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ENVIRONMENTAL AND INDIRECT HUMAN HEALTH RISK ASSESSMENT OF THE GLOFISH® ELECTRIC GREEN® TETRA AND THE GLOFISH® LONG-FIN ELECTRIC GREEN® TETRA (*GYMNOCORYMBUS TERNETZI*): A TRANSGENIC ORNAMENTAL FISH

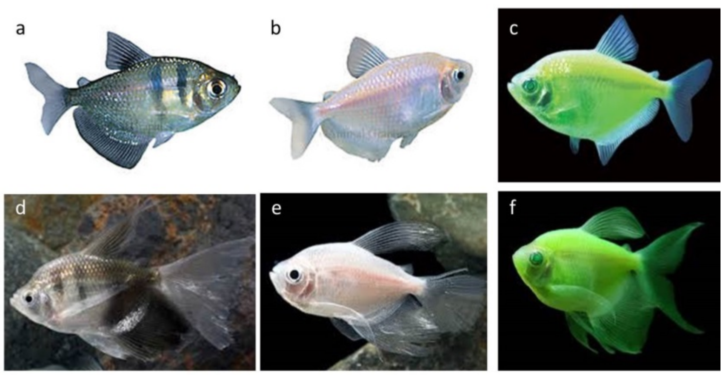

Figure 1. Some variants of Gymnocorymbus ternetzi *available in the ornamental pet trade worldwide (a, b, d, e), and notified transgenic variants (c, f). Wild-type Black Tetra (a, d), White Tetra (b, e) and CGT2016 Electric Green® tetra (c, f) showing normal and long-fin variants, respectively. Taken from commercial websites www.petsmart.com (a, b), www.glofish.com (c, f) and www.segrestfarms.com (d, e).*

Context:

The biotechnology provisions of the Canadian Environmental Protection Act (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" or capable of becoming "toxic". Environment and Climate Change Canada (ECCC) and Health Canada (HC) are mandated to conduct all risk assessments under CEPA.

*On July 5, 2017 a notification under the NSNR(O) was submitted by GloFish LLC to ECCC for the GloFish® Electric Green® Tetra (CGT2016), a genetically engineered white variant of the Black Tetra (*Gymnocorymbus ternetzi*).*

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

This Science Advisory Report is from the September 12-14, 2017 "Environmental and Indirect Human Health Risk Assessments of the GloFish® Electric Green® Tetra and the GloFish® Long-fin Electric Green® Tetra: a 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.

SUMMARY

- 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 a genetically engineered *Gymnocorymbus ternetzi* (GloFish® Electric Green® Tetra (CGT2016)).
- Environmental and indirect human health risk assessments were conducted that included an analysis of potential hazards, likelihoods 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 CGT2016 is **low to medium** as its 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 in the Canadian market.
- The IHH hazard assessment concluded that the indirect human hazard potential of CGT2016 is **low** as the source organisms for the inserted genetic materials are not pathogenic, there are no reported cases of zoonotic infections associated with the CGT2016 or the wild type, and based on the sequence identity and the structure of the inserted transgenes, the production of allergens or toxins is not anticipated.
- Uncertainty associated with the IHH hazard assessment is **low** based on available data on the organism, information from the literature on the unmodified *G. ternetzi* and other ornamental aquarium fishes, and the lack of adverse effects supported by the history of safe use of CGT2016 in the United States and the use of unmodified *G. ternetzi* in Canada and other countries.
- There is a **low** risk of adverse indirect human health effects at the exposure levels predicted for the Canadian population from the use of CGT2016 as an ornamental aquarium fish or other potential uses.

Environmental Risk Assessment

- The **environmental** exposure assessment concluded that the occurrence of CGT2016 in the Canadian environment, outside of aquaria, is expected to be rare, isolated and ephemeral due to their inability to survive typical low winter temperatures in Canada's freshwater environments. Consequently, the likelihood of exposure of CGT2016 to the Canadian environment is ranked **low**.
- The uncertainty associated with this environmental exposure estimation is **low**, given the available data for temperature tolerance of CGT2016 and wild type tetras.
- The environmental hazard assessment concluded **negligible** hazards of CGT2016 through environmental toxicity, interactions with other organisms, hybridization or as a vector for disease, as well as to biodiversity, biogeochemical cycling, and habitat. There

is **low** hazard of CGT2016 through 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 CGT2016, the inconsistent results from studies on other fluorescent transgenic organisms, and the reliance on expert opinion.
- There is **low** risk of adverse environmental effects at the exposure levels predicted for the Canadian environment from the use of CGT2016 as an ornamental aquarium fish or other potential uses.

Conclusions

- The overall assessment of the use of CGT2016 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.

BACKGROUND

Characterization of the notified organism

CGT2016 is a genetically engineered White Tetra (*Gymnocorymbus ternetzi*) possessing two transgenes at a single site of insertion that result in ubiquitous fluorescent green colouration of the organism under white, ultraviolet, or blue light. The purpose of this modification is to create a new colour phenotype of *G. ternetzi* for the ornamental aquarium trade.

The transgene expression construct was injected into newly fertilized eggs of White Tetra, a wild-type white variant of the Black Tetra (*G. ternetzi*, hereafter referred to as White Tetra). In subsequent generations a single insert copy for the construct was confirmed by quantitative PCR against a standard curve, and a single insertion site was confirmed by Southern blot analysis. Segregation of the transgene when bred with wild-type fish was also consistent with a single site of insertion. Greater detail regarding the structure, development, and function of the transgene construct has been provided by the company for the expressed purpose of the current risk assessment and review, but is identified as confidential business information and is not included in this report.

Individual CGT2016 may be hemizygous (i.e., have a single copy of the inserted transgene construct at a locus) or homozygous (i.e., have two copies at that same locus), with both genotypes having identical green phenotypes.

CGT2016 has been in commercial production for the ornamental aquarium trade in the US excluding California since 2012, and in California since 2015. CGT2016 are manufactured for GloFish LLC by two aquarium fish producers in Florida, USA.

Phenotypic Effects of the Modification

The targeted phenotypic effect of the genetic modification is that CGT2016 appears green under ambient light to create a new, bright colour variant for the ornamental aquarium trade. The novel colour phenotype is present in the muscle, as well as skin and eye. The universal nature of the promoter suggests expression may result in green colouration of internal organs, though this has not been specifically reported.

Non-targeted phenotypic effects of the modification

Two non-targeted phenotypic effects have been identified by GloFish LLC in CGT2016: diminished tolerance to low temperature, and decreased competitive reproductive success relative to wild-type siblings. While these changes are slight, and are not expected to impact the organism's fitness in home aquaria, they may have negative impacts on the organism's ability to survive and reproduce in the Canadian environment.

GloFish LLC provided data demonstrating that green fluorescent transgenic tetras do not differ from White Tetras in embryo or juvenile survival. A test of reproductive success in competition with one each of male green transgenic CGT2016, female green transgenic CGT2016, male White Tetra, and female White Tetra indicated that CGT2016 have no reproductive advantage when in competition with wild-type White Tetras.

Though no formal studies have compared potential disease susceptibility of CGT2016 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 CGT2016 or any other commercially available fluorescent line relative to non-transgenic counterparts, and that CGT2016 require the same husbandry care as non-transgenic counterparts. No other studies have examined the phenotype of CGT2016, including potential effects on other ecosystem members in Canadian receiving environments.

Fluorescent protein transgenes in other models

While the gene used in the construct is rarely used as a transgene in other organisms, other fluorescent proteins including eGFP have widespread use in research in a variety of organisms, and some relevant research has been done on various lines of another small tropical fish (Zebrafish, *Danio rerio*) transgenic for red fluorescent protein (RFP) and other fluorescent proteins. Most, but not all, RFP and eGFP Zebrafish lines are slightly less tolerant to extreme cold or heat than wild type (Cortemeglia and Beitinger 2005, 2006a; Leggatt et al. 2018). Most fluorescent transgenic Zebrafish lines have no reported impact on survival, and 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, although a few mice 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). While there are some reports of risk-related alterations in fluorescent transgenic Zebrafish models, there are no consistent 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 CGT2016, were used as a comparator 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 USA. Suggested ideal temperature requirements of Black Tetra in the home aquarium range from 22-26°C, and 27-29°C for reproduction. 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 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, see Leggatt et al. 2018). 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, and climate change may alter long-term temperature patterns in systems. While this temperature heterogeneity is not well characterized or predicted in such systems, it should be considered during risk assessments.

RISK ASSESSMENT – INDIRECT HUMAN HEALTH

Exposure Assessment - Indirect Human Health

Import

Fish will enter Canada through one of four points of entry: Vancouver, BC; Calgary, AB; Toronto, ON; and Montreal, QC. Broodstock are maintained at two separate farms in Florida, where all production 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. CGT2016 will be delivered to retailers in the quantity ordered, where they will be held until sold.

Introduction of the organism

CGT2016 will be marketed at retail outlets where ornamental aquarium fish are sold. The exact number and locations where the notified organism 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 organism. Exposure to CGT2016 by home aquarists that purchase it will most likely be limited to maintenance activities such as water changes and tank cleanings.

Environmental Fate

CGT2106 is not intended for environmental release and is intended to be confined to aquaria 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 considering that no cases of environmental establishment have been reported in the United States where fluorescent *G. ternetzi* have been commercially marketed as an aquarium fish in areas having higher winter minimum temperatures than typical Canadian winter temperatures (see Table 1 and Environmental Exposure Assessment below). If live or dead CGT2016 are released into the environment, it is expected that both CGT2016 and the fluorescent protein would biodegrade normally, and not bioaccumulate or be involved in biogeochemical cycling in a form different from other living organisms.

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

Exposure Ranking	Considerations
High	• The release quantity, duration and/or frequency are high. • The organism is likely to survive, persist, disperse, proliferate and become established in the environment. • Dispersal or transport to other environmental compartments is likely. • The nature of release makes it likely that susceptible organisms or ecosystems will be exposed and/or that releases will extend beyond a region or single ecosystem. • In relation to exposed organisms, routes of exposure are permissive of toxic, zoonotic or other adverse effects in susceptible organisms.

Exposure Ranking	Considerations
Medium	• It is released into the environment, but quantity, duration and/or frequency of release is moderate. • It may persist in the environment, but in low numbers. • The potential for dispersal/transport is limited. • The nature of release and/or use of the organism may result in some exposure to humans that are of intermittent frequency and/or short duration. • In relation to exposed organisms, routes of exposure are not expected to favour toxic, zoonotic or other adverse effects.
Low	• It is used in containment (no authorized or planned intentional release). • The nature of release and/or the biology of the organism are expected to contain the organism such that susceptible populations or ecosystems are not exposed. • Low quantity, duration and frequency of release of organisms that are not expected to survive, persist, disperse or proliferate in the environment where released.

Exposure Characterization

Indirect human health risk assessment looks at potential for CGT2016 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). Risks from workplace exposure to the notified organism are not considered in this assessment[1].

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 CGT2016 is assessed to be low to medium because:

1. The primary source of CGT2016 in Canada is the import fish from the United States;
2. It 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 exposure to the general public primarily to those that possess a home aquarium, which may include immunosuppressed individuals; and
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.

[1] 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.

Uncertainty related to indirect human health exposure assessment

The ranking system 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. There are data on the inability to survive in typical winter water temperatures for both CGT2016 and the wild-type *G. ternetzi.* Human exposure (for general public and immunocompromised individuals) in Canada is expected to occur through home aquaria mainly from maintenance and cleaning activities. The actual number of CGT2016 fish to be imported in the following years is not known at this point. Therefore, because of limited information on exposure scenarios in the Canadian market, the human exposure to CGT2016 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

In-house literature searches found no reports of zoonoses attributed to CGT2016 or to the wild-type *G. ternetzi*, and the notifier provided veterinarian statements that CGT2016 possesses no observed increased susceptibility to pathogens or zoonotic risk compared to non-modified tetras. However, there are reported cases of zoonotic infections from contact with tropical ornamental fish and their housing water (CDC 2015; Gauthier 2015; 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*, and the majority of reported zoonotic cases associated with tropical fish are associated with immunocompromised individuals (Baiano and Barnes 2009; CDC 2015; Gauthier 2015; Lowry and Smith 2007; Roberts et al. 2009). Zoonotic infections from handling of live or recently killed infected fish, which can primarily occur through puncture, cuts, scrapes, abrasions or sores in the skin, can result in cellulitis of the hand or endocarditis, meningitis, and arthritis in severe systemic infections (Boylan 2011). Infections may be prevented through wearing gloves when handling fish or cleaning fish tanks and avoiding contact with any potentially contaminated water for individuals with any open skin wounds (CDC 2015). Washing hands with soap and water after contact with aquarium water is also highly recommended (CDC 2015). As well, people with compromised immune systems or underlying medical conditions should avoid cleaning tanks or handling fish (Haenen et al. 2013).

Allergencity/Toxicity

In-house amino acid sequence analyses of the inserted fluorescent protein using the AllergenOnline Database (v17; January 18, 2017,) found no matches with greater than 35% identity for both 80 and 8 amino acid segments. Basic Local Alignment Search Tool (BLAST) searches on the nucleotide and amino acid sequences of the inserted protein did not detect any homologies to known toxins. One study observed no adverse effects in male rats fed pure green fluorescent protein (GFP) or canola expressing GFP for 26 days (Richards et al. 2003). In-house literature searches found no other reports of adverse effects attributed to the inserted protein in humans.

History of Use

CGT2016 has been commercially marketed as an aquarium fish throughout the United States except California since 2012 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 health effects in humans.

Hazard Characterization

The ranking system used to determine indirect human hazards from the notified organism is given in Table 3, and uncertainty classification associated with hazard ranking given in Table 4. The indirect human hazard potential of CGT2016 is assessed to be **low** because:

1. CGT2016 is a genetically modified tropical fish containing a single inserted construct that was confirmed to be stably integrated through qPCR and multiple crossings;
2. The methods used to produce the notified living organism do not raise any indirect human health concerns and the source organisms of the inserted genetic material are not pathogenic;
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 organism or the wild-type, and no evidence that the notified organism may have higher vector capabilities than wild-type;
4. Sequence identities of the inserted transgene or any potentially expressed proteins from the construct do not match any known allergens or toxins; and
5. There is a safe history of use for the notified line 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. • Prophylactic treatments are not available or are of limited benefit. • 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. • 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. • Effective prophylactic treatments are available. • No potential for community level effects.

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 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 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 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).

Uncertainty related to indirect human health hazard assessment

The ranking system 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 organism, and the method used to genetically modify the wild-type G. ternetzi including the source of genetic materials. Surrogate information from the literature was used from other ornamental fish on the potential for transmission of human pathogens, and anecdotal and expert opinion used for history of adverse indirect human health effects of CGT2016. Consequently, the uncertainty with the low hazard rating is assessed at low since much of the information is based on reports from other ornamental aquarium fish and the fact that, apart from the single study that found no adverse effects in male rats fed pure GFP (Richards et al. 2003), there are no particular studies that have investigated indirect human health effects associated with fluorescent transgenic ornamental fish.

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 can be predicted about exposure from the notified use.

CGT2016 is a genetically modified green-coloured tropical fish derived from a naturally-occurring white variant line of the Black Tetra. The green colour is the result of the introduction of an expression cassette. The notified organism will be marketed throughout Canada for use as an ornamental fish in home aquaria.

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, CGT2016 has been marketed in the U.S. since 2012 with no reported adverse effects. The proteins encoded by the inserted genes and the methods used to modify CGT2016 do not present any pathogenic or toxic potential towards humans.

Owing to the low potential hazard and the low to medium potential exposure, the indirect human health risk associated with the use of CGT2016 for use as an ornamental aquarium fish is assessed to be low.

Other potential uses

The sole intended use for the notified organism is as an ornamental fish for interior home aquaria, and the notifier does not support any uses of the notified organism outside that of being an indoor ornamental aquarium fish. However, the use for other purposes (e.g., in outdoor ponds, as a bait fish, for scientific research) cannot be discounted. Should other potential non-intended uses occur, no additional risks to indirect human health are foreseen that are different from those of any other typical aquarium fish.

Risk Assessment Conclusion

The available evidence suggests low risk of adverse indirect human health effects at the exposure levels predicted for the general Canadian population from use as an ornamental aquarium fish or other potential uses.

RISK ASSESSMENT – ENVIRONMENTAL

Exposure Assessment - Environmental

The exposure assessment for CGT2016 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 organism is for sale in the ornamental tropical fish market, and as such is 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 CGT2016 will be introduced to the Canadian environment and it is appropriate for CGT2016 to be 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. The magnitude of each release event is expected to be very small, although the possibility of larger releases from larger purchases or breeding CGT2016 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. Temperature trials found no fish survived below 9.5°C when temperature was lowered slowly (i.e., 1°C per day, Leggatt et al. 2018), and the notifying company provided data demonstrating CGT2016 and White Tetra had similar cold tolerance (i.e. mid 7°C) when temperature was dropped rapidly. Therefore, it is reasonable to conclude that White Tetra and CGT2016 cannot survive at temperatures below 7°C 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. Consequently, while the temperatures needed for CGT2016 to survive are possible for several Canadian freshwater systems during the spring, summer and fall, it is highly unlikely that CGT2016 can survive the Canadian winter. 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 CGT2016 in the Canadian environment is expected to be rare, isolated, ephemeral, and likely in low numbers. Consequently, the likelihood of exposure of CGT2016 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 CGT2016, 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). 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 CGT2016 and valid surrogate organism (minimum temperature tolerance), evidence of low variability, and data available on the environmental parameters of the receiving environment in Canada (see Table 6).

Hazard Assessment - Environmental

The hazard assessment examined potential impacts of CGT2016 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, and potential to impact biogeochemical cycling, habitat, and biodiversity, above that expected for the unmodified organism. The Hazard rating follows Table 7, and uncertainty for each hazard ranking follows 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 CGT2016. 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 CGT2016 or valid surrogate. Understanding of GxE effects across relevant environmental conditions. Some variability.

Moderate	Limited data on CGT2016, relatives of CGT2016 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 CGT2016 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 of CGT2016 by predators. Exposure of the fluorescent protein to the environment is expected to be lower than exposure of the protein to CGT2016 itself; though different routes to exposure are not necessarily comparable. The expressed transgenic protein is modified from a naturally occurring fluorescent protein. Fluorescent proteins are common in many marine organisms including fishes. There are no noted effects to the notified organism, other than slightly diminished cold tolerance and slightly diminished reproductive success in competition. As well, fluorescent proteins are commonly used as neutral markers in research in a wide range of organisms with almost no reports of toxicity. Of the few reports of negative effects, they are generally specific to transgenic organisms that have high expression of fluorescent transgenes.

The Notification of CGT2016 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. While the potential toxicity through ingestion of the fluorescent protein has not been specifically examined, another green fluorescent protein (GFP) is reported to have no toxic or allergenic effects when fed to rats, and was rapidly degraded in simulated gastric digestion (Richards et al. 2003), suggesting a lack of toxicity or persistence after consumption of fluorescent proteins. This, combined with no reported toxic effects of ubiquitous expression of the fluorescent protein to the CGT2016 organism despite 5 years of commercial production within the US, supports negligible hazard to the environment due to environmental toxicity of CGT2016. The uncertainty associated with this ranking is moderate due to limited direct data from the notified organism or surrogate organisms, but ample indirect evidence from other fluorescent protein models.

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 CGT2016 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 organism via release of mucus, skin cells, gametes, feces, etc. in waste water, disposal of dead CGT2016, and release of CGT2016 to the natural environment. The insert sequence of the CGT2016 transgene does not include any transposable or mobile elements that may enhance HGT. It is not known whether the construct has inserted near native transposable elements in the wild-type fish. However, the transgene is 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. However, the potential rearrangement and consequent expression of the novel DNA by the prokaryote cannot be discounted. Consequently, the potential for the construct to be expressed in a new prokaryotic host cannot be excluded. However, 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 CGT2016 via horizontal gene transfer is low. There is a low uncertainty with the ranking due to lack of data describing the insert site of the transgene within CGT2016, and reliance on surrogate data on impacts should HGT occur.

Potential hazards through interactions with other organisms

CGT2016 is 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 5 years of commercial use in the ornamental aquarium trade in the US, there are no known reports, anecdotal or otherwise, of CGT2016 having different activity levels or behaviour than non-transgenic *G. ternetzi* that may influence competitor or predator success.

Released CGT2016 also have 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 CGT2016 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, although this was not observed in other studies, and the influence of genetic background and rearing history on results have not be examined. Whether RFP studies may also be applied to CGT2016 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 CGT2016 would not have increased hazard above that of wild type. However, this ranking has a moderate level of uncertainty, due to a lack of studies directly examining hazards of CGT2016, lack of understanding of GxE interactions and variability in RFP Zebrafish studies, and lack of understanding of applicability of RFP Zebrafish models to CGT2016.

Potential hazards through hybridization with native species

CGT2016 is 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 for CGT2016 to impact native Canadian species through hybridization. There is negligible uncertainty associated with this rating.

Potential hazards as a vector of disease agents

CGT2016 has negligible potential to harm Canadian environments as a vector of disease. Although disease agents are common in tropical origin freshwater ornamental aquarium fishes, Black Tetra are not listed by the Canadian Food Inspection Agency as a species that is susceptible to diseases of significant importance to aquatic animal health and the Canadian economy, and have not been implicated as vectors for disease agents of concern in Canada. Any disease agents CGT2016 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 CGT2016 once released to cooler Canadian freshwater environments.

Whether CGT2016, or any transgenic fluorescent organism, may have altered ability to act as a vector of disease agents has not been examined. Susceptibility to disease may influence vector capabilities, although this has also not been directly examined in any fish model. GloFish LLC provided statements from veterinarians working with the company that state no evidence for increased susceptibility to, or transmission of, water-borne pathogens, or additional health impediments of CGT2016 or any other commercial fluorescent line relative to non-transgenic counterparts. 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, although some cell line studies have reported alterations in immune-related genes (e.g., Mak et al. 2007). No studies have examined fluorescent protein effects in complex simulated natural conditions.

As vector capabilities of CGT2016 have not been directly examined, there is some reliance on indirect evidence and expert opinion, the uncertainty level for this negligible hazard rating is moderate.

Potential hazards to biogeochemical cycling

CGT2016 has negligible potential to harm biogeochemical cycling in natural environments. This is due to the small size and lack of polluting capacity of Black Tetra in an aquarium setting. The potential effects of fluorescent protein on CGT2016 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). What impacts these changes may have on biogeochemical cycling should CGT2016 have similar influences from fluorescent transgenic gene expression are unknown, but the small size of CGT2016 suggests there will be negligible hazard to biogeochemical cycling even with altered metabolic pathways. This ranking has a moderate level of uncertainty due to lack of studies directly 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. CGT2016 has been in commercial use in the ornamental aquarium trade in the US since 2012, and there have been no reports, anecdotal or otherwise, of CGT2016 having altered behaviour relative to Black Tetra that may influence habitat structure. Consequently, CGT2016 is expected to have negligible impacts on habitat, with low uncertainty associated with this rating due to limited data across different environments.

Potential hazards to biodiversity

There is a negligible hazard of CGT2016 on biodiversity in Canadian ecosystems. Biodiversity can be negatively impacted by numerous drivers including invasive species or the introduction of disease. While the invasiveness of CGT2016 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 entering the environment. As well, Hill et al. (2014) concluded a lack of invasion potential in the USA of fluorescent *G. ternetzi* using the Fish Invasiveness Screening Kit (FISK), and diminished cold tolerance and reproductive success in competition may decrease invasive potential of CGT2016 relative to wild-type. As noted above, CGT2016 is 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, and has negligible potential to impact biodiversity through these routes. The 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 with this rating.

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 CGT2016. The majority of individual hazards assessed have negligible hazard ranking as no effects are expected beyond that of wild-type (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 the construct 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 CGT2016, 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 CGT2016 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 = Hazard x Exposure. Overall Risk is estimated by plotting overall Hazard against Exposure, using a risk matrix or heat map, as illustrated in Figure 2. 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 CGT2016 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 CGT2016 to overwinter in Canadian water systems. As such, any exposure of Canadian freshwater aquatic ecosystems to CGT2016 is expected to be isolated, rare and ephemeral. The quality of data demonstrating diminished cold tolerance in CGT2016 and base species relative to typical Canadian winter freshwater temperatures results in low uncertainty level with this ranking.

The hazard assessment concluded that CGT2016 posed 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 protein 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, CGT2016 used in the ornamental aquarium trade in Canada poses low risk to Canadian environments (Low Exposure x Negligible/Low Hazard = Low Risk). Consequently, use of CGT2016 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 organism.

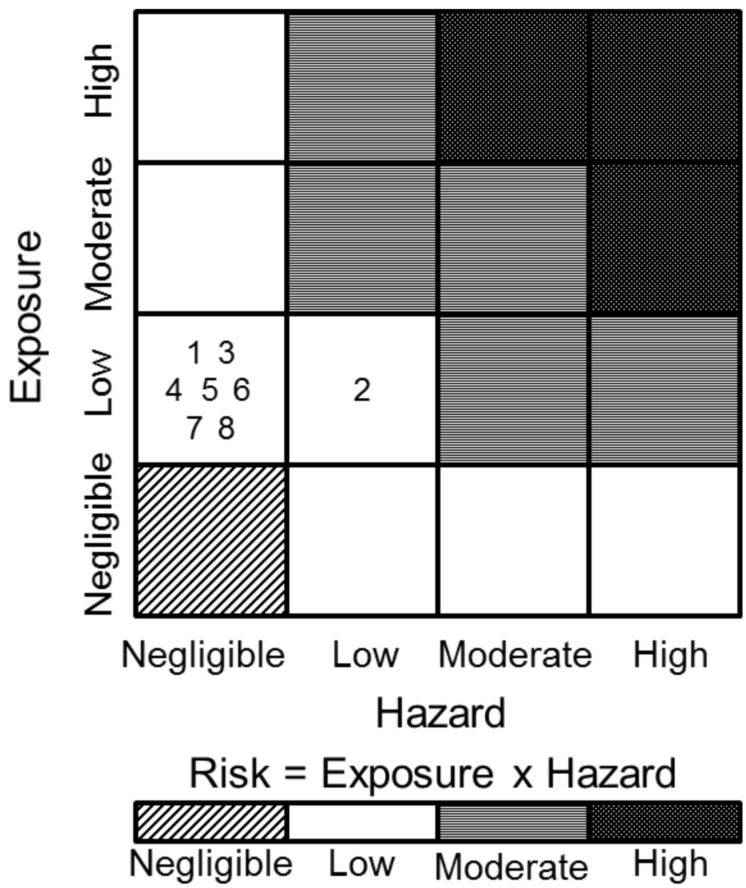

Figure 2: Risk matrix and scale to illustrate how exposure and hazard are integrated to establish a level of risk in the environmental risk assessment. Risk assessments associated with assessed hazard components at the assessed exposure are identified by number: 1) through environmental toxicity; 2) 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 that may influence uncertainty in indirect human health risk assessment include limited information on exposure scenarios in the Canadian market, reliance on reports from surrogate models, and lack of direct data addressing hazards of CGT2016 specifically.

Sources of uncertainty in the environmental exposure and hazard assessment that may influence uncertainty in environmental risk assessment include lack of data directly addressing hazards of the notified organism and comparator species, variability in data taken from surrogate organisms, and lack of understanding of applicability to the notified organism (e.g., 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

Use of CGT2016 for home aquaria is expected to result in low to moderate exposure to humans, primarily through tank maintenance by those who purchase CGT2016. The hazard ranking for CGT2016 to indirect human health is low, due to lack of pathogenicity, allergenicity or toxicity associated with the genetic modification of CGT2016, and history of safe used of the notified organism and wild-type species. Taken together, available evidence does not suggest a risk of adverse indirect human health effects at the exposure levels predicted for the general Canadian population from use of CGT2016 as an ornamental aquarium fish or in other potential uses identified.

Use of CGT2016 for home aquaria is expected to result in potential reoccurring, very small magnitude releases of CGT2016 to the Canadian environment. However, data available indicates CGT2016 does not have capacity to overwinter in Canadian freshwater ecosystems, resulting in low environmental exposure. For potential hazards, the lack of evidence of hazards from the base wild-type species despite long-term extensive use, as well as lack of evidence for increased hazards of CGT2016 relative to wild-type, indicates negligible to low hazard ratings of CGT2016 to Canadian environments. Taken together, the overall risk of CGT2016 to the Canadian environment is low, and the notified organism is not expected to cause harmful effects to Canadian environments at the assessed exposure level.

The import of CGT2016 into Canada, for use in the ornamental aquarium trade and home aquaria, is expected to pose low risk to indirect human health and the Canadian environment. While uncertainty associated with some exposure and hazard classifications is moderate due to limited or no direct data on the notified organism or comparator species, evidence was not identified that suggests CGT2016 under the proposed use, or other potential uses, could cause harm as a result of exposure to Canadian populations or environments.

SOURCES OF INFORMATION

This Science Advisory Report is from the September 12-14, 2017 peer-review meeting, Environmental and Indirect Human Health Risk Assessment of the Glofish® Electric Green® Tetra and the Glofish® Long-Fin Electric Green® Tetra (*Gymnocorymbus ternetzi*): A Transgenic Ornamental Fish. 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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