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Environmental Risk Assessment of the GloFish® Electric Green® Tetra and the GloFish® Long-Fin Electric Green® Tetra: Transgenic Ornamental Fish, Imported to Canada, For Sale in the Pet Trade

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Foreword

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

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ABSTRACT

BACKGROUND

On July 5, 2017, GloFish LLC submitted a regulatory package to Environment and Climate Change Canada (ECCC) under the *New Substances Notification Regulations (Organisms)* [NSNR(O)] of the *Canadian Environmental Protection Act, 1999* (CEPA 1999) for GloFish® Electric Green® Tetra (CGT2016), a green fluorescent genetically engineered Tetra (*Gymnocorymbus ternetzi*), for use as an ornamental fish in home aquaria.

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

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

It is in this context that DFO conducted an environmental risk assessment of the notified organism (CGT2016) under its proposed use which was peer-reviewed by a panel of experts. **Risk** is defined as a function of the potential for Canadian environments to be **exposed** to the notified organism, and the potential for the notified organism to pose **hazards** to the Canadian environment. Exposure and Hazard assessments are conducted separately and then integrated into an assessment of Risk. Uncertainty in Exposure and Hazard assessments are determined, and uncertainty associated with the final risk assessment discussed.

THE NOTIFIED ORGANISM

CGT2016 is a line of genetically engineered diploid, hemizygous or homozygous, long- or regular-fin, green fluorescent transgenic Tetra of the White morph of Black Tetra (*G. ternetzi*), containing transgenes driving expression of a green fluorescent protein gene. Expression of the transgene results in green colouration in natural light and fluorescent green colouration in blue

or UV light. The protein is expressed in the skin, musculature, fins, eyes, and likely other organs of the organism. All CGT2016 individuals are descendants of a single G0 founding individual with the transgene construct microinjected at the single cell stage. Confirmation of a single transgene copy inserted at a single site with Mendelian segregation was confirmed at the F3 generation. CGT2016 has been marketed in the USA ornamental aquarium trade since 2012 as GloFish® Electric Green® Tetra and GloFish® Long-Fin Electric Green® Tetra. The targeted phenotypic change is the presence of green/fluorescent green colouration as a novel colour morph for the ornamental aquarium trade. Other unanticipated phenotypic changes noted by the company include slightly impaired cold tolerance of CGT2016 when temperature was rapidly decreased (i.e., average LD_{50} was 8.11°C relative to 7.94°C for wildtype siblings). As well, CGT2016 had over 40% reduction in expected reproductive success in competition with wild-type siblings, but did not differ in juvenile survival. No other non-target phenotypic changes have been noted in CGT2016.

ENVIRONMENTAL RISK ASSESSMENT

The environmental risk assessment is conducted under GloFish LLC's proposed use scenario: the importation of CGT2016 to four aquarium wholesale locations in Canada, for further distribution to aquarium retail stores across the country for purchase by Canadian consumers for home aquaria.

Exposure

The intended housing for CGT2016 is in indoor, static, physically contained aquaria at wholesalers, retail stores, and in consumer's homes. Based on historical records of aquarium fish in natural ecosystems in Canada and worldwide, it is highly likely that CGT2016 will be introduced purposefully or accidentally into natural freshwater ecosystems in Canada. Based on the expected number of CGT2016 to be purchased by individual consumers, it is expected that release events will be very low magnitude (e.g., 5 or less), although larger magnitude releases cannot be ruled out. Base on temperature preferences and limitations of wild-type *G. ternetzi* and recorded water temperature throughout freshwater systems in Canada, CGT2016 have potential to survive in many natural ecosystems in the summer in Canada, and some ecosystems in the fall and spring. However, the lower temperature tolerance of both CGT2016 and wild-type *G. ternetzi* preclude the fish from surviving over winter in most Canadian freshwater ecosystems. There are some lakes that reach temperatures for a short time in the summer months that are adequate for reproduction of CGT2016. *G. ternetzi* has a relatively long lifecycle (i.e., minimum time to maturation is 4 months in ideal conditions), and as such the potential exists for only one reproductive cycle prior to termination over the winter. Given the above analysis, the occurrence of CGT2016 in the Canadian environment is expected to be rare, isolated and ephemeral and likely in low numbers. Consequently, the likelihood of **exposure** of CGT2016 to the Canadian environment is ranked **low**. The **uncertainty** associated with this estimation is **low**, given the quality of temperature tolerance data available for CGT2016 and valid surrogate organisms, and data available on the environmental parameters of the receiving environment in Canada.

Hazard

The potential for CGT2016 to cause a hazard to Canadian environments was examined in the context of environmental toxicity (i.e., potential to be poisonous), through horizontal gene transfer, through interactions with other organisms including hybridization, as a vector of disease, and through impacts to biogeochemical cycling, habitat, and biodiversity. Wild-type *G. ternetzi* 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 reported in Canada or worldwide despite its wide use in the aquarium trade spanning greater than 65 years. There are no reports of phenotypic effects of the transgene that may increase hazard potential of CGT2016 above that of wild-type domesticated *G. ternetzi*, no evidence of toxicity of the fluorescent protein (i.e., not poisonous to organisms or the environment), and no evidence that potential gene transfer will result in harm to Canadian environments. Some evidence suggests CGT2016 may have lower potential to impact other species through trophic interactions relative to wild-type *G. ternetzi*, as lower cold tolerance may further limit activity in cooler water temperatures. Taken together, CGT2016 is expected to pose **negligible to low environmental hazard** if released to Canadian aquatic ecosystems. The uncertainty rankings associated with individual hazard assessments ranged from negligible to moderate due to limited data specific to CGT2016, limited direct data on the comparator species, variable data from a surrogate model (red fluorescent protein zebrafish), and the reliance on expert opinion for the assessment of some hazards.

CONCLUSIONS ON RISK

Based on a risk matrix integrating the Low Exposure and Negligible to Low Hazards of CGT2016, CGT2016 poses **Low Risk** to Canadian environments under the proposed use for the ornamental aquarium trade. While uncertainty associated with some 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 environments.

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LIST OF ACRONYMS

bp: Base pair

CEPA: *Canadian Environmental Protection Act, 1999*

CGT2016: Notified organism: genetically engineered Tetra of the species *Gymnocorymbus ternetzi*, containing a green fluorescent protein transgene construct

CTmin: Critical thermal minima

CLmin: Chronic lethal minimum temperature

DFO: Fisheries and Oceans Canada

DNA: Deoxyribonucleic acid

dpf: days post fertilization

ECCC: Environment and Climate Change Canada

eGFP: Enhanced green fluorescent protein

GE: Genetically engineered

GFP: Green fluorescent protein, refers to a specific type of green fluorescent protein

GxE: Genotype by environment interaction

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

MOU*:* Memorandum of Understanding

mRNA: Messenger RNA

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

POE: Pathway of effect

RFP: Red fluorescent protein

RNA: Ribonucleic acid

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

SNAc: Significant new activity

GLOSSARY

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

Abundance: the total number of individuals of a taxon or taxa in an area, community or population

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

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

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

CEPA toxic: a substance or an organism that may enter the environment in a quantity or concentration or under conditions that (a) have or may have an immediate or long-term harmful effect on the environment or its biological diversity; (b) constitute or may constitute a danger to the environment on which life depends; or (c) constitute or may constitute a danger in Canada to human life or health

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

Dispersal: movement of an organism in its environment; movement of the organism away from its point of entry into the environment

Distribution: the geographical range of a taxon or group; the spatial pattern or arrangement of the members of a population or group

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

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

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

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

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

Fate: the final outcome or expected result of normal development

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

Frequency: the number of occasions that a given character, species or event occurs in a series of samples or for a given period of time

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

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

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

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

Hazard: potential to cause a harmful effect

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

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

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

Keystone species: a species that has a disproportionately large impact on ecosystem structure and function

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

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

Measurement endpoint: a measurable characteristic of the selected assessment endpoint

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

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

Persist: survives to the reproductive stage

Predator: an organism that kills its victim in order to utilize resources contained in that victim

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

Prey: any animal or animals actually or liable to be killed and consumed by a predator

Primary production: the assimilation of organic or inorganic matter by autotrophs (organisms that can convert inorganic carbon to organic materials and thus do not need to ingest or absorb other living things)

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

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

Selection: non-random differential reproductive success of different genotypes in a population

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

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

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

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

Variable: the property with respect to which parameter values within a sample differ in some discernible way

Variability: the property of being variable in form or quality

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

PART 1: PROBLEM FORMULATION

1.1 PURPOSE OF PART 1

Part 1 of this document elaborates the problem formulation for the environmental risk assessment that will be conducted under the *Canadian Environmental Protection Act* (CEPA), with respect to the GloFish® Electric Green® Tetra (CGT2016), a genetically engineered white variant of the Black Tetra (*Gymnocorymbus ternetzi*) notified by GloFish LLC under the New Substances Notification Regulations (Organisms) [NSNR(O)] for use in the ornamental aquarium trade.

This problem formulation provides a foundation for the risk assessment through identification of environmental protection objectives, and the elaboration of scope. It also identifies protection goals and assessment endpoints that are aligned with the legislative protection goals in CEPA. The Problem Formulation also provides a characterisation of CGT2016, the comparator species, and the potential receiving environment in Canada.

It is critical to accurately reflect the scope and focus of risk assessments conducted under CEPA at the outset, so that an appropriate and scientifically defensible risk assessment can be concluded within the legislated 120-day timeframe specified by the NSNR(O) for notifications under Schedule 5 (Information required with respect to organisms other than micro-organisms). Further information on CEPA and NSNR(O), including guidance on the regulations, detailed guidance for information requirements, use of waivers, significant new activities, risk assessment outcomes and risk management can be found on the Biotechnology page of the Environment Canada website.

1.2 LEGAL CONTEXT UNDER CEPA

CEPA is an act respecting pollution prevention and the protection of the environment and human health in order to contribute to sustainable development. It defines “sustainable development” as development that meets the needs of the present generation without compromising the ability of future generations to meet their own needs.

Under CEPA the “environment” is defined broadly to mean *components of the Earth*, including:

> *(a) air, land and water;*
> *(b) all layers of the atmosphere;*
> *(c) all organic and inorganic matter and living matter and living organisms; and,*
> *(d) the interacting natural systems that include components referred to in (a) to (c).*

The biotechnology provisions of CEPA take a preventative approach to pollution by requiring all new living organism products of biotechnology, including genetically engineered fish, to be notified and assessed prior to import or manufacture, to ultimately determine whether they are “toxic” or capable of becoming “toxic”.

As defined in section 64 of CEPA, an organism is "toxic" if it is entering or may enter the environment in a quantity or concentration or under conditions that:

(a) have or may have an immediate or long-term harmful effect on the environment or its biological diversity;
(b) constitute or may constitute a danger to the environment on which life depends; or,
(c) constitute or may constitute a danger in Canada to human life or health.

Anyone proposing to import or manufacture a living animate product of biotechnology in Canada, including a genetically engineered fish, is required to provide ECCC with the information prescribed in Schedule 5 of the NSNR(O) at least 120 days prior to the commencement of import or manufacture of the organism. This information is used to conduct an environmental risk assessment and an assessment of indirect human health (risk to human health from environmental exposure to the living organism), which is then used as the basis to determine if the organism is CEPA-toxic or capable of becoming CEPA-toxic. Although Schedule 5 allows for the notification of a broad range of activities, the risk assessment may be limited to the activities proposed by the notifier if there is insufficient information to consider all potential applications. In such situations a Significant New Activity (SNAc) Notice is published to limit the activity to the proposed use or uses determined to be not toxic, and request additional information for risk assessment prior to new uses. Organisms for research and development are exempt from the Regulations provided they meet all criteria prescribed in the Regulations, and the organism is imported to, or manufactured in, a facility from which there is no release into the environment of the organism, the genetic material of the organism, or material from the organism that is involved in toxicity.

A waiver for one or more regulatory information requirements specified in Schedule 5 of the NSNR(O) may be requested by the notifier. As specified under paragraph 106(8), waivers may be granted if (a) in the opinion of the Minister of the Environment and Climate Change and the Minister of Health, the information is not needed in order to determine whether the living organism is toxic or capable of becoming toxic; (b) a living organism is to be used for a prescribed purpose or manufactured at location where, in the opinion of the Ministers, the person requesting the waiver is able to contain the living organism so as to satisfactorily protect the environment and human health; or (c) it is not, in the opinion of the Ministers, practicable or feasible to obtain the test data necessary to generate the information. The current notification does not include requests for waivers on any aspect of the risk assessment.

Under its MOU with ECCC and HC, DFO provides science advice through the conduct of an environmental risk assessment, and collaborates with HC to provide advice on an indirect human health risk assessment, for living fish products of biotechnology in accordance with CEPA and the NSNR(O). Under this arrangement, the Minister of ECCC receives scientific advice on risk assessment from DFO, but retains ultimate responsibility for regulatory decision making. Under CEPA, and depending on the risk assessment outcome, options may be available to manage any risks associated with the organism. Environment and Climate Change Canada is responsible for enforcement of the NSNR(O) including adherence to any imposed conditions, or other risk management measures.

The scope of a Schedule 5 assessment under the NSNR(O) must also take into account all likely “potential” use scenarios, not just the specific one(s) being proposed by the notifier. If there is not enough information to consider all potential applications, then a “case-by-case”

approach is taken whereby the specific use scenario notified and elaborated by the Notifier in the regulatory submission, including any containment or mitigation measures, sets the specific parameters around the risk assessments (e.g., possible exposure pathways). However, if the risk assessment concludes no suspicion of 'CEPA toxic' for the proposed use scenario, but there is suspicion that the organism may be toxic under a different use scenario, then the SNAc provisions of CEPA may be used to reassess the import or manufacture of the organism under the circumstances of the significant new activity. While the SNAc is the responsibility of ECCC, DFO may be consulted on various elements.

Further information on CEPA and the NSNR (O), including guidance on the regulations, detailed guidance for information requirements, waivers, significant new activities, risk assessment outcomes and how potential risks are managed, can be found on the Biotechnology page of the ECCC website.

1.3 REGULATORY DECISION MAKING

Regulatory decisions under CEPA are based on whether or not a living organism is "CEPA toxic", as determined through scientific risk assessment, using information that is provided as part of the requirements under the NSNR(O) Schedule 5, and other available information. As depicted in Figure 1.1, risk assessments under CEPA result in one of the following outcomes:

1. A determination that the organism is not suspected of being "CEPA toxic" or capable of becoming "CEPA toxic"; or,

2. A determination that the organism is not suspected of being "CEPA toxic" under the proposed use scenario, but that a significant new activity in relation to the organism may result in the organism becoming "CEPA toxic". In this case, a SNAc notice is issued to gather additional information to support the re-assessment of the organism under its new activity.

3. A suspicion that the organism is "CEPA toxic" or capable of becoming "CEPA toxic", which may require:

    a. the establishment of conditions on the manufacture, import, use or disposal of the organism;

    b. prohibition of the manufacture or import of the organism pending new regulations that are to be put in place by government within a prescribed timeline; or,

    c. prohibition pending submission and assessment of additional information determined to be required.

1.4 RISK ASSESSMENT FRAMEWORK

Risk assessments of GE fish conducted by DFO under the NSNR(O) are carried out in accordance with the following principles:

- Risk assessments are science-based and do not include considerations such as socio-economics, ethics or harm/benefit ratios;
- A comprehensive “cradle-to-grave” approach is taken whereby the organism is assessed from the time it is manufactured through production and use to disposal;
- All life stages, genotypes and genders carrying the transgene are considered in the risk assessment;
- Each event that results in a genetic change, such as the insertion of a transgene or a site-directed mutation, is considered independent and unique, and each GE fish resulting from biotechnology is considered a unique strain, and would be assessed separately under different notifications;
- The characteristics of the notified organism are compared with non-GE wild or non-GE domesticated strains or both, whichever is most relevant;
- Predicted change to measurement endpoints beyond the normal or historical range of variation can be used as an indicator of a novel phenotype, and would be assessed for potential to harm the environment or indirect human health; and
- All available relevant information (e.g., academic, indigenous, governmental) in addition to that submitted by the notifier under the NSNR(O) is used for the assessment.

The environmental risk assessment considers the potential of the organism to cause harmful effects to the aquatic, terrestrial, and atmospheric components of the Canadian environment and follows the classic paradigm in which Risk is proportional to the Exposure and the Hazard ($R \propto E \times H$). Figure 1.2 outlines the overall process by which DFO conducts scientific risk assessment of GE fish.

1.4.1 Protection goals

Protection goals are in accordance with CEPA, and are chosen to protect the Canadian environment and its biological diversity from immediate and long-term harmful effects to the environment on which life depends, as well as human life and health in Canada. Consequently, DFO’s risk assessment conclusions and recommendations to ECCC regarding risk management should be aligned with CEPA and based on the likelihood, severity, and reversibility of the harmful effects that are associated with the release of GM fish to the structure and function of the Canadian ecosystem. Here, harmful effect includes both immediate and long-term detrimental impacts. Structure of the ecosystem refers to the spatial and temporal distribution of both biotic and abiotic factors, including dominant species, rare species, and keystone species, whereas function of the ecosystem refers to interactions among species (e.g.,

competition, predation, disease) and abiotic elements that contribute to the provision of ecosystem services (e.g., nutrient dispersal and cycling, primary production, decomposition, habitat). Changes to the structure or function of the ecosystem are assessed based on changes to assessment endpoints.

1.4.2 Assessment endpoints

Sutter (1990) defines an 'assessment endpoint' as the formal expression of the actual environmental value that is to be protected (i.e., the protection goals); distinct from a measurement endpoint, which he defines as an observed or measured response to the hazard. Assessment endpoints represent ecological entities susceptible to harm upon exposure to a stressor, and should be protected to achieve established protection goals (U. S. EPA 1998; Wolt et al. 2010). They are selected for their relevance to potential interactions between the organism and components of the ecosystem, and are aligned with legislative protection goals under CEPA.

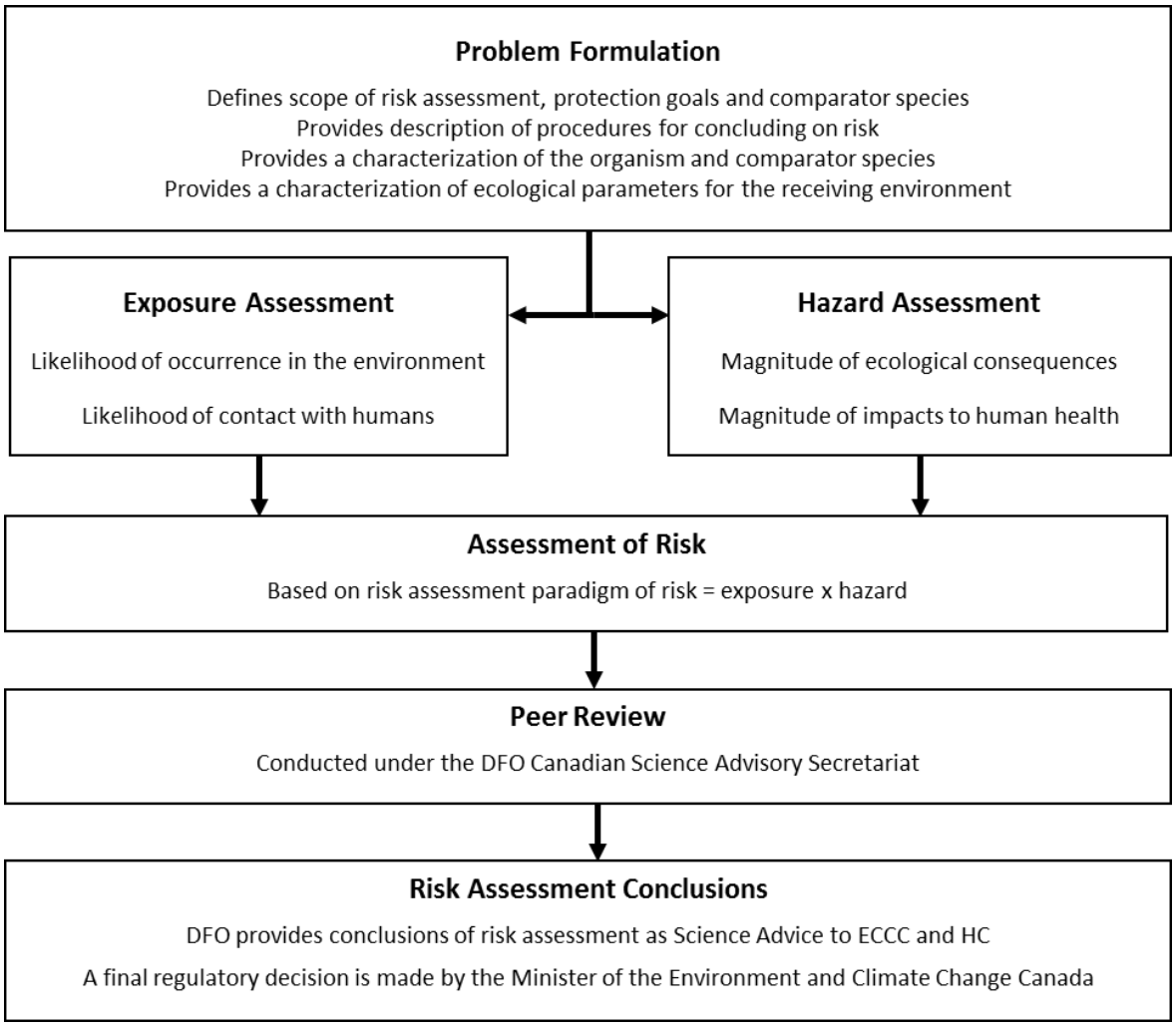

Figure 1.1: *Framework for the DFO risk assessment of GE fish that are notified under Schedule 5 of CEPA.*

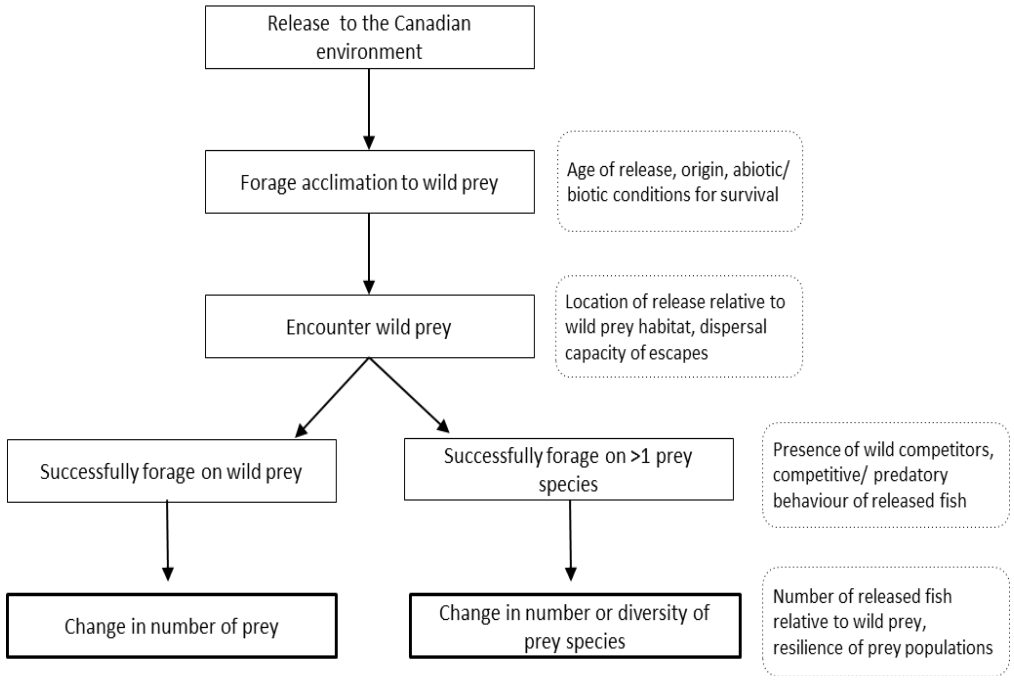

Figure 1.2: *Simplified example adapted from Leggatt et al. (2010), to illustrate a potential Pathways of Effects (POE) for GE fish on Canadian ecosystem components; in this case, the diversity of prey species. Comments in dotted boxes indicate factors that could potentially influence each step of the POE (given in closed boxes).*

1.4.3 Measurement endpoints

Direct measurement endpoints, such as abundance of prey in the presence and absence of the organism in the natural environment, would provide information with direct implications to the organism; without extrapolation, and with the least uncertainty. However, this information may not be available and risk evaluators may therefore rely on data that are indirectly related to the measurement endpoints, as indicators of potential impacts on the assessment endpoints. For example, instead of directly measuring the abundance of a specific prey in the presence and absence of the organism in a freshwater pond, risk evaluators may rely on studies reporting phenotypes likely to affect the abundance of prey in the natural environment, such as competitive and predatory behaviours. Parameters affecting the measurement endpoints are therefore reported for the organism and relevant comparators, and any variation beyond the normal baseline observed in the comparator is considered, and the potential impact on relevant measurement endpoints documented.

Environmental endpoints are selected for their relevance to potential interactions between the organism and components of the ecosystem (Devlin et al. 2007), and are aligned with legislative protection goals under CEPA. Pathways of Effects (POE) can be used to describe the linkages between Activities that are associated with the organism and potential impacts to the environment (DFO 2010; Leggatt et al. 2010). Figure 1.3 illustrates how potential effects, such as a change in the diversity of prey species, can be assessed as a single pathway, composed of relevant ecosystem components, and helps describe the linkages between Activities that are

associated with the organism, and potential effects to the environments, should GE fish be released into the environment. This approach to identification and analysis of key stressor-effect linkages and endpoints is well aligned with frameworks for DFO risk assessments conducted under the *Aquaculture Activities Regulations* for finfish and shellfish aquaculture activities (DFO 2010). However, in the case of GE fish, Stressor Categories identified by POE analysis can be further categorised to align with protection goals under CEPA; to safeguard biodiversity, and the environment on which life and human health depend.

For the environmental hazard assessment, effects are considered harmful if there is potential for an immediate or long-term detrimental impact on the structure or function of the ecosystem. Structure of the ecosystem refers to the spatial and temporal distribution of biotic and abiotic elements, including dominant species, rare species, and keystone species. Function of the ecosystem refers to interactions between species and abiotic elements that contribute to the provision of ecosystem services (e.g., nutrient dispersal and cycling, primary production, decomposition and habitat).

1.4.4 Uncertainty

The risk assessment includes explicit consideration of the uncertainty associated with all elements of the exposure and hazard assessments. Uncertainty has important implications related to regulatory decision making and is closely tied to the application of precaution. In accordance with Canada's policy on the application of precaution in regulatory decision making elaborated in the Government of Canada Framework for the Application of Precaution in Science-based Decision Making about Risk (Government of Canada 2003), in cases where uncertainty about risk is high, precautionary measures that are applied should be proportional to the potential severity of the risk being addressed and to the society's chosen level of protection.

Factors influencing uncertainty include the availability of detailed information about the organism, its history of use, the proposed use scenario, as well as the extent, relevance (e.g., specific to the notified organism rather than surrogate), and quality of peer-reviewed information and/or empirical data. Prevalence of knowledge gaps, inherent variability of biological systems, experimental data, the strength of logical deduction and inferences from expert knowledge of the species will also influence uncertainty. While some forms of uncertainty can be reduced by filling knowledge gaps or through larger data sets, others cannot due to factors such as the inherent complexity and variability of biological systems or the occurrence of chance events.

In conducting environmental risk assessments, the following measures will be employed to ensure accurate identification and understanding of uncertainty, and reduce uncertainty to the greatest extent possible:

- A comprehensive scientific peer-review process will be undertaken on the risk assessment to ensure that unbiased expert advice is available in all key areas and that knowledge gaps and differences in scientific opinion are identified and adequately resolved wherever possible;

- Uncertainty will be explicitly estimated and stated separately for each element of the exposure and hazard assessments so that the overall risk will not be over- or under-represented by the inclusion of cautionary or dismissive assumptions.

1.5 CHARACTERISATION OF CGT2016

CGT2016 is described in the notification as:

"… a line of diploid, hemizygous or homozygous, long- or regular-fin, green Tetra of the species Gymnocorymbus ternetzi*, containing the genetic construct… CGT2016 was derived from a line of White Skirt Tetra, a natural pigment variant of G. ternetzi. Quantitative real-time PCR (qPCR) indicates one copy of the inserted transgenic cassette in the hemizygous case. The integration locus is inherited as a single site.*

The trade names for CGT2016 are GloFish® Electric Green® Tetra and GloFish® Long-Fin Electric Green® Tetra. The proposed Explicit Biological name is *"A genetically modified* Gymnocorymbus ternetzi*, descended from the Green Tetra 0, founder of the GT-0 line, with a single stably integrated insert of the construct"*.

Figure 1.3 demonstrates physical appearance of CGT2016, as well as for wild-type Black Tetra and white morph of the Black Tetra, including long-fin variants for all.

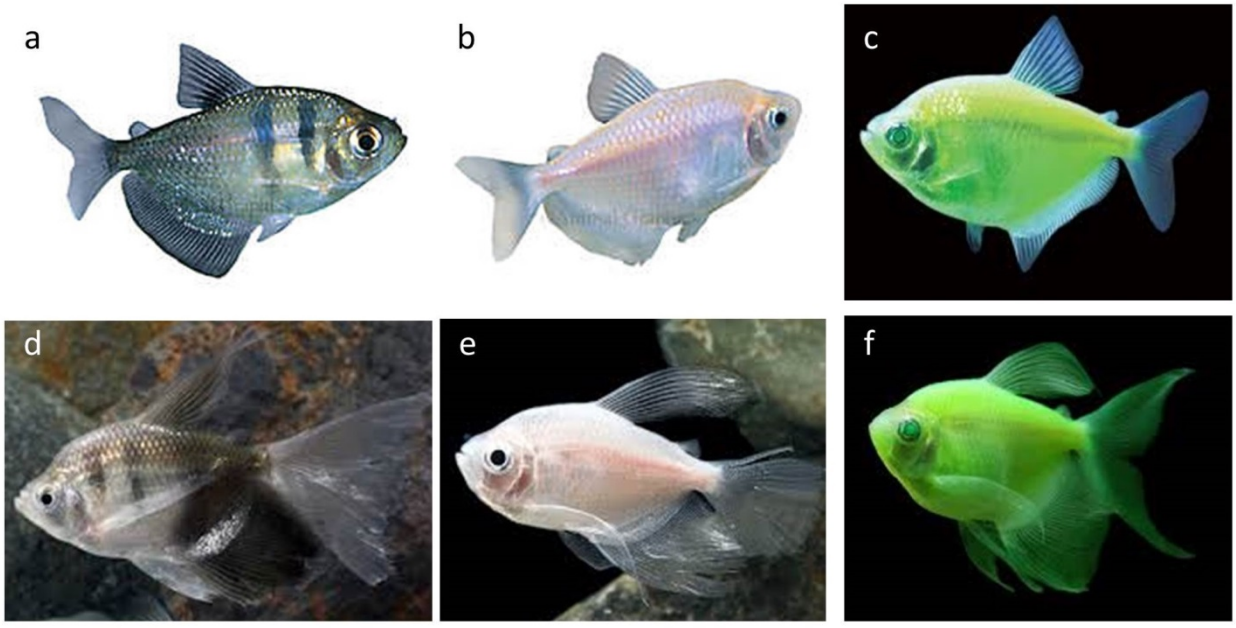

Figure 1.3: *Some variants of Gymnocorymbus ternetzi available in the ornamental pet trade worldwide (a, b, d, e), and notified transgenic variants currently only available in the United States of America (c, f). Wild-type Black Tetra (a, d), White Tetra (b, e) and CGT2016 Electric Green® Tetra (c, f) showing normal and long-fin variants respectively. Taken from PetSmart (a, b), GloFish (c, f) and Segrest Farms (d, e).*

1.5.1 Molecular Characterisation

CGT2016 is a genetically engineered White morph of the Black Tetra (*G. ternetzi*) possessing a single transgenic insert containing fish-origin promoters that drive expression of an exogenous fluorescent protein. This insert results in ubiquitous green colouration of the organism under white light and fluorescent green colouration under ultraviolet or blue light. The purpose of this modification is to create new a 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.

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.

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

1.5.2 Phenotypic Characterization

1.5.2.1 Strain history and production

White Tetras used in CGT2016 production are a naturally occurring colour variant of the Black Tetra (Frankel 2004, see Figure 1.3), generally bred as a separate strain from the Black Tetra that was introduced to North America prior to 1950 (Innes 1950). The individual fish used to produce CGT2016 were sourced from an ornamental aquarium fish producer (5-D Tropical) in 2007. All CGT2016 fish (see Figure 1.3) are descended from a single G0 individual injected with the notified transgene construct at the single cell stage. This G0 individual was crossed to several non-transgenic White Tetras to produce several F1 heterozygous groups.

Continuation of the CGT2016 line has been through batch breeding of CGT2016 individuals, so the CGT2016 population contains a mix of individuals heterozygous or homozygous for the transgene. All white individuals are removed from the CGT2016 population and transferred to separate non-transgenic White Tetra populations. All atypical individuals are culled. CGT2016 has been in commercial production for the ornamental aquarium trade in the US excluding California since 2012, and for California since 2015. CGT2016 are produced by two aquarium fish producers in Florida, USA.

1.5.2.2 Targeted Phenotypic Effects of the Modification

The targeted phenotypic effect of the genetic modification is that CGT2016 appears green under ambient light, including sunlight, to create a new, bright colour variant for the ornamental

aquarium trade (see Figure 1.3). As well, when under blue or UV light (excitation maxima 493 nm) the notified organism fluoresces green (emission maxima 505 nm, clonetech.com). The novel colour phenotype is present in the muscle, as well as skin and eye. Whether the phenotypic green colour is present in internal organs has not been reported but based on transgene promoter use it is expected, though potentially to varying degrees.

GloFish LLC reports that CGT2016 individuals that are hemizygous and homozygous for the transgene insert are indistinguishable from each other phenotypically, and both are part of the commercially available population.

1.5.2.3 Non-targeted phenotypic effects of the modification

Two off-target phenotypic effects have been identified by GloFish LLC in CGT2016: diminished tolerance to low temperature and decreased reproductive success in competition 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 reports that CGT2016 and related green fluorescent Tetras do not differ from White Tetras in embryo or juvenile survival. Though greater detail regarding these studies has been provided by the company for review, it is considered confidential business information and is not included in this report.

The influence of the genetic modification on any other phenotypes has not been formally examined. GloFish LLC provided a summary of diagnostic examination by the Fish Disease Diagnostic Lab, University of Florida from tissues from six CGT2016 individuals. The document did not report any unusual physical appearance or pathological lesions in major organs (e.g., regarding necropsy results: "*All findings were normal except for presence of low numbers of nematodes (Capillaria) in 1 of 6 fish and moderate numbers in 5 of 6 fish*"; regarding microbiology results from brain and posterior kidney "*No bacterial growth was observed*"; regarding histological examination of major organs "*No pathologic lesions are noted in any of the fish examined*". It should be noted that histology was not directly compared to wild-type fish and was looking specifically for gross pathological lesions and signs of disease. In addition, GloFish LLC provided statements from the company's veterinarian and a University of Florida veterinarian involved in health screening for 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 fluorescent line relative to non-transgenic counterparts, and that CGT2016 require the same husbandry care as non-transgenic counterparts, i.e., from the University of Florida veterinarian: "*My observations during evaluation of the above mentioned green fluorescent Tetra is that the expressed fluorescence trait does not confer any significant additional health impediment relative to their non-genetically engineered counterparts. Specifically, based on my personal experience and necropsy results, I have not seen any evidence to suggest that the green fluorescent Tetra differs in susceptibility to, or the transmission of, pathogens. Moreover, it is worthwhile commenting that this is also the case for every line of fluorescent fish from Yorktown Technologies that my laboratory has examined. Similarly, we have not seen any evidence of increased risk of zoonosis posed by any of Yorktown Technologies lines of fluorescent fish, including the green fluorescent Tetra, when compared to wild-type strains.*" From the 5-D Tropical Inc. veterinarian: "*Yorktown's fluorescent*

ornamental fish require the same husbandry practices as their non-fluorescent counterparts, including veterinary care. I have seen no evidence of any increased susceptibility to, or transmission of, water-borne pathogens. Freshwater tropical ornamental fish are not common vectors for zoonotic transmission of disease, and neither the Green Tetra, nor Yorktown Technologies' other fluorescent tropical fish pose any increased zoonotic risk with respect ot their non-genetically engineered counterparts" (From additional information provided by GloFish LLC). No formal studies have compared potential disease susceptibility of CGT2016 and wild-type strains. There are also no formal studies on potential non-target effects of the genetic modification on life-history (other than reproductive success and juvenile viability), environmental tolerances and requirements (other than low temperature tolerance), metabolism, physiology, endocrinology, or behaviour, however there are no anecdotal or otherwise reports of non-target effects other than listed above.

1.5.2.4 Pleiotropic effects of fluorescent protein transgenes in other organisms

Fluorescent protein transgenes in other fish models

While the construct is not commonly used as a transgene in other organisms, other fluorescent proteins including enhanced green fluorescent protein (eGFP) have widespread use in research in a variety or organisms, and some risk assessment relevant research has been done on another small tropical fish (Zebrafish, *Danio rerio*) transgenic for red fluorescent protein (RFP). Zebrafish containing the RFP transgene had a higher critical thermal minima (CTmin) and chronic lethal minimum temperature (CLmin) (i.e., were less cold tolerant), as well as had slightly lower high-temperature tolerance than non-related wild type, examined under different acclimation temperatures (Cortemeglia and Beitinger 2005, 2006a), although differences in strain background and rearing conditions (Schaefer and Ryan 2006) prior to experimentation may have impacted relative extreme temperature tolerance of RFP versus wild-type zebrafish. Amanuma et al. (2008) reported no difference in low temperature tolerance in RFP zebrafish and wild-type siblings in Japan, although the replicate number was low (n=7 fish per genotype) and no statistical comparisons were given so this lack of difference should be taken with caution. Leggatt et al. (2018) reported zebrafish transgenic for eGFP driven by Fli-1 protein promoter were less cold tolerant than source wild-type strain, but two other eGFP lines driven by other promoters did not have diminished cold tolerance, indicating different transgenic lines may have different responses to extreme environmental stressors. In a population of eGFP, RFP, eGFP-RFP (driven by a muscle-specific promoter) and wild-type zebrafish, eGFP fish had lower survival than the other three groups, but there was no effect of RFP or the double transgene on survival (Gong et al. 2003), indicating different transgenes may also have different influences on survival. The lack of survival effect in RFP zebrafish relative to related wild type has also been confirmed in juvenile and adult life stages in laboratory conditions (Howard et al. 2015). The influence of fluorescent transgenes on foraging behaviour has not been reported in any species. The effect of the RFP fluorescent protein on the potential for zebrafish to be preyed upon has been reported to have no effect, increase, or decrease vulnerability to predation. Hill et al. (2011) found GloFish® RFP zebrafish were two times more vulnerable to predation by largemouth bass and Eastern mosquitofish than wild-type zebrafish originating from the same producers. In contrast, Cortemeglia and Beitinger (2006b) found a native wild-caught largemouth bass equally preyed upon a different strain of RFP zebrafish, an unrelated

population of wild-type zebrafish, and wild-caught native mosquito fish, while Jha (2010) found a RFP zebrafish strain in India were less preyed upon by wild-caught snakeheads than unrelated wild-caught zebrafish or wild-caught flying barb, and the two groups of wild-caught prey fish were preyed upon in greater proportions if reared with RFP zebrafish than if reared together, indicating active avoidance of RFP zebrafish by the predator. Factors influence the difference in relative vulnerability of RFP zebrafish to predation are not known, but could include differences in genetic background or rearing history of transgenic and wild-type zebrafish, innate preference or life history of predators used, and/or experimental conditions (e.g., presence of shelter for prey species).

The reported influences of RFP and other fluorescent transgenes on reproductive success or preferences in zebrafish are likewise inconsistent. RFP and wild-type zebrafish had similar age at maturity for females as well as male and female fecundity (Howard et al. 2015). In a population containing equal numbers of eGFP and wild-type zebrafish allowed to breed for one week, the resulting offspring had expected Mendelian ratio of eGFP transgenes indicating there was no reproductive advantage or disadvantage for eGFP zebrafish (Gong et al. 2003). In contrast, Owen et al. (2012) found both wild-type and RFP zebrafish females preferred to associate with RFP rather than wild-type males, regardless of the proportion of wild-type to RFP fish they were raised with. However, in single pair mating trials, there was no difference in female mating latency or number of mating bouts when with wild-type/RFP or preferred/non-preferred male. In another study, RFP males had lower mating success than wild-type males, as well as lower aggression levels to both males and females (Howard et al. 2015). In other behaviours, Jha (2010) found RFP were more aggressive than wild-caught unrelated zebrafish, particularly if reared together. Snekser et al. (2006) found RFP transgene did not influence social partner preferences for either shoaling or in a potential reproductive context in presumably unrelated populations of RFP and wild-type zebrafish.

Howard et al. (2015) examined the fate of the RFP transgene over 15 generations in a serial breeding experiment in 18 populations of zebrafish. For this experiment offspring were collected from tanks daily until there was enough for the next generation, reared separately until big enough to breed, then parental fish removed and next generation added to the tank. In all populations under these conditions the frequency of the RFP transgene declined rapidly, and was eliminated in all populations except one, indicating a strong bias against the RFP transgene in reproduction. This study indicates that RFP zebrafish would be at a large disadvantage relative to wild-type fish, although experimental constraints in the study required a strong human-directed experimental design that would not be present in populations released to nature.

Fluorescent protein transgenes in non-fish models

In general fluorescent proteins are considered neutral – i.e., their presence does not significantly affect cell function, and they are consequently widely used as a neutral marker gene for research. However, there have been some isolated reports of fluorescent transgenes (primarily GFP or eGFP as the most utilized fluorescent transgenes) having negative effects on organisms, particularly in models with high eGFP expression.

In mice, different lines with high eGFP or GFP expression had poor early developmental competence (Devgan et al. 2004), renal defects and high adult mortality due to kidney failure (Guo et al. 2007), and increased cardiomyopathy resulting in high mortality to congestive heart failure (Huang et al. 2000a). In these studies mouse lines with lower GFP expression had normal development, and other studies have reported no effect of GFP on early embryo development in mice (e.g., Sakharova et al. 2011). As well, Tao et al. (2007) found mice transplanted with GFP-expressing haemopoietic stem cells maintained GFP throughout most of their lifespan, while those expressing DsRed quickly disappeared, demonstrating different fluorescent genes or lines may have different success. Li et al. (2013) found mice transgenic for eGFP had altered metabolic pathways including the urea cycle, nucleic acid metabolism, energy utilization, and amino acid catabolism. In terms of fertility, Sakharova et al. (2011) found GFP mice had decreased numbers of viable ova, while GFP expression had no apparent effect on fertility in male mice (Villuendas et al. 2001). In a plant model, GFP expression did not affect pollen fitness under controlled experimental conditions (Hudson and Stewart 2004).

In general, GFP is not considered toxic (i.e., poisonous) as the majority of studies have failed to demonstrate toxicity of GFP (e.g., see Stewart 2001; Stewart 2006), although there have been some reports of GFP and other fluorescent proteins causing increased mortality or poor proliferation in cells, and some promoter-fluorescent protein transgene combinations may more appropriate for some cell lines than others (see Jensen 2012). For example, GFP was reported to decrease viability or induce toxicity in human liver cells (Li et al. 1999), mouse spermatids (Huang et al. 2000b), and CHO cells deficient in Ku80 (NHEF core factor in DNA repair, Koike et al. 2013). However, Koike et al. (2013) found there was no effect of YFP expression, and Tao et al. (2007) found DsRed-expressing haemopoietic stem cells had poor growth, while GFP-expressing cells did not, demonstrating different fluorescent genes in different lines may have different viability.

In cell lines GFP-expression results in some changes in gene expression (Badrian and Bogoyevitch 2007; Kam et al. 2013; Thomas and Nielsen 2005) or proteome patterns (Coumans et al. 2014). The function of several genes altered by GFP have not yet been characterized (Badrian and Bogoyevitch 2007; Thomas and Nielsen 2005), although several genes associated with cell signaling and response to stress/immune function have reported altered expression associated with GFP (Badrian and Bogoyevitch 2007; Baens et al. 2006; Coumans et al. 2014; Mak et al. 2007), including altered response to cytotoxic events (Goto et al. 2003; Kam et al. 2013; Koelsch et al. 2013; Koike et al. 2013). Whether this may translate to altered response to stimulus or stress on a whole animal level has not been examined, but based on cell line studies any effect would be expected to be deleterious to response and survival to fluctuating environments.

Other altered cellular functions noted from fluorescent transgenes include impaired actin-myosin interaction and muscle contractile properties through binding of GFP to myosin (Agbulut et al. 2007), and impaired virulence in GFP-overexpressing *Mycobacterium marinum* infecting medaka, but not in RFP-expressing bacteria (Mutoji and Ennis 2012), although Seif et al. (2016) found no effect of GFP expression on *Leishmania* virulence in mice.

1.5.3 History of Use

The first fluorescent protein discovered was the green fluorescent protein (GFP) naturally occurring in the jellyfish *Aequorea victoria* and first characterized by Shimomura (1979). Since then numerous fluorescent proteins have been characterized from other cnidaria species (see Stewart 2006). Natural fluorescence appears to be a common feature in many marine organisms including fish (Sparks et al. 2014). Since their discovery, fluorescent proteins have been used as neutral markers in many transgenic models for research purposes including examination of developmental biology and gene regulation (see Lin et al. 2016; Udvadia and Linney 2003). Use of fluorescent protein transgenes as neutral reporter genes was first demonstrated in fish with a GFP transgenic zebrafish in 1995 (Amsterdam et al. 1995). Fluorescent protein transgenic use in zebrafish research has expanded greatly since then and includes using multiple fluorescent tags in a single fish such as tagging epithelial cells with dozens of distinguishable tags for quantitative *in vivo* imaging (Chen et al. 2016). A Web of Science scientific article search using topic search string [(eGFP or GFP or fluorescen*) and transgen* and (zebrafish or danio rerio)] resulted in 1546 article hits (accessed July 20, 2017), demonstrating the widespread utility of fluorescent transgenes in research.

Commercial use of fluorescent transgenic fish in the ornamental aquarium trade was first approved and marketed as a RFP transgenic zebrafish (GloFish®) by Yorktown Technologies in the US excluding California in 2003 (see Hill et al. 2011). An updated RFP zebrafish model and additional colour variants followed in subsequent years (see Hill et al. 2011; Howard et al. 2015). CGT2016 was first approved and marketed in the US excluding California in 2012 as GloFish® Electric Green® Tetra, followed by GloFish® Long-fin Electric Green® Tetra (also CGT2016) and additional fluorescent colour variants in the Tetra. Two fluorescent colour variants in tiger barbs were introduced in 2014 (Howard et al. 2015), and two fluorescent colour variants of Red-Tailed Shark introduced in 2017. Approval to sell GloFish® in California, including CGT2016 long and short-fin varieties, was granted in 2015 (California Fish and Game Commission 2015).

GloFish LLC is requesting importation of CGT2016 from the US for the ornamental aquarium trade in Canada. The ornamental aquarium trade is popular internationally, with over 6000 aquarium species traded and estimates of 6 billion fish sold annually (Teletchea 2016). The ornamental aquarium trade in Canada is primarily driven by imports. Canada imported US$ 7.5 million in ornamental fish in 2016, primarily from the US (US$ 3.5 million), followed by Singapore (US$ 1.3 million) and other parts of Asia, the Middle East, South and Central America, Africa, and Europe (from https://comtrade.un.org/db/mr/daCommoditiesResults.aspx?px=H1&cc=030110, accessed July 21, 2017). Total weight imported is not yet available for 2016, in 2015 Canada imported over 104 thousand kg of live ornamental fish, over half of which came from the US (54.6 thousand kg). The exact number of households that contain ornamental fish is not known. Oliveira (2014) reported 9% of Canadians owned “other pets”, including fish, birds, small mammals and herptiles, although other reports cite 10% of North Americans as owning ornamental fish as pets (Rixon et al. 2005), equating to approximately 3.6 million Canadians. *G. ternetzi* is a common

fish in the ornamental aquarium trade in Canada, for example Rixon et al. (2005) found 75% of surveyed pet stores in Ontario carried the species.

1.6 CHARACTERISATION OF COMPARATOR SPECIES

For the purpose of this assessment, the Black Tetra (*G. ternetzi*) will be used as a comparator for the notified organism; however the common names Black Tetra, Black Skirt Tetra, Black Widow Tetra, Petticoat Tetra, White Tetra, White Skirt Tetra, Black Long-Fin Tetra and White Long-Fin Tetra are all accepted as synonymous and morphs of the Black Tetra (see Section 1.6.2, Figure 1.3). It is a small tropical freshwater fish up to 7.5 cm in length, but usually only to 6 cm in aquaria (Innes 1950; Mills and Vevres 1982). It originates from South America, and has been domesticated for use in the ornamental aquarium trade worldwide (Teletchea 2016). Much of the information available on the Black Tetra is from the ornamental aquarium trade, and primarily involves anecdotal information on preferences of domesticated *G. ternetzi* in the home aquarium, rather than scientific studies on habitat or other requirements in the wild.

1.6.1 Taxonomic status

The Black Tetra (*G. ternetzi*) was first described in 1895 by Belgian-British zoologist George Albert Boulenger as *Tetragonopterus ternetzi* based on several specimens from Descalavados, Brazil (Benine et al. 2015; Boulenger 1895). Carl Eigenamnn shifted *T. ternetzi* into *Gymnocorymbus* in 1910 (Benine et al. 2015). As a teleost in the order Characiformes, the Black Tetra is in one of the most diverse and problematic (taxonomically speaking) families: the Characidae (Benine et al. 2015; Oliveira et al. 2011). With nearly 1,100 species in Central America, South America, Mexico and southern US (Frankel 2004; Oliveira et al. 2011), the group represents more than half of the species in Characiformes and is the most active taxon in terms of new species descriptions, adding to its complexity (Boulenger 1895; Oliveira et al. 2011). Because of the breadth of the group's diversity, much of the Characid genera remain unknown or not formally described. Benine et al. (2015) showed through molecular testing that a taxonomic revision was warranted and their results supports the monophylic *Gymnocorymbus*, with currently four valid species including *G. ternetzi*.

The Black Tetra has been bred for the aquarium trade since at least 1950 (Innes 1950). White-coloured strains (White or White Skirt Tetras) with or without long fins have been produced through selection of naturally occurring traits for the aquarium trade (e.g., Axelrod and Vorderwinkler 1976).

1.6.2 Distribution

The known native range of *G. ternetzi* is within the Rio Paraguay river basin in South America. Nearby populations in the upper Rio Parana and Rio Paraiba do Sol systems (Benine et al. 2015), as well as the Amazon River delta including the Paraquai and Rio Negro systems (Brysiewcz et al. 2009), are thought to be introduced and not included in native distributions.

1.6.3 Habitat

The Black Tetra's South American, sub-tropical origins dictate that they prefer warm, freshwater environments (Exotic Aquariums 2009; Jones 2016). Ornamental aquarium websites describe them as an undemanding species that can adapt to a range of conditions (Sharpe 2016), they are a small fish native to predatory waters and are therefore hiders by nature (Exotic Aquariums 2009). As such, they are accustomed to large plants in their natural environment that would provide shelter (Exotic Aquariums 2009; Fish Channel 2015). These plants are often overhanging along the banks of the slow flowing waters of creeks, tributaries and streams, as well as stagnant waters and ponds (Brysiewcz et al. 2009; Seriously Fish 2016). Juveniles and adults have been found together in macrophytes of oxbow lakes of floodplains in Brazil, with higher abundance in the dry season than wet season, and along with other small-sized lentic species, and juveniles of large non-migratory and migratory species (Meschiatti et al. 2000).

The habitat as described in the notification is as follows:

"*In nature, it is associated with rooted plants in slow-moving freshwaters, like oxbow lakes (Meschiattia, Arcifaa, & Fenerich-Veranib, 2000). The striped pattern is thought to be advantageous in avoiding predation under these conditions (Frankel, 2004).*

In its native habitat, this species inhabits relatively cool (22-24°C) slow-flowing freshwaters that are brown, but clear and slightly acidic (pH 5.5-6.3) (Sakurai, Sakamato, & Mori, 1992). It grows to about 5cm in length at maturity; it is omnivorous and in nature feeds on worms, insects, and small planktonic crustaceans (Mills & Vevres, 1989)."

1.6.4 Physiological tolerances

1.6.4.1 Oxygen

There is no reported literature on dissolved oxygen content preferences or tolerances of the Black Tetra.

1.6.4.2 Temperature

There are no known formal studies on the minimum and maximum temperature requirements of the Black Tetra. In the aquarium trade, Black Tetras are thought to prefer a slightly lower temperature than some other Tetras or tropical fish with which they are commonly paired (Aqua-Fish Net 2005) and they are known for their hardiness and can tolerate a normal ambient temperature range of 20-26°C (AC Tropical Fish 2016; Aqua-Fish Net 2005). However, slightly narrower ranges have also been suggested (e.g., 23-25°C by Brysiewcz et al. 2009; 23-26°C by Scheurmann 1990 and Mills and Vevres 1982; 22-24°C by Sakurai et al. 1992) in order to ensure optimum health, although Frank (1980) suggested they could survive down to 16°C. The maximum and minimum temperatures at which Black Tetra can reproduce have not been published, but recommended ranges include 27°C (Axelrod and Vorderwinkler 1976), and 27-29°C (Uma and Chandran 2008). Leggatt et al. (2018) examined the lower temperature tolerances of the white variant of *G. ternetzi* exposed to a gradual decrease in temperature (1°C per day), starting at 20°C, and found the fish lost equilibrium at 9.95 ± 0.03°C. GloFish LLC

provided minimum temperature tolerances of white variants of *G. ternetzi* exposed to rapid drop in temperature, and found White Tetras died at 7.84 ± 0.05°C. The difference in lower temperature tolerance between the two studies is most likely due to differences in the rates of temperature change, where slower temperature change allows for both acclimation to occur, and more time for cold temperature to exert deleterious effects (Beitinger et al. 2000). In this case the latter is likely to account for the higher minimum temperature tolerance of the White Tetra during slower temperature change (Leggatt et al. 2018), and the slower temperature change may more accurately represent the minimum temperature at which the White Tetra may overwinter.

1.6.4.3 pH

Black Tetras appear to prefer soft, acidic water (Jones 2016; Sharpe 2016). That said, it has been noted that these fish are able to tolerate a pH that ranges between 5.0 and 8.5 (AC Tropical Fish 2016; Brysiewcz et al. 2009; Luna and Reyes 2016), and Gonzalez et al. (1997) found Black Tetras could restore ionic balance with exposure to pH 4.5, but not pH 4.0.

1.6.4.4 Salinity

There is no reported literature on salinity preferences or tolerances of the Black Tetra, though Rothen et al. (2002) found extreme mortality in Black Tetra yolk sac larvae when exposed to 1 and 3 ppt salt treatment but very low mortality at the free-swimming larval stage, suggesting salinity tolerance may vary with life stage.

1.6.4.5 Morphology, life-history and growth

The Black Tetra is grey with two prominent vertical black bands located behind the operculum (Frankel 2004; Jones 2016; Sharpe 2016). Juveniles display the most intensive colouration and will become duller as they age, fading to a silvery grey (AC Tropical Fish 2016; Brysiewcz et al. 2009; Exotic Aquariums 2009). In addition, the White Tetra strain has been developed for the aquarium trade by selective breeding of naturally occurring phenotype that is lighter in colouration with no bands (Frankel 2004, see Figure 1.3). There are also White and Black coloured varieties with longer fins that occur naturally in the general population and are selectively bred to produce long-fin variants for the aquarium trade (Axelrod and Vorderwinkler 1976, see Figure 1.3). *G. ternetzi* is a relatively small fish. Its normal length in an aquarium setting is approximately 4-6 cm, while they can reportedly grow to a maximum length of 7.5 cm in the wild (Brysiewcz et al. 2009; Çelik et al. 2012; Innes 1950; Luna and Reyes 2016; Nico and Fuller 2017). The lifespan of the Black Tetra is between 3 and 6 years but could be extended in an aquarium setting if ideal conditions for survival are maintained (Aqua-Fish Net 2005).

All juveniles of the Black Tetra go through a female stage of development, although differential ovary and testes development commences by 42 dpf (Mazzoni et al. 2015). At maturity, there are noticeable morphological differences that help to distinguish male from female Tetras. In the adult life stages, males are visibly slimmer and smaller overall than females, who tend to have a rounder belly (Brysiewcz et al. 2009; Seriously Fish 2016; Sharpe 2016). The male also has a broader anal fin, a dorsal fin that is narrower and more pointed than females, as well as a caudal fin that sometimes has white spots (AC Tropical Fish 2016; Jones 2016; Sharpe 2016).

Benine et al. 2015) also identified a pair of small hooks on the first 5-8 anal fin rays in the males that are absent in the females. A variation in striping where the front edge of the anal fin runs parallel to the second vertical line on the abdomen is another distinguishing feature of the female (AC Tropical Fish 2016; Jones 2016).

There has been some conflicting information as to the age of sexual maturity for Black Tetras, with many reports suggesting maturity ranges anywhere from 1-2 years old (AC Tropical Fish 2016; Brysiewcz et al. 2009; Sharpe 2016). In a study of gonadal development, Mazzoni et al. (2015) found first gonadal maturation was between 150 to 180 dpf (5-6 months), and GloFish LLC states Tetra brood stock reach sexual maturity in 4-5 months, depending on stocking density and season. Once mature, Tetras in general are prolific reproducers, able to double their population in roughly 15 months in a controlled setting and female fish may reproduce every 10-14 days (AC Tropical Fish 2016; Jones 2016). In nature, however, fish larval production is strongly correlated with zooplankton abundance and the resulting biomass is normally not sufficient to handle such large numbers of larvae and would likely limit population growth (Sarma et al. 2003).

While there have been few studies on the reproduction and early development of Black Tetras, aquarium breeding techniques have provided the best proxy for a lack of information on wild conspecifics. Fish of this species are known to scatter eggs and reproduce successfully in densely planted tanks with fine-leaved foliage such as java moss, with the adhesive eggs deposited between leaves (AC Tropical Fish 2016; Jones 2016), and eggs and fry are light sensitive in early stages (Seriously Fish 2016). Males will generally claim and guard a territory during spawning events and the number of laid eggs can total up to 2000 (AC Tropical Fish 2016; Brysiewcz et al. 2009; Jones 2016). Spawning has been noted to occur in the early morning for Tetras, suggesting a diurnal reproduction cycle, (Çelik et al. 2012; Seriously Fish 2016). Adults have commonly been recorded eating eggs in the aquarium setting but it is unknown if this behaviour exists in wild counterparts (Seriously Fish 2016; Sharpe 2016).

The morphological development of Black Tetras is similar to that of zebrafish (see Kimmel et al. 1995), although embryonic development occurs approximately twice as fast up to the hatching stage with the first cell division occurring after only 30 minutes (Pan et al. 2008). Hatching generally occurs after 20-24 hours (AC Tropical Fish 2016; Kimmel et al. 1995; Sharpe 2016), and the embryos spend the next 5-6 days attached to the walls of the tank before filling their swim bladders and hunting for food (Frank 1980).

1.6.4.6 Background genetics

There is no reported literature on the background genetics of Black Tetras. However, Black Tetra have been bred specifically for the home aquaria since before 1950 (Innes 1950), including selective breeding of naturally occurring white morphs and/or long-fin variants, and Teletchea (2016) listed the Black Tetra as being at the highest level of domestication for aquarium fish.

1.6.4.7 History of invasiveness

Black Tetras are thought to have been introduced just outside their native range to the upper Rio Paraná and Rio Paraíba do Sol (Benine et al. 2015), as well as the Amazon River basin in South America (Brysiewcz et al. 2009). They are also reported to be established from released ornamental fish in Colombia (Welcomme 1992), temporarily established in a Colorado hot spring, and isolated occurrences have been reported in Florida and Louisiana (Nico and Fuller 2017; Tuckett et al. 2017), but no occurrences of establishment in temperate climates (US and Europe, Brysiewcz et al. 2009) have been reported over the 70 or more years of its use in the ornamental aquarium trade.

1.6.4.8 Trophic interactions (diet, prey, competitors, predators)

Like most fish, survivability for the Black Tetra depends on the availability of appropriate food during the critical phase in the first few weeks after hatching (May 1974). Ciliate, rotifer, cladoceran and copepod zooplankton are the natural food for many ornamental aquarium fish species and Black Tetras are no exception (Sarma et al. 2003). Normally inhabiting the middle and upper water layers, Black Tetras exhibit a primarily carnivorous lifestyle, feeding on readily available worms, small crustaceans and insects (Galib 2011). However, it is not a particularly selective species, ingesting smaller amounts of algae and plant matter (AC Tropical Fish 2016; Lim 2008). In aquarium settings, hobbyists are advised to feed Black Tetras a diverse diet, including daphnia and mosquito larvae as well as frozen bloodworms and brine shrimp (AC Tropical Fish 2016; Seriously Fish 2016; Sharpe 2016). They will just as easily feed on readily available commercial feeds such as flake food, and spirulina is also a common supplement in aquarium environments (AC Tropical Fish 2016; Sharpe 2016).

It is not known whether the competitors and predators of the Black Tetra have been specifically described, although the Paraquay River basin contains potential competitors such as other Tetra species, and well as potential predators such as various piranha species, wolf fish, and golden dorado (Hales and Petry, Freshwater Ecoregions of the World). Meschiatti et al. (2000) described Black Tetras as part of macrophyte-associated fish communities in oxbow lakes that consisted predominately of small-sized species common in lentic environments (e.g., Black Tetra), juveniles of large non-migratory species and a few juveniles of migratory species. Black Tetras are generally described as a “community tank” fish, and are generally not known to be aggressive to or highly competitive with other aquarium fish although may prey on much SMALLER FISH (FRANK 1980).

1.7 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. Lakes in Canada can range from highly oligotrophic (low productivity) to highly eutrophic (high productivity) depending on the circumstances of their location.

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. Indeed, water temperature is a key abiotic factor that affects both the survival and reproduction of most freshwater fish populations, and is a pervasive determinant of habitat suitability (Amiro 2006; Elliott and Elliott 2010; Jobling 1981; Magnuson et al. 1979).

Lakes in Canada can be categorized into one of four separate types: amictic, monomictic, dimictic, and meromictic. Amictic lakes are found at high altitudes and latitudes, and remain ice covered throughout the year. Most lakes in Canada are holomictic, undergoing physical mixing at least once per year, when a dense surface layer at 4°C, sinks to the bottom of the lake, displacing bottom water to the surface while mixing (Figure 1.4). Monomictic lakes are holomictic lakes that mix layers only once per year, whereas dimictic and polymictic lakes mix layers two or more times per year.

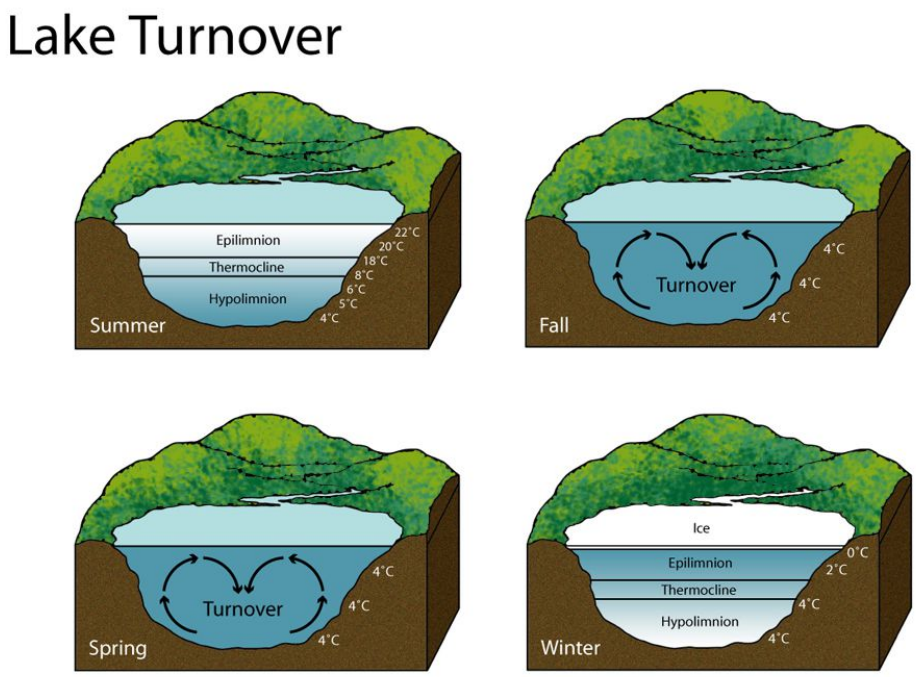

Figure 1.4: *Example of how lake temperatures are affected by turnover (From National Geographic: Lake Turnover).*

One of the warmest lakes in Canada in the winter is Cowichan Lake; a monomictic lake on Vancouver Island, BC, where minimum recorded winter water temperature ranges from 5.8 to 6.8°C (data obtained from BCLSS 2014, and Government of BC Environmental Monitoring System (EMS) database, see Figure 1.5a). As with several other coastal lakes in BC, Cowichan Lake is classified as monomictic, with temperature layers that remain stratifies for most of the

year, except during the winter, when the entire lake is mixed at 4°C or warmer (winter turn-over). This type of lake is rarely frozen and typically remains open during the winter.

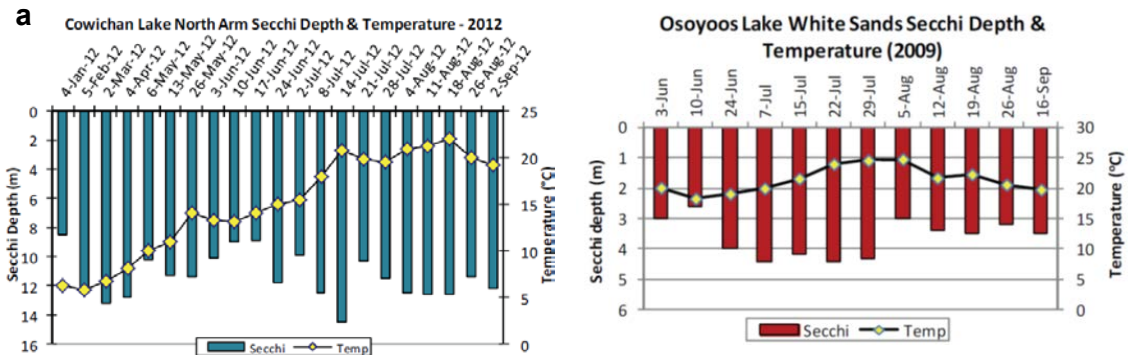

Figure 1.5 *Examples of Canadian lakes with* ***a)*** *relatively warm winter water temperature (Cowichan Lake, taken from BCLSS 2014) and* ***b)*** *warm summers water temperature (Osoyoos Lake, taken from BCLSS 2013).*

Many other warm lakes can be found in the interior of BC, of which Osoyoos Lake is one of the warmest; a dimictic lake with temperatures that remain within the ideal range for Tetras (20 to 26°C) for much of the summer (BCLSS 2013, see Figure 1.5b). As well, many other surveyed lakes have maximum temperatures within this range (see Figure 1.6), although maximum temperatures may not necessarily represent temperatures over much of the summer. For Osoyoos Lake, colder winters temperature of the BC interior mean Osoyoos Lake, as with most lakes in the BC interior and the rest of Canada, is dimictic, such that the water column remains stratified for the summer and winter, but undergoes two turn-overs at 4°C, in the spring and fall, with the warmest water (i.e., 4°C or less) stratifying at the bottom of the lake in the winter (Figure 1.4).

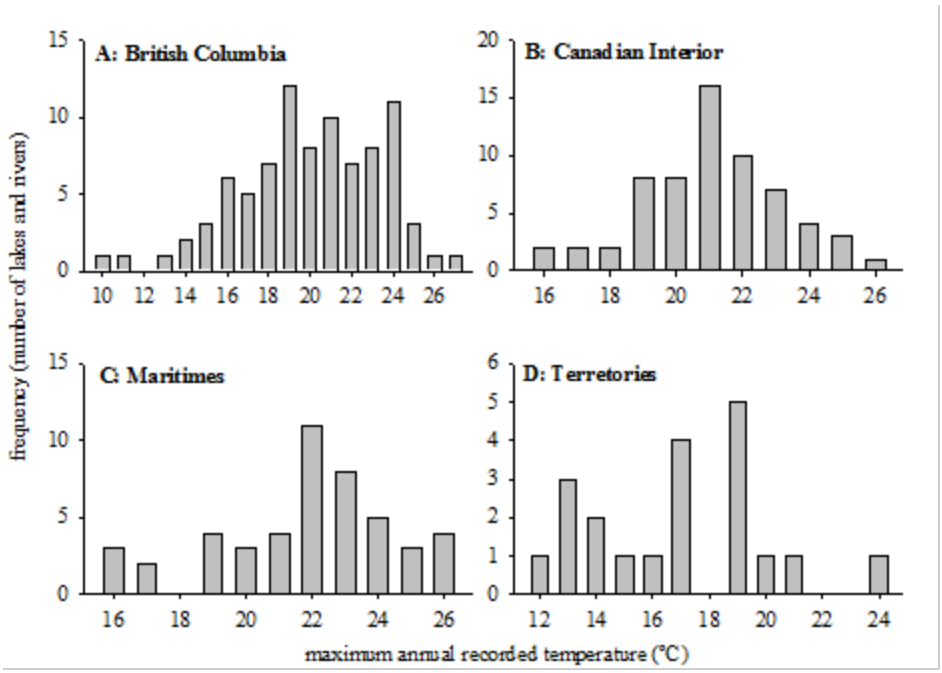

Figure 1.6. *Frequency of maximum temperature ranges during summer months in surveyed lakes and rivers in Canada. From Leggatt and Devlin (2006).*

In both dimictic and monomictic lakes, mixing of the water column brings nutrients at the bottom of the lake up into the water column; an ecologically important event that occurs when heavy (dense) surface water, at 4°C, sinks to the bottom of the lake, breaking the stratified layers. During the turn-over, water in the lake remains at 4°C until temperatures above, or below 4°C prevails, and the water column returns to being stratified. Consequently, 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 (Leggatt et al. 2018, see Figure 1.7), and only a few isolated lakes in Southern Coastal BC have minimum recorded temperatures above this (of these minimum temperatures are below 6°C when measured over several years, see Leggatt et al. 2018). Therefore, if an introduced fish cannot survive at 4°C or below, its occurrence in the Canadian environment will be seasonal at best, with possible localized overwintering pockets if the organism can survive from 4 to 6°C.

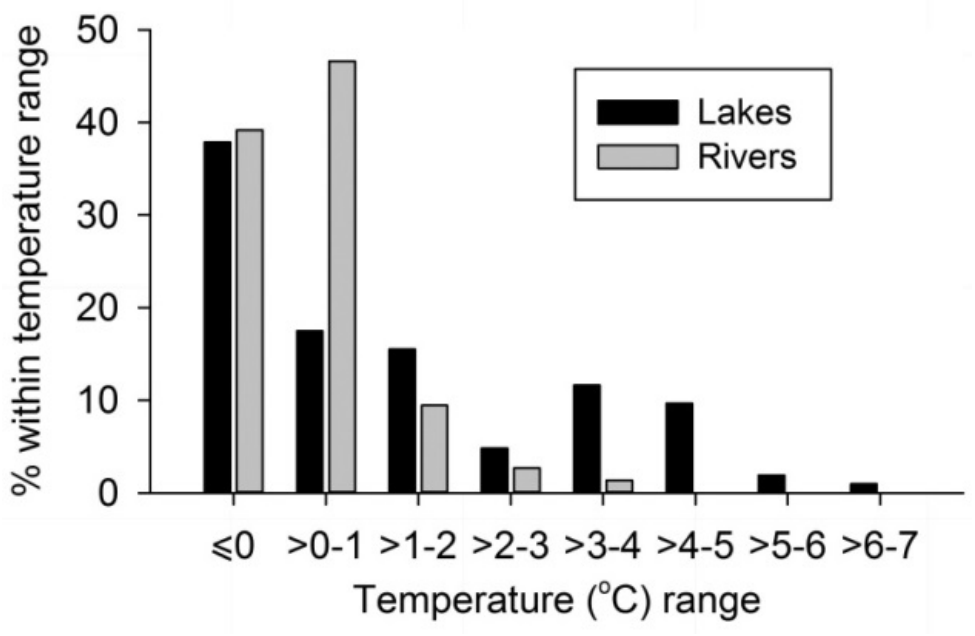

Figure 1.7: *Minimum winter water temperature ranges in surveyed lake and river systems in Canada. Taken from Leggatt et al. (2018).*

Many freshwater systems may have heterogeneity in temperature profiles – for example shoreline regions of lakes may experience more extreme temperatures than central regions (e.g., Trumpickas et al. 2015), or groundwater contributions may increase or decrease temperatures in localized areas of a water body. While such microheterogeneity in thermal profiles is known to exist, it is not widely nor fully defined among Canadian aquatic environments. As well, there are over 40 identified hot springs in Canada with water temperatures higher than ambient temperatures. Many of these have been heavily developed for human use, and/or have temperatures higher than water temperatures expected in tropical regions (e.g., above 40°C). There are also hot springs in Canada with more moderately warm temperatures (e.g., Rabbitkettle Hotsprings in Nahanni National Park Reserve, NT has year-round temperature of 20°C, https://www.pc.gc.ca/en/pn-np/nt/nahanni/decouvrir-discover/natcul1c#rab), and hot springs that feed into surrounding water systems may have temperatures gradients that encompass ideal temperatures for tropical fish persistence. In addition, warm water industrial effluent (e.g., from power plants) can provide areas of refuge for species that require higher than ambient temperature (e.g., see Gozlan et al. 2010).

Climate change is expected to alter long-term temperature patterns in freshwater systems in North America. Predicted changes include increases in surface water temperatures, increases in depth of thermoclines during summer stratification, and increases in ice-free days during the winter (e.g., Missaghi et al. 2017; Santiago et al. 2017; Trumpickas et al. 2015). The impact of climate change on individual water systems is expected to vary, depending on factors such as hydrological processes (e.g., Ficklin et al. 2014 Santiago et al. 2017), location within lakes (e.g., near shore or deep water, elevation, Mountain Research Initiative EDW Working Group 2015; Trumpickas et al. 2015), and lake morphology (e.g., Kraemer et al. 2015). Increases in maximum temperature are expected to be higher than increases in minimum temperature (e.g.,

Ficklin et al. 2014; Trumpickas et al. 2015). Consequently, local and long-term temperature patterns should be considered when assessing environmental risk of novel organisms, and may contribute to uncertainty in risk assessments.

1.8 SUMMARY

Within the legislative context of CEPA and the information requirements of the New Substances Notification Regulations (Organisms) Schedule 5, this document elaborates the Framework for the assessment of potential risks to the Canadian environment that may be associated with the import or manufacture of GE fish. The environmental risk assessment is conducted in accordance with the classical risk assessment paradigm where risk is directly related to the exposure and hazard of the organism. The exposure assessment is based on the likelihood and magnitude of release into the environment, and the likelihood and magnitude of survival, reproduction, establishment, and spread of the organism and potential descendants of the organism in the Canadian environment. The hazard assessment is focused on the potential for the organism to impact: (1) potential prey, predators and competitors of the organism; (2) biological diversity; and, (3) habitat. The level of uncertainty for both exposure and hazard determinations is evaluated and communicated in terms of impact to the final risk assessment. DFO provides science advice in the form of peer-reviewed risk assessments to Environment and Climate Change Canada for regulatory decision-making under CEPA, based on risk to the environment and the uncertainty associated with the conclusion.

PART 2: ENVIRONMENTAL RISK ASSESSMENT

2.1 PURPOSE OF PART 2

Part 2 of this document comprises the environmental risk assessment conducted under the *Canadian Environmental Protection Act*, 1999 (CEPA) with respect to CGT2016, a genetically engineered white variant of the Black Tetra (*Gymnocorymbus ternetzi*) notified by GloFish LLC under the New Substances Notification Regulations (Organisms) [NSNR(O)].

The environmental risk assessment of CGT2016; also known as the GloFish® Electric Green® Tetra or the GloFish® Long-Fin Electric Green® Tetra, was scientifically peer reviewed and concluded within the 120-day legislative timeframe allowed by the NSNR(O) for notifications under Schedule 5.

Further information on CEPA 1999 and NSNR(O), including guidance on the regulations, detailed guidance for information requirements, use of waivers, significant new activities, risk assessment outcomes and risk management can be found on the Biotechnology page of the Environment Canada website (New substances: biotechnology, living organisms) or the Problem Formulation in Part 1 of the document.

2.2 EXPOSURE ASSESSMENT

"*In a 2005 survey of potentially invasive aquatic species available in Canada through the aquarium trade,* G. ternetzi *was excluded from concern as an invasive risk due to its inability to survive Canadian winters and an absence of history as an invasive species (Rixon, Duggan, Bergeron, Ricciardi, & Macissac, 2005)"* (taken from the Notification document for CGT2016).

The exposure assessment for living 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. Figure 2.1 illustrates how, at each step of the exposure assessment, there is a greater potential for the number of organisms that occur in the environment to increase. It also helps to illustrate how the likelihood of occurrence at each step of the exposure pathway will depend on the likelihood of occurrence at the previous step. For example, if the organism is unlikely to survive in the Canadian environment, it is also unlikely to reproduce or proliferate in the environment.

All relevant information regarding physical, biological and geographical containment strategies used at all life stages, and the potential for exposure resulting from the failure of containment during transport, distribution and use of the organism should be evaluated, as well as the potential for both unintentional release resulting from human error or the failure of equipment, and intentional release that may result from cultural or other reasons. When considering the potential for CGT2016 to reproduce and proliferate in the Canadian environment, the reproductive fitness of both the organism and potential descendants of the organism, the stability of the sex-determination systems used by CGT2016, and the influence of propagule

pressure on occurrence should be considered. Rankings for the likelihood of Exposure to the Canadian Environment are provided in Table 2.1.

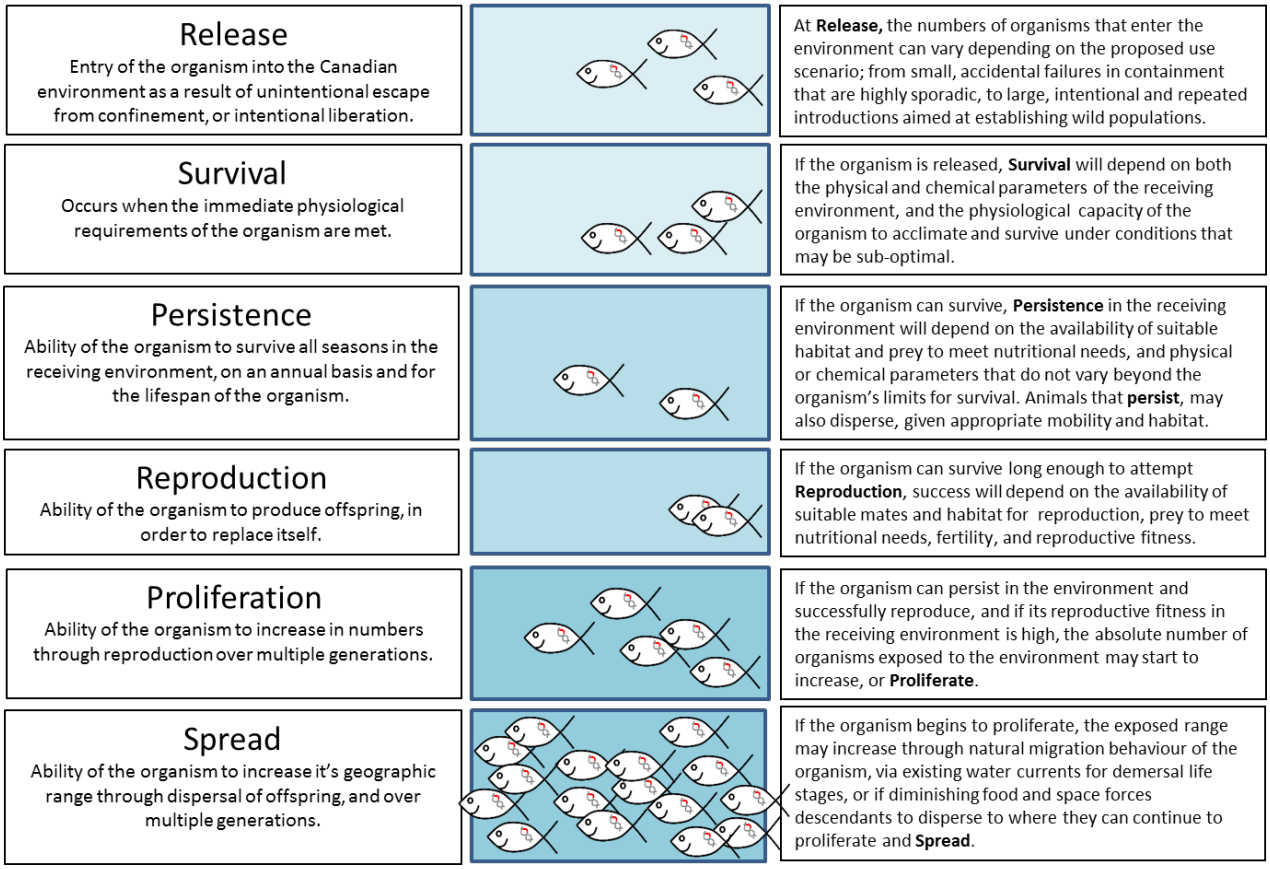

Figure 2.1: *Exposure pathway, where likelihood of each step is limited by the likelihood of the previous step.*

Table 2.1: *Rankings for exposure of CGT2016 to the Canadian environment.*

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

Given the regulatory status of any GE fish undergoing environmental risk assessment under CEPA, a lack of empirical data regarding the survival, fitness and ability of CGT2016 to reproduce in the natural environment will contribute uncertainty to the exposure assessment. Uncertainty associated with the environmental fate of an organism or the failure of biological

and geographical containment may depend on the availability and robustness of the scientific information related to the biological and ecological parameters of the organism, valid surrogates, and the receiving environment. Knowledge gaps or lack of empirical data regarding the survival, fitness, and ability of the CGT2016 to reproduce in the natural environment may also contribute to uncertainty in the assessment of exposure. Table 2.2 ranks uncertainty associated with the likelihood of occurrence and fate of the organism in the Canadian environment.

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

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

2.2.1 Likelihood of Release

Though the stated purpose of the organism is for sale in the ornamental market, and hobbyists who purchase the product do, for the most part, follow the instructions for disposal that are recommended by the retailer or the company itself, there is still a high likelihood that CGT2016 will, sooner or later, be introduced into the Canadian environment. Numerous aquarium fish have established themselves in natural waters in North America, and reoccurring, though isolated reports of aquarium fish in Canadian water suggests the practice of releasing aquarium fish into the environment is common and ongoing (Dumont et al. 2002). Indeed Rixon et al. (2005), and Kerr et al. (2005) both cite the aquarium hobby industry as a significant source for introductions of exotic aquatic organisms into the Great Lakes Basin. In the Pacific Northwest, Strecker et al. (2011) estimated there are 2500 fish released annually into the Puget Sound

region by aquarists, and a survey of aquarium fish owners in Ontario reported 2% of unwanted ornamental aquarium fish had been released to the environment (Marson et al. 2009). Once the organism has been sold into the retail market, it is no longer under the direct control of the importer, and there can be no guarantee of appropriate containment and disposal. Consequently, it's 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 therefore depend heavily on its ability to survive and reproduce in Canadian lakes and rivers. The magnitude of each release event is expected to be very small, although the possibility of larger releases from larger purchases or breeding CGT2016 in the home aquaria cannot be excluded.

Another temporary environmental exposure pathway could be through disposal of CGT2016 carcasses. Potential exposure pathways include disposal of CGT2016 to municipal sewers that could result in exposure to natural water systems if sewage systems have minimal treatment or to open-air landfills or composting areas that can result in exposure to land-based ecosystems. The entire mass of CGT2016 imported into and throughout Canada per year would be less than 160kg, presumably distributed across the country with higher concentrations in urban areas. It is expected that less that the total mass of CGT2016 in Canada would enter the environment via disposal of carcasses, although the proportion of total mass that may enter through this route cannot be estimated at this time.

2.2.2 Likelihood of Survival

Water temperature is a key abiotic factor that affects both the survival and production of most freshwater fish populations, and is a pervasive determinant of habitat suitability (Amiro 2006; Elliott and Elliott 2010; Jobling 1981; Magnuson et al. 1979). As a tropical species, the Black Tetra is not expected to survive in a temperate region where water temperatures are below optimal for survival. Whereas the optimal temperature for Black Tetras may lie somewhere between 20 and 29°C (see Problem Formulation), data collected by DFO for White Tetras indicate a lower lethal temperature of approximately 9.8°C, when temperature is lowered slowly from the optimum, at a rate of 1°C per day (Leggatt et al. 2018). Leggatt et al. (2018) also reported changes in White Tetra activity and feeding level with decreasing temperature, where White Tetras decreased feeding level and activity at 17°C, stopped feeding when temperatures approached 12°C and stopped activity just above 10°C (see Figure 2.2). GloFish Technologies provided data from similar experiments as part of its notification package; however details of the experiment are considered confidential and are not included in this report. Briefly, CGT2016 had slightly impaired cold tolerance when temperature was rapidly decreased (i.e., average LD_{50} was 8.11°C relative to 7.94°C for wildtype siblings). Based on the studies, it is reasonable to conclude that White Tetra and CGT2016 cannot survive at temperatures below 7°C, and likely cannot survive long-term at temperatures lower than 9.5°C.

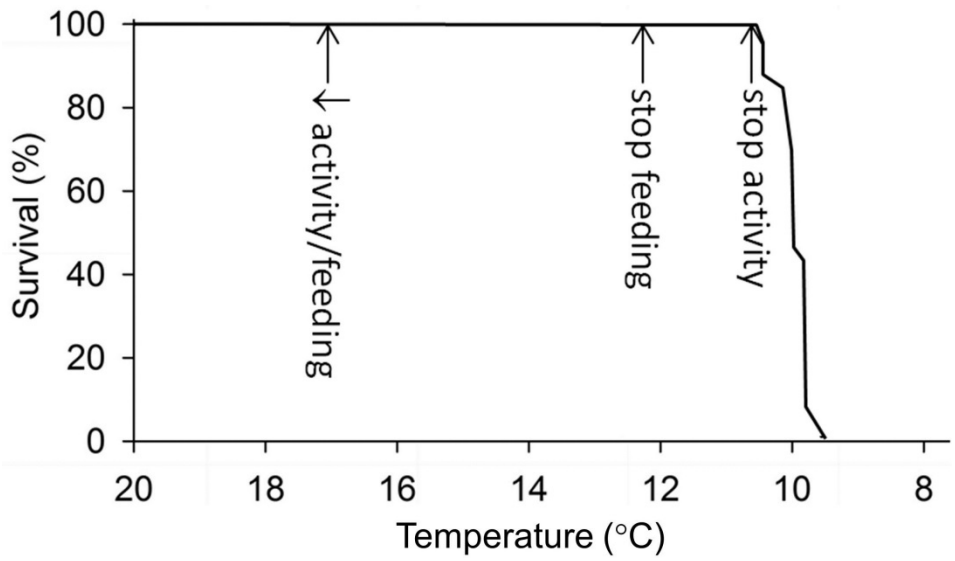

Figure 2.2 *Survival and changes in activity and feeding level in White Tetra when temperatures are lowered gradually from 20°C at a rate of 1°C per day. Modified from Leggatt et al. (2018).*

As discussed in the Problem Formulation (see Characterization of potential receiving environment) there are no lakes in Canada that consistently remain above 6°C throughout the entire course of a year, and most do not remain above 4°C throughout the year. Consequently, while the temperatures needed for CGT2016 to survive are possible for several Canadian lakes during the spring, summer and fall, it is highly unlikely that CGT2016 can survive the Canadian winter. At best, its occurrence in the environment would be seasonal or ephemeral.

2.2.3 Likelihood of Reproduction

Though water temperatures in Canada will limit the occurrence of any CGT2016 that are introduced into the environment (see Section 2.2.2), there may still be time to reproduce, if introduced at the start of a warm season. For example, Osoyoos Lake in the BC interior is one of Canada's warmest lakes in the summer (see Figure 1.5), with an average temperature between 20 and 25°C for about 2 months of the year (mid-July to mid-September, BCLSS 2013). While this may be an ideal temperature range for White Tetra survival, warmer temperatures (27 to 29°C) are more ideal for reproduction. As noted in the Problem Formulation (see section on Comparator Species), Tetras can be prolific breeders under ideal conditions, though in nature, fish larval production is strongly correlated with zooplankton abundance and limits population growth. Under controlled conditions, fish of this species are known to scatter eggs and reproduce successfully in densely planted tanks with fine-leaved foliage, such as java moss, on which adhesive eggs are deposited. Age of sexual maturity for White Tetras may range from four months to over one year depending on stocking density and temperature. Consequently, any reproduction would be limited to a short window of opportunity during the summer, regardless of its age at the time of introduction. For example, any CGT2016 introduced to Osoyoos Lake at the beginning of July would have two months in a novel environment to find habitat and resources needed for reproduction, as well as conspecifics to reproduce with. Though any fertilized eggs that are not eaten as food could hatch in a relatively short period of

time (24 hours), any offspring would not mature prior to onset of cooler temperatures, would not survive the winter, and would no longer occur until the next introduction. Though isolated opportunities for reproduction in the Canadian environment could occur, it would never result in more than a single generation presence in the environment.

2.2.4 Likelihood of Proliferation and Spread

The capacity for CGT2016 to proliferate and spread in the Canadian environment is precluded by the fact that White Tetras cannot survive the Canadian winter. Consequently their occurrence in the environment is expected to be isolated, rare, and ephemeral.

One possible exception to this would be in a minimal number of isolated pockets of warm water (e.g., hot springs or in warm water industrial effluent). For example, establishment of other tropical fish species have been reported in Banff hot springs, BC, where local aquarists purposefully introduced numerous tropical freshwater aquarium species in 1960 (Mayhood 1995). Of the species introduced at this site, almost all are listed by the US Geological Survey of having numerous established populations within the USA (USGS Nonindigenous Aquatic Species Database), but only two, sailfin molly and African Jewelfish, successfully established while other species did not persist past a few years (e.g., guppy, green swordtail, convict cichlid, freshwater angelfish, Mayhood 1995). Black Tetra were introduced into a hot spring in Colorado, USA, and persisted for a few years but failed to establish (Nico and Fuller 2017). In order for CGT2016 to become established in isolated warm thermal areas, the temperatures would have to remain stable enough, and other ecological conditions be appropriate for long-term growth, survival, reproduction, and embryo and juvenile development and growth. While the requirement limits of *G. ternetzi* and CGT2016 for reproduction and survival have not been established, general temperature recommendations for reproduction are 27-29°C, suggesting very specific temperature requirements for reproduction and consequent establishment that may not be present in most thermal pockets in Canada. Combined with the poor success of establishment in hot springs of tropical species that have greater overall establishment success than *G. ternetzi* (see above) and the lack of establishment of *G. ternetzi* when introduced to a Colorado hot spring (Nico and Fuller 2017), the potential for *G. ternetzi* and CGT2016 to establish isolated populations in thermal pockets of freshwater in Canada is expected to be negligible.

It is noted that any released CGT2016 are expected to occupy areas near the shoreline and not in deep-water areas, based on what is known of wild-type habitat preferences. These areas are expected to have more extreme temperature ranges than deep-water or mid-lake areas that are often the source for water temperature measurements (Trumpickas et al. 2015). Consequently, there may be more warm temperature areas suitable for breeding in summer months than indicated by recorded data, but conversely winter temperatures may be colder than recorded which may reduce the potential for overwintering of CGT2016.

2.2.5 Conclusions

Given the above analysis, the occurrence of CGT2016 in the Canadian environment is expected to be rare, isolated and ephemeral and likely in low numbers. Consequently the likelihood of exposure of CGT2016 to the Canadian environment is ranked low (Table 2.1). The uncertainty

associated with this estimation is low, given the quality data available for CGT2016 and valid surrogate organisms (temperature tolerance) and data available on the environmental parameters of the receiving environment in Canada (Table 2.2). This conclusion concurs with those of Hill et al. (2014) that predicted low invasion potential of GloFish® Tetras in the US based on a lack of potential fish to hybridize with, limited history of invasiveness, a lack of traits associated with persistence, and high predation potential based on their small size and fluorescent colouration.

Changing water temperature patterns with climate change has potential to increase uncertainty associated with determining the ability of the notified organism to survive, reproduce, proliferate and spread in Canadian freshwater ecosystems. The predicted increases in temperatures in summer months may increase the number of areas with adequate temperatures for spawning, as well as the length of time adequate conditions are available. However, increases in winter temperatures are predicted to be smaller than in summer months, and are not currently predicted to increase by the 5°C or more needed for *G. ternetzi* to overwinter in most systems, as estimated by their laboratory minimum temperature tolerance. As such, climate change is not expected to add significant uncertainty to, or alter the low environmental exposure ranking for CGT2016 in the near term.

The notifying company identifies the sole intended use for the notified organism as an ornamental fish for interior, static home aquaria. However, once purchased by consumers, other unintended uses cannot be discounted. While some unintended uses may increase release of CGT2016 (e.g., as bait fish, in outdoor ponds), they would not be expected to alter their ability to overwinter in Canadian environments or otherwise alter the low environmental exposure ranking for the organism.

Overall, the likelihood of CGT2016 exposure to the Canadian environment is estimated, with low uncertainty, to be low. This conclusion and its associated uncertainties are discussed further in the Assessment of Risk (Section 2.4).

2.3 HAZARD ASSESSMENT

The hazard assessment examines potential impacts that could result from environmental exposure to CGT2016 in the environment. The hazard identification process considers the potential environmental toxicity (i.e., potential to be poisonous), allergenicity, capacity to act as a vector for pathogens, and capacity to impact ecosystem components. Table 2.3 categorizes the severity of the biological consequences based on the severity and reversibility of effects to the structure and function of the ecosystem. The severity (negligible, low, moderate, high) of the potential impacts to the assessment endpoints is evaluated in the risk assessment as well as the uncertainties; taking into consideration the appropriateness of control experiments and data, rearing conditions, interaction effects, phenotypic plasticity, and genetic background, in order to minimize uncertainty regarding assessment of potential ecological consequences of CGT2016. Any difference in measurement endpoint is evaluated relative to ‘normal’ variation, based on published studies and expert opinion (Table 2.3).

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

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

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

Given the lack of empirical data around the behaviour and fitness of CGT2016 in the natural environment, significant attention to uncertainty considerations in the hazard assessment is required. Uncertainty around the hazard assessment may be significant due to clear knowledge gaps and lack of empirical data around the behaviour and effects of CGT2016 in the natural environment.

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

- The environment in which fish are reared can significantly affect the phenotypic expression of the transgene (e.g., Sundström et al. 2007). The influence of rearing environment limits our ability to extrapolate laboratory data as a reliable indicator of how a GE fish may behave (e.g., compete, survive) in the natural environment unless it can be demonstrated that wild-type controls reared in the laboratory environment behave the same way as wild-type fish in the natural environment, and it is demonstrated that there are no Genotype by Environment (GxE) interactions between wild-type and GE fish, or GxE interactions are well defined. In the absence of such control data, there is uncertainty around the extent to which we can rely upon laboratory data as an accurate indicator of behaviour in the natural environment;

- The phenotypic effects of the transgene can vary significantly with the genetic background of the parent (e.g., wild-type vs. domesticated species). For example, the performance of a wild-type fish with an inserted growth hormone gene construct may be very different from the performance of a domesticated fish of the same species into

which the same construct has been inserted (e.g., Devlin et al. 2001). Consequently, regulators must scrutinize the background genetics of experimental controls when evaluating the scientific validity of experimental data to assess whether the phenotype is durable across multiple genotypes as would be encountered in nature. Experimental data on transgene expression in one species or strain should be interpreted with caution as it may or may not be representative of the expression of the same transgene in a different species or strain; and

- A single transgene may result in several phenotypic expressions, termed pleiotropic effects. For example, some empirical data demonstrates that increased growth due to transgenesis in some fish species may also affect disease resistance (Jhingan et al. 2003). Thus, unless the investigator has specifically directed attention towards an unintended effect, it may go undetected. It should also be noted that different types of modified genes may have vastly different pleiotropic effects. For example, modification of a central fitness trait, such as size or growth rate, is expected to have broad pleiotropic and fitness implications for all traits related to growth, whereas the pleotropic implication for more benign traits, such as eye colour or flesh pigmentation, may be limited.

Criteria for the assessment of uncertainty address potential effects to the environment, which may rely heavily on information and data found in published and peer-reviewed scientific literature. A description of rankings for uncertainty regarding the potential hazards of the organism in the environment is provided in Table 2.4.

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

The proposed use of CGT2016 in Canada (i.e., importation and transport in static containers, holding in static tanks in commercial wholesalers and retailers, rearing in static tanks in home aquaria) provide minimal pathways of effects of CGT2016 to Canadian environments, with the possible exception of release of waste water to Canadian environments indirectly through municipal sewage systems, and release of CGT2016 carcasses to municipal sewers with limited treatment and/or open-air landfills and composts. The majority of potential hazards posed by CGT2016 (e.g., through interactions with other organisms, as a vector for disease, impacts to biogeochemical cycling, habitat and biodiversity) would be through direct release of CGT2016 to natural aquatic ecosystems, although some potential hazards could act indirect release of waste water and carcasses (e.g., environmental toxicity, horizontal gene transfer).

Table 2.4: *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.

2.3.1. Potential hazards through environmental toxicity

Potential routes of environmental toxicity (i.e., potential to be poisonous) of CGT2016 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 or native organisms is expected to be lower, although not necessarily comparable, than within the CGT2016 organism itself. The CGT2016 line is reported to be less cold tolerant and have lower reproductive success in competition than wild-type fish but has no effect on juvenile survival (see Section 1.5.2.3). As well, GloFish LLC submitted statements from two veterinarians involved with CGT2016 and non-transgenic counterparts stating CGT2016 does not have significant health impairments and requires the same husbandry care as non-transgenic counterparts (see Section 1.5.2.3). There are no known major effects of the transgenic fluorescent protein to the viability of the CGT2016 organism, and this suggests toxic effects on native species viability and reproduction are not expected through environmental exposure to the fluorescent protein or the CGT2016 organism.

The expressed transgenic protein is derived from a naturally occurring fluorescent protein from a cnidarian species from the marine environment which is also used in the commercial ornamental aquarium trade worldwide including Canada. Fluorescent proteins are common in many marine organisms, most noted in the Cnidaria phylum, but also occurring in other groups including fishes (Sparks et al. 2014). As well, numerous fluorescent proteins are commonly used as neutral markers in science and the majority of studies do not report toxicity from fluorescent transgenes (e.g., see Stewart 2006). Of the reports of negative effects, they are generally specific to transgenic organisms that have high expression of fluorescent transgenes, where lower expressing lines did not have negative effects (e.g., Devgan et al. 2004; Guo et al. 2007; Huang et al. 2000a).

The Notification of CGT2016 includes a report screening the amino acid sequence of the fluorescent protein for allergenicity on Allermatch. They found no matches with known allergen amino acid sequences using a 7-8 amino acid word length, and seven matches using a six amino acid word length. However, use of amino acid word lengths shorter than eight have high probability of random alignments with no predictive value (see Herman et al. 2009) and consequently these six word length matches are not expected to indicate allergenicity of the fluorescent protein. While the potential toxicity of the fluorescent protein expressed by the construct has not been specifically examined in the scientific literature, another 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 for 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, **indicate negligible potential for environmental toxicity of CGT2016. This ranking has moderate level of uncertainty** due to limited direct data from the notified organism or surrogate organisms, but ample indirect evidence from other fluorescent protein models.

2.3.2. Potential hazards through horizontal gene transfer

Horizontal gene transfer (HGT) is the non-sexual exchange of genetic material between organisms of the same or different species (DFO 2006). Horizontal gene transfer is a rare event, often measured on an evolutionary time frame, and is much more frequent among prokaryotes than eukaryotes (EFSA 2013). Genetic analyses suggest HGT events may have taken place repeatedly in vertebrate evolution, including in fish (e.g., Kuraku et al. 2012; Thomas et al. 2010; Uh et al. 2006), although gene transfers among eukaryotes appear to be primarily limited to filamentous organisms (e.g., oomycetes and fungi, Peccoud et al. 2017). As well, gene transfers in eukaryotes primarily involve transposable elements (i.e., DNA that is able to move from one locus in the genome to another, see Peccoud et al. 2017).

In order for HGT of a specified transgene to take place on a biologically relevant scale and result in hazardous effects, the following steps must occur: exposure and uptake of the free transgene to a novel organism, stability and expression of the gene within the novel organism, neutral or positive selection of the novel organism expressing the transferred gene (DFO 2006), and harmful effects to the environment as a result of expression of the transferred gene in the novel organism.

Exposure: In order for HGT of the transgene to occur, the transgene must be available in free DNA form to a novel susceptible organism. DNA can be released from organisms through release of mucus, skin cells, gametes, feces, etc. (Rees et al. 2014). Routes of DNA release from CGT2016 to the environment could be through disposal of waste aquarium water into municipal sewage systems that feed directly, or with minimal treatment, to natural water systems. If CGT2016 fish were intentionally or accidentally released to natural water systems this could also provide a continuous source of transgene DNA release as long as the fish persisted in the system. Disposed of carcasses in open-air landfills or composts could also be a source of transgene DNA release. Free DNA within water systems decays quickly, within days

or weeks, although can persist longer in sediments (Turner et al. 2015). The small size of CGT2016 indicates the magnitude of free DNA source will be relatively small, and less than 160kg of CGT by mass are expected to be imported per year (see Exposure section) indicating exposure of susceptible organisms to free DNA from CGT2016 would be low, but likely present.

Uptake: A novel organism must take up the DNA segment intact. This includes prokaryotes as the most likely to uptake foreign DNA (DFO 2006), and in higher organisms uptake to germline or reproductive cells would be required for propagation past a single generation. The insert sequence of the CGT2016 transgene does not include any features common to transposable elements (as listed by Sultana et al. 2017) or mobile elements. Consequently the transgene in isolation is not expected to have increased uptake beyond that of wild-type Tetra. Whether the transgene is inserted beside or within a naturally occurring transposable element of *G. ternetzi* is not known. While the presence of transposable elements has not been specifically examined in *G. ternetzi*, they are present in Characiformes species (e.g., Daniel et al. 2015; Schemberger et al. 2016; Yano et al. 2016), and consequently the potential for the transgene to be near transposable elements in the *G. ternetzi* genome cannot be discounted.

Stability, Expression and Selection: DFO (2006) identified stability of DNA in a new host as the most significant barrier to HGT by natural selection. Prokaryotes are the organisms with the most potential for horizontal gene uptake, and the fish and cnidarian origin of the inserted gene construct result in an expected lack of homology between the transgene and bacteria recipient DNA that would assist in in stability of the inserted gene construct. For expression of the transgene to occur in a novel organism, co-transfer of one or both of the regulatory elements would have to occur. While the potential for the fish-origin promoters used to functionally express in prokaryotic organisms has not been directly examined, vertebrate promoters generally have low activity in prokaryotic hosts (see DFO 2006) and are therefore not expected to have significant activity in prokaryotic hosts. However, the potential recombination of the novel DNA by the prokaryotic host cannot be discounted (Brigulla and Wackernagel 2010), and coding regions of the transgene could be inserted next to bacterial promoters. Consequently the potential for the construct to be expressed in a new prokaryotic host cannot be excluded. Should all of the above steps occur, neutral or positive selection for the organisms with the novel phenotype would then have to occur for the mobile transgene to persist in the population, and the potential for this to occur has not been explored. Genes encoding 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 detected, suggesting introduction of the transgene through HGT to novel hosts is not expected to result in harmful effects. While the introduction of the transgene to novel organisms in Canadian environments through HGT cannot be excluded, the lack of expected harmful effect of such an introduction results in **low hazard rating through HGT**. As the insert site of the transgene is not known and there is reliance on surrogate data for impacts should HGT occur, **the uncertainty associated with this rating is low**.

2.3.3. Potential hazards through interactions with other organisms

The trophic interactions of wild-type *G. ternetzi* in its native range are not well documented (see Section 1.6.4.8), nor is there documentation of trophic interactions of escaped ornamental wild-type or CGT2016 *G. ternetzi* in other areas. Should CGT2016 be release to the environment, they have potential to interact with other organisms in Canadian freshwater aquatic ecosystems, including potential prey, competitors, and predators.

In an aquarium setting, Black Tetras will readily feed on live food (e.g., small worms, crustaceans, insect larvae etc.) as well as commercial ornamental fish flakes and very small fish (e.g., larvae, Fish Care Manuals 2012). As such they have potential to impact localized populations of small prey organisms at location of release. The extent to which they could impact such populations has not been examined. However, Black Tetras are not known to be voracious eaters, and do not overeat (Frank 1980). As such they are not expected to impact prey populations more than other small native fish. Released CGT2016 could also potentially impact other small predator species through competition. While the competition ability of CGT2016 or Black Tetras have not been examined, they are generally described in the ornamental aquarium trade as a community tank fish and are generally not reported to be aggressive towards other species of similar or greater size. As such, they are not expected to have exceptional ability to impact native fish populations through strong competitive ability. Suggested optimal temperature for health and activity of Black Tetras for ornamental aquarium culture varies from 20-29°C (see Section 1.6.4.2). While water temperatures during the summer months may be within this range for many water systems in Canada (Leggatt and Devlin 2006, see Figure 1.13), temperatures are expected to be much lower at other times of the year. White Tetras obtained from the ornamental aquarium trade are reported to decrease activity and feeding at approximately 17°C, stop feeding above 12°C and stop activity just above 10°C (Leggatt et al. 2018, see Figure 2.2). As activity and feeding level drop with decreasing temperature, impacts to prey and competitors are also expected to decrease with decreasing temperature. CGT2016 is less cold tolerant than White Tetra (see Section 1.5.2.3), and as such may decrease activity and feeding at a higher temperature than those listed for White Tetra above, although this has not been specifically examined. Given the low temperatures expected for Canadian freshwater systems for most of the year, the potential for released CGT2016 to impact native aquatic species through prey acquisition and competition is expected to be negligible through most of the year, and not expected to be greater than other small fish species during the summer months.

The ability of CGT2016 to prey on or compete for food relative to wild-type Black or White Tetras has not been reported. In another fluorescent fish model, Jha 2010 found co-rearing with RFP transgenic zebrafish obtained from the ornamental pet trade in India greatly decreased aggressive behaviour of wild-caught wild-type zebrafish and flying barb, and wild-caught species avoided interactions with the transgenic zebrafish (Jha 2010). Transgenic zebrafish were also much more aggressive to the wild-caught fish than vice versa (Jha 2010). Whether the differences in aggressive behaviour between the RFP transgenic zebrafish and wild-caught fish were due to the fluorescent phenotype, or to differences in genetic background (domesticated versus wild) or rearing history (aquarium versus nature) are not known. The

study was conducted in bare tanks, and whether similar patterns of behaviour would exist in complex environments such as nature have not been examined. As well, whether similar behaviour patterns would occur in the transgenic Tetra has not been directly examined. In five years of commercial use in the ornamental aquarium trade there are no known reports, anecdotal or otherwise, of CGT2016 having different activity levels or behaviour than non-transgenic *G. ternetzi*, and the veterinarian for GloFish LLC has submitted a letter stating CGT2016 require the same husbandry care as non-transgenic fish (see Section 1.5.2.3). This suggests that CGT2016 has similar potential to impact prey and competitors species as non-transgenic *G. ternetzi*.

Released CGT2016 also have potential to impact native predator populations by acting as a new prey source. This could have a positive effect on predator populations by providing a new food source, or a negative effect on predator populations if consuming CGT2016 causes deleterious effects to the predator populations. The latter is not expected, as CGT2016 are not expected to be environmentally toxic, and the fluorescent protein is not expected to be toxic to organisms that ingest CGT2016 (see Section 2.3.1 above). While the predation pressure on CGT2016 relative to non-transgenic *G. ternetzi* has not been reported, the effect of fluorescent transgenesis in another fish model (RFP zebrafish) is conflicting. Hill et al. (2011) found RFP-expressing zebrafish had twice higher predation pressure than wild-type zebrafish when reared in complex habitats with two different sized North American predators (largemouth bass and Eastern mosquito fish). The authors postulated this was due to the more conspicuous colouration of the RFP zebrafish relative to wild-type. It should be noted that the RFP zebrafish in the paper were developed from the golden morph of the zebrafish which lacks conspicuous stripes and this lack of cryptic colouration may have also contributed to the higher predation on RFP zebrafish independent of the fluorescent colouration. An earlier study (Cortemeglia and Beitinger 2006b) using the originally marketed striped variety of RFP zebrafish (Gong et al. 2003) found RFP transgenic and wild-type zebrafish did not significantly differ in predation susceptibility by largemouth bass (at a ratio of 1.4:1 respectively). The differences in results between these studies could be due to differences in phenotype of the RFP zebrafish (striped versus not striped), a lack of refuge areas in the Cortemeglia and Beitinger trial, and low statistical power to identify potential differences in the Cortemeglia and Beitinger trial (Hill et al. 2011). A third study reported yet another trend in results, where RFP zebrafish from the ornamental pet trade had lower predation susceptibility than wild-caught wild-type zebrafish and flying barb in India (Jha 2010). In this study, the wild-caught snakehead predators appeared to actively avoid RFP transgenic fish, and wild-type zebrafish and flying barb were preyed on more heavily in the presence of RFP transgenic fish than in each other's presence. As with aggressive behaviour listed above, whether the differences in predation vulnerability between the RFP transgenic zebrafish and wild-caught fish were due to the fluorescent phenotype, or to differences in genetic background and/or rearing history are not known. The study was conducted in bare tanks, and whether similar patterns of predation vulnerability would exist in complex environments such as nature have not been examined. In the RFP zebrafish model, the impact of fluorescent transgenesis is not consistent among studies and may be influenced by transgenic line, habitat complexity, background genetics and/or rearing history.

Whether any of the above studies could be applied to CGT2016 predation vulnerability is not known and consequently the predation vulnerability of CGT2016 relative to wild-type counterparts cannot be estimated with reasonable certainty. Wild-type Tetras are reported to decrease activity below 17°C, and cease activity around 10.5°C (Leggatt et al. 2018, see Figure 2.2). The decreased activity with decreasing temperature may increase predation susceptibility of Tetras in non-summer months as they may be slower to respond to predator presence than similar-sized native species adapted to a temperate climate. Combined with the bright green colour and lack of cryptic colour stripes in CGT2016, CGT2016 is expected to be more susceptible to predation than native species, and may temporarily benefit native predators if released to natural ecosystems.

Due to described low aggressive behaviour of Black Tetras, low activity in cooler waters, and lack of noted alterations in trophic-related behaviour, CGT2016 is not expected to influence trophic interactions of native organisms beyond natural fluctuations, with associated **negligible hazard** relative to non-transgenic counterparts. This ranking has a **moderate level of uncertainty**, due to lack of studies directly examining hazards of CGT2016, lack of understanding of GxE interactions in aggression and predation susceptibility in another fluorescent transgenic fish model (RFP zebrafish) and lack of understanding of application of results from the RFP zebrafish model to CGT2016.

2.3.4. Potential hazards through hybridization with native species

The Black Tetra belongs to the Family Characidae, that have a geographical distribution of South and Central America, and North America as far North as southwestern US (Oliveira et al. 2011, see Section 1.6.2). There are only three other species that currently share the *Gymnocorymbus* genus (Benine et al. 2015) indicating limited close relatives even with its native range. Black Tetras are broadcast spawners, and consequently could potentially form hybrids with species that spawn at the same time and place. However, due to the lack of native fish in Canada from the same family as Black Tetras, any hybrids produced in this way are expected to by non-viable. Therefor there is **negligible potential for CGT2016 to cause hazards through viable hybridization with native fish in Canada.** The high quality data on distribution of Characidae and *Gymnocorymbus* distribution result in **negligible uncertainty** associated with the rating.

2.3.5. Potential to act as a vector of disease agents

Commercial ornamental aquarium fish are commonly reported to carry numerous disease agents including viruses, bacteria, fungi, and parasites (e.g., Evans and Lester 2001; Hongslo and Jansson 2009; Řehulka et al. 2006; Rose et al. 2013; Whittington and Chong 2007). While individual aquarium fish species examined are not always listed and Black Tetra is often not among the species examined when listed, a few studies have specifically identified ornamental aquarium-sourced Black Tetra and found symptoms of tuberculosis (Řehulka et al. 2006), although no parasites (Kim et al. 2002; note: parasitic flukes and cysts have been found on gills of ornamental aquarium-sourced White Tetras by Fisheries and Oceans Canada, Leggatt unpublished data, and GloFish LLC submitted a veterinarian assessment that identified

nematodes on all submitted CGT2016 fish, see Section 1.5.2.3). Although disease agents are common on tropical-origin freshwater ornamental aquarium fish, very few species (e.g., goldfish, zebra danio, tank goby, guppy, three spot gourami) are listed as species susceptible to diseases of significant importance to aquatic animal health and the Canadian economy by the Canadian Food Inspection Agency (see Susceptible Species of Aquatic Animals). Black Tetra is not included on the susceptible species list, nor are any other Tetra species, indicating they have not been implicated as vectors for disease agents of concern in Canada. Any disease agents 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. Increased susceptibility to disease may increase vector capabilities through heightened ability to act as a reservoir and increased shedding of disease agents, or decrease vector capabilities by succumbing to disease quickly. Some studies of fluorescent cultured cell models used in research have reported potential alterations in disease susceptibility. For example, GFP expression decreased T-cell activation (Koelsch et al. 2013), induced cytokine IL-6 secretion (Mak et al. 2007), inhibited immune-related signalling pathways (Baens et al. 2006), and altered expression of genes involved in immune function (Coumans et al. 2014) and response to stress (Badrian and Bogoyevitch 2007). Whether these alterations may be observed in whole animal fluorescent transgenic models has not been examined. CGT2016 has been grown on a commercial scale in the US for 5 years, as have numerous other transgenic fluorescent aquarium species and lines starting in 2003. GloFish LLC provided statements from the company's veterinarian and a University of Florida veterinarian involved in health screening for 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 fluorescent line relative to non-transgenic counterparts under standard rearing conditions (see Section 1.5.2.3). In other tropical species (e.g., zebrafish), fluorescent models have been used extensively in laboratory conditions for research with no known reported effects on disease susceptibility, and Howard et al. (2015) tracked wild-type and RFP transgenic zebrafish in 18 populations over 15 generations in laboratory conditions and reported no differences in survival between transgenic and wild-type fish. This indicates there is **negligible potential for CGT2016 to have altered vector capabilities relative to wild-type Tetras**. As this has not been directly examined, and there is reliance on indirect evidence and testimony of experts, the **uncertainty level for this rating is moderate**.

2.3.6. Potential to impact biogeochemical cycling

CGT2016 is expected to contribute to nutrient cycles within released habitats through ingestion of prey and other food items and release of waste (ammonia and feces). In a static aquarium environment, the Black Tetra wild-type counterpart is described as not over eating, and does not cause excessive pollution of its environment (Frank 1980). Combined with its small size this indicates it will have limited capabilities to impact nutrient cycles. The potential effects of the fluorescent protein in CGT2016 on metabolism, and hence nutrient cycling, have not been

examined. In a different model organism, eGFP transgenic mice were found to have alterations in the urea cycle, nucleic acid and amino acid metabolism, and energy utilization (Li et al. 2013). What impacts these changes may have on biogeochemical cycling should CGT2016 have similar influences from fluorescent transgenic gene expression are not known, but the small size and lack of polluting capabilities of Black Tetra indicated CGT2016 has **negligible potential to impact biogeochemical cycling in natural environments** even with altered metabolic pathways. This ranking has a **moderate level of uncertainty** due to lack of studies directly examining this hazard.

2.3.7. Potential to affect habitat

Black Tetra is a small fish with no evidence suggesting they may have effects to 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 since 2012, and there have been no reports, anecdotal or otherwise, of CGT2016 having altered behaviour relative to Black Tetra that may influence effects on habitat structure. Consequently, CGT2016 is expected to have **negligible effects to habitat with low uncertainty associated with this rating**.

2.3.8. Potential to affect biodiversity

Biological diversity (or biodiversity) is defined in CEPA (1999) as “the variability among living organisms from all sources, including, without limiting the generality of the foregoing, terrestrial and marine and other aquatic ecosystems and the ecological complexes of which they form a part and includes the diversity within and between species and of ecosystems”. Biodiversity can be negatively impacted by numerous drivers including invasive species and disease introduction. While the invasiveness of CGT2016 has not been directly assessed, there are no reports of Black Tetra becoming invasive in North America, Europe, or elsewhere worldwide. Black Tetra have been used in the ornamental aquarium trade in North America since at least 1950, and are common in Canadian and the US pet trade (e.g., Rixon et al. 2005 found 75% of surveyed pet stores in Ontario carried *G. ternetzi*, while Strecker et al. 2011 found 96.7% of surveyed pet stores in Washington carried *G. ternetzi*). Occurrences of *G. ternetzi* in natural aquatic systems in the US have been noted in Florida, Colorado, and Louisiana (Nico and Fuller 2017; Tuckett et al. 2017) but all occurrences failed to establish, and Hill et al. (2014) concluded a lack of invasion potential in the USA of fluorescent *G. ternetzi* using the Fish Invasiveness Screening Kit (FISK). Despite the extensive long-term use of *G. ternetzi* in the aquarium trade, and its noted release to the environment, there are no reports of the Black Tetra causing harm to aquatic ecosystems including biodiversity. As noted above, Black Tetras are not expected to impact native species through tropic or hybridization interactions, act as a vector for disease agents of concern in Canada, significant impact biogeochemical cycling, or impact habitat. Addition of the transgenic construct and fluorescent protein in CGT2016 is not expected result in environmental toxicity or cause hazards through HGT of the transgene, and is not expected to increase potential hazards of Black Tetra due to interactions with native species, as a vector of disease, or impact biogeochemical cycling and habitat. Taken together, there is a **negligible hazard of CGT2016 effecting biodiversity of Canadian ecosystems**. The reliance on data

from the comparator species for invasiveness and biodiversity effects results in a **low degree of uncertainty** with this rating.

2.3.9. Conclusions

The Black Tetra is a small, non-aggressive fish with expected limited activity due to low temperatures in most seasons in Canada, 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. The potential environmental hazards posed by CGT2016 above that of wild-type Black Tetra have not been specifically addressed. However there is no evidence of environmental toxicity (i.e., ability to be poisonous) associated with the construct and the majority of other fluorescent models do not report toxicity associated with fluorescent transgenes. As well there is no evidence for potential effects via gene transfer of the transgene to native Canadian species through hybridization or horizontal gene transfer. In some non-fish fluorescent models, there is limited evidence that some fluorescent transgenes have potential to impact vector capabilities by altering response to disease, and impact contribution to biogeochemical cycling by altering metabolic pathways. However, CGT2016 and other fluorescent fish models have no reported differences in survival, disease susceptibility, or husbandry care, and as such are not expected to have altered ability to pose hazards as a vector of disease or impact biogeochemical cycling. Some evidence suggests CGT2016 may have lower potential to impact other species through trophic interactions relative to Black Tetras, as lower cold tolerance may further limit activity in cooler water temperatures, and bright colouration may increase predation as reported in RFP zebrafish (Hill et al. 2011). One report in RFP zebrafish suggests presence of fluorescent fish may result in increased predation on wild fish and decrease aggressive behaviour of wild fish (Jha 2010) which could have negative consequences to wild populations. However, whether noted differences were due to the presence of the transgene, domestication level, or rearing history is not known, and it is not known whether these results would apply to complex natural habitats or specifically to CGT2016. Use of CGT2016 other than the intended use as an ornamental fish in indoor static aquaria (e.g., as a bait fish, rearing in outdoor ponds) is not expected to pose unique hazards beyond those from the intended use.

All specific hazards examined had negligible rating, with the exception of a low hazard rating for impacts through HGT (see Table 2.5). In this latter case, the potential for an effect to occur (i.e., transfer of transgene to prokaryotic populations) cannot be excluded, but the effect is not anticipated to cause detrimental impacts to the structure or function of Canadian ecosystems. The uncertainty ratings associated with the individual hazard classifications ranged from negligible to moderate (see Table 2.5), due to the limited data specific to 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**.**

2.4 ASSESSMENT OF RISK

Risk is the likelihood that a harmful effect is realized as a result of exposure to a hazard. The risk assessment incorporates the nature and severity of the harmful effect, the likelihood that the harmful effect is realized, and the uncertainty associated with each conclusion. DFO’s science

advice to ECCC and HC for a regulatory decision is based on the overall risk of the organism, carried out in the context of the notifier's proposed use scenario, and all other potential use scenarios.

Table 2.5: *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 for Disease	Negligible	Moderate
To Biogeochemical Cycling	Negligible	Moderate
To Habitat	Negligible	Low
To Biodiversity	Negligible	Low

An overall conclusion on Risk is based on the classic paradigm where risk is proportional to Hazard and Exposure:

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

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

2.4.1. Risk Assessment of CGT2016

The exposure assessment of CGT2016 concluded that CGT2016 used in the ornamental aquarium trade or other unintended uses in Canada would have **low likelihood for occurrence in the Canadian environment**. This is due to the high likelihood of release of small numbers of fish from home aquaria, but negligible likelihood for 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 lack of cold tolerance in CGT2016 and base species relative to Canadian winter freshwater temperatures results in **low uncertainty** level with this ranking.

The hazard assessment of CGT2016 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 fluorescent protein would increase hazards relative to wild-type Black Tetra. Uncertainty ranking associated with individual hazard components ranged from negligible to moderate (see Table 2.5), due to the limited data specific

to 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.

Using the risk matrix in Figure 2.3, CGT2016 used in the ornamental aquarium trade or other uses in Canada **pose low risk to Canadian environments** (**Low Exposure x Negligible/Low Hazard** ∝ **Low Risk**). In general, individual hazard assessments are expected to result in no effects beyond natural fluctuations to Canadian environments under the assessed exposure level. One exception to this is hazards associated with horizontal gene transfer, under the assessed exposure level, may result in an effect beyond the normal range (i.e., potential transfer of a marine-derived fluorescent protein gene to prokaryotes in a freshwater environment), but this effect is not expected to be harmful.

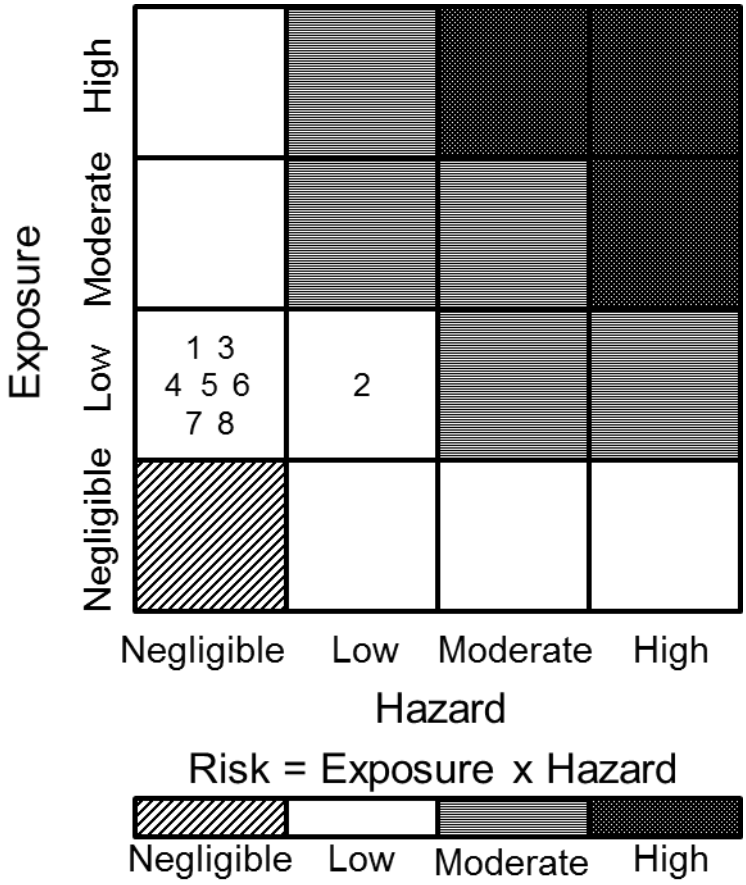

Figure 2.3: *Risk matrix and colour scale to illustrate how exposure and hazard are integrated to establish a level of risk in the environmental risk assessment. Risk assessments associated with assessed hazard components at the assessed exposure are identified by number: 1) through environmental toxicity;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

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 for some hazard assessments (e.g., impacts as vector of disease). Some individual hazard classifications have significant uncertainty level (e.g., moderate uncertainty in negligible hazard through environmental toxicity, through tropic interactions, as a vector or disease or to biogeochemical cycling). The majority of identified environmental hazards of CGT2016 would be expected to require continued exposure of ecosystem components to CGT2016 in order to pose significant risk to the environment, and consequently uncertainty in risk may be more closely aligned with that of exposure rather than hazard. In contrast, hazards through horizontal gene transfer or as a vector of disease could be reliant on initial exposure to CGT2016 to transfer the transgene or agents of disease, which could then have longer-term consequences after CGT2016 has been removed from the environment. As such, uncertainty in risk associated with these hazards may be more closely aligned with uncertainty in hazard assessment. Despite moderate uncertainty in some individual assessment components, there is no current evidence to suggest overall risk ratings of CGT2016 used for the ornamental aquarium trade in Canada may be higher than the assessed low rating for risk to Canadian environments. Future studies should be aimed at decreasing uncertainty in key hazard components, e.g., by directly examining the influence of the genetic modification on potential for environmental impacts through environmental toxicity, trophic interactions, as a vector of disease or to biogeochemical cycling.

2.5. SUMMARY AND CONCLUSIONS

GloFish LLC has requested approval for importation of CGT2016, a fluorescent green transgenic Tetra of species *G. ternetzi*, for use in the ornamental aquarium trade and home aquaria. CGT2016 would be imported to Canada through four import sites, followed by further distribution to home aquaria throughout Canada. The final destination and purpose of the import would be as ornamental fish in the home aquaria.

Use of CGT2016 in the home aquaria in Canada, or in other unintended uses, is expected to result in frequent, very small magnitude releases of CGT2016 to the Canadian environment, although the potential for occasional high magnitude releases cannot be excluded. However, high quality data available indicates CGT2016 does not have capacity to overwinter in Canadian freshwater ecosystems. This results in a Low Exposure ranking with associated uncertainty being low. For potential hazards, the lack of evidence of hazards from base 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 ecosystems. Due to lack of direct information on hazards of base model and/or CGT2016, uncertainty with hazard assessments range from negligible to moderate. 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. While uncertainty associated with some 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 or other potential uses, could cause harm as a result of exposure to Canadian environments.

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