Fisheries and Oceans Canada / Pêches et Océans Canada
Ecosystems and Oceans Science / Sciences des écosystèmes et des océans

National Capital Region
Canadian Science Advisory Secretariat
Science Advisory Report 2019/002

# ENVIRONMENTAL AND INDIRECT HUMAN HEALTH RISK ASSESSMENT OF THE GLOFISH® TETRAS (*GYMNOCORYMBUS TERNETZI*): FIVE LINES OF TRANSGENIC ORNAMENTAL FISH

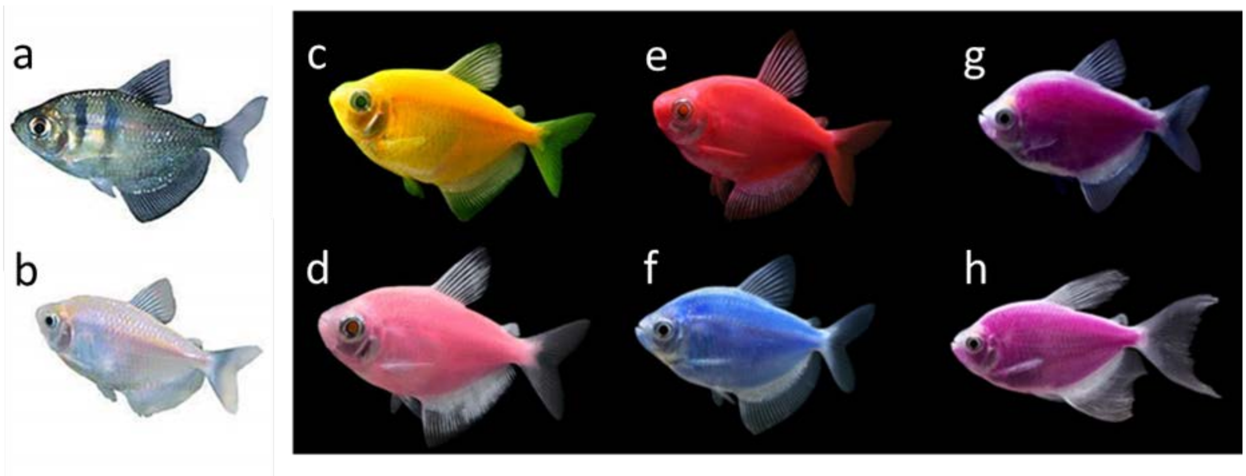


*Figure 1. Some variants of* Gymnocorymbus ternetzi *available in the ornamental pet trade worldwide (a, b), and notified transgenic variants currently only available in the United States of America (c, d, e, f, g, h). Wild-type Black Tetra (a), White Tetra (b), Sunburst Orange® Tetra (c), Moonrise Pink® Tetra (d), Starfire Red® Tetra (e), Cosmic Blue® Tetra (f), and Galactic Purple® Tetra (g, h). Taken from www.petsmart.com (a, b), www.glofish.com (c, d, e, f, g, h). Galactic Purple® Tetra is shown in both regular (g) and long-fin varieties (h). All lines shown are available in long-fin variety except for the Cosmic Blue® Tetra.*

## Context:

*The biotechnology provisions of the* Canadian Environmental Protection Act, 1999 (CEPA*) take a preventative approach to environmental protection by requiring all new living organism products of biotechnology, including genetically engineered fish, to be notified and assessed prior to their import into Canada or manufacture in Canada, to determine whether they are "toxic"[1] or capable of becoming "toxic". Environment and Climate Change Canada (ECCC) and Health Canada (HC) are mandated to conduct all risk assessments under CEPA.*

[1] Under CEPA, "toxic" is a regulatory concept used to describe a substance or 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; (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.

*Under a Memorandum of Understanding (MOU) between the Department of Fisheries and Oceans (DFO), ECCC and HC, DFO conducts an environmental risk assessment, provides science advice, and collaborates with HC to conduct an indirect human health risk assessment*[2] *for any fish products of biotechnology notified under CEPA and the* New Substances Notification Regulations (Organisms) [NSNR(O)*]. The advice is conveyed to ECCC and HC as a Science Advisory Report, to inform the risk assessment they will conduct under CEPA.*

*On June 16, 2018, five notifications under the NSNR(O) were submitted by GloFish LLC to ECCC for five distinct lines of GloFish® Tetras, genetically engineered variants of the Black Tetra (*Gymnocorymbus ternetzi*).This Science Advisory Report summarizes the results of the July 17 - 18, 2018 "Environmental and Indirect Human Health Risk Assessments of the GloFish® Tetras: Five Lines of Transgenic Ornamental Fish" Canadian Science Advisory Secretariat (CSAS) peer-review meeting. Additional publications from this meeting will be posted on the Fisheries and Oceans Canada (DFO) Science Advisory Schedule as they become available.*

*Note that a previous risk assessment was conducted on a related GloFish®, 'Electric Green®', in 2017 and has been published as Science Advisory Report 2018/027.*

# SUMMARY


- Pursuant to the *Canadian Environmental Protection Act* (CEPA), five notifications of living organisms under the *New Substances Notification Regulations (Organisms)* (NSNR(O)) were submitted by GloFish LLC to Environment and Climate Change Canada (ECCC) for five distinct lines of genetically-engineered *Gymnocorymbus ternetzi* (Black Tetra): the Sunburst Orange® Tetra, Moonrise Pink® Tetra, Starfire Red® Tetra, Cosmic Blue® Tetra, and Galactic Purple® Tetra. Here, they are collectively referred to as the GloFish® Tetras.
- Environmental and indirect human health risk assessments were conducted that included an analysis of potential hazards, likelihood of exposure, and associated uncertainties to reach conclusions on risk and to provide science advice to ECCC and Health Canada (HC) to inform their CEPA risk assessment.

## Indirect Human Health Risk Assessment

- The **indirect human health** (IHH) exposure assessment concluded that human exposure potential of the GloFish® Tetras is **low to medium** as their intended use is as an ornamental aquarium fish, thus largely limiting public exposure to those individuals who possess them for use in home aquaria.
- Uncertainty associated with the IHH exposure assessment is **moderate** due to limited information regarding exposure scenarios (i.e., information on number of people purchasing fish and how they will handle them) in the Canadian market.
- The IHH hazard assessment concluded that the indirect human hazard potential of the GloFish® Tetras is **low** as the inserted genetic materials are not known to be toxic or pathogenic to humans, there are no reported cases of zoonotic infections associated with GloFish® Tetras or the wild type (i.e., non-genetically modified Black Tetra), there is safe history of use of the notified lines in the US, and based on the sequence identity and the structure of the inserted transgenes, the production of allergens or toxins is not anticipated.


[2] In this context, an 'indirect' human health risk assessment aims to identify and characterize risks to human health from environmental exposures to the living organism.

- Uncertainty associated with the IHH hazard assessment is **low** based on reliance on information from other ornamental aquarium fish and lack of direct studies investigating human health effects of fluorescent transgenic ornamental fish.
- Therefore, there is a **low** risk of adverse indirect human health effects at the exposure levels predicted for the Canadian population from the use of GloFish® Tetras as an ornamental aquarium fish or other potential uses.

### Environmental Risk Assessment

- The **environmental** exposure assessment concluded that the exposure of GloFish® Tetras to the Canadian environment is **low**. The occurrence outside of aquaria is possible, but it is expected to be rare, isolated and ephemeral due to their inability to survive typical low winter temperatures in Canada's freshwater environments.
- The uncertainty associated with this environmental exposure estimation is **low**, given the available data for temperature tolerance of the five GloFish® Tetras and wild type tetras.
- The environmental hazard assessment concluded **negligible** hazards of the GloFish® Tetras to the Canadian environment with respect to: environmental toxicity, interactions with other organisms, hybridization or as a vector for disease, as well as to biodiversity, biogeochemical cycling, and habitat. There is a **low** hazard rating of GloFish® Tetras with respect to horizontal gene transfer (i.e., no anticipated harmful effects).
- The uncertainty levels, associated with the environmental hazard ratings, range from **negligible** to **moderate** due to the limited data specific to the GloFish® Tetras, the inconsistent results from studies on other fluorescent transgenic organisms, and the reliance on expert opinion.
- Therefore, there is **low** risk of adverse environmental effects at the exposure levels predicted for the Canadian environment from the use of GloFish® Tetras as an ornamental aquarium fish or other potential uses.

### Conclusions

- The overall assessment of the use of GloFish® Tetras in the ornamental aquarium trade or other potential uses in Canada is a **low** risk to the indirect human health of Canadians and to the Canadian environment. Despite moderate level of uncertainty in some individual assessment components, there is no current evidence to suggest overall risk ratings of GloFish® Tetras used for the ornamental aquarium trade in Canada may be higher than the assessed low rating risk to Canadians and to the Canadian environment.

## BACKGROUND

The five GloFish® Tetra strains are independent lines of genetically engineered diploid (two sets of chromosomes), hemizygous (one copy of transgene) or homozygous (two copies of transgene), long- or regular-fin, transgenic colour morphs of the White Tetra (*G. ternetzi*), a white morph of the Black Tetra (also *G. ternetzi*). Trade names (with line names in brackets) for the five notified organisms are the Sunburst Orange® Tetra (OT2018), the Starfire Red® Tetra (RT2018), the Galactic Purple® Tetra (PuT2018), the Moonrise Pink® Tetra (PiT2018), and the Cosmic Blue® Tetra (BT2018). In the following report, they are collectively referred to as the GloFish® Tetras. The purpose of these modifications is to create new colour phenotypes of *G. ternetzi* for the ornamental aquarium trade (Figure 1).

The GloFish® Tetras have been in commercial production for the ornamental aquarium trade in the United States (US) excluding California since 2013 (OT2018, PiT2018, PuT2018) or 2014 (BT2018, RT2018), and in California since 2015. They are manufactured for GloFish LLC by two aquarium fish producers in Florida. A previous risk assessment was conducted on a related GloFish® 'Electric Green®' in 2017 and has been published as Science Advisory Report 2018/027.

## Production of the notified organisms

All five lines of GloFish® Tetras were produced using the same methodologies. In general, a transgene expression cassette was injected into newly fertilized eggs of White Tetras. Confirmation that all F1 and F2 fish are descended from a single founding individual (G0) and constitute a single homogenous line with approximately equal insertion pattern was made via enzyme cleavage and Southern blot analysis, using restriction enzyme *Ase*I and a probe that binds to the expression cassette. More detail regarding the structure, development, and function of the transgene constructs has been provided by the company for the expressed purpose of the current risk assessments and review, but is identified as confidential business information and cannot be released.

Though the interbreeding of F2 hemizygous fish is expected to produce some homozygous F3 fish, to date no homozygous fish have been recovered for BT2018, PiT2018 or PuT2018. No formal investigations of gene silencing or stability of different genotypes are available; however, the company claims that for all lines the phenotypes appear to have remained stable over multiple generations.

Long-fin variants of the GloFish® Tetras were produced by selective breeding within the line (OT2018) or crossing them with long-fin White Tetras (PiT2018, PuT2018, RT2018). Progeny from these crosses were selected for fluorescent colour and long fins to establish long-fin breeding lines. The long-fin trait occurs naturally in Black Tetra and is considered to be within its natural phenotypic range. For the purposes of the CEPA assessment, long-fin variants of the GloFish® Tetras are deemed to be the same as the short-fin variants.

## Characterization of the notified organisms

The GloFish® Tetra lines are maintained through batch breeding with selection of individuals and broodstock based on phenotype, and may include individuals that are hemizygous or homozygous. It is claimed the two genotypes cannot be distinguished phenotypically.

For each of the GloFish® Tetra lines, insert copy number was approximated by quantitative PCR against a standard curve. The absence of vector backbone was confirmed using primer probes specific to four different sections of the vector backbone. In all lines, the segregation of the transgene into approximately 50% of the population when bred in single-pair matings with wild-type fish is consistent with a single locus of insertion; however, the insert site location(s) and final sequence of inserted genetic material have not been determined.

Though no formal studies have compared potential disease susceptibility of the notified organisms and wild-type *G. ternetzi*, GloFish LLC provided veterinarian statements that state no evidence has been noted for increased susceptibility to, or transmission of, water-borne pathogens, or additional health impediments of any commercially available fluorescent line relative to non-transgenic counterparts, and that GloFish® Tetras require the same husbandry and care as non-transgenic counterparts.

**Cosmic Blue® Tetra** (BT2018)

The Cosmic Blue® Tetra, identified in the notification as line BT2018, is a genetically engineered White Tetra (*G. ternetzi*) with an estimated 100 copies of a transgene construct incorporated into its genome at a single site of insertion. The targeted phenotypic effect of the genetic modification is that BT2018 appears blue under ambient light (Figure 1f). Two off-target effects that have been identified by GloFish LLC are diminished tolerance to low temperature and a decrease in reproductive success. In lower temperature tolerance trials, all fish died between 9.4 to 6.9°C (BT2018) and 9.1 to 6.7°C (White Tetra); and BT2018 had a significantly higher median lethal dose (LD50) relative to White Tetras (8.02°C versus 7.64°C respectively $p<0.001$); demonstrating higher cold sensitivity in blue transgenic tetras relative to wild-type White Tetras. In single pair crosses of BT2018 with White Tetras, the proportion of blue offspring was significantly lower than the expected 50% (i.e., 48.4%, $p=0.022$). In reproductive competition trials with White Tetras, the proportion of fluorescent fry at seven days post fertilization (0.386) did not differ from the proportion of 0.4 predicted by random assortment and assuming no homozygous offspring were viable ($p=0.753$).

### Sunburst Orange® Tetra (OT2018)

The Sunburst Orange® Tetra, identified in the notification as line OT2018, is a genetically engineered White Tetra with an estimated 14 copies of a transgene construct incorporated into its genome at a single site of insertion. The targeted phenotypic effect of the genetic modification is that OT2018 appears orange under ambient light (Figure 1c). Two off-target effects that have been identified by GloFish LLC are diminished tolerance to low temperature and a decrease in competitive reproductive success. In lower temperature tolerance trials, all fish died between 9.6°C and 7.9°C for both genotypes; however, OT2018 had a significantly higher LD50 relative to White Tetras (9.07°C versus 8.95°C respectively $p<0.001$); demonstrating higher cold sensitivity in OT2018 relative to wild-type White Tetras. In single pair crosses with OT2018 and White Tetras, the proportion of orange offspring was not significantly different than 50% (49.2%, $p=0.056$). In reproductive success trials with White Tetras, the proportion of fluorescent fry at seven days post fertilization (0.359) was significantly lower than the proportion of 0.4375 predicted by random assortment ($p=0.039$).

### Moonrise Pink® Tetra

The Moonrise Pink® Tetra, identified in the notification as line PiT2018, is a genetically engineered White Tetra with an estimated two copies of a transgene construct incorporated into its genome at a single site of insertion. The targeted phenotypic effect of the genetic modification is that PiT2018 appears pink under ambient light (Figure 1d). For off-target effects, in lower temperature tolerance trials, all fish died between 8.9 and 7.2°C (PiT2018) and 8.6 and 7.0°C (White Tetra). Though PiT2018 had a higher LD50 relative to White Tetras, the difference was not significant (8.03°C versus 7.95°C respectively, $p=0.09$). In single pair crosses of PiT2018 with White Tetras, the proportion of pink offspring was significantly lower than 50% (46.5%, $p=0.031$). In reproductive success trials with White Tetras, the proportion of fluorescent fry at seven days post fertilization (0.351) did not significantly differ from the proportion of 0.4 predicted by random assortment assuming no homozygous offspring were viable ($p=0.263$).

### Galactic Purple® Tetra

The Galactic Purple® Tetra, identified in the notification as line PuT2018, is a genetically engineered White Tetra with an estimated 10 copies of a transgene construct incorporated into its genome at a single site of insertion. The targeted phenotypic effect of the genetic modification is that PuT2018 appears purple under ambient light (Figure 1g and h). One off-target effect investigated by GloFish LLC was decreased cold temperature tolerance. In lower temperature tolerance trials, all fish died between 8.4 and 6.6°C (PuT2018) and 8.4 and 6.2°C (White Tetra); however, PuT2018 had a significantly higher LD50 relative to White Tetras

(7.28°C versus 7.08°C, respectively p<0.001); demonstrating higher cold sensitivity in purple transgenic tetras relative to wild-type White Tetras. In single pair crosses of PuT2018 with White Tetras, the proportion of purple offspring was not significantly different than 50% (48.0%, p=0.231). In reproductive success trials of PuT2018 with White Tetras, the average proportion of fluorescent fry at seven days post fertilization (0.394) was not significantly different than the proportion of 0.4 predicted by random assortment and assuming non-viable homozygous offspring (p=0.974).

#### Starfire Red® Tetra

The Starfire Red® Tetra, identified in the notification as line RT2018, is a genetically engineered White Tetra with an estimated 12 copies of a transgene construct incorporated into its genome at a single site of insertion. The targeted phenotypic effect of the genetic modification is that RT2018 appears red under ambient light (Figure 1e). Two off-target effects investigated by GloFish LLC are cold temperature tolerance and reproductive success. In lower temperature tolerance trials all fish died between 8.5 and 7.2°C (RT2018) and 8.3 and 6.6°C (White Tetra); however, RT2018 had a significantly higher LD50 relative to White Tetras (7.86°C versus 7.40°C respectively, p<0.001); demonstrating higher cold sensitivity in red transgenic tetras relative to wild-type White Tetras. In single pair crosses of RT2018 with White Tetras, the proportion of red offspring was not significantly different than 50% (50.0%, p=0.973). In reproductive success trials with White Tetras, the proportion of fluorescent fry at seven days post fertilization (0.190) was significantly lower than the proportion of 0.4375 predicted by random assortment (p<0.001).

#### Fluorescent protein transgenes in other models

Fluorescent proteins have widespread use in research in a variety of organisms and relevant research has been done on various lines of Zebrafish (*Danio rerio*) transgenic for red fluorescent protein (RFP) and other fluorescent proteins. Most, but not all, RFP and GFP (Green Fluorescent Protein) Zebrafish lines are slightly less tolerant to extreme cold or heat than wild type (Cortemeglia and Beitinger 2005, 2006a; Leggatt et al. 2018). Survival in most fluorescent transgenic Zebrafish lines is similar to their non-transgenic counterparts, but there are inconsistent effects of fluorescent transgenesis on reproductive behaviour, preferences, and success, as well as the ability to avoid predation (Cortemeglia and Beitinger 2006b; Gong et al. 2003; Hill et al. 2011; Howard et al. 2015; Jha 2010; Owen et al. 2012; Snekser et al. 2006). Fluorescent protein transgenes are used extensively as neutral markers for research in diverse organisms including fish, and are generally reported to have no adverse effects to the organisms, though several mouse models with high expression have altered viability (e.g., Devgan et al. 2004), and some cell line models report altered gene expression levels (e.g., Mak et al. 2007). As well, mice transgenic for DsRed or eGFP (enhanced Green Fluorescent Protein) can have altered metabolic enzymes (Chou et al. 2015; Li et al. 2013) or toxicity in cardiac muscle (Chen et al. 2016). While there are some reports of risk-related alterations in fluorescent transgenic Zebrafish models, there are no predictable effects associated with fluorescent protein transgenesis.

### Comparator Species

For the purpose of this assessment, both the Black Tetra (*G. ternetzi*) and its white variant (White Tetra), used to produce the GloFish® Tetras, were used as comparators for the notified organism. The Black Tetra is a small (5-6 cm) tropical freshwater fish from the Rio Paraguay river basin in South America. It has been domesticated for use in the ornamental aquarium trade worldwide since at least 1950 (Innes 1950), including selection for natural white and/or long-fin variants (see Figure 1). Much of the information available on the Black Tetra is from the ornamental aquarium trade, rather than from scientific studies on their natural ecology.

The Black Tetra belongs to the Order Characiformes, Family Characidae. The Characidae Family is distributed throughout the Americas as far north as southern US. Suggested ideal temperature requirements of Black Tetra in the home aquarium range from low to mid 20°C's for maintenance, and mid to high 20°C's for reproduction. The average lower lethal temperature of White Tetra is reported by the notifying company to range from 7.08 to 8.95°C when temperature is dropped rapidly (0.5 to 2°C/hour), and the average non-lethal critical thermal minimum (a proxy for lower lethal temperature) is reported to be 9.95°C when temperature is dropped gradually (1°C/day, Leggatt et al. 2018). This latter study also reported White Tetras decreased feeding and general activity at 17°C and stopped feeding at 12°C.

Average lifespan of Black Tetra in the home aquaria is 3-6 years, and reported maturation age ranges from 5 months to 1-2 years. Black Tetras are scatter spawners. Their eggs hatch after 20-24 hours, and they start feeding at 5-6 days post hatch. Black Tetras are primarily carnivorous, and they are generally not known to be aggressive to or highly competitive with other aquarium fish.

Black Tetras escaped from the aquarium trade are established in Colombia, and have been reported but not established in a Colorado hot spring, as well as in Florida and Louisiana. There are no other reported occurrences or establishments in the over 60 years of its use in the ornamental aquarium trade.

## Characterization of potential receiving environment

The Canadian freshwater environment is comprised of thousands of lakes and rivers, spread across hundreds of drainage basins that cover the entire 9.9 million square kilometres of territory, and from temperate to arctic climate zones. These waterways are highly variable in volume, depth, current velocity, geology and geomorphology, their chemical and physical properties, and overall productivity. Potential receiving environments for ornamental fish in Canada include any freshwater spring, stream, pond, river, lake, or reservoir. While this may encompass an enormous range of possibilities and scenarios, the colder water temperatures experienced in Canada, relative to the geographic origins of ornamental species, will place the most significant limitation on the capacity of tropical freshwater ornamental fish to survive in the Canadian environment. Though the many lakes and rivers of Canada vary in their annual temperature profiles, as well as their average maximum and minimum temperatures, most reach a temperature of 4°C or below at some point annually, and only a few isolated lakes in Southern Coastal BC have minimum recorded temperatures above this (i.e., 6°C or lower). If an introduced fish cannot survive at or below 4°C, its occurrence in the Canadian environment will be seasonal at best, with possible localized overwintering pockets if the organism can survive at 4 to 6°C. It should be noted that many freshwater systems may have heterogeneity in temperature profiles – for example shoreline regions of lakes may experience more extreme temperatures than central regions, or groundwater contributions may increase or decrease temperatures in localized areas of a water body. As well, hot springs or warm water effluent may result in localized areas with year-round temperatures that are higher than typical Canadian temperatures.

# RISK ASSESSMENT – INDIRECT HUMAN HEALTH

## Exposure Assessment - Indirect Human Health

### Import

GloFish® Tetra broodstock are maintained at two separate farms in Florida, where all manufacture of the notified line occurs. Adult fish will be shipped to Canadian distributors for eventual distribution to retail pet stores for purchase by the general public. The notified lines will be delivered to retailers in the quantity ordered where they will be held until sold.

### Introduction of the organism

Notified lines will be marketed at retail outlets where ornamental aquarium fish are sold. The exact number and locations where the notified organisms will be available are not currently known. A 2009 survey estimated 12% of Canadian households owned fish (Whitfield and Smith 2014) but it is not known what percentage of home aquarists may purchase the notified organisms. Exposure to notified lines by home aquarists that purchase them will most likely be limited to maintenance activities such as water changes and tank cleanings.

### Environmental Fate

The notified organisms are not intended for environmental release and will be confined to aquariums in homes and retail outlets. Should any fish be either deliberately or unintentionally released into the environment, the chances of establishing a self-sustaining population are low as no cases of environmental establishment have been reported in United States where fluorescent *G. ternetzi* have been commercially marketed as an aquarium fish in areas having higher minimum winter temperatures than typical Canadian winter temperatures.

The notifier supplied temperature tolerance data demonstrating LD50s ranging between 7°C and 9°C. *G. ternetzi* is not considered a species of concern in Canadian waters due to its lack of a thermal tolerance and no history of invasiveness (Leggatt et al. 2018; Rixon et al. 2005) although various climate projections were not specifically evaluated. If live or dead fish are released into the environment, it is expected that both fish and fluorescent proteins would biodegrade normally, and not bioaccumulate or be involved in biogeochemical cycling in a form different from other living organisms. Therefore, the likelihood of exposure to the notified organisms in the environment is low.

### Other Potential Uses

The sole intended use for the notified lines is as ornamental fish for interior home aquariums. According to the notifier, the lines are not suitable for use in outdoor ponds, as a bait fish, for human consumption, or as an environmental sentinel. As such, the notifier does not support any uses of the notified lines outside that of being an ornamental aquarium fish. An in-house literature search found a study examining the potential use of ornamental fish for mosquito control. Tilak et al. (2007) evaluated the larvivorous potential of Goldfish and Blue Gouramis in a laboratory setting and recommended the introduction of these kinds of fish in ornamental tanks to control mosquito breeding as well as providing aesthetic beauty.

Manufacture of the notified organisms is not anticipated to occur in Canada as the lines are only produced in Florida. However, should manufacture occur, no additional indirect human exposures are foreseen that are different from any other typical aquarium fish. The notifier recommends that individuals who no longer wish to maintain the organisms after purchase either return them to the retailer, give them to another aquarium hobbyist, or humanely euthanize with ice water. According to a patent held by the notifier (U.S. Patent No.: 8,975,467), fluorescent transgenic fish may be used in embryonic studies for tracing cell lineage and migration. As well, they can be used to mark cells in genetic mosaic experiments and in fish cancer models.

**Exposure Characterization**

Indirect human health risk assessment looks at the potential to cause harmful effects to humans in Canada relative to wild-type *G. ternetzi* as a consequence of environmental exposure, including exposure in natural environments and environments under its intended use (i.e., home aquaria). Exposure and risks from workplace exposure to the notified organism are not considered in this assessment[3].

The ranking system used to determine human exposure through release to the environment is given in Table 1, and human exposures through intended and potential uses are also addressed. The human exposure potential of *G. ternetzi* BT2018, OT2018, PiT2018, PuT2018, and RT2018 is assessed to be low to medium because:

1. The primary source of the notified organisms in Canada is the import of adult BT2018, PiT2018, PuT2018, OT2018 and RT2018;
2. The notified organisms will potentially be available for purchase by the public wherever tropical aquarium fish are sold throughout Canada, and not for intentional introduction into the Canadian environment;
3. The sole intended use is as an ornamental aquarium fish, thus limiting potential public exposure to those that possess a home aquarium, which may include immunocompromised individuals;
4. Typical human exposure to live or dead fish in the home is most often related to maintenance activities such as tank cleanings and water changes; and
5. Should other potential uses occur, such as uses as bait fish, in outdoor ponds, mosquito control, and for scientific research, no additional indirect human exposures are foreseen that are different from those of any other typical aquarium fish.

*Table 1. Ranking of human exposure via environmental release considerations.*

| Exposure Ranking | Considerations |
|---|---|
| High | • The release quantity, duration and/or frequency are high.<br>• The organism is likely to survive, persist, disperse, proliferate and become established in the environment.<br>• Dispersal or transport to other environmental compartments is likely.<br>• The nature of release makes it likely that healthy and vulnerable (e.g., immunocompromised) human individuals will be exposed and/or that releases will extend beyond a region or single ecosystem. |

[3] A determination of whether one or more criteria of section 64 of CEPA are met is based on an assessment of potential risks to the environment and/or to human health associated with exposure in the general environment. For humans, this includes, but is not limited to, exposure from air, water and the use of products containing the substances. A conclusion under CEPA may not be reliant to, nor does it preclude, an assessment against the criteria specified in the *Hazardous Products Regulations*, which is part of the regulatory framework for the Workplace Hazardous Materials Information System (WHMIS) for products intended for workplace use.

| Exposure Ranking | Considerations |
|---|---|
| Medium | • It is released into the environment, but quantity, duration and/or frequency of release is moderate.<br>• It may persist in the environment, but in low numbers.<br>• The potential for dispersal/transport is limited.<br>• The nature of release is such that some exposure to healthy and vulnerable human individuals (e.g., immunocompromised) can be expected. |
| Low | • It is used in containment (no authorized or planned intentional release).<br>• The nature of release and/or the biology of the organism are expected to contain the organism such that healthy or vulnerable (e.g., immunocompromised) human individuals are not exposed.<br>• Low quantity, duration and frequency of release of organisms that are not expected to survive, persist, disperse or proliferate in the environment where released. |

#### Uncertainty related to indirect human health exposure assessment

The ranking of uncertainty associated with the indirect human health exposure assessment is presented in Table 2. Adequate information was provided by the notifier on the sources of exposure and factors influencing human exposure including its import, retail distribution and survival in the environment. It was indicated that the notified organisms will not be manufactured in Canada and that the source of exposure will be restricted to the import of fish of each line. The survival of these fish is expected to be limited by their poor tolerance to temperatures below 9°C. Empirical data was presented showing less cold tolerance of the notified lines compared to the wild-type *G. ternetzi*. Human exposure (general public and immunocompromised individuals) in Canada is expected to occur through home aquariums mainly from maintenance and cleaning activities. The percentage of aquarists that will purchase and the actual number of notified organisms to be imported in the following years is not known at this point. Therefore, because of limited information on exposure scenarios of the fluorescent ornamental fish in the Canadian market, the human exposure to the notified organisms is considered low to medium with moderate uncertainty.

*Table 2. Ranking of uncertainty associated with the indirect human health exposure.*

| Uncertainty Ranking | Available Information |
|---|---|
| Negligible | High-quality data on the organism, the sources of human exposure and the factors influencing human exposure to the organism. Evidence of low variability. |
| Low | High-quality data on relatives of the organism or valid surrogate, the sources of human exposure and the factors influencing human exposure to the organism or valid surrogate. Evidence of variability. |
| Moderate | Limited data on the organism, relatives of the organism or valid surrogate, the sources of human exposure and the factors influencing human exposure to the organism. |
| High | Significant knowledge gaps. Significant reliance on expert opinion. |

## Hazard Assessment – Indirect Human Health

### Zoonotic potential

Literature searches found no reports of zoonoses or other adverse effects attributed to the notified organisms or to the wild-type *G. ternetzi*. The notifier provided statements from staff veterinarians at their production farms stating that based on their experience and observations, that the notified lines possess no increased susceptibility to pathogens or zoonotic risk compared to non-modified tetras.

Zoonotic infections primarily occur through puncture, cuts, scrapes, abrasions or sores in the skin (Boylan 2011). Infections may be prevented through wearing gloves when handling fish or cleaning fish tanks and avoiding contact with any potentially contaminated water if any open skin wounds are present. Washing hands with soap and water after contact with aquarium water is also highly recommended. As well, people with compromised immune systems or underlying medical conditions should avoid cleaning tanks or handling fish (Haenen et al. 2013).

Though uncommon, there are reported cases of zoonotic infections from contact with tropical ornamental fish and indirect zoonoses due to ingestion of food or drinking water that has been contaminated with pathogens and parasites associated with ornamental or aquarium fish. Bacterial disease is extremely common in ornamental fish and is most frequently associated with bacteria that are ubiquitous in the aquatic environment acting as opportunistic pathogens secondary to stress (Roberts et al. 2009). Contact is the main route of transmission leading to bacterial infections in humans that develop from handling of aquatic organisms (Lowry and Smith 2007). The most common bacterial species associated with tropical fish capable of causing human illness are *Aeromonas* sp., *Mycobacterium marinum*, *Salmonella* sp., and *Streptococcus iniae* (CDC 2015) with the most commonly reported infections being associated with *M. marinum* (Weir et al. 2012).

### Allergenicity/Toxicity

In-house amino acid sequence analyses of all the inserted proteins using the AllergenOnline Database (v18B; 23 March, 2018) found no matches with greater than 35% identity for both 80 and 8 amino acid segments. The 35% identity for 80 amino acid segments is a suggested guideline proposed by the Codex Alimentarius Commission for evaluating newly expressed proteins produced by recombinant-DNA plants (WHO/FAO 2009). Similar results were provided by the notifier from analyses using the Allermatch website.

Members of the Phylum Cnidaria (source phylum for transgenes) produce toxins that are necessary in prey capture, digestion, and intraspecific aggression, and some of these toxins can be toxic to humans. However, analysis of these sequences did not indicate any homologies to sequences of potential toxins or allergens.

Basic Local Alignment Search Tool (BLAST) searches on the nucleotide and amino acid sequences of the inserted genes and resulting proteins did not detect any homologies to any known toxins. As well, an in-house literature search found no reports of adverse effects attributed to the inserted genes in humans. Furthermore, there is no evidence indicating the potential of the notified lines or *G. ternetzi* producing toxic or other hazardous materials that may accumulate in the environment or be consumed by other organisms in the environment.

### History of Use

The notified lines have been maintained as breeding lines for more than five generations. They have been commercially produced for over five years and marketed as aquarium fish throughout the United States except California since 2013 for the OT2018, PiT2018, and PuT2018 lines and 2014 for the BT2018 and RT2018 lines and in California since 2015 without any reported incidents of adverse health effects in humans. The parental strain, *G. ternetzi* has been available as a home aquarium fish since at least 1950 (Innes 1950) without specific reported incidents of adverse effects in humans.

### Hazard Characterization

The human hazard potential of *G. ternetzi* BT2018, OT2018, PiT2018, PuT2018, and RT2018 is assessed to be **low** because:

1. These genetically modified tropical fish contain a single insert with varying copies of the fluorescence genes that were confirmed to be stably integrated through qPCR and multiple crossings;
2. The methods used to produce the notified living organisms do not raise any indirect human health concerns. Although some of the source organisms from which the inserted genetic material was derived appear to produce toxins, there is no indication that any of the inserted genetic material or expressed proteins in these lines are associated with any toxicity or pathogenicity in humans;
3. While there are reported cases of zoonotic infections associated with tropical aquarium fish, particularly for immunocompromised individuals, there are no reported cases attributed to either the notified organisms or the wild-type, and no reports of the notified organisms having higher vector capabilities than the wild-type;
4. Sequence identities of the inserted transgenes or any potentially expressed proteins from the constructs do not match any known allergens or toxins; and
5. There is a safe history of use for the notified lines in the United States and for the wild-type species as an ornamental aquarium fish globally, with no reported adverse indirect human health effects in the literature.

*Table 3. Considerations for hazard severity (indirect human health).*

| Hazard Ranking | Considerations |
|---|---|
| High | • Effects in healthy humans are severe, of longer duration and/or sequelae in healthy individuals or may be lethal.<br>• Prophylactic treatments are not available or are of limited benefit.<br>• High potential for community level effects. |
| Medium | • Effects on indirect human health are expected to be moderate but rapidly self-resolving in healthy individuals and/or effective prophylactic treatments are available.<br>• Some potential for community level effects. |
| Low | • No effects on indirect human health or effects are expected to be mild, asymptomatic, or benign in healthy individuals.<br>• Effective prophylactic treatments are available.<br>• No potential for community level effects. |

### Uncertainty related to indirect human health hazard assessment

The ranking of uncertainty associated with the indirect human health hazard assessment is presented in Table 4. Adequate information was either provided by the notifier or retrieved from other sources that confirmed the identification of the notified organisms. Adequate information was also provided describing in good detail the methods used to genetically modify the wild-type *G. ternetzi* including the sources of the genetic materials and the stability of the resulting genotypes and phenotypes. However, there were some items requiring clarification and the results from the outcrossing tests were not consistent with the expectations for BT2018 and PiT2018.

Sequence analyses of the inserted genetic material in all the lines did not match any toxins and there were no reports of adverse effects attributed to the inserted proteins in humans. While there were no reports of adverse human health effects directly associated with the notified organisms, surrogate information from the literature on other ornamental fish appear to indicate the potential for transmission of human pathogens. However, such cases of infections are common to all ornamental aquarium fish and not unique to Black Tetras.

Despite more than five years of commercially producing the different colours of fluorescent *G. ternetzi* in the United States, there are no reports of adverse human health effects.

Consequently, combining both empirical data on the organisms, surrogate information from the literature on other ornamental aquarium fish and the lack of adverse effects supported by the history of safe use in the United States, the indirect human health hazard assessment of all the notified lines is considered to be **low with low uncertainty**. The uncertainty is considered low because much of the information on human health effects are based on reports from other ornamental aquarium fish although no particular studies were found investigating human health effects associated more specifically with fluorescent transgenic ornamental fish.

*Table 4. Ranking of uncertainty associated with the indirect human health hazard.*

| Uncertainty Ranking | Description |
|---|---|
| Negligible | There are many reports of indirect human health effects related to the hazard, and the nature and severity of the reported effects are consistent (i.e., low variability); OR<br>The potential for indirect human health effects in individuals exposed to the organism has been monitored and there are no reports of effects. |
| Low | There are some reports of indirect human health effects related to the hazard, and the nature and severity of the effects are fairly consistent; OR<br>There are no reports of indirect human health effects and there are no effects related to the hazard reported for other mammals. |
| Moderate | There are some reports of indirect human health effects that may be related to the hazard, but the nature and severity of the effects are inconsistent; OR<br>There are reports of effects related to the hazard in other mammals but not in humans. |
| High | Significant knowledge gaps (e.g., there have been a few reports of effects in individuals exposed to the organism but the effects have not been attributed to the organism). |

## Risk Characterization

### Notified use

In this assessment, risk is characterized according to a paradigm embedded in section 64 of CEPA that a hazard and exposure to that hazard are both required for there to be a risk. The risk assessment conclusion is based on the hazard, and on what we can predict about exposure from the notified use.

BT2018, OT2018, PiT2018, PuT2018, and RT2018 are genetically modified tropical fish derived from a naturally-occurring albino line of the Black Tetra. Colours are the result of the introduction of expression cassettes containing a fluorescent protein derived from species of sea anemones and corals. The notified organisms will be marketed throughout Canada for use as ornamental fish in home aquariums.

Although there are reported cases of zoonotic infections from exposure to aquarium fish, the Black Tetra is a popular aquarium fish with a long history of safe use with no reported cases in the literature. Similarly, the notified lines (BT2018, OT2018, PiT2018, PuT2018, and RT2018) have been maintained as breeding lines for more than five generations and commercially produced for over four years in the U.S. with no reported adverse effects. The inserted proteins and the methods used to modify the notified lines do not present any pathogenic or toxic potential towards humans.

Owing to the low potential hazard and the low to medium potential exposure, the human health risk associated with the use of *G. ternetzi* BT2018, OT2018, PiT2018, PuT2018, and RT2018 for use as an ornamental aquarium fish is assessed to be low.

### Other potential uses

Other uses that have been identified include the use of the notified organisms in outdoor ponds, as bait fish, and in scientific research. While the notifier is discounting the possibility of some of these uses, the characteristics of the notified organisms do not exclude these possible uses. It is possible that the notified organisms may be used as a bait fish and, when temperatures are favourable, also grown in outdoor ponds as in Florida where the fish is produced. With the published patent, their use as a model research organism is possible; however, this would be done under containment and thereby limiting exposure to the general public. There are no reported cases in the literature of the notified organisms being used as an environmental sentinel but, regardless of the use, available information do not indicate a potential human health implication from any of these uses.

### Risk Assessment Conclusion

There is no evidence to suggest a risk of adverse human health effects at the exposure levels predicted for the general Canadian population from use of the notified lines as ornamental aquarium fish. This risk to human health associated with *G. ternetzi* BT2018, OT2018, PiT2018, PuT2018, and RT2018 is not suspected to meet criteria in paragraph 64(c) of CEPA. No further action is recommended.

# RISK ASSESSMENT – ENVIRONMENTAL

## Exposure Assessment - Environmental

The exposure assessment for the GloFish® Tetras addresses both its potential to enter the environment (release) and its fate once in the environment. The likelihood and magnitude of environmental exposure is determined through an extensive, cradle-to-grave assessment that details the potential for release, survival, persistence, reproduction, proliferation, and spread in the Canadian environment. Exposure ranking classification is given in Table 5, and uncertainty classification of exposure ranking given in Table 6.

*Table 5. Rankings for exposure of genetically engineered fish to the Canadian environment.*

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

*Table 6. 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 (GxE) interactions or complete understanding of GxE effects across relevant environmental conditions. Evidence of low variability. |
| Low | High-quality data on relatives of the organism or valid surrogate. Data on environmental parameters of the receiving environment. Understanding of potential GxE effects across relevant environmental conditions. Evidence of variability. |
| Moderate | Limited data on the organism, relatives of the organism or valid surrogate. Limited data on environmental parameters in the receiving environment. Knowledge gaps. Reliance on history of use or experience with populations in other geographical areas with similar or better environmental conditions than in Canada. |
| High | Significant knowledge gaps. Significant reliance on expert opinion. |

### Likelihood of Release

The stated purpose of the GloFish® Tetras is for sale in the ornamental tropical fish market, and as such they are intended to be maintained in static, indoor aquaria. However, there is abundant evidence that aquarium fish do get released to freshwater environments and that the practice of releasing aquarium fish into the environment is ongoing. 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, there is a high likelihood that GloFish® Tetras will be introduced to the Canadian environment and it is appropriate that they are considered under a scenario of full release. The extent to which the organism is further exposed to the environment will depend heavily on its ability to survive and reproduce in Canadian freshwater ecosystems. Based on typical stocking densities of tropical fish in home aquaria, the magnitude of each release event is expected to be very small, although the possibility of larger releases from larger purchases or from breeding in home aquaria cannot be excluded.

### Likelihood of Survival

As a tropical species, the Black Tetra is not expected to survive in temperate to arctic regions where water temperatures are below optimal for survival. In lower temperature tolerance trials, Leggatt et al (2018) found that no White Tetra individuals could survive below 9.5°C when temperature was lowered slowly (i.e., 1°C per day). In trials conducted by the company on all five of the notified lines, no fish survived below a temperature of 6.2°C, when temperature was dropped rapidly (i.e., 0.5-2°C/hour) and no line had improved lower temperature tolerance relative to wild-type White Tetras. Therefore, it is reasonable to conclude that GloFish® Tetras cannot survive at temperatures below 6.2°C short-term and cannot survive extended periods below 9°C. As discussed earlier, there are no freshwater systems in Canada that regularly remain above 6°C throughout the entire course of a year, and most do not remain above 4°C throughout the year. While the temperatures needed for GloFish® Tetras to survive are possible for several Canadian freshwater systems during the spring, summer and fall, it is highly unlikely they can survive the Canadian winter. Consequently, its occurrence in the environment would be seasonal or ephemeral in the majority of scenarios.

### Likelihood of Reproduction

Isolated opportunities for reproduction may occur in some freshwater systems that have temperatures in the mid-20°C for some of the summer months. Though any fertilized eggs that are not eaten as food could hatch in a relatively short period of time (24 hours), any offspring would require a minimum of 5 months to mature at optimal temperatures not seasonally supported in lakes in Canada, and consequently would not mature prior to onset of cooler temperatures, would likely not survive the winter, and likely would no longer occur until the next introduction. Though isolated opportunities for reproduction in the Canadian environment could occur, it would not result in more than a single generation presence in the environment.

### Exposure Assessment Conclusions

Given the above analysis, the occurrence of GloFish® Tetras in the Canadian environment is expected to be rare, isolated, ephemeral, and likely in low numbers. Consequently, the likelihood of exposure of the GloFish® Tetras to the Canadian environment is ranked low. It should be noted that there are localized areas where water temperatures are above typical Canadian temperatures (i.e., natural hot springs, warm water effluent sources). For such areas to allow for long-term survival and reproduction of GloFish® Tetras, they would require very specific temperature patterns (i.e., stable warm temperature in the 20°Cs throughout the year with periods of temperature from mid to high 20°Cs), as well as other specific biotic and abiotic profiles that would be suitable for the survival of tetras. Such scenarios are expected to be exceedingly rare.

### Uncertainty Associated with Exposure Assessment

The uncertainty associated with the exposure assessment is low, given the available data for GloFish® Tetras and valid surrogate organism (minimum temperature tolerance) and data available on the environmental parameters of the receiving environment in Canada (see Table 06).

## Hazard Assessment - Environmental

The hazard assessment examined potential impacts of GloFish® Tetras to environmental components. The hazard identification process considers the potential to be hazardous through environmental toxicity, horizontal gene transfer, interactions with other organisms, hybridization, and as a vector for pathogens. It also considers their potential to impact biogeochemical cycling, habitat, and biodiversity, above that expected for the unmodified organism. The Hazard rankings are described in Table 7, and uncertainty rankings for environmental hazards are described in Table 8.

*Table 7. 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 8. Ranking of uncertainty associated with the environmental hazard.*

| Uncertainty Ranking | Available Information |
|---|---|
| Negligible | High-quality data on GloFish® Tetras. 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 GloFish® Tetras or valid surrogate. Understanding of GxE effects across relevant environmental conditions. Some variability. |
| Moderate | Limited data on GloFish® Tetras, relatives of GloFish® Tetras 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. |

### Potential hazards through environmental toxicity

The potential for GloFish® Tetras to cause harm to Canadian environments through environmental toxicity is negligible. Potential routes of environmental toxicity include exposure of aquatic ecosystems to the whole animal and its waste, as well as ingestion by predators. Exposure of the fluorescent proteins to the environment is expected to be lower than exposure of the protein to the transgenic fish themselves; though different routes to exposure are not necessarily comparable.

The expressed transgenic proteins are modified from naturally occurring fluorescent proteins that are common in many marine organisms including fishes. Fluorescent proteins are commonly used as neutral markers in research in a wide range of organisms with almost no reports of toxicity. The few reports of negative effects are generally specific to transgenic organisms with especially high expression of fluorescent transgenes. Of the transgenes used, only one has been reported in alternate forms to have toxic effects or physiological alterations in mice (Chen et al. 2016; Chou et al. 2015). 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. There are no noted major effects to the notified organisms, other than inconsistent diminished cold tolerance and reproductive success in competition. Each of the five notifications 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 four or five years of commercial production in the US, there have been no reported toxic effects resulting from exposure to GloFish® Tetras. Consequently, the potential hazard to the environment due to environmental toxicity of the GloFish® Tetras is 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.

### Potential hazards through horizontal gene transfer

Horizontal gene transfer (HGT) is the non-sexual exchange of genetic material between organisms of the same or different species (DFO 2006). Horizontal gene transfer is a rare event among eukaryotes, often measured on an evolutionary time frame, but is much more frequent among prokaryotes (EFSA 2013). In order for HGT of a specified transgene to take place on a biologically relevant scale, the following steps must occur: exposure and uptake of the free transgene to a novel organism, stability and expression of the gene within the novel organism, and neutral or positive selection of the novel organism expressing the transferred gene (DFO 2006). Finally, expression of the transferred gene must have potential to cause harmful effects to the environment in order to constitute a hazard.

The potential for GloFish® Tetras to cause harm to Canadian environments through horizontal gene transfer (HGT) is low. Exposure to prokaryotes with capacity for horizontal gene transfer is expected through release of free DNA from the notified organisms via release of mucus, skin cells, gametes, feces, etc. to the natural environment. The insert sequences of the different transgenes do not include any transposable or mobile elements that may enhance HGT and the transgenes are not expected to have any increased uptake beyond that of the DNA from any wild-type tetra. In general, eukaryotic promoter sequences have minimal activity in prokaryotic hosts, suggesting expression of the transgene in a novel prokaryote is not expected to occur, though the potential rearrangement and consequent expression of the novel DNA by the prokaryote cannot be discounted. Consequently, the potential for gene expression in a new prokaryotic host cannot be disqualified. Genes coding for fluorescent proteins have been introduced to a wide range of organisms and the vast majority report no harmful effects of the introduced fluorescent transgene. As such, the hazard rating of GloFish® Tetras via horizontal gene transfer is low. There is a low uncertainty with the ranking due to lack of data describing the insert site(s) of the transgene(s) within each line, and reliance on surrogate data for impacts, should HGT occur.

**Potential hazards through interactions with other organisms**

The GloFish® Tetras are expected to pose negligible hazards through trophic interactions with other species. Due to the carnivorous nature of Black Tetra, they have potential to impact small prey organisms or through competition with other small predators occupying similar niches. Black Tetras are not known to be voracious eaters, do not overeat (Frank 1980), are not reported to be highly aggressive towards other species, and are not expected to have any greater impact on prey populations than other small native fish species. In five years of commercial use in the ornamental aquarium trade in the US, there are no known reports, anecdotal or otherwise, of different activity levels or behaviour than non-transgenic *G. ternetzi* that may influence competitor or predator success.

There is a potential to impact native predator populations as prey, by providing a new food source, or causing deleterious effects to predator populations through ingestion. Effects of the former are anticipated to be extremely low, and the latter is not expected, as GloFish® Tetras are not predicted to be environmentally toxic (see above). One study found RFP Zebrafish were more aggressive and less preyed upon than wild-caught Zebrafish, but this was not observed in other studies, and the influence of genetic background and rearing history on results have not been examined. Whether RFP studies may also be applied to GloFish® Tetras aggression and predation vulnerability is not known.

Wild-type tetras are reported to decrease activity below 17°C, and cease activity around 10.5°C (Leggatt et al. 2018). The decreased activity with decreasing temperature may increase predation susceptibility and decrease competitive and predation ability in non-summer months. Overall, this indicated Black Tetra would pose negligible hazard to Canadian environments through trophic interactions, and GloFish® Tetras would not have increased hazard above that of the wild type. This ranking, however, has a moderate level of uncertainty, due to the level of studies directly examining the hazards of GloFish® Tetras, limited understanding of GxE interactions (the differential phenotypic plasticity between genotypes among relevant environments), and limited understanding of how RFP Zebrafish models apply to GloFish® Tetras.

**Potential hazards through hybridization with native species**

The GloFish® Tetras are expected to pose negligible hazards through hybridization with other species. The Black Tetra belongs to the Family Characidae, that have a geographical distribution of South and Central America, and North America as far north as southwestern US (Oliveira et al. 2011). The lack of native characids in Canada indicates that there is no potential to impact native Canadian species through hybridization. There is negligible uncertainty associated with this rating.

**Potential hazards as a vector for disease agents**

Though disease agents are common in tropical freshwater ornamental aquarium fishes, Black Tetra are not listed by the Canadian Food Inspection Agency as a species that has known susceptibility to reportable diseases in Canada, and have not been implicated as vectors for disease agents of concern in Canada. Disease agents associated with the GloFish® Tetras are expected to be tropical in origin and would have limited ability to persist within the colder Canadian freshwater environments.

Whether GloFish® Tetras, or any other transgenic fluorescent organism, have altered ability to act as a vector of disease agents has not been examined and no studies have examined fluorescent protein effects in complex natural conditions. GloFish LLC has provided expert opinion statements from veterinarians working with the company that state no evidence for increased susceptibility to, or transmission of, water-borne pathogens for any of the five GloFish® Tetra lines has been observed. Howard et al. (2015) reported no differences in survival between RFP transgenic and wild-type Zebrafish in 18 populations over 15 generations. In Zebrafish and other model research organisms, fluorescent protein transgenes have been used extensively in research with no known reported effects on disease susceptibility. Consequently, the GloFish® Tetras are concluded to have negligible potential to harm Canadian environments as a vector of disease. Since the capacity of GloFish® Tetras to act as a vector for disease agents has not been directly examined, and there is some reliance on indirect evidence and expert opinion, the associated uncertainty is ranked moderate.

**Potential hazards to biogeochemical cycling**

The potential effects of fluorescent protein expression on fish metabolism, and hence nutrient cycling above that of wild-type tetra, 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). Whether GloFish® Tetras have similar influences from fluorescent transgenic gene expression are unknown, but their small size and potential numbers of individuals in an ecosystem suggests there will be negligible hazard to biogeochemical cycling regardless of altered metabolic pathways. This ranking has a moderate level of uncertainty due to the limited level of studies examining this hazard.

**Potential hazards to habitat**

Black Tetra is a small fish with negligible potential to harm habitat structure. Black Tetras spawn in open water and do not build nests or other structures that may impact habitats of other species. GloFish® Tetras have been in commercial use in the ornamental aquarium trade in the US since 2013 (OT2018, Pi2018 and Pu2018) and 2014 (BT2018 and RT2018), and there have been no reports, anecdotal or otherwise, of altered behaviour relative to Black Tetra that may influence habitat structure. Consequently, GloFish® Tetras are expected to have negligible impacts on habitat, though uncertainty associated with this rating is low due to limited availability of data across different environments.

**Potential hazards to biodiversity**

Biodiversity can be negatively impacted by numerous drivers including invasive species or the introduction of disease. While the invasiveness of GloFish® Tetras has not been directly assessed, there are no reports of Black Tetra becoming invasive or causing harm to aquatic ecosystems, despite its common use and reports of it entering the environment. Hill et al. (2014) concluded a lack of invasion potential in the US of fluorescent *G. ternetzi* using the Fish Invasiveness Screening Kit (FISK), a risk identification (screening) tool for freshwater fishes. In addition, the diminished cold tolerance and reproductive success observed in the GloFish® Tetras may diminish invasive potential relative to wild-type.

As noted above, GloFish® Tetras are not expected to harm native species through HGT, trophic interactions or hybridization, act as a vector for disease agents of concern in Canada, or impact biogeochemical cycling or habitat. Consequently, there is negligible potential to impact biodiversity. Reliance on data from the comparator species (i.e., lack of invasiveness and biodiversity effects in Black Tetra) results in a low degree of uncertainty associated with this ranking.

### Hazard Assessment Conclusions

The Black Tetra is a small, non-aggressive fish with expected limited activity due to low temperatures in most seasons in Canada, is not known to be susceptible to diseases of concern in Canada, and has no history of invasiveness in Canada and worldwide despite its wide use. As such, Black Tetra is not expected to pose hazards to Canadian environments. Available evidence does not suggest environmental hazards will arise as a result of the fluorescent phenotype or non-targeted effects in GloFish® Tetras. The majority of individual hazards assessed have negligible hazard ranking as no effects are expected beyond that of wild-type fish (see Table 9). The one exception is a low rating for impacts through horizontal gene transfer, as an effect could potentially occur (i.e., introduction of a fluorescent protein gene to prokaryotes), but this effect is not expected to be harmful (see Table 7). Overall, the notified organism is not anticipated to cause detrimental impacts to the structure or function of Canadian ecosystems beyond natural fluctuations.

### Uncertainty Associated with Hazard Assessments

The uncertainty rating associated with the individual hazard classifications range from negligible to moderate (see Table 9), due to limited data specific to the GloFish® Tetras, limited direct data on the comparator species, variable data from a surrogate model (RFP Zebrafish), and the reliance on expert opinion for the assessment of some hazards.

*Table 9. Summary of hazard rank and uncertainty of GloFish® Tetras to Canadian environments*

| Hazard | Rank | Uncertainty |
|---|---|---|
| Through Environmental Toxicity | Negligible | Moderate |
| Through Horizontal Gene Transfer | Low | Low |
| Through Trophic Interactions | Negligible | Moderate |
| Through Hybridization | Negligible | Negligible |
| As a Vector of Disease | Negligible | Moderate |
| To Biogeochemical Cycling | Negligible | Moderate |
| To Habitat | Negligible | Low |
| To Biodiversity | Negligible | Low |

## Environmental Risk Assessment

An overall conclusion on Risk is based on the classic paradigm where: Risk $\propto$ Hazard x Exposure. Overall Risk is estimated by plotting overall Hazard against Exposure, using a risk matrix or heat map, as illustrated in Figure 3. The matrix can be used as a tool for facilitating communication and discussion on risk. The uncertainty associated with risk is discussed in the context of uncertainty in the hazard and exposure assessments.

The exposure assessment concluded that GloFish® Tetras used in the ornamental aquarium trade in Canada would have low likelihood for occurrence in the Canadian environment. This is due to the high likelihood of release of small numbers of fish from home aquaria, but negligible likelihood for GloFish® Tetras to overwinter in Canadian water systems. As such, any exposure of Canadian freshwater aquatic ecosystems is expected to be isolated, rare and ephemeral.

Uncertainty is low given the quality of data demonstrating diminished cold tolerance in GloFish® Tetras, relative to typical Canadian winter freshwater temperatures.

The hazard assessment concluded that GloFish® Tetras pose negligible to low hazard to the Canadian environment, due to lack of hazards associated with the base Black Tetra species, and no direct evidence that the expressed fluorescent proteins would increase hazards relative to wild-type Black Tetra. Uncertainty ranking associated with individual hazard components ranged from negligible to moderate (see Table 9).

Using the risk matrix in Figure 2, GloFish® Tetras used in the ornamental aquarium trade in Canada pose low risk to Canadian environments (Low Exposure x Negligible/Low Hazard = Low Risk). The use of GloFish® Tetras for the ornamental aquarium trade in Canada is not expected to cause harmful effects to Canadian environments as a result of exposure to the notified organisms.

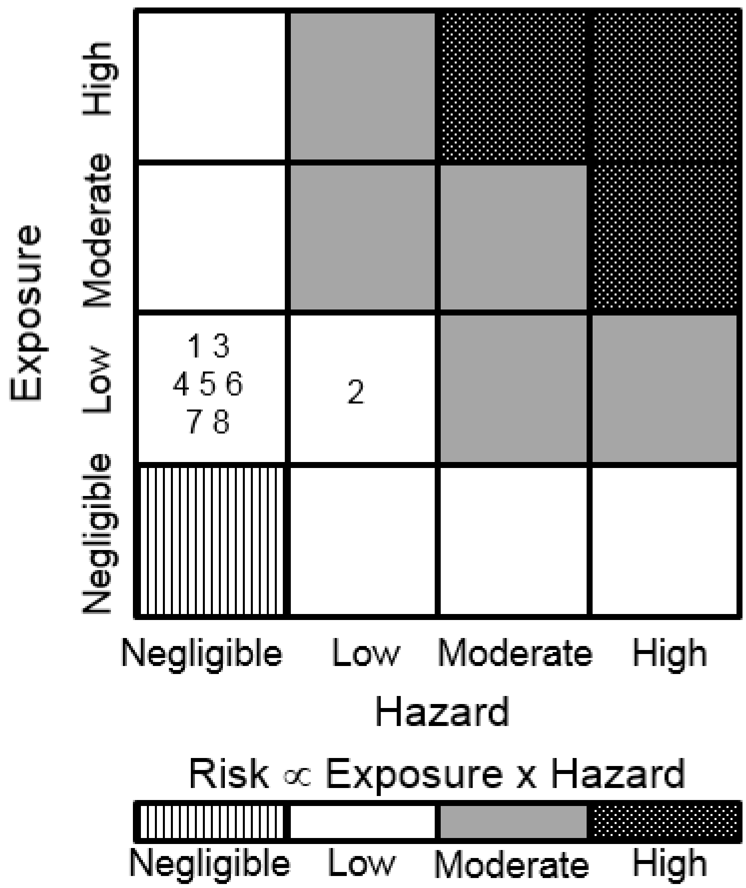


*Figure 2. Risk matrix and colour scale to illustrate how exposure and hazard are integrated to establish a level of risk in the environmental risk assessment. Risk assessments associated with assessed hazard components at the assessed exposure are identified by number: 1) through environmental toxicity;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

## Sources of Uncertainty

Sources of uncertainty in the indirect human health exposure and hazard assessment include limited information on actual market uptake and other potential uses in Canada, reliance on reports from surrogate models, and no particular studies that have investigated human health effects associated with fluorescent transgenic ornamental fish.

Sources of uncertainty in the environmental exposure and hazard assessment include reliance on data directly addressing hazards of the notified organism and comparator species, variability in data taken from surrogate organisms and level of understanding of applicability to the notified organism (e.g., trophic interactions), and some reliance on expert opinion in some hazard assessments (e.g., impacts through vector of disease agents).

Though sources and levels of uncertainty may vary among hazard and exposure rankings, the reported levels of uncertainty are not expected to affect the overall risk estimate.

## CONCLUSIONS AND ADVICE

The import of GloFish® Tetras into Canada, for use in the ornamental aquarium trade and home aquaria, is expected to pose a low risk to human health (including a consideration of vulnerable populations) and the Canadian environment. While uncertainty associated with some exposure and hazard classifications is moderate due to limited direct data on the notified organism or comparator species, there is no evidence to suggest GloFish® Tetras under the proposed use, or other potential uses, could cause harm as a result of exposure to Canadian populations or environments.

## LIST OF MEETING PARTICIPANTS

| Name | Affiliation |
|---|---|
| Gilles Olivier | Chair; Fisheries and Oceans Canada |
| Jay Parsons | Fisheries and Oceans Canada |
| Sophie Foster | Fisheries and Oceans Canada |
| Colin McGowan (Co-author) | Fisheries and Oceans Canada |
| Rosalind Leggatt (Co-author) | Fisheries and Oceans Canada |
| Stephen Dugan (Co-author) | Health Canada |
| Kassim Ali (Co-author) | Health Canada |
| George Arvanitakis | Health Canada |
| Arash Shahsavarani | Environment and Climate Change Canada |
| Jim Louter | Environment and Climate Change Canada |
| Zeina Saikali | Environment and Climate Change Canada |
| Bob Devlin | Fisheries and Oceans Canada |
| Marten Koops | Fisheries and Oceans Canada |
| Anne-Margaret MacKinnon | Fisheries and Oceans Canada |
| Sherry Walker | Fisheries and Oceans Canada |
| Shauna Baillie | Fisheries and Oceans Canada |
| Sylvia Han | Fisheries and Oceans Canada |
| Andrea Raynor Hentschel | Fisheries and Oceans Canada |
| Stephanie McKay | University of Ottawa |
| Marie Breton | Environment and Climate Change Canada |

## SOURCES OF INFORMATION

This Science Advisory Report is from the July 17 -18, 2018 peer-review meeting, Environmental and Indirect Human Health Risk Assessment of GloFish® Sunburst Orange®, Starfire Red®, Galactic Purple®, Cosmic Blue® and Moonrise Pink® Tetras: Transgenic Ornamental Fish. Additional publications from this meeting will be posted on the Fisheries and Oceans Canada (DFO) Science Advisory Schedule as they become available.

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ISSN 1919-5087


Correct Citation for this Publication:

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

*Aussi disponible en français :*

*MPO. 2019. Évaluation des risques pour l'environnement et des risques indirects pour la santé humaine posés par les tétras glofishmd (gymnocorymbus ternetzi) : cinq lignées de poissons d'ornement transgéniques. Secr. can. de consult. sci. du MPO, Avis sci. 2019/002.*