihood of Proliferation and Spread

The capacity for GloFish® Tetras to proliferate and spread in the Canadian environment is precluded by the fact that White Tetras cannot survive the Canadian winter. In order for GloFish® Tetras to become established in isolated warm thermal areas, the temperatures would have to remain stable enough, and other ecological conditions appropriate for long-term growth, survival, reproduction, and embryo and juvenile development and growth. While the requirement limits of *G. ternetzi* and GloFish® Tetras for reproduction and survival have not been established, general temperature recommendations for reproduction are 27-29°C, suggesting very specific temperature requirements for reproduction and consequent establishment that may not be present in most thermal pockets in Canada. Consequently, their occurrence in the environment is expected to be isolated, rare, and ephemeral.

2.2.5. Conclusions

Given the above analysis, the occurrence of GloFish® Tetras in the Canadian environment is expected to be rare, isolated and ephemeral, and likely in low numbers. Consequently, the likelihood of exposure of GloFish® Tetras to the Canadian environment is ranked low (Table 2.1). The uncertainty associated with this estimation is also low, given the quality data available for the GloFish® Tetras and valid surrogate organisms (temperature tolerance) and data available on the environmental parameters of the receiving environment in Canada (Table 2.2). This conclusion concurs with those of Hill et al. (2014) that predicted low invasion potential of the GloFish® Tetras in the US based on a lack of potential fish to hybridize with, limited history of invasiveness, a lack of traits associated with persistence, and high predation potential based on their small size and fluorescent colouration.

Changing water temperature patterns with climate change have potential to increase uncertainty associated with determining the ability of the notified organism to survive, reproduce, proliferate and spread in Canadian freshwater ecosystems. The predicted increases in temperatures in summer months may increase the number of areas with adequate temperatures for spawning, as well as the length of time adequate conditions are available. However, increases in winter temperatures are predicted to be smaller than in summer months, and are not currently predicted to increase by the 5°C or more needed for *G. ternetzi* to overwinter in most systems,

as estimated by their laboratory minimum temperature tolerance. As such, climate change is not expected to add significant uncertainty to, or alter the low environmental exposure ranking for the GloFish® Tetras in the near term.

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. While some unintended uses may increase release of the GloFish® Tetras (e.g., use as bait fish or in outdoor ponds), they would not be expected to alter their ability to overwinter in Canadian environments or otherwise alter the low environmental exposure ranking for the organism.

Overall, the likelihood of GloFish® Tetras exposure to the Canadian environment is estimated, with low uncertainty, to be low. This conclusion and its associated uncertainties are discussed further in the Assessment of Risk (Section 2.4).

2.3. HAZARD ASSESSMENT

The hazard assessment examines potential impacts that could result from environmental exposure to the GloFish® Tetras in the environment. The hazard identification process considers the potential environmental toxicity (i.e., potential to be poisonous), allergenicity, capacity to act as a vector for pathogens, and capacity to impact ecosystem components. 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. The severity (negligible, low, moderate, high) of the potential impacts to the assessment endpoints is evaluated in the risk assessment as well as the uncertainties; taking into consideration the appropriateness of control experiments and data, rearing conditions, interaction effects, phenotypic plasticity, and genetic background, in order to minimize uncertainty regarding assessment of potential ecological consequences of the GloFish® Tetras. Any difference in measurement endpoint is evaluated relative to 'normal' variation, based on published studies and expert opinion (Table 2.3).

Table 2.4: Ranking of hazard to the environment resulting from exposure to the organism.

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

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

Given the lack of empirical data around the behaviour and fitness of the GloFish® Tetras in the natural environment, significant attention to uncertainty considerations in the hazard assessment is required. Uncertainty around the hazard assessment may be significant due to clear knowledge gaps and lack of empirical data around the behaviour and effects of the GloFish® Tetras 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.

Here, 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. Other broad principles that have been demonstrated to influence uncertainty are the differential response of different genotypes to alternate environments (genotype x environment interactions – GxE) that makes it problematic to extrapolate from laboratory data to natural environmental conditions; unexpected pleiotropic effects of the genetic modification; and differential phenotypic effects of the genetic modification in fish with different background genetics (see Devlin et al. 2015; Leggatt et al. 2018b for further details).

The proposed use of the GloFish® Tetras 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 the GloFish® Tetras to Canadian environments. The majority of potential hazards posed by the GloFish® Tetras (e.g., through interactions with other organisms, as a vector for disease, impacts to biogeochemical cycling, habitat and biodiversity) would be through direct release of an organism to natural aquatic ecosystems, although some potential hazards could act indirect release of waste water and carcasses (e.g., environmental toxicity, horizontal gene transfer).

Table 2.5: Ranking of uncertainty associated with the environmental hazard.

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

2.3.1. Potential Hazards Through Environmental Toxicity

Potential routes of environmental toxicity (i.e., potential to be poisonous) of the GloFish® Tetras include exposure of aquatic ecosystems to the whole animal and its waste, as well as ingestion of an organism by predators. Exposure of the fluorescent protein to the environment or native organisms is expected to be lower than exposure of the protein to the organism itself.

Fluorescent proteins are common in many marine organisms, most noted in the cnidaria group, but also occurring in other groups including fishes (Sparks et al. 2014). Numerous fluorescent proteins are commonly used as neutral markers in science and the majority of studies do not report toxicity from fluorescent transgenes to the host organism (e.g., see Stewart 2006). Of the reports of negative effects, they are generally specific to transgenic organisms that have high expression of fluorescent transgenes, where lower expressing lines did not have negative effects (e.g., Huang et al. 2000; Devgan et al. 2004; Guo et al. 2007). Some specific variants of fluorescent proteins have been reported to have toxic effects (Chen et al. 2016) or cause some alterations in immune cells or metabolic enzymes (Chou et al. 2015) in mouse hosts. The toxic effects to host organism are likely due to the production of the fluorescent protein within the host cell, and are not expected to have similar effects to other organisms exposed to the protein through ingestion or contact with the notified transgenic organism.

The lack of reports of toxic effects of the fluorescent proteins used in the notified organisms to non-host organisms, combined with no anecdotal reported toxic effects of expression of the proteins to the notified organisms, aquarium cohabitants, or human owners, despite four to five years of commercial production within the US, indicate **negligible potential for environmental toxicity** of the GloFish® Tetras. This ranking has **moderate level of uncertainty** due to limited direct data from the notified organisms or surrogate organisms, and reliance on anecdotal evidence and indirect evidence from other fluorescent protein models.

2.3.2. Potential Hazards Through Horizontal Gene Transfer

Horizontal gene transfer (HGT) is the non-sexual exchange of genetic material between organisms of the same or different species (DFO 2006). A detailed assessment of the potential for horizontal gene transfer (HGT) of fluorescent protein transgenes from in Canada is presented in the Electric Green® GloFish® Tetra assessment (Leggatt et al. 2018b), and the current assessment follows this previous assessment. A brief summary follows.

In order for HGT of a specified transgene to take place on a biologically relevant scale and result in hazardous effects, the following steps must occur: exposure and uptake of the free transgene to a novel organism, stability and expression of the gene within the novel organism, neutral or positive selection of the novel organism expressing the transferred gene (DFO 2006), and harmful effects to the environment as a result of expression of the transferred gene in the novel organism. Pathways of exposure of free transgenic DNA from the GloFish® Tetras to new organisms (most likely prokaryotes) include exposure within the GloFish® Tetras guts, or through feces, mucus, and other waste sloughed off by the fish into the water. The transgene constructs do not contain viral vectors, transposable elements, or other known factors that may increase the potential for DNA uptake/mobility to a new organism. In order for the transgene to be expressed resulting in phenotypic change, it requires co-transfer of regulatory elements or insertion of the coding regions in functional proximity to native regulatory elements. The close proximity of the promoters to the pigment transgenes in the GloFish® Tetras could increase the likelihood of them being co-transferred and expressed, though vertebrate promoters generally have poor activity in prokaryotes.

Genes encoding fluorescence have been introduced to a wide range of organisms with few reports of harmful effects from the introduced transgenes, although this has not been directly examined in chromoprotein transgenesis. This suggests that the introduction of the transgenes through HGT to a novel host is not expected to result in harmful effects. While the introduction of a fluorescent transgene to novel organisms in Canadian environments through HGT cannot be excluded, the lack of expected harmful effect of such an introduction results in **low hazard rating through HGT**. As the insert sites of the transgenes are not known and there is reliance

on surrogate data for impacts should HGT occur, **the uncertainty associated with this rating is low**.

2.3.3. Potential Hazards Through Interactions with other Organisms

The trophic interactions of non-transgenic *G. ternetzi* in its native range are not well documented (see Section 1.6.4.8), nor is there documentation of trophic interactions of escaped ornamental non-transgenic or GloFish® *G. ternetzi* in other locations outside of their native range. Should GloFish® Tetras be released to the environment, they have potential to interact with other organisms in Canadian freshwater aquatic ecosystems, including potential prey, competitors, and predators. The potential hazards of the current GloFish® Tetras to Canadian environments through trophic interactions follows that of the Electric Green® Tetra and further details can be found in Leggatt et al. (2018b).

Black Tetras have potential to impact localized populations of small prey organisms (worms, crustaceans, insect and fish larvae) at location of release. The extent to which they could impact such populations has not been examined; however, Black Tetras are not known to be voracious eaters, do not overeat (Frank 1980) and are generally described as not aggressive towards other species of similar or greater size. As such, they are not expected to have exceptional ability to impact native fish populations using similar resources through competition. White Tetras obtained from the ornamental aquarium trade are reported to decrease activity and feeding at approximately 17°C, stop feeding above 12°C and stop activity just above 10°C (Leggatt et al. 2018a, see Figure 2.1). Given the low temperatures expected for Canadian freshwater systems for most of the year, the potential for released GloFish® Tetras to impact native aquatic species through trophic interactions is expected to be negligible through most of the year, and not expected to be greater than other small fish species during the summer months.

The ability of the GloFish® Tetras to prey on or compete for food relative to non-transgenic Black or White Tetras has not been reported. In another fluorescent fish model, domesticated RFP transgenic Zebrafish were more aggressive than wild-caught, non-domesticated non-transgenic Zebrafish and Flying Barb (Jha 2010), but it is not known if this was due to the fluorescent phenotype, or to differences in genetic background (domesticated versus wild) or rearing history (aquarium versus nature). Whether similar behaviour patterns would occur in the GloFish® Tetras has not been directly examined, but there are no known reports, anecdotal or otherwise, of GloFish® Tetras having different activity levels or behaviour than non-transgenic *G. ternetzi* in four to five years of commercial use. This suggests that notified GloFish® Tetra lines have similar potential to impact prey and competitors species as non-transgenic *G. ternetzi*.

Released GloFish® Tetras 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® Tetras causes deleterious effects to the predator populations. The latter is not expected, as GloFish® Tetras are not expected to be environmentally toxic, and the fluorescent proteins are not expected to be toxic to organisms that ingest them (see Section 2.3.1 above). While the predation pressure on GloFish® Tetras relative to non-transgenic *G. ternetzi* has not been reported, the reported effects of fluorescent transgenesis in another fish model (RFP Zebrafish) are conflicting. RFP-expressing Zebrafish have been reported to be preyed upon at a higher rate (Hill et al. 2011), equally, (Cortemeglia and Beitinger 2006), or at a lower rate (Jha 2010) than non-transgenic Zebrafish. The differences in results between these studies could be due to differences in background strains of both the transgenic and wild-type fish (striped versus golden, domestic versus wild), transgenic strains used, rearing history of the fish, types of predators and environmental complexity in the studies, and demonstrates altered predator susceptibility of

fluorescent fish may be influenced by numerous factors. There are no known studies on impacts of other fluorescent protein transgenes on predator susceptibility, and it is not known if any of the above studies could be applied to the GloFish® Tetras predation vulnerability.

Due to described low aggressive behaviour of Black Tetras, low activity in cooler waters and lack of noted alterations in trophic-related behaviour, GloFish® Tetras are not expected to influence trophic interactions of native organisms beyond natural fluctuations, with associated **negligible hazard** relative to non-transgenic counterparts. This ranking has a **moderate level of uncertainty**, due to lack of studies directly examining hazards of GloFish® Tetras, lack of understanding of GxE interactions in aggression and predation susceptibility in another fluorescent transgenic fish model (RFP Zebrafish), and lack of understanding the applicability of results from the RFP Zebrafish model to the GloFish® Tetras.

2.3.4. Potential Hazards Through Hybridization with Native Species

The Black Tetra belongs to the Family Characidae, that have a geographical distribution of South and Central America, and North America as far north as the southwestern US (Oliveira et al. 2011). There are only three other species that currently share the *Gymnocorymbus* genus (Benine et al. 2015) indicating limited close relatives even within its native range. Black Tetras are broadcast spawners and, consequently, could potentially form hybrids with species that spawn at the same time and place. However, due to the lack of native fish in Canada from the same family as Black Tetras, any hybrids produced in this way are expected to be non-viable. Therefore, there is **negligible potential for the GloFish® Tetras to cause hazards through viable hybridization with native fish in Canada.** The high quality data on distribution of Characidae and *Gymnocorymbus* distribution result in **negligible uncertainty** associated with the rating.

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). Although disease agents are common on tropical-origin freshwater ornamental aquarium fish, very few species (e.g., Goldfish, Zebrafish, Tank Goby, Guppy, Three Spot Gourami) are listed as species susceptible to diseases of significant importance to aquatic animal health and the Canadian economy by the Canadian Food Inspection Agency. Black Tetra is not included on the susceptible species list, nor are any other tetra species, indicating they have not been implicated as vectors for disease agents of concern in Canada. Any disease agents GloFish® Tetras 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® Tetras once released to cooler Canadian freshwater environments.

Whether the GloFish® Tetras, or any transgenic fluorescent organism, may have altered ability to act as a vector of disease agents has not been 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 (see Leggatt et al. 2018 for details). As well, mice transgenic for DsRed monomer had decreased monocyte and increased lymphocyte count relative to non-transgenic mice (Chou et al. 2015). Whether observed differences are common in fluorescent models, or may result in whole animal impacts to disease susceptibility and transmission has not been examined. GloFish® Tetras have been grown on a commercial scale in the US since

2012 to 2014 depending on the line, as have numerous other transgenic fluorescent aquarium species and lines starting in 2003. GloFish LLC provided statements from the company's veterinarian that state no evidence for increased susceptibility to, or transmission of, water-borne pathogens (see Section 1.3.1.2.3). Pathology screening of individuals from the notified lines found varying incidences of parasites and bacteria, but nothing unusual for typical non-transgenic aquarium fish, though notified lines were not directly compared with non-transgenic relatives. In other tropical species (e.g., Zebrafish), fluorescent models have been used extensively in laboratory conditions for research with no known reported effects on disease susceptibility, and Howard et al. (2015) tracked non-transgenic and RFP transgenic Zebrafish in 18 populations over 15 generations in laboratory conditions and reported no differences in survival between transgenic and non-transgenic fish. This indicates there is **negligible potential for GloFish® Tetras to have altered vector capabilities relative to non-transgenic tetras**. As this has not been directly examined, and there is reliance on indirect evidence and testimony of experts, the **uncertainty level for this rating is moderate**.

2.3.6. Potential to Impact Biogeochemical Cycling

Released GloFish® Tetras are expected to contribute to nutrient cycles in aquatic habitats through ingestion of prey and other food items and release of waste (ammonia and feces). In a static aquarium environment, the Black Tetra non-transgenic counterpart is described as not over eating, and does not cause excessive pollution of its environment (Frank 1980). Combined with its small size this indicates it will have limited capabilities to impact nutrient cycles. The potential effects of fluorescent proteins 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® Tetras have similar influences from fluorescent transgenic gene expression are not known, but the small size and lack of polluting capabilities of Black Tetra indicated they have **negligible potential to impact biogeochemical cycling in natural environments** even with altered metabolic pathways. This ranking has a **moderate level of uncertainty** due to lack of studies directly examining this hazard.

2.3.7. Potential to Affect Habitat

The Black Tetra is a small fish with no evidence suggesting they may have effects on habitat structure. Black Tetras spawn in open water and do not build nests or other structures that may impact habitats of other species. GloFish® Tetras have been in commercial use in the ornamental aquarium trade since between 2012 and 2014, and there have been no reports, anecdotal or otherwise, of GloFish® Tetras having altered behaviour relative to Black Tetra that may influence effects on habitat structure. Consequently, GloFish® Tetras are expected to have **negligible effects to habitat**. Although this has not been directly examined, there is no evidence of the ability to alter habitat in the base species over many decades of commercial use, resulting in **low uncertainty associated with this rating**.

2.3.8. Potential to Affect Biodiversity

Biological diversity (or biodiversity) is defined in CEPA as "the variability among living organisms from all sources, including, without limiting the generality of the foregoing, terrestrial and marine and other aquatic ecosystems and the ecological complexes of which they form a part and includes the diversity within and between species and of ecosystems". Biodiversity can be negatively impacted by numerous drivers including invasive species and disease introduction. While the invasiveness of GloFish® Tetras has not been directly assessed, there are no reports

of Black Tetra becoming invasive in North America, Europe, or elsewhere worldwide. Black Tetras have been used in the ornamental aquarium trade in North America since at least 1950, and are common in the Canadian and the US pet trade (e.g., Rixon et al. 2005 found 75% of surveyed pet stores in Ontario carried *G. ternetzi*, while Strecker et al. 2011 found 96.7% of surveyed pet stores in Washington carried *G. ternetzi*). Occurrences of *G. ternetzi* in natural aquatic systems in the US have been noted in Florida, Colorado, and Louisiana (Nico and Fuller 2017; Tuckett et al. 2017) but all occurrences failed to establish, and Hill et al. (2014) concluded a lack of invasion potential in the USA of fluorescent *G. ternetzi* using the Fish Invasiveness Screening Kit (FISK). Despite the extensive long-term use of *G. ternetzi* in the aquarium trade, and its noted release to the environment, there are no reports of the Black Tetra causing harm to aquatic ecosystems including biodiversity. As noted above, Black Tetras are not expected to impact native species through trophic or hybridization interactions, act as a vector for disease agents of concern in Canada, significantly impact biogeochemical cycling, or impact habitat. The addition of transgenic fluorescent genes and proteins in GloFish® Tetras is not expected to result in environmental toxicity or cause hazards through HGT of the transgenes, interactions with native species, as a vector of disease, or through impacts to biogeochemical cycling and habitat. Taken together, there is a **negligible hazard of GloFish® Tetras affecting biodiversity of Canadian ecosystems**. The reliance on data from the comparator species for invasiveness and biodiversity effects results in a **low degree of uncertainty** with this rating.

2.3.9. Conclusions

The Black Tetra is a small, non-aggressive fish with expected limited activity due to low temperatures in most seasons in Canada, has not been identified to be susceptible to diseases of concern in Canada, and has no history of invasiveness in Canada and worldwide despite its wide use. As such, Black Tetra is not expected to pose hazards to Canadian environments. The potential environmental hazards posed by GloFish® Tetras above that of non-transgenic Black Tetra have not been specifically addressed. However, there is no evidence of environmental toxicity (i.e., ability to be poisonous) associated with the fluorescent proteins used in the notified lines, and the majority of other fluorescent models do not report toxicity associated with fluorescent transgenes. As well, there is no evidence for potential effects via gene transfer of the transgenes to native Canadian species through hybridization or horizontal gene transfer. In some non-fish fluorescent models, there is limited evidence that some fluorescent transgenes have potential to impact vector capabilities by altering response to disease, and impact contribution to biogeochemical cycling by altering metabolic pathways. However, GloFish® Tetras and other fluorescent fish models have no reported increases in survival, or differences in disease susceptibility or husbandry care, and, as such, are not expected to have altered ability to pose hazards as a vector of disease or impact biogeochemical cycling. Some evidence suggests GloFish® Tetras may have lower potential to impact other species through trophic interactions relative to Black Tetras, as lower cold tolerance in most lines may further limit activity in cooler water temperatures, and bright colouration may increase predation as reported in RFP Zebrafish (Hill et al. 2011). One report in RFP Zebrafish suggests presence of fluorescent fish may result in increased predation on wild fish and decrease aggressive behaviour of wild fish (Jha 2010) which could have negative consequences to wild populations. However, whether noted differences were due to the presence of the transgene, domestication level, or rearing history is not known, and it is not known whether these results would apply to complex natural habitats or specifically to GloFish® Tetras. Use of GloFish® Tetras other than the intended use as an ornamental fish in indoor static aquaria (e.g., as a bait fish, rearing in outdoor ponds) is not expected to pose unique hazards beyond those from the intended use.

All specific hazards examined had negligible ratings, with the exception of a low hazard rating for impacts through HGT (see Table 2.5). In this latter case, the potential for an effect to occur

(i.e., transfer of transgene to prokaryotic populations) cannot be excluded, but the effect is not anticipated to cause detrimental impacts to the structure or function of Canadian ecosystems. The uncertainty ratings associated with the individual hazard classifications ranged from negligible to moderate (see Table 2.5), due to the limited data specific to GloFish® Tetras, limited direct data on the comparator species, variable data from a surrogate model (RFP Zebrafish), and the reliance on expert opinion for the assessment of some hazards**.**

Table 2.6: Summary of hazard rank and uncertainty of GloFish® Tetras to Canadian environments.

Hazard	Rank	Uncertainty
1. Through Environmental Toxicity	Negligible	Moderate
2. Through Horizontal Gene Transfer	Low	Low
3. Through Trophic Interactions	Negligible	Moderate
4. Through Hybridization	Negligible	Negligible
5. As a Vector for Disease	Negligible	Moderate
6. To Biogeochemical Cycling	Negligible	Moderate
7. To Habitat	Negligible	Low
8. To Biodiversity	Negligible	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:

$$\text{Risk} \propto \text{Exposure} \times \text{Hazard}$$

For each endpoint, hazard and exposure are ranked as: negligible, low, moderate, or high, and include an analysis of uncertainty for both. Overall Risk is estimated by plotting Hazard against Exposure, using a matrix or heat map, as illustrated in Figure 2.2. The matrix cannot be used as a tool for establishing a discreet conclusion or decision on risk, but can be used as a device 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.

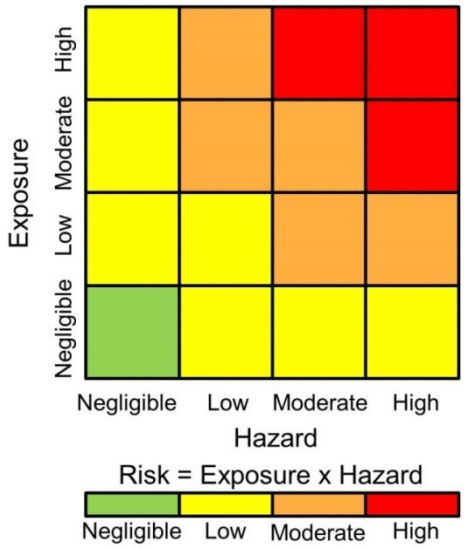

Figure 2.2: Risk matrix and colour scale to illustrate how exposure and hazard are integrated to establish a level of risk in the environmental risk assessment.

The exposure assessment of GloFish® Tetras concluded that for all GloFish® Tetras (BT2018, OT2018, PiT2018, PuT2018, and RT2018) used in the ornamental aquarium trade or other unintended uses in Canada would have **low likelihood for occurrence in the Canadian environment**. This is due to the high likelihood of release of small numbers of fish from home aquaria, but negligible likelihood for GloFish® Tetras to overwinter in Canadian aquatic systems. As such, any exposure of Canadian freshwater aquatic ecosystems to GloFish® Tetras is expected to be isolated, rare and ephemeral. The quality of data demonstrating lack of cold tolerance in the GloFish® Tetras and comparator species relative to Canadian winter freshwater temperatures results in **low uncertainty.**

The hazard assessment of GloFish® Tetras concluded that GloFish® Tetras **posed negligible to low hazard to the Canadian environment**, due to lack of hazards associated with the non-transgenic Black Tetra species, and no direct evidence that the expressed fluorescent proteins would increase hazards of notified lines relative to non-transgenic Black Tetra. **Uncertainty ranking associated with individual hazard components ranged from negligible to moderate** (see Table 2.5) due to the limited data specific to GloFish® Tetras, limited direct data on the comparator species, variable data from a surrogate model (e.g., RFP Zebrafish), and the reliance on expert opinion for the assessment of some hazards.

As indicated in Figure 2.3, GloFish® Tetras used in the ornamental aquarium trade or other uses in Canada **pose low risk to Canadian environments** (**Low Exposure x Negligible/Low Hazard ∝ Low Risk**).

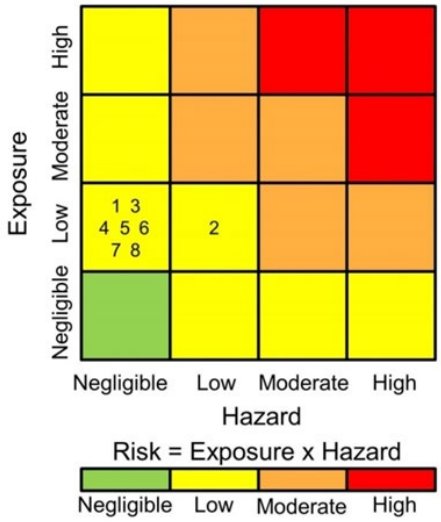

Figure 2.3: Risk matrix and scale to illustrate how exposure to and hazard of GloFish® Tetras are integrated to establish a level of risk in the environmental risk assessment. Risk assessments associated with assessed hazard components at the assessed exposure are identified by number: 1) through environmental 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.

In general, individual assessed hazards are expected to result in no effects beyond natural fluctuations to Canadian environments under the assessed exposure level. One exception to this is the hazards associated with horizontal gene transfer, under the assessed exposure level, may result in an effect beyond the normal range (i.e., potential transfer of a marine-derived fluorescent protein gene to prokaryotes in a freshwater environment), but this effect is not expected to be harmful.

Sources of uncertainty in the environmental exposure and hazard assessment that may influence uncertainty in environmental risk assessment include: lack of data directly addressing hazards of the notified organism and comparator species, variability in data taken from surrogate organisms, and lack of understanding of applicability to the notified organism (e.g., hazards through trophic interactions), and some reliance on expert opinion for some hazard assessments (e.g., hazards as vector of disease). Some individual hazard classifications have significant uncertainty level (e.g., moderate uncertainty in negligible hazard through environmental toxicity, through trophic interactions, as a vector of disease or to biogeochemical cycling). The majority of identified environmental hazards of GloFish® Tetras would be expected to require continued exposure of ecosystem components to GloFish® Tetras in order to pose significant risk to the environment, and, consequently, uncertainty in risk may be more closely aligned with that of exposure rather than hazard. In contrast, hazards through horizontal gene transfer or as a vector of disease could result from initial exposure to GloFish® Tetras to transfer the transgene or agents of disease, which could then have longer-term consequences after GloFish® Tetras have been removed from the environment. As such, uncertainty in risk associated with these hazards may be more closely aligned with uncertainty in hazard assessment. Despite moderate uncertainty in some individual assessment components, there is no current evidence to suggest overall risk ratings of GloFish® Tetras used for the ornamental aquarium trade in Canada may be higher than the assessed rating of low risk to Canadian environments. Future studies should be aimed at decreasing uncertainty in key hazard components by directly examining the influence of the genetic modification on potential for

environmental impacts through environmental toxicity, trophic interactions, biogeochemical cycling, or as a vector of disease.

2.5. SUMMARY AND CONCLUSIONS

Use of GloFish® Tetras in the home aquaria in Canada, or in other unintended uses, is expected to result in infrequent, very small magnitude releases of GloFish® Tetras to the Canadian environment outside of aquaria, though the potential for occasional high magnitude releases cannot be excluded. Regardless, high quality data available indicates GloFish® Tetras do not have the capacity to overwinter in Canadian freshwater ecosystems. This results in a Low Exposure ranking with associated uncertainty being low. For potential hazards, the lack of evidence of hazards from base non-transgenic species despite long-term extensive use, as well as lack of evidence for increased hazards of GloFish® Tetras relative to non-transgenic, indicates Negligible to Low Hazard ratings of GloFish® Tetras to Canadian ecosystems. Due to lack of direct information on hazards of base model and/or GloFish® Tetras, uncertainty with hazard assessments range from negligible to moderate. Taken together, the overall Risk of GloFish® Tetras to the Canadian environment is Low, and the notified organisms are not expected to cause harmful effects to Canadian environments at the assessed exposure level. Specifically:

The low exposure ranking and negligible to low hazard ranking of notified GloFish® Tetra lines results in overall **Low Risk of BT2018 to Canadian environments**.

The low exposure ranking and negligible to low hazard ranking of notified GloFish® Tetra lines results in overall **Low Risk of OT2018 to Canadian environments**.

The low exposure ranking and negligible to low hazard ranking of notified GloFish® Tetra lines results in overall **Low Risk of PiT2018 to Canadian environments**.

The low exposure ranking and negligible to low hazard ranking of notified GloFish® Tetra lines results in overall **Low Risk of PuT2018 to Canadian environments**.

The low exposure ranking and negligible to low hazard ranking of notified GloFish® Tetra lines results in overall **Low Risk of RT2018 to Canadian environments**.

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 notified GloFish® Tetras under the proposed or other potential uses, could cause harm as a result of exposure to Canadian environments.

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