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Environmental Risk Assessment of the GloFish® Sunburst Orange® Danio: a Transgenic Ornamental Fish, imported to Canada, For Sale in the Pet Trade

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Foreword

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

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LIST OF ACRONYMS

bp: Base pair

CEPA: *Canadian Environmental Protection Act, 1999*

CTmin: Critical thermal minima

CLmin: Chronic lethal minimum temperature

DFO: Fisheries and Oceans Canada

DNA: Deoxyribonucleic acid

dpf: Days post fertilization

ECCC: Environment and Climate Change Canada

eGFP: Enhanced green fluorescent protein

GE: Genetically engineered

GxE: Genotype by environment interaction

HC: Health Canada

HGT: Horizontal gene transfer

hpf: Hours post fertilization

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

MOU: Memorandum of Understanding

mRNA: Messenger RNA

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

RFP: Red fluorescent protein

RNA: Ribonucleic acid

s.e.m.: Standard error of the mean

GLOSSARY

Abiotic factors: physical, chemical and other non-living environmental factors

Aquarium trade: the commercial industry that lawfully trades in aquatic live organisms with its customers

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

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

Competition: the simultaneous demand by two or more organisms (competitors) or species for an essential common resource that is actually or potentially in limited supply (exploitative competition), or the detrimental interaction between two or more organisms or species seeking a common resource that is not limiting (interference competition)

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

Established: growing and reproducing successfully in a given area as a self-sustaining population

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

Genotype × Environment (GxE) interactions: 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

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

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

Hazard: potential to cause a harmful effect

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

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

Likelihood: the degree of belief warranted by evidence; the degree to which a proposition, model or hypothesis fits the available data

Measurement endpoint: a measurable characteristic of the selected assessment endpoint

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

Persist: survives to the reproductive stage

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

Spread: movement of a successfully established population beyond its distribution limit

Survival: occurs when the immediate physiological requirements of the organism are met

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

ABSTRACT

Pursuant to the *Canadian Environmental Protection Act* (CEPA), a notification under the *New Substances Notification Regulations (Organisms)* (NSNR(O)) was submitted by GloFish LLC to Environment and Climate Change Canada (ECCC) for the import of a genetically engineered *Danio rerio* called the GloFish® Sunburst Orange® Danio (YZ2018) for commercial sales in Canada. The environmental risk assessment analyzed potential hazards, likelihood of exposure and associated uncertainties, to reach a conclusion on risk. The environmental exposure assessment concluded that the occurrence of YZ2018 in the Canadian environment, outside of aquaria, is expected to be rare, isolated, and ephemeral due to its inability to survive typical low winter temperatures in Canada's freshwater environments. Consequently, the likelihood of exposure of YZ2018 to the Canadian environment is ranked low. Uncertainty associated with the exposure assessment is low, given the available data for temperature tolerance of the notified line and relevant comparators, and lack of establishment of non-transgenic *Danio rerio* in North America despite a long history of use. The environmental hazard assessment concluded that potential hazards linked with environmental toxicity, trophic interactions, hybridization, disease, biodiversity, biogeochemical cycling, and habitat are negligible. There is low hazard (i.e., no anticipated harmful effects) related with horizontal gene transfer. Uncertainty associated with the environmental hazard ratings range from low to moderate due to data limitations for the notified and surrogate organisms, and some reliance on expert opinion and anecdotal evidence. There is low risk of adverse environmental effects at the exposure levels predicted for the Canadian environment from the use of YZ2018 as an ornamental aquarium fish or other potential uses.

EXECUTIVE SUMMARY

BACKGROUND

On March 4, 2019, GloFish LLC submitted a regulatory package to Environment and Climate Change Canada (ECCC) under the *New Substances Notification Regulations (Organisms)* [NSNR(O)] of the *Canadian Environmental Protection Act, 1999* (CEPA) for import of the GloFish® Sunburst Orange® Danio (YZ2018); a fluorescent orange, genetically engineered Zebrafish (*Danio rerio*), for use as an ornamental fish in home aquaria.

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

Under a memorandum of understanding with ECCC and 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 ECCC receives scientific advice from DFO and retains ultimate responsibility for regulatory decision making on the use of the notified fish.

It is in this context that DFO conducted an environmental risk assessment of the notified organism (YZ2018) under its 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 discussed in the context of the final conclusion on risk.

THE NOTIFIED ORGANISM

YZ2018 is a line of genetically engineered diploid, hemizygous or homozygous, orange fluorescent transgenic Zebrafish, derived from the Golden Zebrafish (*Danio rerio*). Expression of the transgenes results in orange colouration of the fish under natural light. The protein is expressed in the skin, musculature, fins, eyes, and likely other organs of the organism. All YZ2018 individuals are descendants of a single G0 founding individual with the tandem transgene construct microinjected at the single cell stage. Confirmation of multiple transgene copies inserted at a single site was confirmed at the F1 generation.

YZ2018 has been marketed in the USA ornamental aquarium trade since 2006 as the GloFish® Sunburst Orange® Danio. The targeted phenotypic change is the display of fluorescent orange colouration; providing a novel colour morph 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 is conducted under GloFish LLC's proposed use scenario: the importation of YZ2018 into Canada for distribution to aquarium retail stores across the country and for purchase by Canadian consumers for home aquaria.

Exposure

The intended housing for YZ2018 is indoor, static, physically contained aquaria at wholesalers, retail stores, and in consumer homes. Based on historical records of aquarium fish in natural ecosystems in Canada and worldwide, it is highly likely that YZ2018 will be introduced purposefully or accidentally into natural freshwater ecosystems in Canada. Though it is expected that release events will be sporadic and of very low magnitude (e.g., five or less fish per release), the potential for larger magnitude releases cannot be ignored.

As a tropical species, the Zebrafish is not expected to persist in temperate regions, where water temperatures are below optimal for its survival and reproduction. While the vast majority of lakes in Canada will reach temperatures of 4°C or less over the winter, *D. rerio* will stop feeding at temperatures below 8°C, and cannot survive at temperatures below 5°C; making it unlikely to survive the Canadian winter. Relative to non-transgenic Zebrafish, YZ2018 demonstrated diminished cold temperature tolerance and diminished reproductive success. Therefore, the occurrence of YZ2018 in the Canadian environment is expected to be rare, isolated and ephemeral, and likely in low numbers. The likelihood of exposure of YZ2018 to the Canadian environment is ranked low. The uncertainty associated with this estimate is low, given the quality of data available for YZ2018 and valid surrogate organisms (temperature tolerance) and data available on parameters of the receiving environment in Canada.

Hazard

The potential for YZ2018 to cause a hazard to Canadian environments was examined in the context of environmental toxicity (i.e., potential to be poisonous), horizontal gene transfer, interactions with other organisms including hybridization, disease vectors, and impacts to biogeochemical cycling, habitat, and biodiversity. Non-transgenic *D. rerio* is a small, non-aggressive fish, and is expected to have limited activity due to low water temperatures 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 YZ2018 above that of non-transgenic *D. rerio*, and no evidence that potential gene transfer will result in harm to the Canadian environment.

Rankings for specific hazards examined ranged from negligible to low. Uncertainty ranged from low to moderate, due to limited data specific to YZ2018, limited direct data on comparator species, variable data from surrogate models (Zebrafish that ubiquitously express red fluorescent protein), and the reliance on expert opinion for the assessment of some hazards. Outside of its intended use as an ornamental fish in static aquaria, YZ2018 is not expected to pose unique hazards beyond those of the intended use.

CONCLUSIONS ON RISK

The overall risk of YZ2018 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 direct data on the notified organism, no evidence was identified to suggest YZ2018, under the proposed or other potential uses, could cause harm as a result of exposure to the Canadian environment.

PART 1: PROBLEM FORMULATION

1.1. PURPOSE OF PART 1

Part 1 of this document elaborates the problem formulation for the environmental risk assessment that will be conducted under the *Canadian Environmental Protection Act* (CEPA), with respect to the GloFish® Sunburst Orange® Danio (YZ2018), a genetically engineered Zebrafish (*Danio rerio*) 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 also identifies protection goals and assessment endpoints that are aligned with the legislative protection goals in CEPA, and provides a characterisation of YZ2018, the comparator species, and the potential receiving environment in Canada.

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

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

A detailed overview of the legal context for the risk assessment process, the risk assessment framework, and regulatory decision-making process under CEPA is provided in Leggatt et al. (2018a).

Briefly, the risk assessment is conducted within the legislative context of CEPA and the information requirements of the *New Substances Notification Regulations (Organisms)* 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 natural environment in Canada. The hazard assessment is focused on the potential for the organism to impact: (1) potential prey, predators, and competitors of the organism; (2) biological diversity; and, (3) habitat. The level of uncertainty for both exposure and hazard determinations is evaluated and communicated in terms of impact to the final risk assessment. DFO provides science advice in the form of peer-reviewed risk assessments to Environment and Climate Change Canada for regulatory decision-making under CEPA, based on risk to the environment and the uncertainty associated with the conclusion.

1.3. CHARACTERISATION OF YZ2018

1.3.1. Molecular Characterization

YZ2018 is a genetically engineered 'Golden Zebrafish' low pigment morph of the striped Zebrafish possessing multiple copies of transgenic insert containing fish-origin promotors that drive the expression of exogenous proteins. The insert results in a ubiquitous yellow/orange colouration of the organism under ambient light, including sunlight. The purpose of this modification is to create a new orange colour phenotype of *D. rerio* for the ornamental aquarium trade (Figure 1.1).

Figure 1.1.: Some variants of Danio rerio available in the ornamental pet trade worldwide (a, b), and the notified transgenic variant currently only available in the United States (c). Non-transgenic Zebrafish (a), Golden Zebrafish (b), GloFish® Fluorescent Danio YZ2018 Sunburst Orange® (Images obtained from (a) www.petsmart.ca; (b) DFO; (c) www.glofish.com).

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

1.3.1.1 Production of the Notified Organism

The transgene expression cassette described above was injected into newly fertilized eggs of the non-transgenic Golden Zebrafish (*D. rerio*). A single founding (i.e., one G0) individual was identified by phenotypic yellow fluorescence, and separately crossed to three non-transgenic Golden Zebrafish to produce several F1 groups that were visually screened for fluorescence and non-transgenic fish removed. Transgenic F1 male individuals were confirmed to have Mendelian (50:50) segregation of the transgene at a single locus, and were used to calculate transgene copy number. These F1 fish were crossed to an unspecified number of non-transgenic Golden Zebrafish to produce F2 hemizygous fish, a sample of which was used to confirm a single genetic lineage by Southern blot hybridization. Though all fish had identical banding patterns, indicating identical transgene copy insertion sites, the small sample size prevents a definitive conclusion regarding the presence of alternate genotypes within the F2 population.

1.3.1.2. Characterization of the Transgene Integrant

The sequence of the gene construct as it is inserted into YZ2018 has not been determined, and the specific location of the insert within the organism's genome has not been presented.

Consequently, any potential rearrangements to the construct or any potential effects it may have on the transcription of genes close to the site of insertion are unknown.

YZ2018 may include individuals that are hemizygous (i.e., have a single copy at a locus) or homozygous (i.e., have two copies at a single locus) for the transgenic sequence. According to the notifier, hemizygous and homozygous individuals are not distinguishable phenotypically.

1.3.1.3. Inheritance and Stability of the Transgene

The specific insert location of the transgene has not been determined, and consequently, it is unknown whether the transgene is inserted into a stably expressed genome location, or in an area prone to silencing.

Several F1 individuals used to develop the YZ2018 line were confirmed to have expected Mendelian segregation of the transgene as indicated by presence or absence of yellow fluorescence as a phenotype of the offspring. This indicated that the inheritance of the transgene was stable in this generation of fish, though phenotype and genotype were not compared to assess the presence of silenced transgenes in non-fluorescent fish. As well, inheritance and stability have not been examined in subsequent generations. Should transgene expression be silenced in an individual, this individual would not display phenotypic yellow colouration and would, consequently, be removed from the YZ2018 population (the frequency with which this would occur is unknown). YZ2018 broodstock are consciously chosen, and positive selective pressure for bright coloured broodstock would presumably prevent individuals with partially silenced transgenes from propagating to the next generation. According to the notifier, non-fluorescent siblings are culled from the population and are not sold as Golden Zebrafish.

Yorktown Technologies and GloFish LLC have maintained the breeding line for over five generations and the line has been commercially produced for over five years with no evidence of gene silencing reported. In addition, GloFish LLC states, “5-D Tropical Inc., one of the largest tropical ornamental fish producers in the world, has produced YZ2018 (and all other GloFish® lines sold commercially) for several years and has found the phenotype to be durable and stable across generations.” However, no formal evaluation is available.

1.3.1.4. Methods to Detect YZ2018

YZ2018 individuals are easily distinguished from golden variety Zebrafish by their phenotypic uniform bright fluorescent yellow/orange colouration under ambient light, including sunlight. There are currently no known non-transgenic Zebrafish species with the ability to fluoresce under ambient light or sunlight, making YZ2018 individuals phenotypically distinguishable from other non-transgenic Zebrafish species. In addition, YZ2018 fish can be distinguished genetically by PCR amplification of the transgene insert.

1.3.2. Phenotypic Characterization

Golden Zebrafish used in YZ2018 production are a naturally occurring, stripe free colour variant of the pigmented wild type Zebrafish (Clark and Ekker 2015, see Figure 1.1). The individual fish used to produce YZ2018 were sourced from an ornamental aquarium fish producer (5-D Tropical) in 2007. All YZ2018 fish are descended from a single G0 individual injected with the notified transgene construct at the single cell stage. This G0 individual was crossed with several non-transgenic Golden Zebrafish to produce several F1 hemizygous groups, of which several individuals were bred with non-transgenic Golden Zebrafish to produce the F2 hemizygous group. Continuation of the YZ2018 line has been through batch breeding in populations that contain a mix of individuals hemizygous and homozygous for the transgene, with wild type

Golden Zebrafish removed from the population as they occur. Broodstock of YZ2018 are maintained separately by two aquarium fish producers in Florida.

1.3.2.1. Targeted Phenotypic Effects of the Modification

The targeted phenotypic effect of the genetic modification is that YZ2018 appears orange under ambient light. The novel colour phenotype is present in muscle, as well as skin, and eyes. GloFish LLC reports that YZ2018 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. Non-targeted Phenotypic Effects of the Modification

Two off-target effects identified by GloFish LLC in YZ2018 are a diminished tolerance to low temperature, and a decrease in competitive reproductive success when competing with non-transgenic Zebrafish. The influence of the genetic modification on any other phenotypes, including survival, fecundity and behaviour, has not been formally examined.

1.3.2.3. Pleiotropic Effects of Fluorescent Protein Transgenes in Zebrafish

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

Zebrafish containing the RFP transgene were observed to be less cold tolerant than non-related wild type, when examined under different acclimation temperatures (Cortemeglia and Beitinger 2005, 2006a), though differences in strain background and rearing conditions (Schaefer and Ryan 2006) prior to experimentation may have impacted relative extreme temperature tolerance. Similarly, Leggatt et al. (2018b) reported Zebrafish transgenic for eGFP driven by the *fli1a* promoter were less cold tolerant than the source non-transgenic strain, however, two other eGFP lines driven by other promoters did not have diminished cold tolerance. This indicates that different transgenic lines may have different responses to extreme environmental stressors. Five of six previously notified GloFish® Tetras containing similar constructs as YZ2018 were also reported to have diminished cold tolerance (DFO 2019).

No effect of fluorescence protein transgenesis was observed on survival of RFP expressing Zebrafish relative to related wild type fish under laboratory conditions (Howard et al. 2015). In a population of eGFP, RFP, eGFP-RFP, and non-transgenic Zebrafish, eGFP fish had lower survival, but there was no effect of RFP or the double transgene on survival (Gong et al. 2003), indicating different transgenes or insert sites may also have different influences on survival. The influence of fluorescent transgenes on foraging behaviour has not been reported in any species.

Reports describing the effects of RFP transgenesis on vulnerability to predation have varied. Cortemeglia and Beitinger (2006b) found that RFP and wild type Zebrafish were equally preyed upon. Hill et al. (2011) found that GloFish® RFP expressing Zebrafish were two times more vulnerable to predation than unrelated wild-caught Zebrafish. In contrast, Jha (2010) found a RFP expressing Zebrafish strain in India was less preyed upon by wild-caught Snakeheads than unrelated wild-caught Zebrafish. Factors influencing the difference in relative vulnerability of RFP expressing Zebrafish to predation are not known, but could include differences in genetic background or rearing history of transgenic and non-transgenic Zebrafish, innate preference or life history of predators used, transgene promoters used or transgene insertion sites, and/or experimental conditions (e.g., presence of shelter for prey species).

The reported influences of RFP and other fluorescent transgenes on reproductive success or preferences in Zebrafish are likewise inconsistent. RFP and non-transgenic Zebrafish had similar age at maturity for females, as well as similar male and female fecundity (Howard et al. 2015). In a population containing equal numbers of muscle-expressing eGFP and non-transgenic Zebrafish eGFP offspring had no reproductive advantage or disadvantage (Gong et al. 2003). In contrast, Owen et al. (2012) found both non-transgenic and RFP expressing Zebrafish females preferred to associate with RFP rather than non-transgenic males, regardless of the proportion of non-transgenic to RFP fish they were raised with. In another study, RFP males had lower mating success than non-transgenic males, as well as lower aggression levels to both males and females (Howard et al. 2015).

Jha (2010) found RFP were more aggressive than wild-caught unrelated Zebrafish. Snekser et al. (2006) found the RFP transgene did not influence social partner preferences for either shoaling or in a potential reproductive context in presumably unrelated populations of RFP and non-transgenic Zebrafish. Howard et al. (2015) examined the fate of the RFP transgene over 15 generations in a serial competitive breeding experiment in 18 populations of GloFish® Zebrafish. In all populations, the frequency of the RFP transgene declined rapidly, and was eliminated in all populations except one, indicating a strong bias against the RFP transgene insert in reproduction. In previously notified GloFish® Tetras, only three of six lines were reported to have decreased reproductive success in competition (DFO 2019). Of these lines, the GloFish® Tetra containing RFP had the most extreme decrease in competitive reproductive success, suggesting the results presented in Howard et al. (2015) may represent the most extreme example of diminished reproductive success from fluorescent protein transgenesis, and this may not be present in all existing GloFish® lines.

Relevant published studies using other fluorescent protein transgenic Zebrafish, and data from other notified GloFish® lines may be used as surrogate data demonstrating potential general effects of fluorescent protein transgenesis on risk-relevant phenotypes of the notified organism. However, use of this data must consider potential differences between the notified organism and surrogate organisms, including transgenes used (both fluorescent protein genes and promoters, and whether transgene type is known), background genetics (e.g., domestic versus wild), effect of potential insertion sites, etc.

1.4. CHARACTERISATION OF COMPARATOR SPECIES

For the purpose of this assessment, the non-transgenic Zebrafish will be used as a comparator for the notified organism. Other common names used for the Zebrafish include *Danio*, Zebra Danio, and Striped Danio. This tropical freshwater fish, native to the subcontinent of India (Lessman 2011), is a popular aquarium fish and is ubiquitous in pet stores across Canada. Its small size (approximately 4 cm fully grown), variable diet, and low cost make it accessible to home aquaria with minimal maintenance and care. Since the Zebrafish is a well used model species for scientific inquiry, much is known of its natural history and the influence of domestication on its phenotype and genotype.

1.4.1. Taxonomic Status

Danio rerio is one of approximately 45 *Danio* species located throughout India, Pakistan, Nepal, Bangladesh, Sri Lanka, and Myanmar, Thailand, Malaysia, Sumatra, and the Yunnan Province in China (Fang 2003). It belongs to the Cyprinidae family, in the order Cypriniformes, which includes carp and minnows (Spence et al. 2008). Believed to have evolved in South Asia around 320 million years ago (Ribas and Piferrer 2014), *D. rerio* was first described by Francis Hamilton in 1822. It was later assigned to the subgenus *Brachydanio* by Weber and de Beaufort (1916), together with the other small *Danio* species; with *Danio* being reserved for the larger species of the genus (Barman 1991). *Danio* and *Brachydanio* were aligned by Barman (1991) primarily because there were no diagnostic characters that separated the two genera (Spence et al. 2008). The first molecular phylogeny of the group proposed that *Danio* was monophyletic with two subclades that were either deep-bodied or slender-bodied (Meyer et al. 1993; Meyer et al. 1995). However, a more complete phylogeny based on morphological analysis proposed that *Danio* was paraphyletic (Fang 2003). The deep-bodied clade has now been assigned to its own distinct genera of *Devario* (currently with 35 valid species) and *Danio* is reserved for the slender-bodied species (Fang 2003). Many different strains of *D. rerio* have been created for laboratory and aquarium purposes (Howe et al. 2013).

1.4.2. Distribution

The distribution of *D. rerio* includes India, Pakistan, Nepal, Bangladesh, Sri Lanka, Myanmar, Thailand, Malaysia, Sumatra, and the Yunnan Province in China (Barman 1991, Fang 2003). The natural habitat of the Zebrafish is centred on tropical areas subjected to monsoon climate fluctuations, a range that includes the Ganges and Brahmaputra river basins in north-eastern India, Bangladesh and Nepal, the Krishna watersheds, and river basins draining into the Arabian Sea (Barman 1991; Engeszer et al. 2007; Spence et al. 2008; Ribas and Piferrer 2014). Zebrafish may also be widely distributed over the Indian subcontinent but are simply overlooked in surveys on account of their small size and lack of value as a food fish (Spence et al. 2008). The city of Khulna in Bangladesh, probably represents the southern limit of their range (Spence et al. 2006).

1.4.3. Habitat

Zebrafish typically inhabit stagnant or standing ponds, shallow ditches, slow moving streams or rivers, often connected to rice paddies though not in the fields themselves (McClure et al. 2006; Spence et al. 2006; Engeszer et al. 2007; Spence et al. 2008). Their association with rice paddies is postulated to be due to fertilizers that contribute to the growth of zooplankton, a major source of food for Zebrafish (Spence et al. 2007; Ribas and Piferrer 2014). These environments are likely to be free of predatory fish, especially in aquatic areas resulting from monsoon fluctuations. Along the Ganges and Brahmaputra drainages, Zebrafish are present in shallow, silt-bottomed waters that are generally clear (visibility to 30 cm), often in open, and unshaded locations with aquatic vegetation on the margins (McClure et al. 2006; Spence et al. 2006; Engeszer et al. 2007). They are known to be highly adaptable to different conditions and habitats, displaying strong behavioural plasticity to different environments (Suriyampola et al. 2016; Ribas and Piferrer 2014).

1.4.4. Physiological Tolerances

1.4.4.1. Oxygen

Zebrafish have a higher metabolic rate than larger fish and consequently consume more oxygen per unit weight (Lawrence 2007). The dissolved oxygen requirements of wild Zebrafish have not been determined (Lawrence 2007). Studies simulating environmental hypoxia in culture show that the ideal dissolved oxygen content is 7.5 mg/L with tolerated levels (i.e., no observable effects to the fish, at least in the short term) that range from 5.5 – 9 mg/L (Cortemeglia and Beitinger 2006a; Lawrence 2007; Lawrence et al. 2012; Ribas and Piferrer 2014). Larvae tolerate oxygen levels as low as 3.3 mg/L without developmental defects (Strecker et al. 2011b).

1.4.4.2. Temperature

Zebrafish are considered to be eurythermic, tolerating 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). Laboratory studies indicate that temperate climates below 24°C may reduce breeding incidences and induce developmental defects at various embryonic and larval stages (Schirone and Gross 1968; Barrionuevo and Burggren 1999; Hallare et al. 2005), and reproduction in extreme temperatures has been reported to skew the sex ratio in laboratory strains (Sfakianakis et al. 2012). Leggatt et al. (2018b) observed that Golden Zebrafish reduced their activity and feeding at 16°C, stopped feeding at 8°C, and stopped all activity at 7°C. Field and microcosm studies indicate that Zebrafish would not survive in waters at temperatures less than or equal to 5°C (Cortemeglia et al. 2008; Ribas and Piferrer 2014). Leggatt et al. (2018b) also reported non-transgenic and eGFP-transgenic research strains of Zebrafish could not withstand long-term rearing (80-100% mortality within one week) at 6°C.

1.4.4.3. pH

Zebrafish can tolerate a wide range of pH levels, but a range between 7 and 8.2 will ensure successful rearing and breeding of Zebrafish in a laboratory setting (Brand et al. 2002; Cortemeglia and Beitinger 2006a). An average pH of approximately 8.0 is reported in natural habitats in Bangladesh and India (McClure et al. 2006; Spence et al. 2006; Lawrence 2007), although Zebrafish have also been reported in pH ranges from 5.9 to 9.8 (Engeszer et al. 2007; Arunachalam et al. 2013; Parichy 2015), demonstrating that Zebrafish can withstand a wide range pH in their natural habitat.

1.4.4.4. Salinity

Zebrafish is a freshwater species, but can tolerate brackish water (Lawrence 2007). This adaptation is likely due to the variability of their natural habitat; underlying geology in combination with seasonal rainfall fluctuations can dramatically affect salinity variability in the slow moving waters that Zebrafish prefer (Lawrence 2007). While Spence et al. (2006) recorded salinities in Bangladesh that ranged between 0 and 0.8 ppt, it has been shown that Zebrafish embryos raised in salinities as high as 2 ppt showed similar survival and hatching rates as controls held at 0.3 ppt (Sawant et al. 2001).

1.4.5. Morphology, Life-history and Growth

The Zebrafish body shape is fusiform and laterally compressed, with a terminal oblique mouth directed upwards (Spence et al. 2008). Other diagnostic features for the species include an incomplete lateral line extending to the pelvic fin base as well as two pairs of barbels and five to seven longitudinal stripes extending from behind the operculum into the caudal fin (Barman 1991). These stripes occur at different development stages with two stripes first forming centrally and then subsequent stripes being added sequentially above and below (McClure 1999). The anal fin is similarly striped, while the dorsal fin has a dark blue upper edge, bordered with white (Spence et al. 2008). The Golden Danio strain is similar in morphology to non-transgenic Zebrafish, but lacks blue/black pigmentation, so that its primary colouration is gold with white stripes (Laale 1977; Yossa et al. 2013).

Males and females tend to have slightly different colouration with the males displaying gold and blue stripes whereas females have a whitish belly with silver and blue stripes (Parichy 2006; Ribas and Piferrer 2014). While the sex of juveniles cannot be reliably distinguished, mature, gravid females are normally bigger with a more rounded belly versus the smaller more elongated males (Parichy 2006; Ribas and Piferrer 2014). The males also tend to have larger anal fins while females display a small genital papilla in front of the anal fin origin (Laale 1977; Yossa et al. 2013). Breeding tubercles present on the pectoral fins is another distinguishing feature of the male (McMillan et al. 2015).

Zebrafish in the wild are primarily an annual species, where the main recruitment period occurs from April to August and spawning coincides with the beginning of monsoon season in August (Spence et al. 2007; Ribas and Piferrer 2014). This suggests that a seasonal change is the primary influence on reproductive behaviours, though food availability is likely as strong a driver for reproductive success since gravid females have been found in the wild in January and cultured Zebrafish spawn all year long (Spence et al. 2006; Spence et al. 2007; Clelland and Peng 2009).

Wild Zebrafish are thought to have WZ ♀ / ZZ ♂ sex chromosome determination, although genetic WZ females can develop as functional males (Wilson et al. 2014). In contrast, domesticated Zebrafish seem to follow a polygenic sex determination system that is influenced by multiple genetic factors that may vary from strain to strain, and that can be influenced by environmental factors such as temperature, hypoxia, food availability and population density (Liew et al. 2012; Liew and Orban 2014; Dranow et al. 2016). The environmental mechanisms controlling sex determination in Zebrafish are not known, but poor or harsh conditions can increase the proportion of fish that develop into males (Wilson et al. 2014).

Zebrafish are oviparous and in the laboratory are characterized by asynchronous, batch spawning, usually breeding in small groups (Laale 1977; Lawrence 2007), although Hutter et al. (2010) suggest group spawning was an artifact of high density and low volume aquaria in the laboratory, and Zebrafish in the wild were more likely to spawn in pairs with sexual selection. Reproductive maturity occurs within 75 days when females reach a standard length of approximately 25 mm (Eaton and Farley 1974; Laale 1977). Normally just after dawn, mature females scatter clutches of eggs over substratum with aquatic vegetation in shallow water and provide no accompanying parental care (Sessa et al. 2008; Adatto et al. 2011). Spawning frequency occurs every 4-7 days but greater clutch size has been noted in cultured animals when there are at least 10 days between spawning events (Niimi and LaHam 1973; Laale 1977). Clutch size can vary dramatically ranging from less than 200 to as high as 1800 (Laale 1977; Spence and Smith 2006; Kurtzman et al. 2010). Daylight, and temperature, also appears to affect development and hatching of eggs as both are accelerated in the presence of proper lighting and optimum temperature (Laale 1977). Eggs are demersal and, depending on environmental conditions, usually hatch within 3-7 days post-fertilization (see Figure 1.2 for lifecycle details) (Kimmel et al. 1995; Lawrence 2007). The larval stage lasts about two weeks, transitioning from a metamorphosis stage into juveniles at around four weeks (Ribas and Piferrer 2014). The next two months are characterized by rapid, sometimes exponential, growth (Spence et al. 2007; Chen and Ge 2012, 2013). This corresponds to high temperatures and greater food availability during the monsoon months in the wild (Ribas and Piferrer 2014).

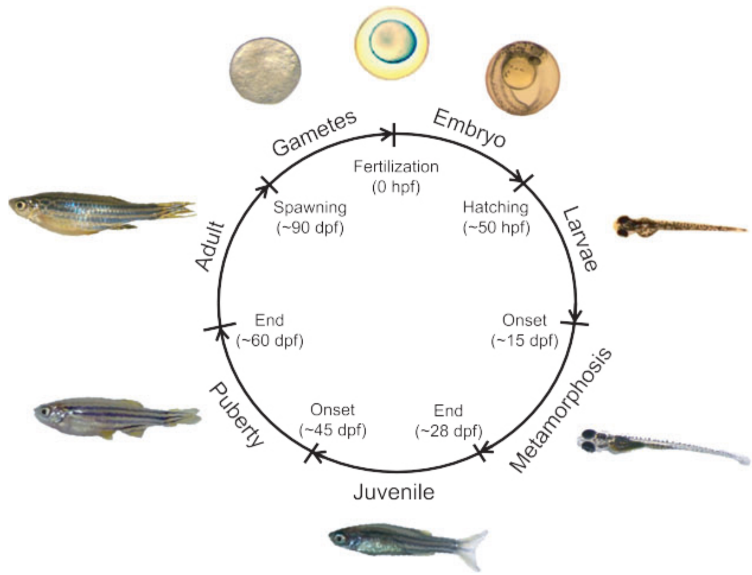

Figure 1.2.: From Ribas and Piferrer (2014). Life cycle of the Zebrafish reared under laboratory conditions. hpf = hours post-fertilization; dpf = days post-fertilization.

Growth rates in laboratory domesticated strains have been reported as higher than wild strains and these differences were maintained when the two strains are raised together, with F1 hybrids possessing an intermediate growth rate between wild and laboratory strains (Eaton and Farley 1974; Robison and Rowland 2005; Spence et al. 2007). This is likely due to inadvertent selection for rapid growth as a result of greater food abundance in a laboratory or cultured setting (Robison and Rowland 2005; Spence et al. 2008). Although domesticated strains have a larger mean body size than wild-type, domestication has no significant bearing on the maximum body length of the species (Wright et al. 2006; Suriyampola et al. 2016). In fact, domestication may impede overall growth as the average adult laboratory Zebrafish measures 22 to 30 mm and wild Zebrafish up to 38 mm (Spence et al. 2007; Spence et al. 2008; Siccardi et al. 2009 Augustine et al. 2011; Yossa et al. 2011). The mean lifespan of domesticated Zebrafish is 42 months with the oldest surviving to 66 months. There are no reported studies detailing the lifespan of Zebrafish in the wild (Spence et al. 2006).

1.4.6. Background Genetics

An analysis of genetic variation within and among wild Zebrafish in Nepal, India and Bangladesh, compared these wild populations to several commonly used lab strains (Whiteley et al. 2011). By examining the genetic variation among nearly 2000 single nucleotide polymorphisms (SNPs) and the cytochrome B mitochondrial gene, wild populations were subdivided into three major mitochondrial DNA classes based in Bangladesh and Southern India; Central Nepal; and Northern India with Eastern and Western regions of Nepal (see also Gratton et al. 2004). The physical and climatic-caused (i.e., monsoons) interconnections between geographically proximate locations appears to have ensured high gene flow, keeping divergence at a minimum within these groups. Any noted substructure was attributed to isolation by distance or an effect of the rivers themselves on dispersal. Detailed studies on potential causes for divergence among groups can be found in Silas (1952), Daniels (2001), Gupta et al. (2003), Karanth (2003), and Whiteley et al. (2011).

The origins of aquarium and laboratory strains are often not known, although Whiteley et al. (2011) found three laboratory strains derived from two aquarium populations likely originated from the Ganges/Brahmaputra region. Population genomics demonstrate that wild populations are genetically distinct from laboratory Zebrafish and in most cases genetic variation is much lower in laboratory strains than wild strains (Spence et al. 2008; Coe et al. 2009; Whiteley et al. 2011). Genetic diversity of domesticated strains may vary greatly depending on their origin and propagation practices.

1.4.7. History of Invasiveness

The ubiquitous presence of Zebrafish in the aquarium trade as well as in research has not translated to a large environmental presence outside of its natural distribution in Southeast Asia. Thought to be restricted mostly by its limited ability to adapt to cold weather environments, most reports of nonindigenous occurrences have been located in Southern U.S. Temporary local establishment was reported in New Mexico, and isolated occurrences have been reported in California, Florida and Connecticut (Nico et al. 2016), as well as in Columbia, though current status is unknown (Welcomme 1998). Whereas individuals from the southern States were thought to originate from aquarium trade fish farms, often located near the site of discovery, the Connecticut occurrence is believed to originate from a possible aquarium release (Nico et al. 2016). Although confirmed to be eradicated in New Mexico, the other releases have not been confirmed as failures (Nico et al. 2016). Tuckett et al. (2017) found both non-transgenic and GloFish® Zebrafish in natural water bodies less 500 m from aquarium farms in Florida, but the species was not found farther than 500 m from farms, indicating a lack of dispersal, establishment and spread post-release. There has been a record of intentional release and establishment of Zebrafish in at least one Japanese reservoir pond in North Okinawa.

1.4.8. Trophic Interactions (Diet, Prey, Competitors, Predators)

In the wild, the adult Zebrafish diet is based mainly on zooplankton and insects (Ribas and Piferrer 2014). Gut content analysis of Zebrafish in its natural habitat indicate the main components of their diet are zooplankton and aquatic insects while phytoplankton, filamentous algae, vascular plant material, water flea eggs and some terrestrial insects and arachnids were also found (Spence et al. 2007; Arunachalam et al. 2013). This suggests that the Zebrafish feeds directly in the entire water column and substratum as well as at the water surface.

Zebrafish are preyed on by various piscine (e.g., Snake Head, Knifefish, Catfish, Garfish), insect (dragonfly larvae), avian (kingfisher, herons, egrets), and water snake predators (Gerlai 2013; Parichy 2015; Suriyampola et al. 2016).

Competitors are not well documented, although Zebrafish have been found shoaling with *Rasbora* spp. in the wild. Domesticated Zebrafish have changed their behaviour from non-transgenic Zebrafish in that they exhibit greater surface feeding and a decreased startle response (Robison and Rowland 2005), both of which could impact foraging and predator avoidance success.

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. (2018a).

Though the many lakes and rivers of Canada vary in their annual temperature profiles, as well as their average maximum and minimum temperatures, most that have been surveyed reach 4°C or below at some point annually, and only a few isolated lakes in Southern Coastal British Columbia have minimum recorded temperatures above this. Consequently, if an introduced fish cannot survive at 4°C or below, its occurrence in the Canadian environment will be seasonal at best. However, it should be noted that mean freshwater surface temperatures in Canada are rising as a result of global climate change and are projected to increase by 1.5 to 4.0°C over the next 50 years (DFO 2013). Consequently, local and long-term temperature patterns should be considered when assessing environmental risk of novel organisms, and may contribute to uncertainty in risk assessments.

1.6. SUMMARY

The Problem Formulation provides a foundation for the risk assessment through identification of environmental protection objectives and the elaboration of scope. It also identifies protection goals and assessment endpoints that are aligned with the legislative protection goals in CEPA, and provides a characterisation of the notified organism, the comparator species, and the potential receiving environment in Canada.

The notified organism, YZ2018, is a genetically engineered Zebrafish (*D. rerio*), possessing multiple copies of an expression cassette that results in ubiquitous yellow/orange colouration of the living organism under ambient light, including sunlight. Other changes to the organism include diminished tolerance for cold temperatures and reduced reproductive success, relative to an appropriate comparator.

The comparator species, non-transgenic Zebrafish, is a tropical freshwater fish native to the subcontinent of India (Lessman 2011), and a popular aquarium fish that is ubiquitous in pet stores across Canada, where it has no history of invasiveness.

Most lakes and rivers in Canada reach a temperature of 4°C or below at some point annually. This will limit the persistence of any tropical freshwater fish that cannot survive at or below this temperature.

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*, 1999 (CEPA) with respect to YZ2018, a genetically engineered golden variant of the striped non-transgenic Zebrafish (*Danio rerio*) notified by GloFish LLC under the *New Substances Notification Regulations (Organisms*) [NSNR(O)].

The environmental risk assessment of YZ2018, also known as the GloFish® Sunburst Orange® Danio, was scientifically peer reviewed and concluded within the 120-day legislative timeframe allowed by the NSNR(O) for notifications under Schedule 5.

2.2. EXPOSURE ASSESSMENT

The exposure assessment for living YZ2018 addresses both their potential (likelihood) to enter the natural environment (release) through deliberate human action, and fate (survival, reproduction, proliferation and spread) once in the natural 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 the organism to the Canadian environment.

Exposure Ranking	Assessment
Negligible	No occurrence; Not observed[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

A lack of empirical data regarding the survival, fitness and ability of YZ2018 to reproduce in the natural environment will contribute uncertainty to the exposure assessment. Uncertainty associated with the environmental fate of an organism may depend on the availability and robustness of scientific information regarding 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 Ranking	Available Information
Negligible	High-quality data on the organism (e.g., sterility, temperature tolerance, fitness). Data on environmental parameters of the receiving environment and at the point of entry. Demonstration of absence of Genotype by Environment Interaction (GxE) effects or complete understanding of GxE effects across relevant environmental conditions. Evidence of low variability.
Low	High-quality data on relatives of the organism or valid surrogate. Data on environmental parameters of the receiving environment. Understanding of potential GxE effects across relevant environmental conditions. Evidence of variability.
Moderate	Limited data on the organism, relatives of the organism or valid surrogate. Limited data on environmental parameters in the receiving environment. Knowledge gaps. Reliance on history of use or experience with populations in other geographical areas with similar or better environmental conditions than in Canada. Limited or no understanding of GxE effects across relevant environmental conditions.
High	Significant knowledge gaps. Significant reliance on expert opinion.

2.2.1. Likelihood of Release

Though the stated purpose of the organism is for sale in the ornamental market, and hobbyists who purchase the product do, for the most part, follow the instructions for disposal that are recommended by the retailer or the company itself, there is still a high likelihood that some YZ2018 will be introduced into the Canadian environment intentionally by humans. Numerous aquarium fish have established themselves in natural waters in North America, and reoccurring, though isolated reports of aquarium fish in Canadian water suggests the practice of releasing aquarium fish into the environment is common and ongoing (Dumont et al. 2002). Indeed Rixon et al. (2005), and Kerr et al. (2005) both cite the aquarium hobby industry as a significant source for introductions of exotic aquatic organisms into the Great Lakes Basin. In the Pacific Northwest, Strecker et al. (2011a) estimated there are 2500 fish released annually into the Puget Sound region by aquarists, and a survey of aquarium fish owners in Ontario reported 2% of unwanted ornamental aquarium fish had been released to the environment (Marson et al. 2009). Once the organism has been sold into the retail market, it is no longer under the direct control of the importer, and there can be no guarantee of appropriate containment and disposal. Consequently, it's appropriate for YZ2018 to be considered under a scenario of full release. The extent to which the organism is further exposed to the environment will, therefore, depend heavily on its ability to survive and reproduce in Canadian lakes and rivers. Though the magnitude of each release event is expected to be very small, the possibility of larger releases from larger purchases or breeding of YZ2018 in the home aquaria cannot be excluded.

2.2.2. Likelihood of Survival

Water temperature is a key abiotic factor that affects both the survival and production of most freshwater fish populations, and is a pervasive determinant of habitat suitability (Magnuson et al. 1979; Jobling 1981). As a tropical species, the Zebrafish is not expected to survive in a temperate region where water temperatures are below optimal for survival. Though 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 (Essner 2003; Cortemeglia and Beitinger 2005, 2006a; Schaefer and Ryan 2006). Since many thermal tolerance studies are performed in a laboratory setting where the abiotic and biotic properties may differ from those found in the wild, studies have been performed in the field to confirm the cold tolerance of Zebrafish (Cortemeglia et al. 2008; Ribas and Piferrer 2014). These studies demonstrated that both non-transgenic and transgenic Zebrafish would not survive in waters at temperatures less than or equal to 5°C and are unlikely to survive the Canadian winter.

Data collected by DFO on the overwintering potential in Canada of transgenic Zebrafish indicates a minimum lethal temperature range of 6.6 to 4.8°C for Golden Zebrafish (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) and cannot survive long-term rearing at 6°C (Leggatt et al. 2018b), although temperature tolerance may vary at different life stages. Other laboratory studies indicate that temperate climates below 24°C may reduce breeding incidences, alter sex ratios, and induce developmental defects at various embryonic and larval stages (Schirone and Gross 1968; Barrionuevo and Burggren 1999; Hallare et al. 2005; Sfakianakis et al. 2012). Thus, the transgenic Zebrafish tested are not expected to persist over winter in Canadian aquatic ecosystems (Leggatt et al. 2018b).

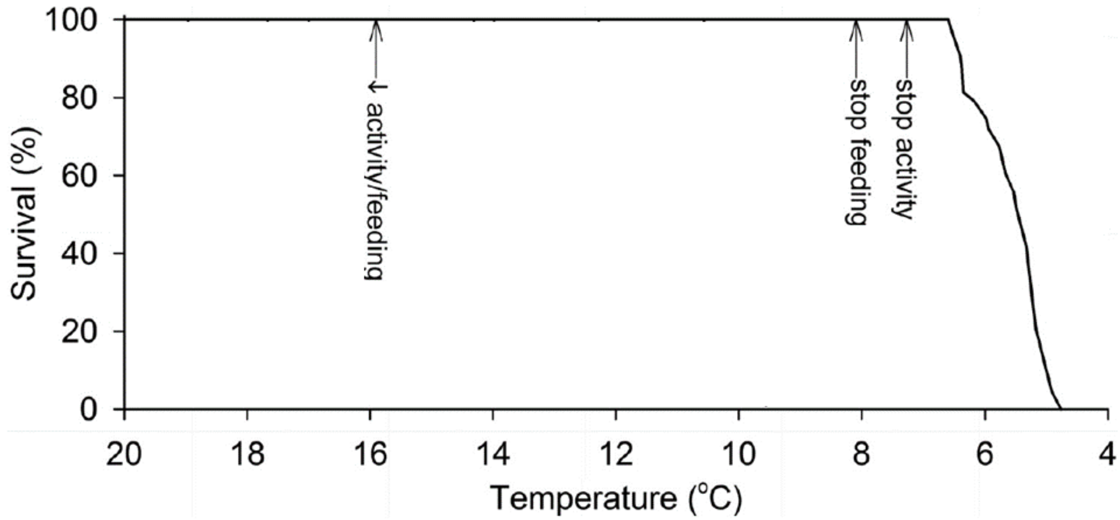

Figure 2.1.: Survival and changes in activity and feeding level in non-transgenic golden variety Zebrafish when temperatures are lowered gradually from 20°C at a rate of 1°C per day (40 Zebrafish, Danio rerio, divided into two tanks). Modified from Leggatt et al. 2018b.

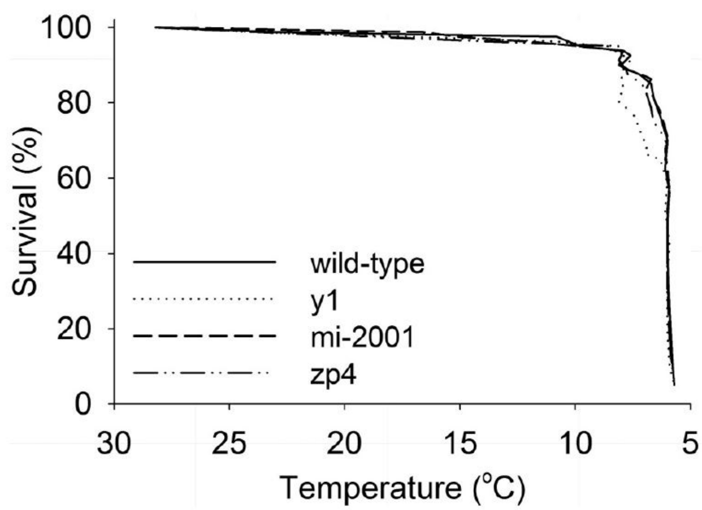

*Figure 2.2.: Survival during gradual cold exposure for three lines of green fluorescent protein (GFP) transgenic (y1, mi2001, zp4) Zebrafish (*Danio rerio*) and their non-transgenic progenitor line (AB). Temperature was dropped from 28 to 12°C at a rate of approximately 1°C per day, then from 12 to 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. 2018b.*

GloFish® Technologies have also conducted research on the lower temperature tolerance of Golden Zebrafish and YZ2018, and provided data from their experiments as part of its notification package. These experiments conclude that the lower temperature tolerance of YZ2018 is significantly higher than non-transgenic Golden Zebrafish when temperatures drop rapidly. 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, and may allow for lower temperatures to be achieved. Regardless of the difference in methodology and results, when these studies are taken together, it is reasonable to conclude that Golden Zebrafish and

YZ2018 cannot survive at temperatures below 4°C, and likely cannot survive long-term at temperatures lower than 6°C.

As discussed in the Problem Formulation (see Characterization of potential receiving environment), there are no lakes in Canada that consistently remain above 6°C throughout the entire course of a year, and most do not remain above 4°C throughout the year. Consequently, while the temperatures needed for YZ2018 to survive are possible for several Canadian lakes during the spring, summer and autumn, it is highly unlikely that YZ2018 can survive the Canadian winter. At best, its occurrence in the environment would be seasonal or ephemeral. In Florida, Tuckett et al. (2017) observed Zebrafish surviving in natural systems, 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.

It should be noted that mean freshwater surface temperatures in Canada are rising as a result of global climate change, and are projected to increase by 1.5 to 4.0°C over the next 50 years (DFO 2013). Consequently, annual minimum temperatures of some Canadian lakes may, in the future, become tolerable to Zebrafish, allowing them to survive the winter, provided they can acquire the necessary resources. If this were to occur, the organism may require reassessment.

2.2.3. Likelihood of Reproduction

Though water temperatures in Canada will limit the occurrence of any YZ2018 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 two months of the year (mid-July to mid-September, (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 be prolific breeders under ideal conditions. In nature, fish larval production occurs from April to August and spawning coincides with the beginning of monsoon season in August (Spence et al. 2007; Ribas and Piferrer 2014). This suggests that a seasonal change is the primary influence on reproductive behaviours. Additionally, food availability is likely as strong a driver for reproductive success since gravid females have been found in the wild (within their natural range) in January, while cultured Zebrafish spawn all year long (Spence et al. 2006; Spence et al. 2007; Clelland and Peng 2009). Zebrafish undergo external fertilization by scattering their eggs, and, thus reproduce successfully in tanks with substrates that protect the eggs from the parents since Zebrafish consume their own embryos and provide no parental care (Axelrod and Vorderwinkler 1976; Mills and Vevers 1989; Hill and Yanong 2002; Spence and Smith 2006). In nature, normally just after dawn, mature females scatter clutches of eggs over substratum with aquatic vegetation in shallow water and provide no accompanying parental care (Sessa et al. 2008; Adatto et al. 2011). Spawning frequency occurs every 4-7 days but greater clutch size has been noted in cultured animals when there are at least 10 days between spawning events (Niimi and LaHam 1973; Laale 1977). Age of sexual maturity for Zebrafish occurs within 75 days when females reach a standard length of approximately 25 mm (Eaton and Farley 1974; Laale 1977). Consequently, any reproduction would be limited to a short window of opportunity during the summer, regardless of its age at the time of introduction. Any YZ2018 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. Though any fertilized eggs that are not eaten as food 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 and would not survive the winter. Though isolated opportunities for reproduction in the Canadian environment could occur, it is unlikely to result in more than a single generation presence in the environment.

Interspecific hybrids have been reported between *D. rerio* and *Danio albolineatus* (synonym *Brachydanio albolineatus*, common name Pearl Danio) (Axelrod and Vorderwinkler 1976). Although hybridization between the two species does occur, the F1 hybrid is sterile. Several genera of the Cyprinidae family are found in Canada, but it is not known if they could inter-mate with Zebrafish, however, the notifier mentions that it seems unlikely given the differences in adaptation. Additionally, data from the notifier suggest that YZ2018 has lower reproductive success than non-transgenic Golden Zebrafish making it even less likely to mate successfully with native Cyprinidae.

2.2.4. Likelihood of Proliferation and Spread

The capacity for YZ2018 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 YZ2018 are expected to occupy areas near the shoreline, based on what is known of non-transgenic 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). Though summer temperatures may be warmer than indicated by recorded data, winter temperatures may be colder, which may further reduce the potential for overwintering of YZ2018. The potential for establishment in isolated thermal pockets (e.g., hot springs, industrial effluent) is expected to be small and confined to very localized areas (see Leggatt et al. 2018a).

2.2.5. Conclusions

Given the above analysis, the occurrence of YZ2018 in the Canadian environment is expected to be rare, isolated and ephemeral, and likely in low numbers. Consequently, the likelihood of exposure of YZ2018 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 available for YZ2018 and valid surrogate organisms (temperature tolerance) and data available on the environmental parameters of the receiving environment in Canada.

The notifying company identifies the sole intended use for the notified organism as an ornamental fish for interior, static home aquaria. However, once purchased by consumers, other unintended uses cannot be discounted. While some unintended uses will lead to the release of YZ2018, 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 YZ2018 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 YZ2018 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 YZ2018 in the natural environment. Criteria for the assessment of uncertainty address potential effects to the environment, which may rely heavily on information and data found in published and peer-reviewed scientific literature. A description of rankings for uncertainty regarding the potential hazards of the organism in the environment is provided in Table 2.4.

Table 2.3.: 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 YZ2018. 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 YZ2018 or valid surrogate. Understanding of GxE effects across relevant environmental conditions. Some variability.
Moderate	Limited data on YZ2018, relatives of YZ2018 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.

Here, the quality of data refers to the data or information available for each parameter being examined, the integration of this information and breadth of experimental conditions examined, sample size, appropriateness of controls, statistical analysis, as well as the experimental design and interpretations of the results. Variability refers to both the range of phenotypic differences among individuals or strains within the same environment as well as the range of physical, chemical, and biological conditions that may be experienced by a GE fish in the receiving environment.

The proposed use of YZ2018 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 YZ2018 to Canadian environments. The majority of potential hazards posed by YZ2018 (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 YZ2018 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).

Fisheries and Oceans Canada's (DFO) Centre for Aquatic Biotechnology Regulatory Research has conducted a significant amount of laboratory research on the fitness and behaviour of GE fish to aid in estimating the fitness of GE fish in the natural environment through use and comparison of results of studies conducted in tanks, semi-natural streams, and mesocosms, so many of the sources of uncertainty are well understood (see Devlin et al. 2015). Though research may not be conducted on the organism per se, it has highlighted several broad principles that may be applicable to the organism and that represent potential sources of uncertainty about the extent to which laboratory data can be depended upon as a reliable indicator of how GE fish would behave in the natural environment. These findings are described below:

- The environment in which fish are reared can significantly affect the phenotypic expression of the transgene (Sundström et al. 2007). The influence of rearing environment limits our ability to extrapolate laboratory data as a reliable indicator of how a GE fish may behave (e.g., compete, survive) in the natural environment unless it can be demonstrated that non-transgenic controls reared in the laboratory environment behave the same way as non-transgenic fish in the natural environment, and it is demonstrated that there are no Genotype by Environment (GxE) interactions between non-transgenic and GE fish, or GxE interactions are well defined. In the absence of such control data, there is uncertainty around the extent to which we can rely upon laboratory data as an accurate indicator of behaviour in the natural environment;
- The phenotypic effects of the transgene can vary significantly with the genetic background of the parent (e.g., non-transgenic vs. domesticated species). For example, the performance of a non-transgenic fish with an inserted growth hormone gene construct may be very different from the performance of a domesticated fish of the same species into which the same construct has been inserted (e.g., Devlin et al. 2001). Consequently, regulators must scrutinize the background genetics of experimental controls when evaluating the scientific validity of experimental data to assess whether the phenotype is durable across multiple genotypes as would be encountered in nature. Experimental data on transgene expression in one species or strain should be interpreted with caution as it may or may not be representative of the expression of the same transgene in a different species or strain; and
- A single transgene may result in several phenotypic expressions, termed pleiotropic effects. For example, some empirical data demonstrates that increased growth due to transgenesis in some fish species may also affect disease resistance (Jhingan et al. 2003). Thus, unless the investigator has specifically directed attention towards an unintended effect, it may go undetected. It should also be noted that different types of modified genes may have vastly different pleiotropic effects. For example, modification of a central fitness trait, such as size or growth rate, is expected to have broad pleiotropic and fitness implications for all traits

related to growth, whereas the pleiotropic implication for more benign traits, such as eye colour or flesh pigmentation, may be limited.

2.3.1. Potential Hazards Through Environmental Toxicity

A detailed assessment of the potential for environmental toxicity in Canada relevant to the introduction of fluorescent transgenic tropical freshwater fish is presented in Leggatt et al. (2018a). 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 protein to the environment is expected to be lower than exposure of the protein to YZ2018; 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 YZ2018. Consequently, the potential hazard to the environment due to environmental toxicity of YZ2018 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.

2.3.2. Potential Hazards Through Horizontal Gene Transfer

A detailed assessment of the potential for horizontal gene transfer (HGT) in Canada relevant to the introduction of tropical freshwater fish is presented in Leggatt et al. (2018a). 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 YZ2018 gut, or through feces, mucus, and other waste sloughed off by the fish into the water. The transgene construct does not contain viral vectors, transposable elements, or other known factors that may increase the potential for DNA uptake/mobility to a new organism. In order for the transgene to be expressed resulting in phenotypic change, it requires co-transfer of regulatory elements, or insertion of the coding region functionally near native regulatory elements. The close proximity of the promoters to the zsYellow1 gene could increase the likelihood of them being co-transferred and expressed, though vertebrate promoters generally have poor activity in prokaryotes.

Genes encoding fluorescence have been introduced to a wide range of organisms with few reports of harmful effects from the introduced transgenes. This suggests that the introduction of the transgene through HGT to a novel host is not expected to result in harmful effects. Though the introduction of a fluorescent transgene to another organism in Canadian environments through HGT cannot be excluded, the absence of 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 expression in an unknown genetic background, results in **moderate** uncertainty.

2.3.3. Potential Hazards Through Interactions with Other Organisms

The trophic interactions of non-transgenic *D. rerio* in its native range are not well documented (see Section 1.4.8), nor is there documentation of trophic interactions of escaped ornamental non-transgenic or YZ2018 *D. rerio* in other areas. Should YZ2018 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.

Zebrafish are omnivorous and feed on zooplankton, phytoplankton, insects and insect larvae, worms and small crustaceans. In an aquarium setting, Zebrafish will feed on live food (e.g., brine shrimp, *Artemia* sp.), as well as commercial ornamental fish flakes and very small fish (e.g., larvae, Westerfield 2000; Avdesh et al. 2012). As such they have the potential to impact localized populations of small prey organisms at the location of release. The extent to which they could impact such populations has not been examined. Released YZ2018 could also potentially impact other small predator species through competition. While the competitive ability of YZ2018 has not been examined, Zebrafish are generally described in the aquarium trade as a hardy fish, considered good for beginner aquarists and interact well with other fish species in the aquarium. Interestingly, a study was conducted using GloFish® to examine the persistence of a transgene over multiple generations when mixed with non-transgenic Zebrafish (Howard et al. 2015). Non-transgenic males demonstrate aggressive behaviour when mating with females, thus excluding transgenic males from mating, and applying a selective pressure resulting in the eventual loss of the transgene (Howard et al. 2015). As such, YZ2018 are not expected to have exceptional ability to impact native fish populations through strong competitive ability. An unpublished study by DFO found a strain of GloFish® Tetra did not differ in competitive success or aggressive behaviour from sibling non-transgenic tetras (R. Leggatt, DFO, pers. comm.). A suggested optimal temperature for health and activity of Zebrafish for ornamental aquarium culture varies from approximately 24-28°C (see Section 1.4.4.2). While water temperatures during the summer months may be within this range for many water systems in Canada, temperatures are expected to be much lower at other times of the year. Non-transgenic Zebrafish decrease activity and feeding at approximately 16°C, and stop feeding and stop activity below 8°C (Leggatt et al. 2018b, see Figure 2.2). As activity and feeding levels drop with decreasing temperature, impacts to prey and competitors are also expected to decrease with decreasing temperature. Research conducted by GloFish® Technologies demonstrates that YZ2018 is less cold tolerant than non-transgenic Golden Zebrafish (see Section 2.2.2). Given the low temperatures expected for Canadian freshwater systems for most of the year, the potential for released YZ2018 to impact native aquatic species through prey acquisition and competition is expected to be negligible through most of the year, and not expected to be greater than other small fish species during the summer months.

The ability of YZ2018 to prey on or compete for food relative to non-transgenic or Golden Zebrafish has not been reported. Jha (2010) found co-rearing with RFP transgenic Zebrafish, obtained from the ornamental pet trade in India, greatly decreased aggressive behaviour of wild-caught non-transgenic Zebrafish and flying barb, and wild-caught species avoided interactions with the transgenic Zebrafish. Transgenic Zebrafish were also much more aggressive to the wild-caught fish than vice versa (Jha 2010). Whether the differences in aggressive behaviour between the RFP transgenic Zebrafish and wild-caught fish were due to the fluorescent phenotype, or to differences in genetic background (domesticated versus wild) or rearing history (aquarium versus nature) are not known. The study was conducted in bare tanks, and whether similar patterns of behaviour would exist in complex environments such as nature have not been examined. As well, whether similar behaviour patterns would occur in YZ2018 transgenic Zebrafish have not been directly examined. In over five years of commercial use in the ornamental aquarium trade there are no known reports, anecdotal or otherwise, of YZ2018 having different activity levels or behaviour than non-transgenic *D. rerio*, under typical conditions.

Released YZ2018 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 YZ2018 causes deleterious effects to the predator populations. The latter is not expected as YZ2018 are not expected to be environmentally toxic, and the ZsYelllow1 protein is not expected to be toxic to organisms that ingest YZ2018 (see Section 2.3.1). While the predation pressure on YZ2018 relative to non-transgenic *D. rerio* has not been reported, the effect of fluorescent transgenesis in another transgenic model (RFP expressing Zebrafish) is conflicting. Hill et al. (2011) found RFP-expressing Zebrafish had twice higher predation pressure than non-transgenic Zebrafish when reared in complex habitats with two different sized North American predators (Largemouth Bass and Eastern Mosquitofish). The authors postulated this was due to the more conspicuous colouration of the RFP expressing Zebrafish relative to non-transgenic. It should be noted that the RFP expressing Zebrafish in the paper were developed from the golden morph of the Zebrafish which lacks conspicuous stripes and this lack of cryptic colouration may have also contributed to the higher predation on RFP expressing Zebrafish independent of the fluorescent colouration. An earlier study (Cortemeglia and Beitinger 2006b) using the originally marketed striped variety of RFP expressing Zebrafish (Gong et al. 2003) found RFP transgenic and non-transgenic Zebrafish did not significantly differ in predation susceptibility by Largemouth Bass (at a ratio of 1.4:1, respectively). The differences in results between these studies could be due to differences in the expression of RFP (type of RFP and promoter(s) used are not known), differences in the phenotype of the RFP expressing Zebrafish (striped versus not striped), a lack of refuge areas, and low statistical power to identify potential differences in the Cortemeglia and Beitinger trial (Hill et al. 2011). A third study reported yet another trend in results, where RPF expressing Zebrafish from the ornamental pet trade had lower predation susceptibility than wild-caught non-transgenic Zebrafish and flying barb in India (Jha 2010). In this study, the wild-caught Snakehead predators appeared to actively avoid RFP transgenic fish, and non-transgenic Zebrafish and Flying Barb were preyed on more heavily in the presence of RFP transgenic fish than in each other's presence. As with aggressive behaviour listed above, whether the differences in predation vulnerability between the RFP transgenic Zebrafish and wild-caught fish were due to the fluorescent phenotype, or to differences in genetic background and/or rearing history are not known. The study was conducted in bare tanks, and whether similar patterns of predation vulnerability would exist in complex environments such as nature have not been examined. In the RFP expressing Zebrafish model, the impact of the fluorescent transgene is not consistent among studies and may be influenced by transgenic line (i.e., site of insertion in the genome), habitat complexity, background genetics and/or rearing history.

Whether any of the above studies could be applied to YZ2018 predation vulnerability is not known and, consequently, the predation vulnerability of YZ2018 relative to non-transgenic counterparts cannot be estimated with reasonable certainty. Non-transgenic Zebrafish decrease activity and feeding at approximately 16°C, and stop feeding and stop activity below 8°C (Leggatt et al. 2018b, see Figure 2.2). The decreased activity with decreasing temperature may increase predation susceptibility of Zebrafish in non-summer months as they may be slower to respond to predator presence than similar-sized native species adapted to a temperate climate. Combined with the bright yellow/orange colour and lack of cryptic colour stripes in YZ2018, YZ2018 is expected to be more susceptible to predation than native species, and may temporarily benefit native predators if released to natural ecosystems.

Due to described low aggressive behaviour of Zebrafish, low activity in cooler waters, and lack of noted alterations in trophic-related behaviour, YZ2018 is not expected to influence trophic interactions of native organisms beyond natural fluctuations, with associated **negligible** hazard relative to non-transgenic counterparts. Despite a lack of studies directly examining the hazards of YZ2018, availability of data on a surrogate (RPF expressing Zebrafish) and poor understanding of GxE interactions in aggression and predation susceptibility in another fluorescent transgenic Zebrafish model, result in a **moderate** level of uncertainty.

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, see Section 1.4).

There are several other species that currently share the *Danio* genus (Fang 2003). Zebrafish are scatter breeders, and consequently could potentially form hybrids with 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.

Several genera of fish from the Cyprinidae family are found in Canada, though it is not known if these could successfully breed with Zebrafish. The notifier states that inter-breeding may be unlikely given: 1) the probable differences in adaptation; and 2) the fact that YZ2018 has lower competitive reproductive success than non-transgenic Golden Zebrafish. Overall, these data indicate that should Zebrafish be able to successfully hybridize with Canadian Cyprinidae species, YZ2018 would not have greater, and may have lower hybridization potential, than non-transgenic Zebrafish. Consequently, there is **negligible** potential for YZ2018 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 intra-Family hybridization result in **moderate** uncertainty associated with the rating.

2.3.5. Potential to Act as a Vector of Disease Agents

Commercial ornamental aquarium fish are commonly reported to carry numerous disease agents including viruses, bacteria, fungi, and parasites (e.g., Evans and Lester 2001; Řehulka et al. 2006; Whittington and Chong 2007; Hongslo and Jansson 2009; Rose et al. 2013). GloFish LLC submitted a veterinarian assessment that identified the presence of low to moderate numbers of the external parasite *Piscinoodinium* (a parasitic dinoflagellate) in four of six fish and heavy numbers in two of six fish. It is stated that this finding is unrelated to the transgenic nature of YZ2018. 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 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. This conclusion, however, was based on experimental concentrations of SVCV that would never be achieved in a natural setting. No natural SVCV infections have been reported in Zebrafish, including in the wild, in the hobbyist community, and in the laboratory setting (see Hanwell et al. 2016). In addition, any disease agents YZ2018 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 YZ2018 once released to cooler Canadian freshwater environments.

Whether YZ2018, 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 *in vitro* 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). Whether these alterations may be observed in whole animal fluorescent transgenic models has not been examined. YZ2018 has been grown on a commercial scale in the US since 2006 (California since 2015), as have numerous other transgenic fluorescent aquarium species and lines starting in 2003. Fluorescent Zebrafish have been used extensively in laboratory conditions for research with no known reported effects on disease susceptibility, and Howard et al. (2015) tracked non-transgenic and RFP transgenic Zebrafish in 18 populations over 15 generations in laboratory conditions and reported no differences in survival between transgenic and non-transgenic fish. This indicates there is **negligible** potential for YZ2018 to have altered vector capabilities relative to non-transgenic Zebrafish. As this has not been directly examined in YZ2018, and there are limited data on a valid surrogate and a reliance on expert opinion, the uncertainty level for this rating is ranked **moderate.**

2.3.6. Potential to Impact Biogeochemical Cycling

YZ2018 is 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 ZsYellow1 protein in YZ2018 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 YZ2018 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 YZ2018 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.

2.3.7. Potential to Affect Habitat

Zebrafish are a small species that do not build nests of other structures that may impact habitats of other species. YZ2018 has been in commercial use in the ornamental aquarium trade since 2006, and there have been no reports, anecdotal or otherwise, of YZ2018 having altered behaviour, relative to Golden Zebrafish, that may influence effects on habitat structure. Consequently, YZ2018 is expected to have **negligible** effects to habitat with **low** uncertainty associated with this rating.

2.3.8. Potential to Affect Biodiversity

Biological diversity (or 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, and non-transgenic and GloFish® Zebrafish have been rated as having low invasiveness potential in Continental USA using the Fish Invasiveness Screening Kit (Hill et al. 2014, 2017). As elaborated above, YZ2018 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 resulting fluorescent protein in YZ2018, 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 YZ2018 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.

2.3.9. Conclusions

YZ2018 is not expected to be hazardous to Canadian environments. It has no history of invasiveness despite its widespread use in the United States. 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. YZ2018 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. There is evidence to suggest that, compared to non-transgenic Zebrafish and other fluorescent fish models, YZ2018 may have lowered potential to affect other species through trophic interactions, as lower cold tolerance may further limit activities in cooler water temperatures.

The examined hazards had negligible to low hazard ranking (Table 2.5), while uncertainty ranged from low to moderate, due to limited data specific to YZ2018, limited direct data on comparator species, variable data from surrogate model (RFP expressing 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, YZ2018 is not expected to pose unique hazards beyond those of the intended use.

Table 2.5.: Summary of hazard rank and uncertainty of YZ2018 to Canadian environments.

Hazard	Rank	Uncertainty
1. Through environmental toxicity	Negligible	Moderate
2. Through horizontal gene transfer	Low	Moderate
3. Through trophic interactions	Negligible	Moderate
4. Through hybridization	Negligible	Moderate
5. As a vector for disease	Negligible	Moderate
6. To Biogeochemical cycling	Negligible	Moderate
7. To habitat	Negligible	Low
8. To Biodiversity	Negligible	Low

2.4 ASSESSMENT OF RISK

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

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 both. Overall Risk is estimated by plotting Hazard against Exposure, using a matrix or heat map, as illustrated in Figure 2.3. The matrix cannot be used as a tool for establishing a discreet conclusion or decision on risk, but can be used as a device to facilitate communication and discussion. The uncertainty associated with overall Risk rating is not estimated, rather uncertainty in the hazard and exposure assessments are discussed in the context of a final conclusion on risk.

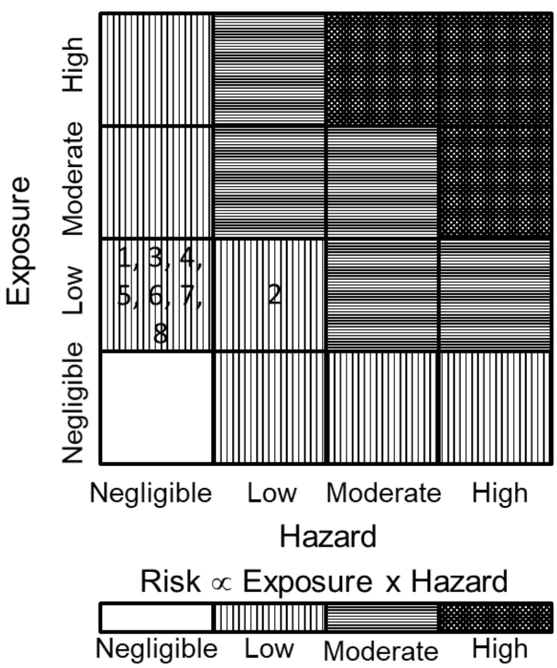

Figure 2.3.: Risk matrix and colour scale to illustrate how exposure and hazard are integrated to establish a level of risk in the environmental risk assessment. Risk assessments associated with assessed hazard components at the assessed exposure are identified by number: 1) through environmental toxicity;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

2.4.1. Risk Assessment of YZ2018

The exposure assessment concluded that YZ2018 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 YZ2018 to overwinter in Canadian water systems. As such, any exposure to Canadian freshwater ecosystems to YZ2018 is expected to be isolated, rare, and ephemeral. The quality of data demonstrating lack of cold tolerance in YZ2018 and Golden Zebrafish, relevant to Canadian freshwater temperatures result in low uncertainty associated with this ranking.

The hazard assessment concluded that YZ2018 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 non-transgenic Golden Zebrafish. Uncertainty ranking associated with individual hazard components ranged from low to moderate, due to limited data specific to YZ2018, limited direct data on comparator species, variable data from surrogate model (RFP expressing Zebrafish), and the reliance on expert opinion for the assessment of some hazards.

Using the risk matrix seen in Figure 2.3, YZ2018 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 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. The majority of identified environmental hazards of YZ2018 would be expected to require continuous exposure of ecosystem components to YZ2018 in order to pose significant risk to the environment. Consequently, uncertainty in risk may be more closely aligned with that of exposure rather than hazard.

Despite moderate uncertainty in some of the individual assessment components, there is no current evidence to suggest that overall risk ratings of YZ2018 may be higher than the assessed low ranking for risk to Canadian environments.

2.5. SUMMARY AND CONCLUSIONS

GloFish LLC has requested approval for the import of YZ2018, a fluorescent orange transgenic Zebrafish of species *D. rerio* for use in the ornamental aquarium trade and home aquaria. Use of YZ2018 in home aquaria in Canada, or in other unintended uses, is expected to result in frequent, very small magnitude releases of YZ2018 to the Canadian environment, although the potential for occasional high magnitude releases cannot be ruled out. Available high quality data indicates that YZ2018 does 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 base non-transgenic species despite long-term extensive use, and a lack of evidence for increased hazards of YZ2018 relative to non-transgenic, indicates negligible to low hazard ranking to Canadian ecosystems. Due to a lack of direct information on the hazards of base models or YZ2018, uncertainty with hazard assessments ranged from low to moderate. Taken together, the overall risk of YZ2018 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 YZ2018 under the proposed or other potential uses, could cause harm as a result of exposure to the Canadian environment.

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