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Environmental Risk Assessment of the GloFish® Galactic Purple® and Cosmic Blue® Danios: Transgenic Ornamental Fish

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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

CFIA: Canadian Food Inspection Agency

DNA: Deoxyribonucleic acid

eGFP: Enhanced green fluorescent protein

GE: Genetically engineered

GFP: Green fluorescent protein

GxE: Genotype by environment interaction

HGT: Horizontal gene transfer

kb: Kilobase – 1000 base pairs of DNA

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

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

mRNA: Messenger RNA

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

RFP: Red fluorescent protein

RNA: Ribonucleic acid

SVCV: Spring viremia of carp virus

UAS: Upstream activation sequence

UV: Ultraviolet

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

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

Hybridization: any crossing of individuals of different genetic composition, typically belonging to different strains or species

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

Life cycle: The sequence of events from the origin as a zygote, to the death of an individual; those stages through which an organism passes between the production of gametes by one generation and the production of gametes by the next

Ornamental: all small water living animals of class Pisces (fish) which are kept as pets and as decorative pieces

Persist: survives to the reproductive stage

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

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), two notifications under the New Substances Notification Regulations (Organisms) (NSNR(O)) were submitted by GloFish LLC to Environment and Climate Change Canada (ECCC) for genetically engineered *Danio rerio* (GloFish® Cosmic Blue® Danio (BZ2019) and GloFish® Galactic Purple® Danio (PZ2019)). The environmental risk assessment was conducted that included an analysis of potential hazards, likelihoods of exposure, and associated uncertainties to reach conclusions on risk. Assessments were compared with previously notified GloFish® Sunburst Orange® Danio (YZ2018). The environmental exposure assessment concluded that the occurrence of BZ2019 and PZ2019 in the Canadian environment, outside of aquaria, is expected to be rare, isolated, and ephemeral due to their inability to survive typical low winter temperatures in Canada's freshwater environments. Consequently, the likelihood of exposure of BZ2019 and PZ2019 to the Canadian environment is ranked low. The uncertainty associated with this environmental exposure estimation is low, given the available data for temperature tolerance of the notified lines and relevant comparators and the lack of establishment through the long history of use of non-transgenic *Danio rerio* in North America. The environmental hazard assessment concluded that the hazards of BZ2019 and PZ2019 associated with environmental toxicity, trophic interactions, hybridization, vector for disease, biodiversity, biogeochemical cycling, and habitat are negligible. There is low hazard (i.e., no anticipated harmful effects) associated with horizontal gene transfer. The uncertainty levels, associated with the environmental hazard ratings, range from low to moderate due to data limitations and quality for the notified and surrogate organisms, or some reliance on expert opinion and anecdotal evidence. There is low risk of adverse environmental effects at the exposure levels predicted for the Canadian environment from the use of BZ2019 and PZ2019 as an ornamental aquarium fish or other potential uses.

EXECUTIVE SUMMARY

BACKGROUND

On May 8, 2019, GloFish LLC submitted two 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® Galactic Purple® Danio, and the GloFish® Cosmic Blue® Danio. These ornamental fish are Golden Zebrafish (*Danio rerio*) that have been genetically engineered to fluoresce different colours for use in home aquaria. Note that similar risk assessments have been conducted on the related GloFish® Sunburst Orange® Danio (DFO 2020), as well as six different colours of GloFish® Tetras (DFO 2018, 2019).

The biotechnology provisions of CEPA take a preventative approach to pollution by requiring all new living 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 CEPA (Section 64), an organism is considered "toxic" if it can enter the environment in a quantity or concentration or under conditions that (a) have or may have an immediate or long-term harmful effect on the environment or its biological diversity; (b) constitute or may constitute a danger to the environment on which life depends; or (c) constitute or may constitute a danger in Canada to human life or health. Anyone proposing to import or manufacture a living animal product of biotechnology in Canada, including genetically engineered fish, is required to provide ECCC with the information prescribed in NSNR(O) at least 120 days prior to the commencement of import or manufacture of the organism. This information is used to conduct an environmental risk assessment and an assessment of indirect human health (risk to human health from environmental exposure to the living organism), which are then used as the basis to determine if the organism is CEPA-toxic or capable of becoming CEPA-toxic.

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

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

THE NOTIFIED ORGANISMS

The two GloFish® Danio strains are independent lines of genetically engineered diploid, hemizygous or homozygous, transgenic colour morphs of the Golden Zebrafish (*D. rerio*). Each line possesses a different transgene for which expression results in a unique colour under natural light, and becomes fluorescent under blue or UV light. The 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), with the transgene construct microinjected at the single cell stage. Uniform insert location(s) of the transgene(s), copy number, and Mendelian segregation were examined at the F2 generation.

The Cosmic Blue® Danio (BZ2019) and Galactic Purple® Danio (PZ2019) have been marketed in the United States (except California) since 2010 and 2011, respectively, and in California since 2015, without incident. The targeted phenotypic change is the presence of a unique fluorescent colouration as novel colour morphs for the ornamental aquarium trade. Other unanticipated phenotypic changes noted by the company include slightly impaired cold tolerance and a reduction of reproductive success in competition with non-transgenic siblings.

ENVIRONMENTAL RISK ASSESSMENT

The environmental risk assessment was conducted under GloFish LLC's proposed use scenario: the import of BZ2019 and PZ2019 to aquarium wholesale locations in Canada, with further distribution to aquarium retail stores across the country, to be purchased by Canadian consumers for home aquaria.

Exposure

The intended housing for BZ2019 and PZ2019 is indoor, static, physically contained aquaria at wholesalers, retail stores, and in consumer's homes. Based on historical records of aquarium fish in natural ecosystems in Canada and worldwide, it is highly likely that the organisms will be introduced purposefully or accidentally into natural freshwater ecosystems in Canada. Based on the expected number of fish to be purchased by individual consumers, it is expected that release events will be very low magnitude (e.g., five fish or less per release), though larger magnitude releases cannot be ruled out.

Based on temperature preferences and limitations of non-transgenic and notified *D. rerio,* and recorded water temperature throughout freshwater systems in Canada, BZ2019 and PZ2019 could survive in Canadian ecosystems during the summer, spring and autumn, but could not survive over winter. Indeed, there are no reports of established populations of non-transgenic *D. rerio* in either Canada or the United States, despite decades of sales and trading across North America and occasional reports of transient occurrences.

The occurrence of BZ2019 and PZ2019 in the Canadian environment is expected to be rare, isolated and ephemeral. Consequently, the likelihood of **exposure** to the Canadian environment is ranked **low**. The **uncertainty** associated with this estimation is **low**, given the quality of temperature tolerance data available for each line and valid surrogate organisms, and data available on the environmental parameters of the receiving environment in Canada.

Hazard

The potential for BZ2019 and PZ2019 to be hazards to Canadian environments was examined in the context of environmental toxicity, horizontal gene transfer, interactions with other organisms including hybridization, as a vector of disease, and through impacts to biogeochemical cycling, habitat, and biodiversity. Non-transgenic *D. rerio* is a small, non-aggressive fish with expected limited activity due to low temperatures in most seasons in Canada. It has no history of invasiveness in Canada or worldwide despite its widespread use in the aquarium trade. There are no reports of phenotypic effects of the transgene that may increase the hazard potential of BZ2019 or PZ2019 above that of non-transgenic *D. rerio*, and no evidence that potential gene transfer will result in harm to the Canadian environment.

Rankings for the specific **hazards** examined ranged from **negligible to low**. **Uncertainty** ranged from **low to moderate**, due to limited data specific to the organisms, limited direct data on comparator species, variable data from surrogate models (RFP Zebrafish), and the reliance on expert opinion for the assessment of some hazards. BZ2019 and PZ2019 are not expected to pose additional hazards if used in applications other than the intended use of ornamental fish for home aquaria.

CONCLUSIONS ON RISK

The overall **risk** of BZ2019 and PZ2019 to the Canadian environment is ranked **low**, and the notified organisms are not expected to cause harmful effects to Canadian environments at the assessed exposure level. While the uncertainty associated with some hazard classifications is moderate due to limited or no direct data on the notified organisms or comparator species, no evidence was identified to suggest that BZ2019 or PZ2019, under the proposed or other potential uses, could cause harm as a result of exposure to Canadian environments. These conclusions concur with previous assessments of notified GloFish® Danio and Tetra lines (DFO 2018, 2019, 2020).

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® Galactic Purple® Danio (PZ2019) and Cosmic Blue® Danio (BZ2019); genetically engineered variants of the Golden Zebrafish (*Danio rerio*), also known as the Golden Danio, notified by GloFish LLC under the *New Substances Notification Regulations (Organisms)* [NSNR(O)] for use in the ornamental aquarium trade. The problem formulation provides a foundation for the risk assessment through identification of environmental protection objectives and the elaboration of scope. It identifies protection goals and assessment endpoints that are aligned with the legislative protection goals in CEPA. The Problem Formulation also provides a characterisation of the two GloFish® Danio strains, the comparator species, and the potential receiving environment in Canada. Notification of PZ2019 and BZ2019 under CEPA follows previous similar GloFish® notifications for Sunburst Orange® Danio (YZ2018) and six lines of GloFish® Tetras. The current assessment follows the previous assessments (DFO 2018, 2019, 2020) and identifies information that might alter assessment conclusions from those of previous conclusions.

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

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

A detailed overview of the legal context for the risk assessment process, the risk assessment framework, and regulatory decision making process under CEPA is provided in Leggatt et al. (2018b). Briefly, the risk assessment is conducted within the legislative context of CEPA and the information requirements of the NSNR(O) Schedule 5. Potential risks to the Canadian environment that may be associated with the import or manufacture of GE fish is determined in accordance with the classical risk assessment paradigm, where risk is directly related to the exposure and hazard of the organism. The exposure assessment is based on the likelihood and magnitude of release into the environment, and the likelihood and magnitude of survival, reproduction, establishment, and spread of the organism and potential descendants of the organism in the Canadian environment. The hazard assessment is focused on the potential for the organism to impact: (1) potential prey, predators, and competitors of the organism; (2) biological diversity; and, (3) habitat. The level of uncertainty for both exposure and hazard determinations is evaluated and communicated in terms of impact to the final risk assessment. DFO provides science advice in the form of peer-reviewed risk assessments to ECCC for regulatory decision-making under CEPA, based on risk to the environment and the uncertainty associated with the conclusion.

1.3 CHARACTERISATION OF THE ORGANISMS

In its current notifications, GloFish LLC is requesting the import of two new transgenic strains of Zebrafish (*D. rerio*) from the US, for the ornamental aquarium trade in Canada. Trade names for the transgenic organisms are the Galactic Purple® Danio and the Cosmic Blue® Danio. Figure 1.1 demonstrates the physical appearance of the two notified GloFish® Danio strains, as well as the domesticated non-transgenic Zebrafish (also known as Striped Danio or Zebra Danio), and the non-transgenic Golden Zebrafish (also known as the Golden Danio), a low pigment morph of the Striped Danio.

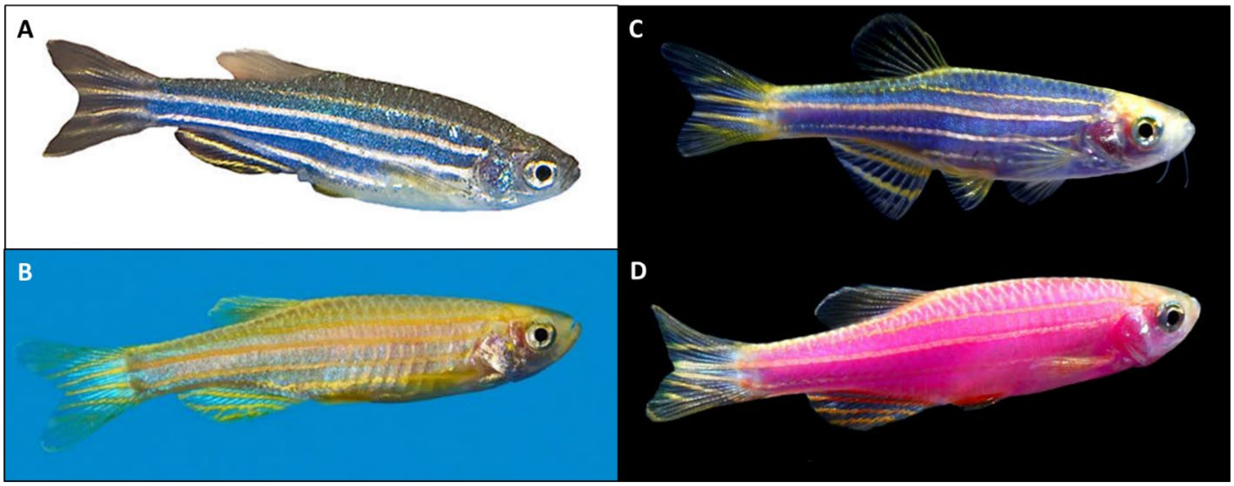


Figure 1.1: Some variants of Danio rerio *available in the ornamental pet trade worldwide (A, B), and notified transgenic variants currently only available in the United States of America (C, D). Domesticated Striped Zebrafish (A), Golden Zebrafish (B), Cosmic Blue® Danio (C), and Galactic Purple® Danio (D). Images taken from PetSmart (A), All Pond Solutions (B), Tampa Bay Cichlids (C), and Liveaquaria.com (D).*

1.3.1 Cosmic Blue® Danio (BZ2019)

1.3.1.1 Molecular Characterisation

BZ2019 is a genetically engineered Golden Zebrafish (*D. rerio*) possessing multiple copies of two transgene constructs containing transgenes for either a blue fluorescent protein or a non-fluorescent protein, respectively. The complete genetic constructs used in BZ2019, including both the genes and regulatory components, are the same as those used for the previously assessed Cosmic Blue® Tetra (DFO 2019). The inserted genetic material results in blue colouration of BZ2019 under ambient light and fluorescent blue colouration under UV light. The purpose of this modification is to create a new blue colour phenotype of *D. rerio* for the ornamental aquarium trade.

Though greater detail regarding the structure, development, and function of the transgene constructs used to create BZ2019 has been provided by the company for review, it is considered confidential business information and cannot be included in this report.

1.3.1.1.1 Production of the notified organism

The purified transgene expression cassettes described above were injected into newly fertilized eggs of non-transgenic Golden Zebrafish (*D. rerio*). Random batches of F1 offspring were produced from pair-wise crossing of a single blue mosaic founding individual (i.e., one G0) to several non-transgenic Golden Zebrafish. Transgenic individuals were identified by presence of blue fluorescence and raised to maturity. At maturity, transgenic F1 fish were crossed to non-transgenic Golden Zebrafish to produce a population of F2 fish segregating for fluorescence and colour that became the founding population used for line propagation. F2 fish were also used to generate information regarding the molecular and physical characteristics of the organism.

Confirmation that all F2 fish contain homogeneous insert sites and constitute a single homogeneous line was accomplished via enzyme cleavage and Southern blot analysis of all F2 fish. A test to verify that vector backbone was not incorporated into the organism was performed on F2 generation fish via PCR, using primers specific to four different locations of the vector backbone. No vector was detected, however, a bacteriophage T3 RNA polymerase promoter was identified in the sequences of both transgene constructs, suggesting that some of the vector backbone was present as part of the final purified transgene constructs. Re-analysis of previously notified lines confirmed this T3 promoter is also present in the construct sequences of GloFish® Electric Green®, Cosmic Blue®, and Galactic Purple® Tetras.

Continuation of the BZ2019 line has been through batch breeding in populations that contain a mix of individuals hemizygous and homozygous for the transgene, with non-transgenic Golden Zebrafish removed from the population as they occur. Broodstock of BZ2019 are maintained separately by two aquarium fish producers in Florida.

1.3.1.1.2 Characterization of the transgene integrant

The sequence of the gene constructs as they are inserted into BZ2019 has not been determined, and the specific location of the inserts within the organism's genome has not been presented. Crossing of F2 generation fish hemizygous for the transgene with non-transgenic fish resulted in approximately 50% transgenic offspring at four days post fertilization. This indicates either a single insert location of the transgenes or multiple insertion sites that are closely located with Mendelian segregation.

Transgene copy number was estimated using quantitative real-time PCR (qPCR) on F2 generation hemizygous BZ2019, and a competitive PCR assay was used to estimate the ratio of one construct to the other. Results indicate that multiple copies of both transgene cassettes were incorporated into the genome of BZ2019 fish.

1.3.1.1.3 Inheritance and stability of the transgene

The specific insert location of the transgene is unknown and 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). 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). The potential for reactivation after such silencing is not known.

In single pair matings of F2 hemizygous individuals with non-transgenic fish, the resulting offspring were not significantly different than 50% fluorescent in most families, indicating the constructs are likely inherited as a single locus. However, overall the proportion of fluorescent offspring was slightly but significantly lower than 50%. As the genotype of non-fluorescent offspring was not determined, it cannot be concluded if the lower percentage was due to decreased viability of fluorescent gamete or larvae, or due to transgene silencing in some individuals. Genetic stability was only examined at a single generation and has not been demonstrated over multiple generations.

1.3.1.1.4 Methods to detect BZ2019 fish

BZ2019 individuals are easily distinguished from non-transgenic Golden Zebrafish by their phenotypic blue colouration under natural/normal light. Although BZ2019 is similar in colouration to non-transgenic striped Zebrafish, it can be distinguished by its fluorescence under blue or UV light. No known non-transgenic cyprinid species has similar blue fluorescent colouration, making BZ2019 individuals easily phenotypically distinguishable from other non-transgenic species, unless the gene has been silenced. BZ2019 fish can also be distinguished genetically by PCR amplification and detection of unique fragments that result from restriction enzyme digest of the transgene insert.

1.3.1.2 Phenotypic Characterization

1.3.1.2.1 Targeted phenotypic effects of the modification

The targeted phenotypic effect of the genetic modification is that BZ2019 appears blue under ambient light. The novel colour phenotype is present in muscle as well as skin and eye. GloFish LLC reports that BZ2019 individuals that are hemizygous and homozygous for the transgene insert are indistinguishable from each other phenotypically and are both part of the commercially available population.

1.3.1.2.2 Non-targeted phenotypic effects of the modification

Two off-target effects identified by GloFish LLC in BZ2019 are diminished fluorescent offspring during paired trials with non-transgenic fish, and reduced occurrences of reproductive success in competition with non-transgenic siblings for mates. In paired trials, the overall proportion of fluorescent offspring was less than the expected 50%, suggesting decreased viability of gametes and/or larvae containing the fluorescent transgene (hemizygous) relative to those without the transgene, or silencing of the transgene in some individuals. In competitive reproductive success tests, there were some trials where the proportion of blue offspring were greater than expected, but overall, and for most trials, the blue proportion was significantly lower than what was expected from random assortment alone, indicating BZ2019 may be reproductively disadvantaged compared to non-transgenic Zebrafish. This difference was still significant when decreased viability of blue gametes or larvae was taken into consideration ($p<0.001$).

The company also conducted a low-temperature tolerance test comparing the survival of hemizygous BZ2019 with sibling non-transgenic Golden Zebrafish during a decrease in temperature. However, an analysis of data provided by the company found the difference in cold tolerance between the two groups to be insignificant ($p = 0.800$), while the difference in LD_{50} between genotypes was very small (0.12°C). The LD_{100} (lethal dose at which 100% of fish have died) of the two genotypes was similar and varied from 4.5 to 5.0°C between trials.

The notifier submitted a diagnostics report examining necropsy, microbiology and histology of BZ2019. Other than the presence of nematode parasites in two of six fish that was stated to be unrelated to the transgenic nature of the fish, no usual findings were reported. BZ2019 fish were not compared with non-transgenic sibling fish.

No formal studies have compared the potential disease susceptibility of BZ2019 with that of 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.

1.3.1.2.3 Pleiotropic effects of fluorescent protein transgenes in other Zebrafish

A detailed overview of the pleiotropic effects of fluorescent protein transgenes that have been observed in other Zebrafish is presented in Noble-Brzezinski et al. (2021).

1.3.2 Galactic Purple® Danio (PZ2019)

1.3.2.1 Molecular Characterisation

PZ2019 is a genetically engineered "Golden Zebrafish" possessing a single area of insertion that contains multiple copies of an expression cassette. This genetic change results in purple colouration of the organism under ambient white light, and fluorescent purple under UV light. The purpose of the modification is to create a new colour phenotype of *D. rerio* for the ornamental aquarium trade (Figure 1.1).

Though greater detail regarding the structure, development, and function of the transgene constructs used to create BZ2019 has been provided by the company for review, it is considered confidential business information and cannot be included in this report.

1.3.2.1.1 Production of the notified organism

The purified transgene expression cassette was injected into newly fertilized eggs of Golden Zebrafish. A single founding individual (G0) was identified by phenotype (purple colour) and separately crossed to several non-transgenic Golden Zebrafish to produce several F1 progeny. A batch of F1 fry expressing purple colour were raised to maturity then crossed with non-transgenic Golden Zebrafish to produce a population of F2 fish.

Confirmation that all F2 fish contain homogeneous insert sites and constitute a single homogeneous line was made via enzyme cleavage and Southern blot analysis. Of the 133 F2 fish that were tested, four had alternative genetic lineage and were not used in PZ2019 line propagation or further testing.

Confirmation that the vector backbone was not incorporated into the organism was performed on several F2 generation fish via PCR using primers specific for four different locations of the vector backbone. As with BZ2019, a bacteriophage T3 promoter was identified upstream of the CBA promoter, indicating some vector backbone was present in the final inserted construct.

Continuation of the PZ2019 line has been through batch breeding in populations that contain a mix of individuals hemizygous and homozygous for the transgene, with non-transgenic Golden Zebrafish removed from the population as they occur. Broodstock of PZ2019 are maintained separately by two aquarium fish producers in Florida.

1.3.2.1.2 Characterization of the transgene integrant

The sequence of the cassette as it is inserted into the genome of PZ2019 has not been determined, and the specific location of the insert within the PZ2019 genome is unknown.

Transgene copy number was estimated using quantitative real-time PCR (qPCR) on six F2 generation hemizygous PZ2019. Results indicate that multiple copies of the transgene constructs were incorporated into the genome of PZ2019 fish.

1.3.2.1.3 Inheritance and stability of the transgene

The specific insert location of the transgene has not been determined and it is unknown whether it has inserted into a stable genome location or in an area prone to silencing. Should transgene expression be silenced in an individual, it would not display the purple colouration and would, consequently, be removed from the breeding population and terminated. Four F2 individuals had alternate Southern blot banding patterns and were not used in line propagation. Two of these fish had distinctly different banding patterns from all other F2 fish, indicating different integration sites at the G0 stage. However, the other two have similar banding patterns as those used in line propagation, but with one band missing and another with fainter intensity. While these two fish may also be the result of different integrations at the G0 stage, it could also be indicative of potential loss of transgene copy number between the G0 and F2 stages and possible line instability.

In single pair matings between hemizygous individuals and non-transgenic fish, the resulting offspring were not significantly different than 50% fluorescent in most families, indicating the construct is likely inherited as a single locus. However, overall the proportion of fluorescent offspring was slightly but significantly lower than 50%. As the genotype of non-fluorescent offspring was not determined, it cannot be concluded if the lower percentage was due to decreased viability of fluorescent gamete or larvae, or due to transgene silencing in some individuals. Inheritance and stability have not been examined in subsequent generations.

1.3.2.1.4 Methods to detect PZ2019 fish

PZ2019 individuals are easily distinguished from non-transgenic Zebrafish by their uniform purple colouration under natural light, and fluorescent colouration under blue or UV light. No known Zebrafish species has similar colouration, making PZ2019 individuals easy to distinguish from non-transgenic Zebrafish. PZ2019 fish can also be distinguished genetically by PCR amplification and detection of unique fragments that result from restriction enzyme digest of the transgene insert.

1.3.2.2 Phenotypic Characterization

1.3.2.2.1 Targeted phenotypic effects of the modification

The targeted phenotypic effect of the genetic modification is that PZ2019 appears purple under ambient light. The novel colour phenotype is present in muscle as well as skin and eye. GloFish LLC reports PZ2019 individuals that are hemizygous and homozygous for the transgene insert are indistinguishable from each other phenotypically and are both part of the commercially available population.

1.3.2.2.2 Non-targeted phenotypic effects of the modification

Two off-target effects identified by GloFish LLC in PZ2019 are diminished fluorescent offspring during paired trials with non-transgenic fish, and a decrease in reproductive success in competition for mates with non-transgenic siblings. In paired trials, the overall proportion of fluorescent offspring was less than the expected 50%, suggesting decreased viability of gametes and/or larvae containing the fluorescent transgene relative to those without the transgene, or silencing of the transgene in some individuals. In competitive reproductive success tests, the observed proportion of transgenic (purple fluorescent) offspring was significantly lower than the proportion expected from random assortment alone (0.3661 versus 0.4375, respectively, $p<0.001$), indicating that PZ2019 may be reproductively disadvantaged compared to non-transgenic Zebrafish; though individually, half of the groups showed no significant difference. The overall difference was still significant when decreased viability of purple gametes or larvae was taken into consideration ($p<0.001$). The influence of the genetic modification on any other phenotypes, including survival, fecundity and behaviour, has not been formally examined.

The notifier has also looked at lower temperature tolerance in PZ2019 relative to non-transgenic Golden Zebrafish, but found no significant difference in LD_{50} ($p=0.300$). The LD_{100} (lethal dose at which 100% of fish have died) of the two genotypes was similar and varied from 4.6 to 4.8°C between trials.

The notifier submitted a diagnostics report examining necropsy and microbiology, and histology of PZ2019. No usual findings were reported, though PZ2019 fish were not compared with non-transgenic sibling fish.

No formal studies have compared potential disease susceptibility of PZ2019 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 reports, anecdotal or otherwise, of any non-target effects other than those listed above.

1.3.2.2.3 Pleiotropic effects of fluorescent protein transgenes in Zebrafish

A detailed overview of the pleiotropic effects of fluorescent protein transgenes that have been observed in other Zebrafish is presented in Noble-Brzezinski et al. (2021).

1.3.3 Characterization Relative to Previously Notified and Assessed GloFish®

BZ2019 and PZ2019 were produced using the same methodologies and testing protocols as for previously notified and assessed GloFish® Danio and Tetra lines. All previous and current notified GloFish® Danio and Tetra lines have used the same transgene expression cassette production and elements (promoters, terminator sequences), although the pigment genes used vary between colours. Molecular and phenotypic characterization tests conducted by the company were equal among current and previously notified GloFish® lines, and results from BZ2019 and PZ2019 overlap with some or all of previously notified lines (see Table 1.1). One exception to this is the estimated copy number of the inserted transgenic constructs, as determined through quantitative PCR, and specific genetic lineage as determined by Southern blots, although banding pattern of the currently notified lines fall within the range of previously notified lines (Table 1.1).

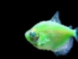
1.3.4 Characterization of Notified Organisms – Summary

The molecular and phenotypic characterizations of BZ2019 and PZ2019 fall within the range of previously notified and assessed GloFish® Danio and Tetra lines (see Table 1.1). Characterization of both lines was well-described with respect to function of the transgene constructs, genetic lineage of progenitor fish, targeted phenotypic effect (colour) and some off-target effects (cold tolerance, reproduction). However, uncertainty remains regarding some aspects of both the molecular characterization (e.g., line stability, copy number) and phenotypic characterization (e.g., effects on disease resistance, behaviour, other traits that may influence fitness or potential risk), resulting in overall moderate uncertainty in the characterization of BZ2019 and PZ2019. This is equivalent to uncertainty in characterization of previously notified and assessed GloFish® Tetras and Danios, though there is lower uncertainty in some characteristics of current lines relative to some previous lines (i.e., genetic lineage relative to YZ2018).

1.4 CHARACTERISATION OF COMPARATOR SPECIES

For the purpose of this assessment, non-transgenic domesticated and/or wild Zebrafish will be used as a comparator for the notified organism. This is the same comparator species used for the previously notified YZ2018 (GloFish® Sunburst Orange® Danio). A detailed overview of the Zebrafish is provided in Noble-Brzezinski et al. (2021).

*Table 1.1: Summary of characterization of all GloFish® lines notified under CEPA for sale in Canada in the ornamental pet trade. * indicates significant differences ($p<0.05$). Data are given as average ± standard error of the mean where appropriate.*

Characterization	BZ2019	PZ2019	YZ2018	BT2018	OT2018	PiT2018	PuT2018	RT2018	CGT2016
									
Commercial name	Cosmic Blue® Danio	Galactic Purple® Danio	Sunburst Orange® Danio	Cosmic Blue® Tetra	Sunburst Orange® Tetra	Moonrise Pink® Tetra	Galactic Purple® Tetra	Starfire Red® Tetra	Galactic Green® Tetra
Species	*Danio rerio*	*D. rerio*	*D. rerio*	*Gymno-corymbus ternetzi*	*G. ternetzi*	*G. ternetzi*	*G. ternetzi*	*G. ternetzi*	*G. ternetzi*
Long-fin variant present	no	no	no	no	yes	yes	yes	yes	yes
% fluorescent offspring in paired crosses with non-transgenic (=diff from expected 50%)*	47.1±1.1*	47.4±0.9*	48.2±0.6	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-transgenic (=diff from expected 40 or 43.75%)*	40.2±4.4*	38.6±3.1*	20.5±4.2*	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 fish during decrease in temperature	5.66 vs 5.54ºC*	5.71 vs 5.52ºC	5.87 vs 5.56ºC*	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	yes	yes	yes	no	yes	no	no	yes	yes
Commercial production date - USA	2010	2011	2012	2014	2013	2013	2013	2014	2012
Approved for sale in Canada	2019	2019	2019	2018	2018	2018	2018	2018	2017

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).

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 two GloFish® 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 and ecological similarities between the two lines, the following section will consider both lines at the same time. The environmental risk assessment format follows that used for previously notified GloFish® Tetras (DFO 2019) and GloFish® Sunburst Orange® Danio (DFO 2020) and results of the current assessment are equivalent to those from previous GloFish® assessments unless otherwise stated.

2.2 EXPOSURE ASSESSMENT

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

Table 2.1: Rankings for likelihood of exposure of genetically engineered fish to the Canadian environment.

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

[1]extremely unlikely or unforeseeable

Given the regulatory status of any GE fish undergoing environmental risk assessment under CEPA, a lack of empirical data regarding the survival, fitness and ability of BZ2019 and PZ2019 to reproduce in the natural environment will contribute uncertainty to the exposure assessment. Uncertainty associated with the environmental fate of an organism or the failure of biological and geographical containment may depend on the availability and robustness of the scientific information related to the biological and ecological parameters of the organism, valid surrogates, and the receiving environment. Table 2.2 ranks uncertainty associated with the likelihood of occurrence and fate of the organism in the Canadian environment.

Table 2.2: Ranking of uncertainty associated with the likelihood of occurrence and fate of the organism in the Canadian environment (environmental exposure).

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

All previous assessments of notified and assessed GloFish® Danio and Tetra lines concluded low rating for environmental exposure with low uncertainty (DFO 2018, 2019, 2020). There are no known molecular or phenotypic characteristics of BZ2019 or PZ2019 that suggest a different rating than previously assessed lines (including another Zebrafish line, YZ2018), and no new scientific literature has been published that would alter the previous ratings. Consequently, the environmental exposure assessments for BZ2019 and PZ2019 are low, with low uncertainty that is consistent with previously notified lines. Details supporting this conclusion follow.

2.2.1 Likelihood of Release

Though the stated purpose of the organism is for sale in the ornamental market, and hobbyists who purchase the product do, for the most part, follow the instructions for disposal that are recommended by the retailer or the company itself, there is still a high likelihood that BZ2019 and PZ2019 will be introduced into the Canadian environment. Once the organism has been sold into the retail market, it is no longer under the direct control of the importer, and there can be no guarantee of appropriate containment and disposal. Numerous aquarium fish have established themselves in natural waters in North America, and reoccurring, though isolated, reports of aquarium fish in Canadian water suggest the practice of releasing aquarium fish into the environment is common and ongoing (Kerr et al. 2005; Rixon et al. 2005; Marson et al. 2009; Strecker et al. 2011). This concurs with a high likelihood of release for previously notified GloFish® Tetras and Sunburst Orange® Danio. The extent to which BZ2019 and PZ2019 may be further exposed to the environment will, therefore, depend heavily on their ability to survive and reproduce in Canadian lakes and rivers.

2.2.2 Likelihood of Survival

As a tropical species, the Zebrafish is not expected to survive in a temperate region where water temperatures are below optimal for survival. Indeed, water temperature is a key abiotic factor that affects both the survival and production of most freshwater fish populations, and is a pervasive determinant of habitat suitability (Magnuson et al. 1979; Jobling 1981). Whereas the optimal temperature for breeding Zebrafish is 26 to 28.5°C, they can tolerate a wide range of temperatures in their natural range, from as low as 6°C in winter to over 38°C in summer (Spence et al. 2008; López-Olmeda and Sánchez-Vázquez 2011; Arunachalam et al. 2013; Little et al. 2013). Adult non-transgenic Zebrafish and transgenic Zebrafish (with a gene coding for a fluorescent protein) both survive a broad range of temperatures in the lab from 5.3 to 41.7°C and 5.6 to 41.4°C, respectively, during extreme temperature tolerance trials (Essner 2003; Cortemeglia and Beitinger 2005, 2006a; Schaefer and Ryan 2006). Data collected by DFO report minimum temperature tolerance range of 6.6 to 4.8°C for Golden Zebrafish (LD_{50} = 5.59, see Figure 2.1), while non-transgenic and transgenic lines of Zebrafish produced for research lose equilibrium on average between 5.38 and 5.90°C (see Figure 2.2, Leggatt et al. 2018a). These data show Zebrafish consistently have average minimum temperature tolerances in the mid 5°C, regardless of genotype, when temperature is dropped relatively rapidly in laboratory conditions (i.e., 1°C per day or faster). Studies in the field confirmed both non-transgenic and transgenic Zebrafish would not survive in waters at temperatures less than or equal to 5°C (Cortemeglia et al. 2008; Ribas and Piferrer 2014). As well, when fish were removed during temperature trials and acclimated at steady low temperatures, non-transgenic and three lines of fluorescent transgenic Zebrafish survived and recovered from four months rearing at 8°C, but were not able to survive beyond one week rearing at 6°C. This indicates despite reported tolerances in the mid 5°C, the effective cold tolerance limit of fluorescent protein transgenic and non-transgenic Zebrafish is likely between 6 and 8°C exclusively (Leggatt et al. 2018a), although temperature tolerance may vary at different life stages.

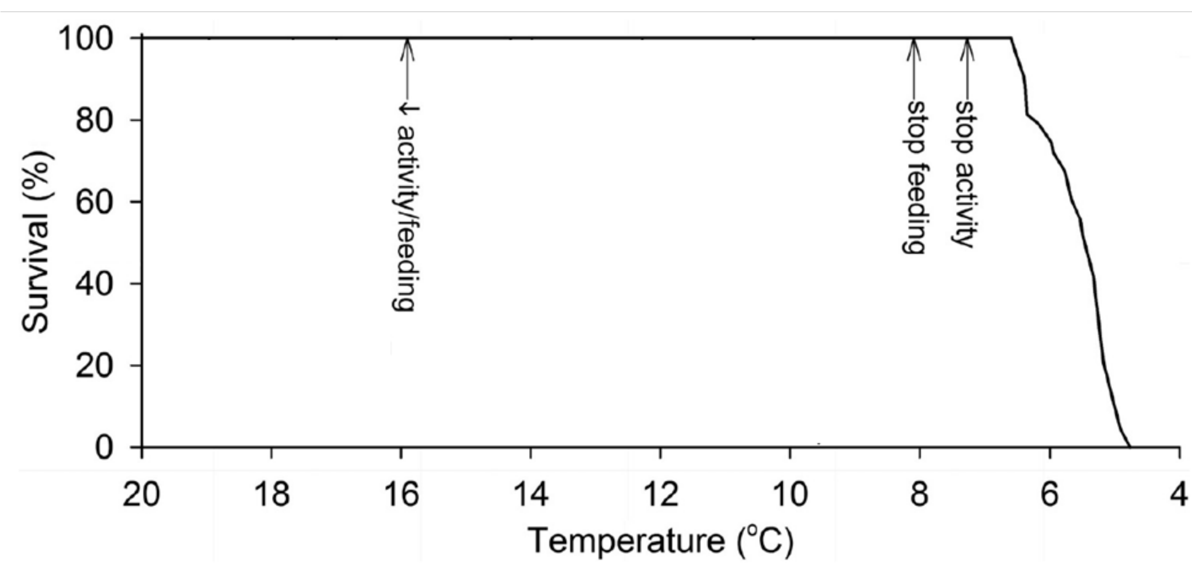


Figure 2.1: Survival and changes in activity and feeding level in non-transgenic golden variety Zebrafish when temperatures are lowered gradually from 20°C at a rate of 1°C per day (40 Zebrafish, Danio rerio, divided into two tanks). Modified from Leggatt et al. (2018a).

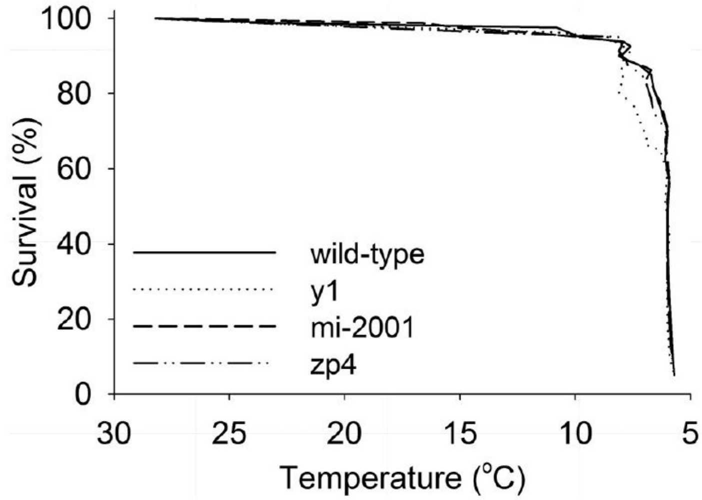


Figure 2.2: Survival during gradual cold exposure for three lines of green fluorescent protein (GFP) transgenic (y1, mi2001, zp4) Zebrafish (Danio rerio) and their non-transgenic progenitor line (AB, wild-type). Temperature was dropped from 28 to 12°C at a rate of approximately 1°C per day, then from 12 to 5.1°C at a rate of approximately 0.1°C per day (80 Zebrafish per line, divided into four tanks). Modified from Leggatt et al. (2018a).

GloFish Technologies have also conducted research on the lower temperature tolerance of Golden Zebrafish, BZ2019 and PZ2019, and provided data from their experiments as part of its notification package. These experiments reported that BZ2019 (LD_{50}=5.66°C) and PZ2019 (LD_{50}=5.71°C) both had higher low-temperature tolerance than non-transgenic Golden Zebrafish (LD_{50}=5.54-5.52°C) when temperatures drop rapidly at a rate of 0.5-1°C per hour (a difference of 0.12-0.19°C, see Figures 1.6, 1.11), although this was only significant for BZ2019. Though the observed lower lethal temperature ranged from 4.4 to 7.9°C, the rapid change in temperature may not be representative of a natural system. Regardless of the difference in methodology and results, when these studies are taken together, it is reasonable to conclude that Golden Zebrafish, BZ2019 and PZ2019 cannot survive at temperatures below 4°C, and likely cannot survive long-term at 6°C or lower.

There are no known lakes in Canada that consistently remain above 7°C throughout the entire course of a year, or above 6°C across multiple years, and almost all do not remain above 4°C throughout the year. Consequently, while the temperatures needed for BZ2019 and PZ2019 to survive are possible for several Canadian lakes during the spring, summer and autumn, it is highly unlikely that BZ2019 and PZ2019 can survive the Canadian winter. At best, its occurrence in the environment would be seasonal or ephemeral. This is further supported by lack of establishment of Zebrafish after noted occurrences in much warmer climates (e.g., Florida, USA, where temperatures are generally above 8°C year round, see below).

Mean freshwater surface temperatures in Canada are rising as a result of global climate change, and are projected to increase by 1.5 to 4.0°C over the next 50 years (DFO 2013). Increased winter water temperatures in the few isolated lakes with infrequent ice coverage in Southwestern BC could increase the potential for overwinter survival in these isolated lakes. However, for the majority of freshwater systems experiencing significant ice coverage in the winter, climate change is expected to decrease the number of ice-days in these systems (DFO 2013), but temperatures would still be expected to be at or below 4°C at some point during the winter, preventing year-round survival of BZ2019 and PZ2019. As well, in Florida, Tuckett et al. (2017) observed Zebrafish surviving in natural systems where water temperatures ranged from

8.7°C to 32.5°C (mean 21.7°C), but only in close proximity (<500 m) of ornamental aquaculture facilities from which they presumably escaped. The authors speculate that the observed lack of dispersal may be the result of relatively cold water in sub-tropical Florida, predatory fish, and additional factors related to physical or biological habitat. Cold tolerance data combined with the lack of establishment of Zebrafish in regions warmer than Canada suggest limited potential to survive in Canadian waters even with increased water temperatures associated with climate change.

2.2.3 Likelihood of Reproduction

Though water temperatures in Canada will limit the persistence of any BZ2019 and PZ2019 that are introduced into the environment (see Section 2.2.2), there may still be time to reproduce, if introduced at the start of a warm season. For example, Osoyoos Lake in the BC interior is one of Canada's warmest lakes in the summer, with an average temperature between 20 and 25°C for about 2 months of the year (mid-July to mid-September), with higher temperatures (e.g., 25°C) restricted to an even shorter window (e.g., end of July – beginning of August, BCLSS 2013). While this may be an ideal temperature range for Zebrafish survival, warmer temperatures (27-30°C) are more ideal for reproduction (López-Olmeda and Sánchez-Vázquez 2011). Zebrafish can spawn at temperatures lower than this, but temperatures below 24°C may reduce breeding incidences, induce developmental defects in offspring, and skew sex-ratios of offspring (Schirone and Gross 1968; Barrionuevo and Burggren 1999; Hallare et al. 2005; Sfakianakis et al. 2012). Seasonal cues, diurnal cues, and food availability have also been demonstrated to influence Zebrafish spawning and success.

Zebrafish are prolific spawners and spawning frequency can occur every 4-7 days, with offspring maturing in as little as 2.5 months under ideal laboratory conditions. However, the lack or limited prevalence of ideal reproduction temperatures in Canada indicate any reproduction would be limited to a short window of opportunity during part of the summer, regardless of age at the time of introduction. For example, any BZ2019 or PZ2019 fish introduced to Osoyoos Lake at the beginning of July would have two months in a new environment to find habitat and resources needed for reproduction, as well as conspecifics to reproduce with, and the window for optimal spawning temperatures may be only a couple of weeks long. Though any fertilized eggs that are not eaten by predators could hatch in a relatively short period of time (3-7 days, Kimmel et al. 1995; Lawrence 2007), any offspring would not mature prior to onset of cooler temperatures in the late summer and would not survive the winter. The reported decreased reproductive success in competition for both PZ2019 and BZ2019 indicates there is no greater potential for reproduction of these lines relative to non-transgenic Zebrafish. Though isolated opportunities for reproduction in the Canadian environment could occur, it would never result in more than two generations present in the environment at a time.

Interspecific hybrids have been reported between *D. rerio* and *Danio albolineatus* (synonym *Brachydanio albolineatus*, common name Pearl Danio, Axelrod and Vorderwinkler 1976), however, the F1 hybrid is sterile. Several genera of the Cyprinidae family are found in Canada, but it is not known if they could reproduce with Zebrafish or produce fertile offspring.

2.2.4 Likelihood of Proliferation and Spread

The capacity for BZ2019 and PZ2019 to proliferate and spread in the Canadian environment is precluded by the fact that Zebrafish cannot survive the winter. It should be noted that any released BZ2019 and PZ2019 are expected to occupy areas near the shoreline, based on what is known of wild-type habitat preferences (see Section 1.4.3). These areas are expected to have more extreme temperature ranges than deep water or mid-lake areas that are often the source of water temperature measurements (Trumpikas et al. 2015). Consequently, winter temperatures may be colder than indicated by recorded data, which may further reduce the potential for overwintering of BZ2019 and PZ2019, though fish may move to follow warmer water as temperatures drop. Warmer summer temperatures in these areas may increase potential for single generation spawning.

2.2.5 Conclusions of Exposure Assessment

Given the above analysis, the occurrence of BZ2019 and PZ2019 in the Canadian environment is expected to be rare, isolated and ephemeral. Consequently, the likelihood of exposure of BZ2019 and PZ2019 to the Canadian environment is ranked **low** according to Table 2.1. The uncertainty associated with this estimate is **low** (Table 2.2), given the quality of data (temperature tolerance) available for BZ2019 and PZ2019 and valid surrogate organisms, evidence of low variability, and data available on the environmental parameters of the receiving environment in Canada. This rating is consistent with the low exposure rating with low uncertainty concluded on for the Sunburst Orange® Danio (DFO 2020), as well as six lines of GloFish® Tetras (DFO 2018, 2019).

The notifying company identifies the sole intended use for the notified organism as an ornamental fish for interior, static home aquaria. However, once purchased by consumers, other unintended uses cannot be discounted (e.g., rearing in outdoor ponds, as bait fish, etc.). While some unintended uses may lead to the release of BZ2019 and PZ2019, they would not be expected to alter the organism's ability to overwinter in Canadian environments, or otherwise alter the low environmental exposure ranking for the organism.

Changing water temperature patterns associated with global climate change have the potential to increase uncertainty when determining the ability of the notified organism to survive, reproduce, proliferate and spread in Canadian freshwater ecosystems.

2.3 HAZARD ASSESSMENT

The hazard assessment examines potential impacts that could result from environmental exposure to BZ2019 and PZ2019 in the environment. The hazard identification process considers potential pathways to harm including through environmental toxicity (i.e., potential to be poisonous), gene transfer, trophic interactions, and as a vector for pathogens, as well as capacity to impact ecosystem components (e.g., habitat, nutrient cycling, biodiversity). Table 2.3 categorizes the severity of the biological consequences based on the severity and reversibility of effects to the structure and function of the ecosystem. Any difference in measurement endpoint is evaluated relative to 'normal' variation, based on published studies and expert opinion.

Given the lack of empirical data around the behaviour and fitness of BZ2019 and PZ2019 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 BZ2019 and PZ2019 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.

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

Table 2.3: Ranking of hazard to the environment resulting from exposure to the organism.

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

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

Table 2.4: Ranking of uncertainty associated with the environmental hazard.

Uncertainty Ranking	Available Information
Negligible	High quality data on BZ2019 and PZ2019. 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 BZ2019 and PZ2019 or valid surrogate. Understanding of GxE effects across relevant environmental conditions. Some variability.
Moderate	Limited data on BZ2019 and PZ2019, relatives of BZ2019 and PZ2019 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.

The proposed use of BZ2019 and PZ2019 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 BZ2019 and PZ2019 to Canadian environments. The majority of potential hazards posed by BZ2019 and PZ2019 (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 BZ2019 and PZ2019 to natural aquatic ecosystems, although some potential hazards could act indirectly through the release of waste water and carcasses (e.g., environmental toxicity, horizontal gene transfer).

In assessments of previously notified and assessed GloFish® Danio and Tetra lines, all concluded with negligible ratings for most environmental hazard pathways and low hazard ratings through horizontal gene transfer (HGT), with uncertainty ranging from negligible to moderate (DFO 2018, 2019, 2020). There are no known molecular or phenotypic characteristics of BZ2019 or PZ2019 that suggest a different rating than previously assessed lines (including another Zebrafish line YZ2018), and no new scientific literature has been published that would alter the previous ratings. Consequently, the environmental hazard assessments for BZ2019 and PZ2019 follow those of the previously notified YZ2018, and follow those of previously notified GloFish® Tetras with the exception of two differences in uncertainty that were identified in the assessment of YZ2018. Details supporting these conclusions follow, and greater detail for each hazard assessment can be found in Leggatt et al. (2018b).

2.3.1 Potential Hazards Through Environmental Toxicity

Potential routes of environmental toxicity include exposure of aquatic ecosystems to the whole animal and its waste, as well as ingestion by predators. Exposure of the fluorescent proteins to the environment is expected to be lower than exposure of the proteins to BZ2019 and PZ2019; though different routes to exposure are not necessarily comparable. Fluorescent proteins are commonly used as neutral markers in research in a wide range of organisms with almost no reports of toxicity (Stewart 2006). The few reports of negative effects are generally specific to transgenic organisms with especially high expression of fluorescent transgenes (Huang et al. 2000; Devgan et al. 2004; Guo et al. 2007). Any toxic effects to host organisms are likely due to production of the protein within the host cell, and are not expected to have equal effects from contact or ingestion exposure.

The notification includes a report screening the amino acid sequence of the fluorescent protein for allergenicity on Allermatch that found no functional matches to known human allergen amino acid sequences. After several years of commercial production in the US, there have been no reported toxic effects resulting from exposure to BZ2019 or PZ2019. Consequently, the potential hazard to the environment due to environmental toxicity of BZ2019 or PZ2019 is ranked **negligible**. The uncertainty associated with this ranking is **moderate** due to limited direct data from the notified organisms or surrogate organisms, and reliance on anecdotal evidence and indirect evidence from other organisms. This concurs with assessment rankings for previously notified YZ2018 Zebrafish and six lines of GloFish® Tetras (DFO 2018, 2019, 2020).

2.3.2 Potential Hazards Through Horizontal Gene Transfer

Horizontal gene transfer is the non-sexual exchange of genetic material between organisms of the same or different species (DFO 2006). Pathways of exposure of free transgenic DNA to novel organisms (most likely prokaryotes) include exposure within the BZ2019 and PZ2019 gut, or through feces, mucus, and other waste sloughed off by the fish into the water. The transgene construct does not contain transposable elements that may increase the potential for DNA uptake/mobility to a new organism, although transposable elements are present in the Zebrafish genome (e.g., Hagemann and Hammer 2006). In order for the transgene to be expressed resulting in phenotypic change, it requires co-transfer of regulatory elements. The close proximity of the promoters to the pigment transgenes could increase the likelihood of them being co-transferred and expressed, though vertebrate promoters generally have poor activity in prokaryotes. The identified presence of the bacteriophage T3 promoter in the transgene constructs of the current and some previously notified lines may increase the potential for functional HGT to occur, and the promoter has been shown to result in expression of TagBFP in *Escherichia coli* (Wu et al. 2015). As well, the ribosomal binding site upstream of aeCP597 may increase the chance of expression should HGT occur.

Genes encoding fluorescence have been introduced to a wide range of organisms with few reports of harmful effects from the introduced transgenes. This suggests that the introduction of the transgene through HGT to a novel host is not expected to result in harmful effects, should it occur. Though the introduction of a fluorescent transgene to a novel organism in Canadian environments through HGT cannot be excluded, the absence of expected harmful effects from such an introduction result in a hazard ranking of **low**. While the transgene is well defined, the limited knowledge of the location of the transgene within the Zebrafish genome, and lack of studies examining HGT of the transgene and resulting consequences, results in **moderate** uncertainty. This concurs with the previous assessment for the GloFish® Sunburst Orange® Danio (YZ2018), and rankings concur with that assessed for six lines of GloFish® Tetras, though in the latter group uncertainty was assessed at low (DFO 2018, 2019, 2020). The uncertainty rating was increased in the current and YZ2018 assessment to better reflect the lack of relevant studies of HGT and resulting consequences.

2.3.3 Potential Hazards Through Interactions with Other Organisms

Should BZ2019 and PZ2019 be released to the environment, they have the potential to interact with other organisms in Canadian freshwater aquatic ecosystems, including potential prey, competitors, and predators. The trophic interactions of wild-type *D. rerio* in its native range are not well documented, nor is there documentation of trophic interactions of escaped ornamental domesticated non-transgenic or BZ2019 and PZ2019 *D. rerio* in other areas. Limited data described below indicate non-transgenic Zebrafish may have limited potential to impact Canadian species through trophic interactions, and BZ2019 and PZ2019 would have equal or less potential to impact through trophic interactions than non-transgenic Zebrafish.

Zebrafish are omnivorous and feed on zooplankton, phytoplankton, insects and insect larvae, worms and small crustaceans and larval fish. As such, they have the potential to impact localized populations of small prey organisms or competitors occupying similar niches at the location of release. Non-transgenic Zebrafish are generally described as a “peaceful” fish, and interact well with other ornamental fish species. While they can tolerate a wide range of temperatures, non-transgenic Golden Zebrafish decrease activity and feeding at approximately 16°C, and stop feeding and stop activity below 8°C (Leggatt et al. 2018a, see Figure 2.2). Consequently, activity and feeding levels of Zebrafish are expected to be low during most seasons in Canada’s temperate waters. Research conducted by GloFish Technologies demonstrates that BZ2019 is significantly less cold tolerant than non-transgenic siblings, which may further limit feeding and competitive activities of this line should it be released to Canadian waters, although differences in cold tolerance may not be functionally significant (i.e., LD_{50} differed by only 0.12°C). In other fluorescent protein transgenic models, an unpublished study by DFO found GloFish® Electric Green® Tetras had similar aggressive behaviour and foraging success as non-transgenic siblings in feeding trials (Leggatt, pers. comm.), while RFP Zebrafish had lower male mating aggression and success than non-transgenic siblings (Howard et al. 2015), suggesting fluorescent protein transgenesis may decrease or not affect competitive success in tropical fish. In contrast to this, Jha (2010) reported domesticated RFP transgenic Zebrafish were more aggressive than unrelated wild-type, wild-caught non-transgenic Zebrafish – although these results may have been influenced by genetic background among other factors (e.g., rearing history), as domestication has been reported to increase aggression in fish (Einum and Fleming 1997). In over five years of commercial use in the ornamental aquarium trade there are no known reports, anecdotal or otherwise, of BZ2019 and PZ2019 having different activity levels or behaviour than non-transgenic *D. rerio*. Given the low temperatures expected for Canadian freshwater systems for most of the year, the potential for released BZ2019 and PZ2019 to impact native aquatic species through prey acquisition and competition is expected to be negligible through most of the year, and is expected to be no greater than for non-transgenic Golden Danio.

Released BZ2019 and PZ2019 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 BZ2019 or PZ2019 causes deleterious effects to the predator populations. The latter is not expected as BZ2019 and PZ2019 are not expected to be environmentally toxic (see Section 2.3.1 above). While the predation pressure on BZ2019 and PZ2019 relative to non-transgenic *D. rerio* has not been reported, the effect of fluorescent transgenesis in another transgenic model (RFP Zebrafish) is conflicting, with RFP-expressing Zebrafish having higher (Hill et al. 2011), equal (Cortemeglia and Beitinger 2006b), or lower (Jha 2010) predation susceptibility relative to non-related non-transgenic fish. These variable findings may be due to differences in rearing history, genetic background, experimental conditions among studies, or genotype x environment interactions. Whether any of the above studies could be applied to BZ2019 and PZ2019 predation vulnerability in Canadian environments is not known and, consequently, the predation vulnerability of BZ2019 and PZ2019 relative to non-transgenic counterparts cannot be estimated with reasonable certainty. However, due to the lack of expected toxicity from ingesting BZ2019 or PZ2019, the notified lines are not expected to pose a hazard as prey to native predators, regardless of potential predation sensitivity.

Based on the non-aggressive behaviour of Zebrafish, low activity in cooler waters, and lack of noted alterations in trophic-related behaviour of the notified lines, BZ2019 and PZ2019 are not expected to influence trophic interactions of native organisms beyond natural fluctuations, with associated **negligible** hazard relative to non-transgenic counterparts. The lack of studies directly examining the hazards of BZ2019 and PZ2019, limited available data on a valid surrogate (RFP Zebrafish) and poor understanding of GxE interactions in aggression and predation susceptibility in surrogate fluorescent transgenic Zebrafish model, result in a **moderate** level of uncertainty. This concurs with assessment rankings for previously notified YZ2018 Zebrafish and six lines of GloFish® Tetras (DFO 2018, 2019, 2020).

2.3.4 Potential Hazards Through Hybridization with Native Species

The tropical freshwater Zebrafish belongs to the family cyprinidae, and has a geographical distribution native to the subcontinent of India within the Ganges and Brahmaputra river basins in north-eastern India, Bangladesh and Nepal (Spence et al. 2008; Lessman 2011). There are several other species that currently share the *Danio* genus (Fang 2003). Zebrafish are scatter breeders, and, consequently, could potentially form hybrids with related species that spawn at the same time and place. The notifier states that interspecific hybrids have been reported between *D. rerio* and *D. albolineatus* (synonym *Brachydanio albolineatus*, common name Pearl Danio, Axelrod and Vorderwinkler 1976), which is also native to the Indian Subcontinent. Though hybrids have been found between the two similar species, the F1 hybrid is sterile.

While there are no species of *Danio* native to Canada, there are several genera of fish from the cyprinidae family, although it is not known if these could successfully breed with Zebrafish. Intergeneric hybrids have been noted for two genera of cyprinidae in Europe (Hayden et al. 2010), and in the fish family mormyridae survival of intergeneric hybrids was related to the phylogenetic distance of the parent species (i.e., greater phylogenetic distance resulted in decreased viability, and increased occurrence of malformations, Kirschbaum et al. 2016). Inter-breeding is unlikely given the probable phylogenetic difference and adaptive differentiation between native Canadian cyprinidae genera and Zebrafish, and any successful intergeneric hybridization would be expected to be sterile, as is the case with hybridization with the more closely related *Danio albolineatus.* BZ2019 and PZ2019 would have further lower hybridization potential than non-transgenic Zebrafish given their decreased reproductive success. Consequently, there is **negligible** potential for BZ2019 and PZ2019 to cause hazards through viable hybridization with native fish in Canada. The high quality data on distribution of cyprinidae but lack of data on potential for intergeneric hybridization result in **moderate** uncertainty associated with the rating. This concurs with assessment rankings for previously notified YZ2018 Zebrafish (DFO 2020), although conclusions for six lines of GloFish® Tetras were negligible potential with negligible uncertainty due to lack of native Canadian species within the Tetra family (DFO 2018, 2019).

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). As part of its notifications, GloFish LLC provided summaries of diagnostic examinations undertaken by the Fish Disease Diagnostic Lab, University of Florida, for both PZ2019 and BZ2019. All necropsy findings were normal except for the presence of low numbers of nematodes (species not specified) in two of six BZ2019 examined. It's stated, however, that this finding is unrelated to the transgenic nature of BZ2019. No bacterial growth was observed from any samples of brain and posterior kidney, and histological examination of major organs found no pathologic lesions in any of the fish examined. It should be noted that histology was not directly compared to non-transgenic fish, instead looking specifically for gross pathological lesions and signs of disease.

Disease agents are common in tropical-origin freshwater ornamental aquarium fish and Zebrafish is listed among very few species (e.g., Goldfish, Tank Goby, Guppy, Three Spot Gourami) as species susceptible to diseases of significant importance to aquatic animal health and the Canadian economy by the Canadian Food Inspection Agency (CFIA). In 2012, the Canadian Food Inspection Agency (CFIA) placed Zebrafish on its list of susceptible species, expressing concern that Zebrafish could be a vector for Spring Viremia of Carp Virus (SVCV), a hemorrhagic disease of freshwater finfish. However, no natural SVCV infections have been reported in Zebrafish, including in the wild, in the hobbyist community, and in the laboratory setting (Hanwell et al. 2016). Since the principal mode of entry of BZ2019 and PZ2019 will be through importation from the US, the CFIA will play a critical role in regulating disease agents of *Danio rerio* that are imported into Canada. In addition, any disease agents BZ2019 and PZ2019 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 BZ2019 and PZ2019 once released to cooler Canadian freshwater environments. Zebrafish can be infected with cold-water disease agents through experimental procedures (e.g., SVCV), but the susceptibility of Zebrafish to disease agents relevant for Canada under natural conditions is not known.

Whether BZ2019 and PZ2019, 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. For example, GFP expression decreased T-cell activation (Koelsch et al. 2013), induced cytokine IL-6 secretion (Mak et al. 2007), inhibited immune-related signalling pathways (Baens et al. 2006), and altered expression of genes involved in immune function (Coumans et al. 2014) and response to stress (Badrian and Bogoyevitch 2007). As well, Chou et al. (2015) reported mice transgenic for DsRed had alterations in some white blood cell numbers (lymphocytes and monocytes) but not others. BZ2019 and PZ2019 have been grown on a commercial scale in the US since 2010 and 2011, respectively, as have numerous other transgenic fluorescent aquarium species and lines starting in 2003. In GloFish® Tetra notifications GloFish LLC provided statements from veterinarians that stated they had not seen increases in susceptibility to, or the transmission of, pathogens in any GloFish® line, although no empirical evidence was provided. Fluorescent Zebrafish have been used extensively in laboratory conditions for research with no known reported effects on disease susceptibility. 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 suggests there is **negligible** potential for BZ2019 and PZ2019 to have altered vector capabilities relative to non-transgenic Zebrafish. As this has not been directly examined in BZ2019 and PZ2019, there are limited data on a valid surrogate, and reliance on expert opinion, the uncertainty level for this rating is **moderate**. This concurs with assessment rankings for previously notified YZ2018 Zebrafish and six lines of GloFish® Tetras (DFO 2018, 2019, 2020).

2.3.6 Potential to Impact Biogeochemical Cycling

BZ2019 and PZ2019 are expected to contribute to nutrient cycles within habitats through ingestion of prey and other food items and release of waste (ammonia and feces). The potential effects of fluorescent protein in BZ2019 and PZ2019 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 BZ2019 and PZ2019 have similar influences from fluorescent transgenic gene expression are not known, but the small size of Zebrafish and potential low numbers of individuals in an ecosystem indicates a **negligible** potential for BZ2019 and PZ2019 to impact biogeochemical cycling in natural environments, even with altered metabolic pathways. Uncertainty is **moderate** due to a lack of studies directly examining this hazard. This concurs with assessment rankings for previously notified YZ2018 Zebrafish and six lines of GloFish® Tetras (DFO 2018, 2019, 2020).

2.3.7 Potential to Affect Habitat

Zebrafish are a small species that do not build nests or other structures that may impact habitats of other species. BZ2019 and PZ2019 have been in commercial use in the ornamental aquarium trade since 2006 and 2011, respectively, and there have been no reports, anecdotal or otherwise, of BZ2019 or PZ2019 having altered behaviour, relative to Golden Zebrafish, that may influence effects on habitat structure. Consequently, BZ2019 and PZ2019 are expected to have **negligible** effects to habitat with **low** uncertainty associated with this rating. This concurs with assessment rankings for previously notified YZ2018 Zebrafish and six lines of GloFish® Tetras (DFO 2018, 2019, 2020).

2.3.8 Potential to Affect Biodiversity

Biodiversity can be negatively impacted by numerous drivers, including invasive species and the introduction of disease. Despite their long standing use in the ornamental aquarium trade and as models for research and repeated occurrence in natural systems, there have been no reports of Zebrafish becoming invasive in North America, Europe, or elsewhere worldwide. As elaborated above, BZ2019 and PZ2019 are not expected to negatively impact native species through trophic or hybrid interactions, act as a vector for disease agents of concern in Canada, impact biogeochemical cycling, or impact habitat. Addition of the transgenic construct and fluorescent protein in BZ2019 and PZ2019 is not expected to result in environmental toxicity, or cause hazards through HGT of the transgene, and is not expected to increase potential hazards through interactions with native species. Taken together, there is a **negligible** hazard of BZ2019 and PZ2019 affecting biodiversity of Canadian ecosystems. Reliance on data from the comparator species for invasiveness and biodiversity effects results in a **low** degree of uncertainty with this ranking. This concurs with assessment rankings for previously notified YZ2018 Zebrafish and six lines of GloFish® Tetras (DFO 2018, 2019, 2020).

2.3.9 Conclusions of Hazard Assessment

BZ2019 and PZ2019 are not expected to be hazardous to Canadian environments. These species have no history of invasiveness despite widespread use. There is no evidence of environmental toxicity associated with the construct, and the majority of other fluorescent models do not report toxicity associated with fluorescent transgenes. There is also no indication of potential effects to the environment via transfer of the transgene to native Canadian species through hybridization, or HGT. BZ2019 and PZ2019 and other fluorescent fish models have no reported differences in survival, disease susceptibility, or husbandry care, and are not expected to have an altered ability to act as a vector for disease or impact biogeochemical cycling.

The examined hazards have negligible to low hazard rankings (Table 2.5), while uncertainty ranged from low to moderate due to limited data specific to BZ2019 and PZ2019, limited direct data on comparator species, variable data from surrogate models (e.g., RFP Zebrafish), and the reliance on expert opinion for the assessment of some hazards. Outside of its intended use as an ornamental fish in static aquaria, PZ2019 and BZ2019 are not expected to pose unique hazards beyond those of the intended use. Hazard ranking concurred with that previously assessed for YZ2018 Zebrafish and six lines of GloFish® Tetras, although uncertainty differed from that assessed in GloFish® Tetras in two hazard categories due to increased acknowledgement of data limitations (through HGT), or differences in family distributions (through hybridization, see Table 2.5).

2.4 ASSESSMENT OF RISK

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

Risk $\propto$ Exposure $\times$ Hazard

For each endpoint, hazard and exposure are ranked as: negligible, low, moderate, or high, and include an analysis of uncertainty for each. Overall Risk is estimated by plotting Hazard against Exposure, using a matrix or heat map, as illustrated in Figure 2.3. Though the matrix cannot be used as a tool for establishing a discreet conclusion or decision on risk, it can be used to facilitate communication and discussion. The uncertainty associated with overall Risk rating is not estimated, rather uncertainty in the hazard and exposure assessments are discussed in the context of a final conclusion on risk.

Table 2.5: Summary of all ranks and uncertainty rating for environmental risk assessments of currently notified Zebrafish lines (BZ2019, PZ2019), a previously notified Zebrafish line (YZ2018), and six previously notified lines of GloFish® Tetras (DFO 2018, 2019, 2020). Italics indicate where previous assessments differ from the current assessment.

Assessment	**Rank / Uncertainty**		
	BZ2019 & PZ2019	**YZ2018**	**GloFish® Tetras**
Exposure	**Low / Low**	**Low / Low**	**Low / Low**
Hazards:			
1.Environmental toxicity	Negligible / Moderate	Negligible / Moderate	Negligible / Moderate
2. HGT	Low / Moderate	Low / Moderate	Low / *Low*
3. Trophic interactions.	Negligible / Moderate	Negligible / Moderate	Negligible / Moderate
4. Hybridization	Negligible / Moderate	Negligible / Moderate	Negligible / *Negligible*
5. Vector for disease	Negligible / Moderate	Negligible / Moderate	Negligible / Moderate
6. Biogeochemical	Negligible / Moderate	Negligible / Moderate	Negligible / Moderate
7. Habitat	Negligible / Low	Negligible / Low	Negligible / Low
8. Biodiversity	Negligible / Low	Negligible / Low	Negligible / Low
Environmental Risk	**Low**	**Low**	**Low**

2.4.1 Risk Assessment of PZ2019 and BZ2019

The exposure assessment concluded that BZ2019 and PZ2019 used in the ornamental aquarium trade or other unintended uses would have a low likelihood of occurrence in the Canadian environment. This is due to the high likelihood of release of small numbers from home aquaria, but negligible likelihood for BZ2019 and PZ2019 to overwinter in Canadian aquatic ecosystems. As such, any exposure to Canadian freshwater ecosystems to BZ2019 and PZ2019 is expected to be isolated, rare, and ephemeral. The quality of data demonstrating lack of cold tolerance in BZ2019 and PZ2019 and Golden Zebrafish, relevant to Canadian freshwater temperatures result in low uncertainty associated with this ranking.

The hazard assessment concluded that BZ2019 and PZ2019 poses negligible to low hazard to the Canadian environment due to the lack of hazard associated with Golden Zebrafish and no direct evidence that the expressed fluorescent protein would increase hazard, relative to Golden Zebrafish. Uncertainty ranking associated with individual hazard components ranged from low to moderate, due to limited data specific to BZ2019 and PZ2019, limited direct data on comparator species, variable data from surrogate model (RFP Zebrafish), and the reliance on expert opinion for the assessment of some hazards.

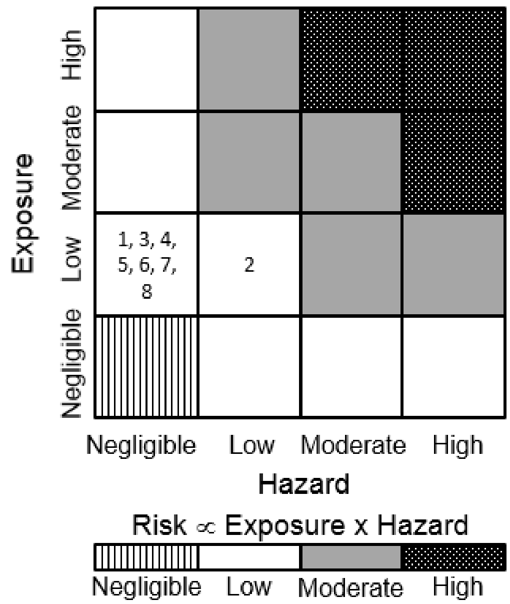


Figure 2.3: Risk matrix to illustrate how exposure and hazard are integrated to establish a level of risk in the environmental risk assessment. Risk assessments associated with assessed hazard components at the assessed exposure are identified by number: 1) through environmental toxicity; 2) through horizontal gene transfer; 3) through interactions with other organisms; 4) through hybridization; 5) as a vector of disease; 6) to biogeochemical cycling; 7) to habitat; and 8) to biodiversity.

Using the risk matrix seen in Figure 2.3, BZ2019 and PZ2019 used in the ornamental aquarium trade or other uses in Canada pose **low risk** to Canadian environments. Individual hazards are expected to result in no harmful effects beyond natural fluctuations to Canadian environments under the assessed level of exposure. Sources of uncertainty in the environmental exposure and hazard assessments that may influence uncertainty in environmental risk assessment include a lack of data directly addressing hazards of the notified organism and comparator species, variability in data taken from surrogate organisms, and in some cases reliance on expert opinion.

Despite moderate uncertainty in some of the individual assessment components, there is no current evidence to suggest that overall risk ratings of BZ2019 and PZ2019 may be higher than the assessed low ranking for risk to Canadian environments. This concurs with low risk assessment rankings for previously notified YZ2018 Zebrafish and six lines of GloFish® Tetras (DFO 2018, 2019, 2020, see Table 2.5).

2.5 SUMMARY AND CONCLUSIONS

Use of BZ2019 and PZ2019 in home aquaria in Canada, or in other unintended uses, is expected to result in frequent, very small magnitude releases of BZ2019 and PZ2019 to the Canadian environment, although the potential for occasional high magnitude releases cannot be ruled out. Available high quality data indicates that BZ2019 and PZ2019 do not have the capacity to overwinter in Canadian freshwater ecosystems. This results in an exposure ranking of low, with associated uncertainty being low. The lack of evidence of hazards from non-transgenic comparator species despite long-term extensive use, and a lack of evidence for increased hazards of BZ2019 and PZ2019 relative to non-transgenic Zebrafish, indicates negligible to low hazard ranking to Canadian ecosystems. Due to a lack of or limited direct information on the hazards of base models or BZ2019 and PZ2019, uncertainty with hazard assessments ranged from low to moderate. Taken together, the overall risk of BZ2019 and PZ2019 to the Canadian environment is ranked low, and the notified organism is not expected to cause harmful effects to the Canadian environment at the assessed exposure level. Though uncertainty with some of the hazard estimates is moderate due to limited and or no direct data on the notified organism or comparator species, no evidence was identified to suggest BZ2019 and PZ2019 under the proposed or other potential uses, could cause harm as a result of exposure to the Canadian environment.

REFERENCES CITED

Akitake, C.M., Macurak, M., Halpern, M.E., and Goll, M.G. 2011. Transgenerational analysis of transcriptional silencing in zebrafish. Dev. Biol. 352(2): 191-201.

Arunachalam, M., Raja, M., Vijayakumar, C., Malaiammal, P., and Mayden, R.L. 2013. Natural history of zebrafish (*Danio rerio*) in India. Zebrafish 10(1): 1-14.

Axelrod, H.R., and Vorderwinkler, W. 1976. Encyclopedia of Tropical Fishes with Special Emphasis on Breeding. T.F.H. Plublishing, Neptune City, NJ.

Badrian, B., and Bogoyevitch, M.A. 2007. Changes in the transcriptional profile of cardiac myocytes following green fluorescent protein expression. DNA Cell Biol. 26(10): 727-736.

Baens, J., Noels, H., Broeckx, V., Hagen, S., Fevery, S., Biliau, A.D., Vankelecom, H., and Marynen, P. 2006. The dark side of EGFP: Defective polyubiquitination. PLoS ONE 1(1): e54.

Barrionuevo, W.R., and Burggren, W.W. 1999. O_2 consumption and heart rate in developing zebrafish (*Danio rerio*): influence of temperature and ambient O_2. Am. J. Physiol. 276(2): R505-R513.

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

Burgman, M. 2005. Risk and Decisions for Conservation and Environmental Managers. Cambridge University Press. 504 p.

Chou, C.J., Peng, S.Y., Wan, C.H., Chen, S.F., Cheng, W.T.K., Lin, K.Y., and Wu, S.C. 2015. Establishment of a DsRed-monomer-harboring ICR transgenic mouse model and effects of the transgene on tissue development. Chinese J. Physiol. 58(1): 27-37.

Cortemeglia, C., and Beitinger, T.L. 2005. Temperature tolerances of wild-type and red transgenic zebra danios. Trans. Am. Fish. Soc. 134(6): 1431-1437.

Cortemeglia, C., and Beitinger, T.L. 2006a. Projected US distributions of transgenic and wildtype zebra danios, *Danio rerio*, based on temperature tolerance data. J. Therm. Biol. 31(5): 422-428.

Cortemeglia, C., and Beitinger, T.L. 2006b. Susceptibility of transgenic and wildtype zebra danios, *Danio rerio*, to predation. Environ. Biol. Fish. 76(1): 93-100.

Cortemeglia, C., Beitinger, T.L., Kennedy, J.H., and Walters, T. 2008. Field confirmation of laboratory-determined lower temperature tolerance of transgenic and wildtype zebra danios, *Danio rerio*. Am. Midl. Nat. 160(2): 477-479.

Coumans, J.V.F., Gau, D., Polijak, A., Wasinger, V., Roy, P., and Moens, P.R. 2014. Green fluorescent protein expression triggers proteome changes in breast cancer cells. Exp. Cell Res. 320: 33-45.

Devgan, V., Rao, M.R.S., and Seshagiri, P.B. 2004. Impact of embryonic expression of enhanced green fluorescent protein on early mouse development. Biochem. Biophys. Res. Comm. 313(4): 1030-1036.

Devlin, R.H., Sundström, L.F., and Leggatt, R.A. 2015. Assessing ecological and evolutionary consequences of growth-accelerated genetically engineered fishes. BioScience 65(7): 685-700.

DFO, 2006. Proceedings of the Expert Panel Meeting on the Potential Risks Associated with Horizontal Gene Transfer from Novel Aquatic Organisms. DFO Can. Sci. Advis. Sec. Proceed. Ser. 2006/036.

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

DFO. 2018. Environmental and Indirect Human Health Risk Assessment of the Glofish® Electric Green® Tetra and the Glofish® Long-Fin Electric Green® Tetra (*Gymnocorymbus ternetzi*): A Transgenic Ornamental Fish. DFO Can. Sci. Advis. Sec. Sci. Advis. Rep. 2018/027.

DFO. 2019. Environmental and Indirect Human Health Risk Assessment of the GloFish® Tetras (*Gymnocorymbus ternetzi*): Five Lines of Transgenic Ornamental Fish. DFO Can. Sci. Advis. Sec. Sci. Advis. Rep. 2019/002.

DFO. 2020. Environmental and Indirect Human Health Risk Assessment of the GloFish® Sunburst Orange® Danio (*Danio rerio*): A Transgenic Ornamental Fish. DFO Can. Sci. Advis. Sec. Sci. Advis. Rep. 2020/015.

Einum, S., and Fleming, I.A. 1997. Genetic divergence and interactions in the wild among native, farmed and hybrid Atlantic salmon. J. Fish Biol. 50: 634-651.

Essner, J.J. 2003. Temperature Sensitivity of Fluorescent Transgenic Zebrafish. Discovery Genomics, Inc., Minneapolis, MN.

Evans, B.B., and Lester, R.J.G. 2001. Parasites of ornamental fish imported into Australia. Bull. Eur. Assoc. Fish Pathol. 21(2): 51-55.

Fang, F. 2003. Phylogenetic analysis of the asian cyprinid genus *Danio* (Teleostei, Cyprinidae). Copeia. 4: 714-728.

Gerlai, R. 2013. Antipredatory behavior of Zebrafish: Adaptive function and a tool for translational research. Evol. Psychol. 11(3): 591-605.

Guo, J.K., Cheng, E.C., Wang, L., Swenson, E.S., Ardito, T.A., Kashgarian, M., Cantley, L.G., and Krause, D.S. 2007. The commonly used beta-actin-GFP transgenic mouse strain develops a distinct type of glomerulosclerosis. Trans. Res. 16(6): 829-834.

Hagemann, S., and Hammer, S.E. 2006. The implications of DNA transposons in the evolution of *P* elements in zebrafish (*Danio rerio*). Genomics 88(5): 572-579.

Hallare, A.V., Schirling, M., Luckenbach, T., Köhler, H.R., and Triebskorn, R. 2005. Combined effects of temperature and cadmium on developmental parameters and biomarker responses in zebrafish (*Danio rerio*) embryos. J. Therm. Biol. 30(1): 7-17.

Hanwell, D., Hutchinson, S.A., Collymore, C., Bruce, A.E., Louis, R., Ghalami, A., Allison, W.T., Ekker, M., Eames, B.F., Childs, S., Kurrasch, D.M., Gerlai, R., Thiele, T., Scott, I., Ciruna, B., Dowling, J.J., McFarlane, S., Huang, P., Wen, X.Y., Akimenko, M.A., Waskiewicz, A.J., Drapeau, P., Babiuk, L.A., Dragon, D., Smida, A., Buret, A., O'Grady, E., Wilson, J., Sowden-Plunkett, L., and Tropepe, V. 2016. Restrictions on the importation of Zebrafish into Canada associated with Spring Viremia of Carp Virus. Zebrafish 13: S153-S163.

Hayden, B., Pulcini, D., Kelly-Quinn, M., O'grady, M., Caffrey, J., McGrath, A., and Mariani, S. 2010. Hybridisation between two cyprinid fishes in a novel habitat: genetics, morphology and life-history traits. BMC Evol. Biol. 10: 169.

Hill, J.E., Kapuscinski, A.R., and Pavlowich, T. 2011. Fluorescent transgenic zebra danio more vulnerable to predators than wild-type fish. Trans. Am. Fish. Soc. 140(4): 1001-1005.

Hongslo, T., and Jansson, E. 2009. Health survey of aquarium fish in Swedish pet-shops. Bull. Eur. Assoc. Fish Pathol. 29(5): 163-174.

Howard, R.D., Rohrer, K., Liu, Y., and Muir, W.M. 2015. Mate competition and evolutionary outcomes in genetically modified zebrafish (*Danio rerio*). Evolution 69(5): 1143-1157.

Huang, W.Y., Aramburu, J., Douglas, P.S., and Izumo, S. 2000. Transgenic expression of green fluorescence protein can cause dilated cardiomyopathy. Nat. Med. 6(5): 482-483.

Jha, P. 2010. Comparative study of aggressive behaviour in transgenic and wildtype zebrafish *Danio rerio* (Hamilton) and the flying barb *Esomus danricus* (Hamilton), and their susceptibility to predation by the snakehead *Channa striatus* (Bloch). Ital. J. Zool. 77(1): 102-109.

Jobling, M. 1981. Temperature tolerance and the final preferendum--rapid methods for the assessment of optimum growth temperatures. J. Fish Biol. 19: 439-455.

Kapuscinski, A.R., Hayes, K.R., Li, S., and Dana, G. 2007. Environmental Risk Assessment of Genetically Modified Organisms. Methedologies for Transgenic Fish Vol. 3. CABI publishing.

Kerr, S.J., Brousseau, C.S., and Muschett, M. 2005. Invasive aquatic species in Ontario. Fisheries 30(7): 21-30.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., and Schilling, T.F. 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203: 253-310.

Kirschbaum, F., Nguyen, L., Baumgartner, S., Chi, H.W.L., Wolfart, R., Elarbani, K., Eppenstein, H., Korniienko, Y., Guido-Böhm, L., Mamonekene, V., Vater, M., and Tiedemann, R. 2016. Intragenus (*Campylomormyrus*) and intergenus hybrids in mormyrid fish: Physiological and histological investigations of the electric organ ontogeny. J. Physiol. (Paris) 110: 281-301.

Koelsch, K.A., Wang, Y., Maier-Moore, J.S., Sawalha, A.H., and Wren, J.D. 2013. GFP affects human T cell activation and cytokine production following *in vitro* stimulation. PLoS ONE 8(4): e50068.

Lawrence, C. 2007. The husbandry of zebrafish (*Danio rerio*): a review. Aquaculture 269(1-4): 1-20.

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

Leggatt, R. Johnson, N., and McGowan, C. 2018b. Environmental Risk Assessment of the Glofish® Electric Green® Tetra and the Glofish® Long-Fin Electric Green® Tetra: Transgenic Ornamental Fish, Imported to Canada, For Sale in the Pet Trade. DFO Can. Sci. Advis. Sec. Res. Doc. 2018/049. xii + 54 p.

Lessman, C.A. 2011. The Developing Zebrafish (*Danio rerio*): A vertebrate model for high-throughput screening of chemical libraries. Birth Defects Res. C 93(3): 268-280.

Levin, S.A. 2009. Princeton Guide to Ecology. Princeton University Press, Princeton, NJ. 848 p.

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

Lincoln, R.G., Boxshall, G., and Clark, P. 1988. A Dictionary of Ecology, Evolution and Systematics. 2nd ed. Cambridge University Press.

Little, A.G., Kunisue, T., Kannan, K., and Seebacher, F. 2013. Thyroid hormone actions are temperature-specific and regulate thermal acclimation in zebrafish (*Danio rerio*). BMC Biol. 11: 26.

López-Olmeda, J.F., and Sánchez-Vázquez, F.J. 2011. Thermal biology of zebrafish (*Danio rerio*). J. Therm. Biol. 36(2): 91-104.

Magnuson, J.J., Crowder, L.B., and Medvick, P.A. 1979. Temperature as an ecological resource. Amer. Zool. 19(1): 331-343.

Mair, G.C., Nam, Y.K., and Solar, I.I. 2007. Risk management: reducing risk through confinement of transgenic fish. *In* Environmental Risk Assessment of Genetically Modified Organisms. Methodologies for Transgenic Fish. *Edited by* A.R. Kapuscinski and K.R. Hayes and S. Li and G. Dana. CABI Publishing. pp. 209-238.

Mak, G.W.-Y., Wong, C.-H., and Tsui, S.K.-W. 2007. Green fluorescent protein induces the secretion of inflammatory cytokine interleukin-6 in muscle cells. Anal. Biochem. 362: 296-298.

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

Moon, D.C., Moon, J., and Keagy, A. 2010. Direct and indirect interactions. Nat. Edu. Know. 3(10): 50.

Noble-Brzezinski, S., Leggatt, R., Johnson, N., and McGowan, C. 2021. Environmental risk assessment of the Glofish® Sunburst Orange® Danio: a transgenic ornamental fish, imported to Canada, for sale in the pet trade. DFO Can. Sci. Advis. Sec. Res. Doc. 2021/013. In Press.

Řehulka, J., Kaustová, J., and Řehulková, E. 2006. Causal agents of mycobacterial diseases in freshwater ornamental fish and their importance for human health in the Czech Republic. Acta Vet. Brno 75: 251-258.

Ribas, L., and Piferrer, F. 2014. The zebrafish (*Danio rerio*) as a model organism, with emphasis on applications for finfish aquaculture research. Rev. Aquacult. 6(4): 209-240.

Rixon, C.A.M., Duggan, I.C., Bergeron, N.M.N., Ricciardi, A., and Macisaac, H.J. 2005. Invasion risks posed by the aquarium trade and live fish markets on the Laurentian Great Lakes. Biodivers. Conserv. 14(6): 1365-1381.

Rose, S., Hill, R., Bermudez, L.E., and Miller-Morgan, T. 2013. Imported ornamental fish are colonized with antibiotic-resistant bacteria. J. Fish Dis. 36(6): 533-542.

Schaefer, J., and Ryan, A. 2006. Developmental plasticity in the thermal tolerance of zebrafish *Danio rerio*. J. Fish Biol. 69(3): 722-734.

Schirone, R.C., and Gross, L. 1968. Effect of temperature on early embryological development of the zebra fish, *Brachydanio rerio*. J. Exp. Zool. 169(1): 43-52.

Sfakianakis, D.G., Leris, I., Mylonas, C.C., and Kentouri, M. 2012. Temperature during early life determines sex in zebrafish, *Danio rerio* (Hamilton, 1822). J Biol Res-Thessalon 17: 68-73.

Spence, R., Gerlach, G., Lawrence, C., and Smith, C. 2008. The behaviour and ecology of the zebrafish, *Danio rerio*. Biol. Rev. Camb. Philos. Soc. 83(1): 13-34.

Stewart, C.N. 2006. Go with the glow: fluorescent proteins to light transgenic organisms. Trends Biotechnol. 24(4): 155-162.

Strecker, A.L., Campbell, P.M., and Olden, J.D. 2011. The aquarium trade as an invasion pathway in the Pacific Northwest. Fisheries 36(2): 74-85.

Trumpikas, J., Shuter, B.J., Minns, C.K., and Cyr, H. 2015. Characterizing patterns of nearshore water temperature variation in the North American Great Lakes and assessing sensitivities to climate change. Great Lakes Res. 41: 53-64.

Tuckett, Q.M., Ritch, J.L., Lawson, K.M., and Hill, J.E. 2017. Landscape-scale survey of non-native fishes near ornamental aquaculture facilities in Florida, USA. Biol. Invasions 19: 223-237.

Uh, M., Khattra, J., and Devlin, R.H. 2006. Transgene constructs in coho salmon (*Oncorhynchus kisutch*) are repeated in a head-to-tail fashion and can be integrated adjacent to horizontally-transmitted parasite DNA. Transgen. Res. 15(6): 711-727.

Whittington, R.J., and Chong, R. 2007. Global trade in ornamental fish from an Australian perspective: The case for revised import risk analysis and management strategies. Prev. Vet. Med. 81(1-3): 92-116.

Wu, F., Rijn, E.V., Van Schie, B.G.C., Keymer, J.E., and Dekker, C. 2015. Multi-color imaging of the bacterial nucleoid and division proteins with blue, orange, and near-infrared fluorescent proteins. Front. Microbiol. 6: 607.