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

Canadian Science Advisory Secretariat (CSAS)

Research Document 2026/028

National Capital Region

Environmental Risk Assessment of the GloFish®, Cosmic Blue®, Electric Green®, Sunburst Orange®, and Galactic Purple® Sharks (*Epalzeorhynchos frenatum*): Transgenic Ornamental Fishes

Kamya Bhatnagar and Colin McGowan

Fisheries and Oceans Canada
Aquaculture, Biotechnology and Aquatic Animal Health Science
200 Kent Street
Ottawa, ON K1A 0E6

April 2026

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.

Published by:

Fisheries and Oceans Canada
Canadian Science Advisory Secretariat
200 Kent Street
Ottawa ON K1A 0E6

http://www.dfo-mpo.gc.ca/csas-sccs/
DFO.CSAS-SCAS.MPO@dfo-mpo.gc.ca

ISSN 1919-5044
ISBN 978-0-660-98897-9 Cat. No. Fs70-5/2026-028E-PDF

Correct citation for this publication:

Bhatnagar, K., and McGowan, C. 2026. Environmental Risk Assessment of the GloFish® Cosmic Blue®, Electric Green®, Sunburst Orange®, and Galactic Purple® Sharks (*Epalzeorhynchos frenatum*): Transgenic Ornamental Fishes. DFO Can. Sci. Advis. Sec. Res. Doc. 2026/028. vi + 29 p.

Aussi disponible en français :

*Bhatnagar, K., et McGowan, C. 2026. Évaluation des risques pour l'environnement posés par les labéos frenatus (*Epalzeorhynchos frenatum*) GloFish^MD Cosmic Blue^MD, Electric Green^MD, Sunburst Orange^MD et Galactic Purple^MD, des poissons d'ornement transgéniques. Secr. can. des avis sci. du MPO. Doc. de rech. 2026/028. vi + 32 p.*

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Pursuant to the *Canadian Environmental Protection Act 1999* (CEPA), a notification under the *New Substances Notification Regulations (Organisms)* (NSNR(O)) was submitted by Spectrum Brands to Environment and Climate Change Canada (ECCC) for the import of four genetically engineered *Epalzeorhynchos frenatum* (Rainbow Shark) called the GloFish® Cosmic Blue® Shark (BS2017), Electric Green® Shark (GS2017), Sunburst Orange® Shark (OS2016), and Galactic Purple® Shark (PS2016), for commercial sales in Canada. The environmental risk assessment analyzed potential hazards, likelihood of exposure and associated uncertainties, to reach a conclusion on risk. The environmental exposure assessment concluded that the occurrence of BS2017, GS2017, OS2016, and PS2016 in the Canadian environment, outside of aquaria, is expected to be rare, isolated, and ephemeral due to its inability to survive typical low winter temperatures in Canada's freshwater environments. Consequently, the likelihood of exposure to the Canadian environment is ranked low. Uncertainty associated with the exposure assessment is low, given the available data for temperature tolerance of the notified line and relevant comparators, and lack of establishment of non-transgenic *E. frenatum* in North America despite a long history of use. The environmental hazard assessment concluded that potential hazards linked with environmental toxicity, trophic interactions, hybridization, disease, biodiversity, biogeochemical cycling, and habitat are negligible. There is low hazard (i.e., no anticipated harmful effects) related with horizontal gene transfer. Uncertainty associated with the environmental hazard ratings range from negligible to moderate due to data limitations for the notified and surrogate organisms, and some reliance on expert opinion and anecdotal evidence. There is low risk of adverse environmental effects at the exposure levels predicted for the Canadian environment from the use of BS2017, GS2017, OS2016, and PS2016 as ornamental aquarium fish, or other potential uses.

1. INTRODUCTION

On October 3, 2023, the GloFish brand of Spectrum Brands LLC submitted four regulatory packages (notifications) to Environment and Climate Change Canada (ECCC) under the *New Substances Notification Regulations (Organisms)* [NSNR(O)] of the *Canadian Environmental Protection Act, 1999* (CEPA) for the GloFish® Cosmic Blue®, Electric Green®, Sunburst Orange®, and Galactic Purple® Rainbow Sharks; herein referred to collectively as the GloFish® Sharks. These ornamental fish are domesticated *Epalzeorhynchos frenatum* (Rainbow Shark) that have been genetically engineered to fluoresce different colours in home aquaria. Notification of the GloFish® Sharks under CEPA follows previous similar GloFish® notifications for six lines of GloFish® Tetra (DFO 2018, 2019), three lines of GloFish® Danio (DFO 2020a, 2020b), three lines of GloFish® Betta (DFO 2021a), four lines of GloFish® Barb (DFO 2023a), and four lines of GloFish® Pristella (DFO 2024).

The biotechnology provisions of CEPA take a preventative approach to pollution by requiring all new living products of biotechnology, including genetically-engineered fish, to be notified and assessed prior to import or manufacture in Canada, to ultimately determine whether they are "toxic" or capable of becoming "toxic". Under CEPA (Section 64), an organism is considered "toxic" if it can enter the environment in a quantity or concentration or under conditions that (a) have or may have an immediate or long-term harmful effect on the environment or its biological diversity; (b) constitute or may constitute a danger to the environment on which life depends; or (c) constitute or may constitute a danger in Canada to human life or health. Anyone proposing to import or manufacture a living animal product of biotechnology in Canada, including genetically-engineered fish, is required to provide ECCC with the information prescribed in NSNR(O) at least 120 days prior to the commencement of import or manufacture of the organism. This information is used to conduct an environmental risk assessment and an assessment of indirect human health (risk to human health from environmental exposure to the living organism), which are then used to determine whether the organism is CEPA-toxic or capable of becoming CEPA-toxic. Further information on CEPA and NSNR(O), including guidance on the regulations, detailed guidance for information requirements, use of waivers, significant new activities, risk assessment outcomes, and risk management, can be found on the Biotechnology page of the Environment and Climate Change Canada (ECCC) website.

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

It is in this context that DFO conducted this environmental risk assessment of the notified organisms under the proposed use. Here, Risk is defined as a function of the potential for Canadian environments to be exposed to the notified organisms and the potential for the notified organisms 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 exposure and hazard assessments are discussed in the context of the final risk assessment. A detailed overview of the legal context for the risk assessment process, the risk assessment framework, and regulatory decision making process under CEPA is provided in Leggatt et al. (2018a).

2. CHARACTERIZATION OF THE ORGANISMS

In the current notifications, Spectrum Brands is seeking to import four new transgenic strains of Rainbow Shark (*Epalzeorhynchos frenatum*) to Canada from the United States, for sale in the ornamental aquarium industry. Trade names for the transgenic organisms are; GloFish® Cosmic Blue® Shark (BS2017), GloFish® Electric Green® Shark (GS2017), GloFish® Sunburst Orange® Shark (OS2016), and GloFish® Galactic Purple® Shark (PS2016); herein referred to collectively as the GloFish® Sharks. The four lines were developed at the GloFish® main R&D facility in Riverview, Florida and are commercially produced by New Age Fisheries, also located in Florida.

The four lines are genetically engineered variants of the domesticated albino Rainbow Shark and differ from one another in the expression of distinct fluorescent proteins that result in novel colour phenotypes. Figure 1 (C-F) demonstrates the physical appearance of the four notified GloFish® *E. frenatum* strains, as well as a non-transgenic, domesticated *E. frenatum* (A), and an albino variant of domesticated *E. frenatum* (B).

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

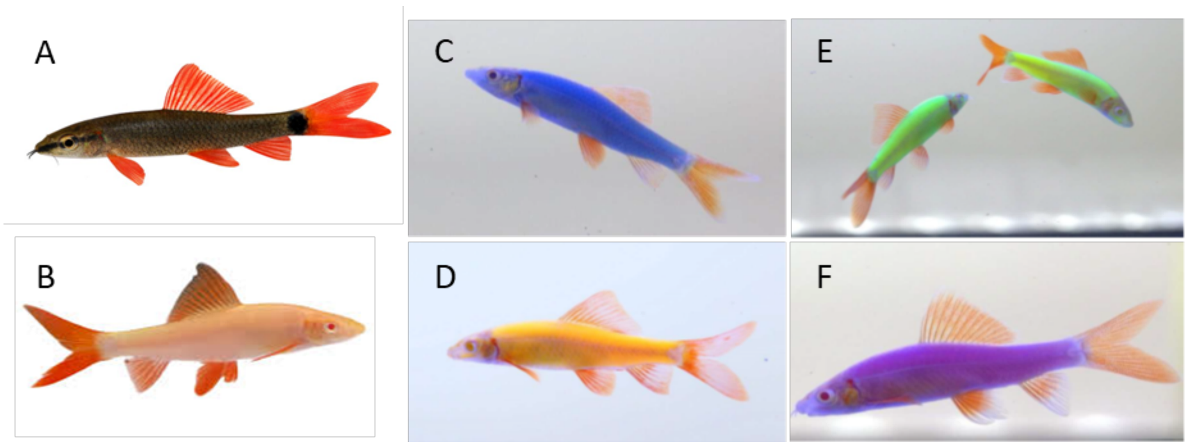

Figure 1. The current notification's transgenic fluorescent GloFish® E. frenatum *variants. Non-transgenic domesticated* E. frenatum *(A), non-transgenic albino E. frenatum (B), GloFish® Cosmic Blue® Shark (C), GloFish® Sunburst Orange® Shark (D), GloFish® Electric Green® Shark (E), and GloFish® Galactic Purple® Shark (F). All images are provided by Spectrum Brands and taken under blue light, except for A, which is taken from Pets on Mom, and B, which is taken from Navya Aquarium.*

2.1. GLOFISH® COSMIC BLUE® SHARK (BS2017)

BS2017 is a genetically engineered *E. frenatum*. The genetic modification results in ubiquitous blue colouration of the organism under ambient light, including sunlight (Figure 1C). The purpose of the modification is to create a new colour phenotype of *E. frenatum* for the ornamental aquarium trade.

2.1.1. Molecular Characterization

2.1.1.1. Production of the notified organism

Purified transgene expression cassette was injected into newly fertilized embryos of Rainbow Shark. Further details provided by the company that describe line development and analysis to confirm that BS2017 constitutes a single homogeneous line and that the vector backbone was not incorporated along with the transgenes are considered confidential business information and are not reported here.

2.1.1.2. Characterization of the transgene integrant

The sequence of the cassette as it is inserted into the genome of the BS2017 line has not been determined, and the specific location of the insert within the organism's genome is unknown. Details regarding the analysis to confirm that multiple copies of the transgene cassette were incorporated at a single insert location are considered confidential business information and are not reported here.

2.1.1.3. Inheritance and stability of the transgene

The BS2017 Rainbow Shark line was created by introducing the genetic constructs into non-genetically engineered *E. frenatum*, resulting in a blue fluorescent phenotype. The desired trait has been maintained through inbreeding and selection of the blue fluorescent phenotype. GloFish® has been using this breeding line for approximately ten generations, and it has been commercially produced since 2018, ensuring genetic consistency and stability across generations.

2.1.1.4. Methods to detect BS2017 fish

BS2017 individuals are distinguished from non-transgenic domesticated *E. frenatum* by their blue colouration under natural light and fluorescence under blue or UV light. BS2017 can be further identified genetically by PCR amplification of a section of the injected expression cassette to distinguish BS2017 from other blue transgenic *E. frenatum* carrying a different cassette.

2.1.2. Phenotypic Characterization

2.1.2.1. Targeted phenotypic effects of the modification

The targeted phenotypic effect of the genetic modification is that Cosmic Blue® Shark (BS2017) appears blue under ambient light and fluorescent under UV or blue light. The novel colour phenotype is present in muscle as well as the skin and eyes. The notifier reports that BS2017 individuals that are hemizygous and homozygous for the transgene insert are indistinguishable from each other phenotypically and are both part of the commercially available population.

2.1.2.2. Additional phenotypic effects of modification

The company provided information and data from a low-temperature tolerance experiment used to identify potential differences between BS2017 and non-transgenic domesticated *E. frenatum* that may impact their ability to survive and reproduce in the wild. Results indicate that the transgenic Cosmic Blue® Shark is more sensitive to cold than their non-transgenic siblings. The influence of genetic modification on any other phenotypes has not been formally examined.

No formal studies have compared the potential disease susceptibility of BS2017 and non-transgenic strains. There are also no formal studies on potential unintended phenotypic effects of genetic modification on life history, environmental tolerances and requirements (other than low-temperature tolerance), metabolism, physiology, endocrinology, or behaviour; however, there are no anecdotal or otherwise reports of any unintended phenotypic effects other than the one described above.

2.2. GLOFISH® ELECTRIC GREEN® SHARK (GS2017)

GS2017 is a genetically engineered *E. frenatum*. The genetic modification results in ubiquitous green colouration of the organism under ambient light, including sunlight (Figure 1E). The purpose of the modification is to create a new colour phenotype of *E. frenatum* for the ornamental aquarium trade.

2.2.1. Molecular Characterization

2.2.1.1. Production of the notified organism

The purified transgene expression cassette was injected into newly fertilized embryos of albino Rainbow Shark. Further details provided by the company that describe line development and analysis to confirm that GS2017 constitutes a single homogeneous line and that the vector backbone was not incorporated along with the transgenes are considered confidential business information and are not reported here.

2.2.1.2. Characterization of the transgene integrant

The sequence of the cassette as it is inserted into the genome of the GS2017 line has not been determined, and the specific location of the insert within the organism's genome is unknown. Details regarding the analysis to confirm that multiple copies of the transgene cassette were incorporated at a single insert location are considered confidential business information and are not reported here.

2.2.1.3. Inheritance and stability of the transgene

The GS2017 Rainbow Shark line was created by introducing a genetic construct into non-genetically engineered *E. frenatum*, resulting in a green fluorescent phenotype. The desired trait has been maintained through inbreeding and selection of the green fluorescent phenotype.

GloFish® has been using this breeding line for approximately ten generations, and it has been commercially produced since 2018, ensuring genetic consistency and stability across generations.

2.2.1.4. Methods to detect GS2017 fish

GS2017 individuals are distinguished from non-transgenic domesticated *E. frenatum* by their green colouration under natural light and fluorescence under blue or UV light. GS2017 can be further identified genetically by PCR amplification of a section of the injected expression cassette to distinguish GS2017 from other green transgenic *E. frenatum* carrying a different cassette.

2.2.2. Phenotypic Characterization

2.2.2.1. Targeted phenotypic effects of the modification

The targeted phenotypic effect of the genetic modification is that Electric Green® Shark (GS2017) appears green under ambient light and fluorescent under UV or blue light. The novel colour phenotype is present in muscle as well as the skin and eyes. The notifier reports that GS2017 individuals that are hemizygous and homozygous for the transgene insert are indistinguishable from each other phenotypically and are both part of the commercially available population.

2.2.2.2. Additional phenotypic effects of modification

The company provided information and data from a low-temperature tolerance experiment used to identify potential differences between GS2017 and non-transgenic domesticated *E. frenatum* that may impact their ability to survive and reproduce in the wild. The low-temperature tolerance test compared the survival of low temperature sensitivity of the GS2017 line in comparison to the non-fluorescent siblings. Results indicate that the transgenic Electric Green® Shark is more sensitive to cold than their non-transgenic siblings. The influence of genetic modification on any other phenotypes has not been formally examined.

No formal studies have compared the potential disease susceptibility of GS2017 and non-transgenic strains. There are also no formal studies on potential unintended phenotypic effects of genetic modification on life history, environmental tolerances and requirements (other than low-temperature tolerance), metabolism, physiology, endocrinology, or behaviour; however, there are no anecdotal or otherwise reports of any unintended phenotypic effects other than the one described above.

2.3. GLOFISH® SUNBURST ORANGE® SHARK (OS2016)

OS2016 is a genetically engineered *E. frenatum*. The genetic modification results in ubiquitous orange colouration of the organism under ambient light, including sunlight (Figure 1D). The purpose of the modification is to create a new colour phenotype of *E. frenatum* for the ornamental aquarium trade.

2.3.1. Molecular Characterization

2.3.1.1. Production of the notified organism

Purified transgene expression cassette was injected into newly fertilized embryos of albino Rainbow Shark. Further details provided by the company that describe line development and analysis to confirm that OS2016 constitutes a single homogeneous line and that the vector backbone was not incorporated along with the transgenes are considered confidential business information and are not reported here.

2.3.1.2. Characterization of the transgene integrant

The sequence of the cassette as it is inserted into the genome of the OS2016 line has not been determined, and the specific location of the insert within the organism's genome is unknown. Details regarding the analysis to confirm that multiple copies of the transgene cassette were incorporated at a single insert location are considered confidential business information and are not reported here.

2.3.1.3. Inheritance and stability of the transgene

The OS2016 Rainbow Shark line was created by introducing genetic constructs into non-genetically engineered *E. frenatum*, resulting in an orange fluorescent phenotype. GloFish® has been using this breeding line for approximately ten generations, and the orange Rainbow Shark line has been commercially produced since 2017, ensuring genetic consistency and stability. There is no evidence of gene silencing or off-target mutations, and the fluorescent phenotypes have remained stable across generations.

2.3.1.4. Methods to detect the OS2016 fish

OS2016 individuals are distinguished from non-transgenic domesticated *E. frenatum* by their orange colouration under natural light and fluorescence under blue or UV light. OS2016 can be further identified genetically by PCR amplification of a section of the injected expression cassette to distinguish OS2016 from other orange transgenic *E. frenatum* carrying a different cassette.

2.3.2. Phenotypic Characterization

2.3.2.1. Targeted phenotypic effects of the modification

The targeted phenotypic effect of the genetic modification is that Sunburst Orange® Shark (OS2016) appears orange under ambient light and fluorescent under UV or blue light. The novel colour phenotype is present in muscle as well as the skin and eyes. The notifier reports that OS2017 individuals that are hemizygous and homozygous for the transgene insert are indistinguishable from each other phenotypically and are both part of the commercially available population.

2.3.2.2. Additional phenotypic effects of modification

The company provided information and data from the low-temperature tolerance experiment to identify potential differences between OS2016 and non-transgenic domesticated *E. frenatum* that may impact their ability to survive and reproduce in the wild. Results indicate that the temperature tolerance of the transgenic Sunburst Orange® Shark is similar to that of their non-transgenic siblings. No formal studies have compared the potential disease susceptibility of OS2016 and non-transgenic strains. There are also no formal studies on potential unintended phenotypic effects of genetic modification on life history, environmental tolerances and requirements (other than low-temperature tolerance), metabolism, physiology, endocrinology, or behaviour; however, there are no anecdotal or otherwise reports of any unintended phenotypic effects.

2.4. GLOFISH® GALACTIC PURPLE® SHARK (PS2016)

PS2016 is a genetically engineered *E. frenatum*. The genetic modification results in ubiquitous purple colouration of the organism under ambient light, including sunlight (Figure 1F). The purpose of the modification is to create a new colour phenotype of *E. frenatum* for the ornamental aquarium trade.

2.4.1. Molecular Characterization

2.4.1.1. Production of the notified organism

The purified transgene expression cassette was injected into newly fertilized embryos of albino Rainbow Shark. Further details provided by the company that describe line development and analysis to confirm that BS2017 constitutes a single homogeneous line and that the vector backbone was not incorporated along with the transgenes are considered confidential business information and are not reported here.

2.4.1.2. Characterization of the transgene integrant

The sequence of the cassette as it is inserted into the genome of the PS2016 line has not been determined, and the specific location of the insert within the organism's genome is unknown. Details regarding the analysis to confirm that multiple copies of the transgene cassette were incorporated at a single insert location are considered confidential business information and are not reported here.

2.4.1.3. Inheritance and stability of the transgene

The PS2016 Rainbow Shark line was created by introducing genetic constructs into non-genetically engineered *E. frenatum*, resulting in a purple fluorescent phenotype. The desired trait has been maintained through inbreeding and selection of the purple fluorescence phenotype. GloFish® has been using this breeding line for approximately ten generations and the purple Rainbow Shark line had been commercially produced since 2017, ensuring genetic consistency and stability across generations.

2.4.1.4. Methods to detect PS2016 fish

PS2016 individuals are distinguished from non-transgenic domesticated *E. frenatum* by their purple colouration under natural light and fluorescence under blue or UV light. PS2016 can be further identified genetically by PCR amplification of a section of the injected expression cassette to distinguish PS2016 from other purple transgenic *E. frenatum* carrying a different cassette.

2.4.2. Phenotypic Characterization

2.4.2.1. Targeted phenotypic effects of the modification

The targeted phenotypic effect of the genetic modification is that Galactic Purple® Shark (PS2016) appears purple under ambient light and fluorescent under UV or blue light. The novel colour phenotype is present in muscle as well as the skin and eyes. The notifier reports that PS2016 individuals that are hemizygous and homozygous for the transgene insert are indistinguishable from each other phenotypically and are both part of the commercially available population.

2.4.2.2. Additional phenotypic effects of modification

The company provided information and data from a low-temperature tolerance experiment to identify potential differences between PS2016 and non-transgenic domesticated *E. frenatum* that may impact their ability to survive and reproduce in the wild. Results indicate that the transgenic Galactic Purple® Shark is more sensitive to cold than their non-transgenic siblings. The influence of genetic modification on any other phenotypes has not been formally examined. No formal studies have compared the potential disease susceptibility of PS2016 and non-transgenic strains. There are also no formal studies on potential unintended phenotypic effects of the genetic modification on life history, environmental tolerances and requirements (other than low-temperature tolerance), metabolism, physiology, endocrinology, or behaviour; however, there are no anecdotal or otherwise reports of any unintended phenotypic effects other than the one listed above.

2.5. PLEIOTROPIC EFFECTS OF FLUORESCENT TRANSGENES IN OTHER FISH

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

Zebrafish containing a RFP transgene were observed to be less cold tolerant than unrelated non-transgenic Zebrafish, when examined under different acclimation temperatures (Cortemeglia and Beitinger 2005, 2006b), though differences in strain background and rearing conditions (Schaefer and Ryan 2006) prior to experimentation may have impacted relative extreme temperature tolerance. Similarly, Leggatt et al. (2018b) reported that Zebrafish transgenic for eGFP, driven by the Fli-1 protein promoter, were less cold tolerant than the source non-transgenic strain. However, Leggatt et al. (2018b) also reported on two other eGFP lines, driven by different promoters, that did not exhibit diminished cold tolerance. This indicates that different transgenic lines may have different responses to extreme environmental stressors. Five of six previously notified GloFish® Tetras, three previously notified lines of GloFish® Danios, and two of three GloFish® Betta lines were also reported to have diminished cold tolerance (DFO 2018, 2019, 2020a, 2020b, 2021a).

No effect of fluorescence protein transgenesis was observed on survival of RFP Zebrafish relative to related non-transgenic fish under laboratory conditions (Howard et al. 2015). In a population of eGFP, RFP, eGFP-RFP, and non-transgenic Zebrafish, eGFP fish had lower survival, but there was no effect of RFP or the double transgene on survival (Gong et al. 2003), indicating different transgenes or transgenic lines may also have different influences on survival. Paired crosses with non-transgenic siblings resulted in fewer fluorescent offspring than expected in two of six lines of GloFish® Tetras, two of three lines of GloFish® Danios, and one of four GloFish® Barbs (DFO 2019, 2020b, 2023), indicating decreased viability of fluorescent gametes or larvae in some fluorescent models. These tests could not be carried out with the GloFish® Sharks as they are seasonal spawners and reproduction under hatchery conditions is accomplished *in vitro*.

Reports describing the effects of RFP transgenesis on vulnerability to predation have shown varied outcomes. Cortemeglia and Beitinger (2006a) found that RFP and unrelated non-transgenic Zebrafish were equally preyed upon. Hill et al. (2011) found that GloFish® RFP Zebrafish were two times more vulnerable to predation than unrelated non-transgenic Zebrafish. In contrast, Jha (2010) found a domesticated RFP Zebrafish strain in India was less preyed upon by wild-caught Snakeheads than were wild-caught non-transgenic Zebrafish. Factors influencing 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 non-transgenic Zebrafish, innate preference or life history of predators, and/or experimental conditions (e.g., presence of shelter for prey species). Jha (2010) found RFP were more aggressive than wild-caught unrelated Zebrafish, although this may have been due to differences in domestication and/or rearing. GloFish® Electric Green® Tetra did not differ from non-transgenic Tetras in foraging success or aggression levels in paired foraging competition trials (Leggatt and Devlin 2020).

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

Snekser et al. (2006) found the RFP transgene did not influence social partner preferences for either shoaling or in a potential reproductive context in presumably unrelated populations of RFP and non-transgenic Zebrafish. Jiang et al. (2011) reported sex-specific differences in non-transgenic Cloud Mountain Minnow (*Tanichthys albonubes*) preference for non-transgenic or RFP transgenic conspecifics, where non-transgenic males tended to prefer transgenic females, while non-transgenic females preferred non-transgenic males and had no preference for shoal type. Howard et al. (2015) examined the fate of the RFP transgene over 15 generations in a serial competitive breeding experiment in 18 populations of GloFish® Zebrafish. In all populations, the frequency of the RFP transgene declined rapidly, and was eliminated in all populations except one, indicating a strong bias against the RFP transgene in reproduction. In previously notified GloFish®, three of six lines of Tetras, all three GloFish® Danio lines, two of three GloFish® Betta lines, all four GloFish® Tiger Barb lines, and Pristella lines were reported to have decreased reproductive success in competition (DFO 2018, 2019, 2020a, 2020b, 2021a, 2023, 2024). These tests could not be carried out with the GloFish® Sharks as they are seasonal spawners and reproduction under aquaculture conditions is accomplished *in vitro*.

Overall, there are inconsistent reports of pleiotropic effects in other fluorescent protein transgenic models, and, for the most part, these effects would be considered detrimental to the organism (e.g., diminished cold tolerance, diminished reproductive success).

2.6. CHARACTERIZATION RELATIVE TO PREVIOUSLY ASSESSED GLOFISH®

The GloFish® Sharks were produced using similar methodologies and testing protocols as previously notified and assessed GloFish® Tetra, Danio, Betta, Tiger Barb, and Pristella lines. All previously notified GloFish® lines have used similar transgene expression cassette production and elements (promoters, terminator sequences), though the pigment genes vary between colours of fish. Similar molecular and phenotypic characterization tests have been conducted by the company for the current and previously notified GloFish® lines, and results from tests conducted on the GloFish® Shark overlap with some or all of the previously notified lines (data considered confidential business information and not given here).

3. CHARACTERIZATION OF THE COMPARATOR SPECIES

For the purpose of this risk assessment, the domesticated Epalzeorhynchos frenatum (Fowler, 1934), commonly known as the Rainbow Shark, Red Fin Shark or Rainbow Shark minnow, was selected as a comparator. E. frenatum is a popular ornamental species that has been bred and traded in North America for many years. It is native to southeast Asia (Cambodia, Viet Nam, Thailand, Lao People's Democratic Republic) where it lives in freshwater rivers, floodplains and marshlands (Vidthayanon 2012). It grows to a maximum size of approximately 15 cm and can live for up to 15 years in captivity (Seriously Fish). While E. frenatum is popular and is of economic importance, it is not used as a model for scientific research and is not a prevalent species in the scientific literature.

3.1. TAXONOMIC STATUS

E. frenatum is a freshwater fish of the family Cyprinidae (also known as carps or minnows). The title of ‘shark’ has likely resulted from its elongated body shape and fins, and possibly their aggressive behaviour toward similarly shaped fish. However, whereas true sharks are in the Chondrichthyes class of jawed fish that have skeletons primarily composed of cartilage, *Epalzeorhynchos* are in the Osteichthyes Class of jawed fish with a bony endoskeleton. Cyprinidae is the largest and most diverse family of vertebrates with over 1200 extant species distributed world-wide. The genus *Epalzeorhynchos* consists of four species, all native to southeast Asia. Three of the species, *E. frenatum*, *E. bicolor* and *E. kalopterum*, are bred and sold as aquarium ornamentals. The fourth species, *E. munense*, is not part of the ornamental trade. Populations of all four species are in decline in the wild, with *E. bicolor* listed as critically endangered (Vidthayanon 2012). The loss has been blamed in part on overfishing, but is more likely the result of habitat destruction and pollution. Large scale environmental change such as the damming of major rivers, drainage of swampland, and clearance of forest near rivers have had significant impacts on the genus.

Most members of *Epalzeorhynchos* were formerly regarded as *Labeo* spp. and are referred to as such in older literature (FishBase). *Epalzeorhynchos* itself has undergone recent changes with a handful of species having been reassigned to *Crossocheilus*, and *E. bicornis* moved to the genus *Akrokolioplax* (Zhang and Kottelat 2006).

Through the process of domestication, an albino variant of *E. frenatum* was identified and has become popular within the ornamental fish trade (See Figure 1). This variant is also called *E. frenatum* and is fully interfertile with the wild-type.

3.2. PHYSICAL, CHEMICAL AND BIOLOGICAL REQUIREMENTS

E. frenatum has been observed over sandy substrates, near any type of solid surface, at mid-water or bottom depths of rivers and streams (Seriously Fish, FishBase). They feed primarily on algae, phytoplankton, some zooplankton, periphyton, and detritus. *E. frenatum* migrates seasonally, during the rainy season, into flooded habitats, and returns to the river as floodwaters recede (Seriously Fish, FishBase). Disruption of this migratory pattern by human development may be contributing to the decline of this species in the wild (Vidthayanon 2012).

E. frenatum is not a model organism and is rarely the subject of scientific enquiry, either in the field or in the lab. Most studies involving *E. frenatum* are relevant to aquaculture and commercial production scenarios, such as its response to gonadotropic hormones (cGnRH IIa, sGnRH IIIa) for induction of spawning (Sipos et al. 2020), or the study of viruses known to cause significant disease in aquaculture fish stocks (spleen and kidney necrosis virus) (Koda et al. 2021). Studies relevant to the biology of *E. frenatum* in the wild are absent from the scientific literature, with the exception of Leggatt (2019), who considered temperature tolerance.

In the aquarium, *E. frenatum* prefer a neutral pH and water temperatures between 24 and 27°C (FishBase, Seriously Fish). In experiments at DFO (see Leggatt 2019), when water temperatures were dropped relatively slowly (decrease of 1°C/day from 20.5°C), non-transgenic albino *E. frenatum* stopped feeding around 13°C, and lost equilibrium between 16 and 9.6°C, though the majority (80%) of fish lost equilibrium between 11.4 and 9.6°C, with a calculated LD_{50} of 10.7±0.1°C.

3.3. LIFE-HISTORY

In the wild, *E. frenatum* are seasonal spawners, though it is not clear whether its reproductive cycle is tied in any way to its seasonal migration into flooded habitat. Reproduction involves females scattering large numbers of eggs that are fertilized externally by males. In the hatchery, spawning is induced by injecting fish with gonadotropic hormone. Eggs and milt are collected, and eggs are fertilized *in vitro*. At 25°C, eggs hatch in about one day and fry are ready to begin feeding by day seven (information provided in notification).

3.4. BACKGROUND GENETICS

There is little published information regarding the genetics of *E. frenatum*. Most recently, Phimphan et al. (2020) examined the *E. frenatum* karyotype in comparison with two other members of the subfamily Ciprininae. They determined a 2n chromosomal number of 50, which is in keeping with most other members of the subfamily.

3.5. HISTORY OF INVASIVENESS

E. frenatum has no history of invasiveness and there are no reports of establishment anywhere outside of its natural range. Though Tuckett et al. (2017) observed *E. frenatum* in the wild, at distances less than 500 meters from the effluent of aquaculture facilities in Florida, none were observed at distances greater than 500 meters, suggesting a limited capacity to spread and establish in Florida.

4. CHARACTERIZATION OF POTENTIAL RECEIVING ENVIRONMENT

Though the many lakes and rivers of Canada vary in their annual temperature profiles, as well as their average maximum and minimum temperatures, most reach 4°C or below at some point annually, and only a few isolated lakes in Southern Coastal British Columbia have minimum recorded temperatures above this. Of these latter lakes all but one has a minimum temperature recorded below 6°C, and the one lake with minimum temperature recording above 6°C (Cowichan Lake) has, on occasion, minimum temperatures recorded below 6°C (see Leggatt et al. 2018b). It is worth noting that temperature recordings of these warmer lakes are often restricted to a single measurement per winter, and recorded temperatures may not represent the coldest or warmest temperature obtained during winter months.

Given the above, 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 (e.g., industrial effluent, hot springs etc., isolated lakes, if species can survive between 4-6°C). It should be noted that many freshwater systems may have heterogeneity in temperature profiles; for example, groundwater contributions may increase or decrease temperatures in localized areas of a water body, and shorelines are expected to have more extreme temperatures than deep waters. Also, hot springs or warm water effluent may result in localized areas with year-round elevated temperatures. Finally, mean freshwater surface temperatures in Canada are rising as a result of global climate change and are projected to increase by 1.5 to 4.0°C over the next 50 years (DFO 2013) and therefore, could increase the number of possible lakes in which organisms with moderate cold tolerance could survive.

A more detailed description of potential receiving environments in Canada relevant to the introduction of tropical freshwater fish is presented in Leggatt et al. (2018a).

5. ENVIRONMENTAL RISK ASSESSMENT

The environmental risk assessment is conducted under the *Canadian Environmental Protection Act* (CEPA) with respect to the four GloFish® Shark lines that are described in the first part of this document, and have been notified by Spectrum Brands under the *New Substances Notification Regulations (Organisms)*. Given the common comparator species, and the physiological and ecological similarities between the four lines, the following section will consider all lines at the same time. The environmental risk assessment format follows that used for previously notified GloFish® Tetras (DFO 2018, 2019), Danios (DFO 2020a, 2020b), Bettas (DFO 2021a), Tiger Barb (DFO 2023), and Pristella Tetras (DFO 2024). Results of the current assessment are equivalent to those from previous GloFish® assessments unless otherwise stated.

5.1. EXPOSURE ASSESSMENT

The exposure assessment for the four living organisms addresses both their potential to enter the environment (release) and fate once in the environment. The likelihood and magnitude of environmental exposure is determined through an extensive assessment that details the potential for release, survival, persistence, reproduction, proliferation, and spread in the Canadian environment. Rankings for the likelihood of exposure to the Canadian environment are provided in Table 1.

Table 1. Rankings for likelihood of exposure of genetically engineered fish to the Canadian environment.

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

[1]extremely unlikely or unforeseeable

Given the regulatory status of any GE fish undergoing environmental risk assessment under CEPA, a lack of empirical data regarding the survival, fitness and ability of GloFish® Sharks to reproduce in the natural environment will contribute uncertainty to the exposure assessment. Uncertainty associated with the environmental fate of an organism or the failure of biological and geographical containment may depend on the availability and robustness of the scientific information related to the biological and ecological parameters of the organism, valid surrogates, and the receiving environment. Table 2 ranks uncertainty associated with the likelihood of occurrence and fate of the organism in the Canadian environment.

Table 2. Ranking of uncertainty associated with the likelihood of occurrence and fate of the organism in the Canadian environment (environmental exposure).

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

All previous assessments of notified and assessed GloFish® lines concluded low rating for environmental exposure with low uncertainty (DFO 2018, 2019, 2020a, 2020b, 2021a, 2023, 2024). There are no known molecular or phenotypic characteristics of GloFish® Sharks that suggest a different rating than previously assessed lines, and no new scientific literature has been published that would alter the previous ratings. Consequently, the environmental exposure assessments for GloFish® Sharks are low, with low uncertainty that is consistent with previously notified lines. Details supporting this conclusion follow.

5.1.1. 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 GloFish® Sharks will be introduced into the Canadian environment. Once the organism has been sold into the retail market, it is no longer under the direct control of the importer, and there can be no guarantee of appropriate containment and disposal. Numerous aquarium fish have established themselves in natural waters in North America, and reoccurring, though isolated, reports of aquarium fish in Canadian water suggest the practice of releasing aquarium fish into the environment is common and ongoing (Kerr et al. 2005; Rixon et al. 2005; Marson et al. 2009; Strecker et al. 2011; DFO. 2021b; Dickey et al. 2023). This concurs with a high likelihood of release for previously notified GloFish® lines. The extent to which GloFish® Sharks may be further exposed to the environment will, therefore, depend heavily on their ability to survive and reproduce in Canadian lakes and rivers.

5.1.2. Likelihood of Survival

As a tropical species, *E. frenatum* is not expected to survive in a temperate region, where water temperatures are below optimal for survival. Indeed, water temperature is a key abiotic factor that affects both the survival and production of most freshwater fish populations, and is a pervasive determinant of habitat suitability (Jobling 1981; Magnuson et al. 1979).

In the aquarium, *E. frenatum* do best at temperatures between 24 and 27°C (see Section 1.6.2). Data provided by the notifier indicate that three of the four lines of GloFish® Sharks were less tolerant of cold than the domesticated non-transgenic control fish, a trend that has been observed in other species of GloFish® (DFO 2018, 2019, 2020a, 2020b, 2021a, 2023, 2024).

In experiments at DFO (see Leggatt 2019), when water temperatures were dropped relatively slowly (decrease of 1°C/day from 20.5°C), non-transgenic albino *E. frenatum* stopped feeding around 13°C, and lost equilibrium between 16 and 9.6°C, though the majority (80%) of fish lost equilibrium between 11.4 and 9.6°C, with a calculated LD_{50} of 10.7±0.1°C.

As discussed in Section 4, there are no known lakes in Canada that consistently remain above 7°C throughout the entire course of a year, or above 6°C across multiple years, and almost all do not remain above 4°C throughout the year (with the exception of hot springs and industrial effluent).

While the temperatures needed for GloFish® Sharks to survive may be possible for several Canadian lakes during the summer, it is extremely unlikely that GloFish® Sharks can survive the Canadian winter. At best, its occurrence in the environment would be seasonal or ephemeral. This is further supported by lack of establishment of *E. frenatum* after noted occurrences in much warmer climates (e.g., in Florida, Tuckett et al. 2017).

Though mean freshwater surface temperatures in Canada are rising as a result of global climate change, and are projected to increase by 1.5 to 4.0°C over the next 50 years (DFO 2013), the majority of freshwater systems experiencing significant ice coverage in the winter are expected to see a decrease in the number of ice-days in these systems (DFO 2013). However, continuation of any winter ice coverage would result in temperatures at or below 4°C at some point during the winter, preventing year-round survival of GloFish® Sharks.

Cold-tolerance data combined with the lack of establishment of *E. frenatum* in regions warmer than Canada suggest negligible potential for survival in Canadian waters, even when the increased water temperatures associated with climate change are taken into account.

5.1.3. Likelihood of Reproduction

Though water temperatures in Canada will prevent the persistence of any GloFish® Sharks that are introduced (see Section 2.2.2), there may still be time during the summer for survival and reproduction to occur, provided adults are introduced at the start of a warm season and the environmental conditions required for spawning are met. *E. frenatum* are seasonal spawners, though it is not clear if this seasonality is associated in any way with their seasonal migration onto flooded plains. In the wild, females scattering large numbers of eggs that are fertilized externally by males (see Section 1.6.3). In the hatchery, spawning must be induced by injecting fish with gonadotropic hormone. Eggs and milt are collected, and eggs are fertilized *in vitro*. At 25°C, eggs hatch in about one day and fry are ready to begin feeding by day seven.

While some lakes in Canada may reach 25°C for short periods during the summer (see Section 1.7), it is highly unlikely that all other environmental conditions required *E. frenatum* to reproduce will occur in a temperate environment where there is no rainy season.

5.1.4. Likelihood of Proliferation and Spread

The capacity for GloFish® Sharks to proliferate and spread in the Canadian environment is limited by the fact that *E. frenatum* cannot survive the cold winter temperatures and is unlikely to reproduce when temperatures in the summer are more favorable.

5.1.5. Conclusions of the Exposure Assessment

Given the above analysis, the occurrence of GloFish® Sharks in the Canadian environment is expected to be rare, isolated and ephemeral. Consequently, the likelihood of exposure of GloFish® Sharks to the Canadian environment is ranked **low** according to Table 1. The uncertainty associated with this estimate is **low** (Table 2), given the quality of data (temperature tolerance) available for GloFish® Sharks and valid surrogate organisms, and data available on the environmental parameters of the receiving environment in Canada. This rating is consistent with the low exposure rating with low uncertainty concluded on for six lines of GloFish® Tetra (DFO 2018, 2019), three lines of Danio (DFO 2020a, 2020b), three lines of GloFish® Betta (DFO 2021a), four lines of GloFish® Tiger Barb (DFO 2023), and four lines of GloFish® Pristella Tetras (DFO 2024).

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

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

5.2. HAZARD ASSESSMENT

The hazard assessment examines potential impacts to the environment that could result from exposure to GloFish® Sharks. The hazard identification process considers potential pathways to harm including through environmental toxicity (i.e., potential to be poisonous), gene transfer, trophic interactions, and as a vector for pathogens, as well as capacity to impact ecosystem components (e.g., habitat, nutrient cycling, biodiversity). Table 3 categorizes the severity of the biological consequences based on the severity and reversibility of effects to the structure and function of the ecosystem. Any difference in measurement endpoint is evaluated relative to 'normal' variation, based on published studies and expert opinion.

Table 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.

Uncertainty around the hazard assessment may be significant due to clear knowledge gaps and lack of empirical data around the behaviour and effects of GloFish® Sharks in the natural environment. Criteria for the assessment of uncertainty address potential effects to the environment, which may rely heavily on information and data found in published and peer-reviewed scientific literature. A description of rankings for uncertainty regarding the potential hazards of the organism in the environment is provided in Table 4.

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

Table 4. Ranking of uncertainty associated with the environmental hazard.

Uncertainty Ranking	Available Information
Negligible	High quality data on the organisms. 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 the organisms or valid surrogate. Understanding of GxE effects across relevant environmental conditions. Some variability.
Moderate	Limited data on the organisms, relatives of the organisms or valid surrogate. Limited understanding of GxE effects across relevant environmental conditions. Knowledge gaps. Reliance on expert opinion.
High	Significant knowledge gaps. Significant reliance on expert opinion.

The proposed use of GloFish® Sharks 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 GloFish® Sharks to Canadian environments. The majority of potential hazards posed by GloFish® Sharks (e.g., through interactions with other organisms, impacts to biogeochemical cycling, habitat and biodiversity) would be through direct release of GloFish® Sharks into natural aquatic ecosystems, although some potential hazards could act indirectly through the release of waste water and carcasses into the environment (e.g., environmental toxicity, horizontal gene transfer, as a vector for disease).

In assessments of previously notified and assessed GloFish® lines, all concluded with negligible rating for most environmental hazard pathways and low hazard rating through horizontal gene transfer (HGT), with uncertainty ranging from negligible to moderate (DFO 2018, 2019, 2020a, 2020b, 2021a, 2023, 2024). Though E. frenatum may differ from previously notified species in some phenotypes (i.e. aggression), there are no known molecular or phenotypic characteristics of GloFish® Sharks derived from the genetic modifications that suggest a different rating than previously assessed lines, and no new scientific literature has been published that would alter the previous ratings. Consequently, the environmental hazard assessments for GloFish® Sharks follow those of the previously notified GloFish® lines. Details supporting these conclusions follow, and greater detail for each hazard assessment endpoint can be found in Leggatt et al. (2018a).

5.2.1. Potential Hazards Through Environmental Toxicity

Potential routes of environmental toxicity include exposure of aquatic ecosystems to the whole animal and its waste, as well as ingestion by predators. Exposure of the fluorescent proteins to the environment is expected to be lower than exposure of the proteins to GloFish® Shark lines; though different routes of exposure are not necessarily comparable. Fluorescent proteins are commonly used as neutral markers in research in a wide range of organisms with almost no reports of toxicity (Stewart 2006). The few reports of negative effects are generally specific to transgenic organisms with especially high expression of fluorescent transgenes (Huang et al.

2000; Devgan et al. 2004; Guo et al. 2007). Any toxic effects to host organisms are likely due to production of the protein within the host cell, and are not expected to have equal effects from contact or ingestion exposure.

The notifications include a report screening the amino acid sequence of the fluorescent protein for allergenicity on Allermatch™ and AllergenOnline that found no functional matches to known human allergen amino acid sequences. After several years of commercial production in the US, there have been no reported toxic effects resulting from exposure to other species of GloFish® containing transgenes coding the same proteins as those in the GloFish® Shark lines in both Canada and the United States. Consequently, the potential hazard to the environment due to environmental toxicity of GloFish® Sharks is ranked negligible. The uncertainty associated with this ranking is moderate due to limited direct data from the notified organisms or surrogate organisms, and reliance on anecdotal evidence and indirect evidence from other organisms. This concurs with assessment rankings for previously notified GloFish® lines (DFO 2018, 2019, 2020a, 2020b, 2021a, 2023, 2024), and no new relevant data has become available since analyses of previous GloFish® lines.

5.2.2. 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). Pathways of exposure of free transgenic DNA to novel organisms (most likely prokaryotes) include exposure within the gut, or through feces, mucus, and other waste sloughed off by the fish into the water. The transgene constructs do not contain transposable elements that may increase the potential for DNA uptake/mobility to a new organism. In order for the transgene to be expressed and result in phenotypic change, it requires co-transfer of regulatory elements. The close proximity of the promoters to the pigment transgenes could increase the likelihood of them being co-transferred and expressed, though vertebrate promoters generally have poor activity in prokaryotes. As well, the identified presence of the bacteriophage T3 promoter in the transgene constructs of most of the current and some previously notified lines may increase the potential for functional HGT to occur as the T3 promoter has been shown to result in expression of cnidarian fluorescent protein transgenes in *Escherichia coli* (Wu et al. 2015). One recent study examined the potential for HGT of fluorescent protein transgenes using genetically engineered fruit flies (transgenic for DsRed) and its parasitoid (Ramirez-Santos et al. 2018). The authors did not find any evidence of HGT of the fluorescent protein transgene over 16 generations of experimental rearing, although cautioned their experimental design may not have detected rare events of HGT or transfer of mutated transgenes.

Genes encoding fluorescence have been introduced to a wide range of organisms with few reports of harmful effects from the introduced transgenes. This suggests that the introduction of the transgene through HGT to a novel host is not expected to result in harmful effects, should it occur. Graham and Davis (2021) recently demonstrated HGT of an environmentally advantageous gene (antifreeze protein) between two fish species at an evolutionary scale. While this demonstrates HGT can occur very infrequently with advantageous genes between higher organisms, the lack of fitness advantage (e.g., cold tolerance) conferred by the present fluorescent protein transgenes suggests that if HGT transfer occurred it would likely be on an individual organism level. Though the introduction of a fluorescent transgene to a novel organism in Canadian environments through HGT cannot be excluded, the absence of expected harmful effects from such an introduction result in a hazard ranking of **low**. While the transgenes are well defined, the limited knowledge of the location of the transgenes within the *E. frenatum* genome, and lack of studies examining HGT of the transgenes and resulting consequences, results in **moderate** uncertainty. This concurs with the previous assessments for the GloFish® lines, though in some groups (Tetras) uncertainty was assessed as low (DFO 2018, 2019). Here, as with the GloFish® Danios, Bettas, Barbs, and Pristella Tetras, the uncertainty rating was increased to moderate to better reflect the lack of or limited number of relevant studies of HGT and resulting consequences (DFO 2020a, 2020b, 2021a, 2023, 2024).

5.2.3. Potential Hazards Through Interactions with Other Organisms

Should GloFish® Sharks be released to the environment, they have the potential to interact with other organisms in Canadian freshwater aquatic ecosystems, including potential prey, competitors, and predators. The trophic interactions of wild *E. frenatum* in its native range are not well documented (see Section 1.6), nor is there documentation of trophic interactions of escaped domesticated non-transgenic *E. frenatum* in other areas. Limited data described below indicate non-transgenic *E. frenatum* may have limited potential to impact Canadian species through trophic interactions, and GloFish® Sharks would likely have equal or less potential to impact through trophic interactions.

Wild *E. frenatum* are omnivorous, feeding mostly on algae and detritus found at the bottom of rivers and flood plains. As such, they have the potential to impact localized populations of competitors occupying similar niches at the location of release. Non-transgenic *E. frenatum* are generally described as aggressive to conspecifics, or fish that resemble conspecifics (see Section 1.6). However, activity and hence feeding levels of *E. frenatum* are expected to be low during most seasons in Canada's temperate waters due to limited activity at temperatures below 17°C (Leggatt 2019).

In other fluorescent protein transgenic models, GloFish® Electric Green® Tetras had similar aggressive behaviour and foraging success as non-transgenic siblings in paired feeding trials (Leggatt and Devlin 2019), whereas RFP Zebrafish had lower male mating aggression and success than non-transgenic siblings (Howard et al. 2015). Though both of these studies are on typically non-aggressive fish, they suggest that fluorescent protein transgenesis may decrease or not affect competitive success in tropical fish. The notifying company has indicated that there is no information available to support any claim of differences in behaviour between GloFish® Sharks and non-transgenic *E. frenatum*.

Given the low temperatures expected for Canadian freshwater systems for most of the year, and lack of evidence of altered behaviour from genetic modifications, the potential for anticipated numbers of released GloFish® Sharks to impact native aquatic species through prey acquisition and competition is expected to be negligible through most of the year, and is expected to be no greater than for non-transgenic *E. frenatum*.

Released GloFish® Sharks may 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 GloFish® Sharks causes deleterious effects to the predator populations. The latter is not expected as GloFish® Sharks are not expected to be environmentally toxic (see Section 2.3.1 above).

The effects of predation on GloFish® Sharks relative to non-transgenic *E. frenatum* has not been reported. In other transgenic models (RFP Zebrafish), results are conflicting, with RFP-expressing Zebrafish having higher (Hill et al. 2011), equal (Cortemeglia and Beitinger 2006a), or lower (Jha 2010) predation susceptibility relative to non-related, non-transgenic fish. These variable findings may be due to differences in rearing history, genetic background, experimental conditions among studies, or GxE interactions. Whether any of the above studies could be applied to the GloFish® Sharks predation vulnerability in Canadian environments is not known and, consequently, the predation vulnerability of GloFish® Sharks relative to their non-transgenic counterparts cannot be estimated with reasonable certainty. However, due to the expected lack of toxicity from ingesting GloFish® Sharks, the notified lines introduced at anticipated scales are not expected to pose a hazard as prey to native predators, regardless of potential predation sensitivity.

Based on low activity of *E. frenatum* in cooler waters, and lack of noted alterations in trophic-related behaviour of the notified lines, GloFish® Sharks are not expected to influence the trophic interactions of native organisms beyond natural fluctuations, with an assessment of **negligible** hazard relative to non-transgenic counterparts. The lack of studies directly examining the hazards of GloFish® Sharks, conflicting data on surrogate (RFP Zebrafish) models, and poor understanding of GxE interactions in aggression and predation susceptibility in surrogate fluorescent transgenic models, result in a **moderate** level of uncertainty. This concurs with assessment rankings for previously notified GloFish® Tetras, Danios, Bettas, Tiger Barbs and Pristella Tetras (DFO 2018, 2019, 2020a, 2020b, 2021a, 2023, 2024).

5.2.4. Potential Hazards Through Hybridization with Native Species

The genus *Epalzeorhynchos* consists of four species, all of which are endemic to Southeast Asia and none of which are established in North America. There are no records or reports of hybridization between species within this genus, either in the wild or under culture. While there are no species of *Epalzeorhynchos* native to Canada, there are several genera of the cyprinidae family. Intergeneric hybrids have been noted for two genera of cyprinidae in Europe (Hayden et al. 2010), and in the family mormyridae, where survival of intergeneric hybrids was related to the phylogenetic distance of the parent species (i.e., greater phylogenetic distance resulted in decreased viability, and increased occurrence of malformations (Kirschbaum et al. 2016).

It is unknown if Canadian cyprinids could successfully breed with Rainbow Sharks, though it is highly unlikely given the phylogenetic difference and adaptive differentiation between native Canadian cyprinidae genera and *Epalzeorhynchos*. Rainbow Sharks are scatter breeders, making it easier for potential hybridizations to occur with related species that may spawn at the same time and place. However, Rainbow Sharks are also seasonal breeders, making it difficult to naturally breed Rainbow Sharks under commercial conditions, as is done with many other tropical freshwater aquarium species. Instead, ovulation must be induced hormonally and fertilization carried out *in vitro* (see Section 1.6.3). The chance of release into a Canadian environment that can support the natural reproduction process for Rainbow Sharks is remote.

Consequently, there is **negligible** potential for GloFish® Sharks to cause hazard through natural hybridization with native fish in Canada. High quality data on the distribution of *Epalzeorhynchos* species and related genera, but lack of data on the potential for intergeneric hybridization, results in a **moderate** uncertainty associated with the rating. This concurs with the assessment ranking for previously notified GloFish® *Danios* that also belong to the family Cyprinidae and are also scatter breeders (DFO 2020a, 2020b). Uncertainty differs from that of the GloFish® Tiger Barb that are in the family Cyprinidae, but pair spawn, making hybridization less likely. All other GloFish® lines (Tetras, Bettas, and Pristella) that are not in the family cyprinidae and do not have other family members in North America, have uncertainty rankings of negligible.

5.2.5. Potential to Act as a Vector of Disease Agents

Commercial ornamental aquarium fish are commonly reported to carry numerous disease agents including viruses, bacteria, fungi, and parasites (e.g., Evans and Lester 2001; Řehulka et al. 2006; Whittington and Chong 2007; Hongslo and Jansson 2009; Rose et al. 2013; Walczak et al. 2017). While disease agents are common in tropical-origin freshwater ornamental aquarium fish, any disease agents GloFish® Sharks 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 GloFish® Sharks if released into cooler Canadian freshwater environments.

E. frenatum is not listed among the few tropical species susceptible to diseases of significant importance to aquatic animal health and the Canadian economy by the Canadian Food Inspection Agency (CFIA) (Susceptible Species of Aquatic Animals). Since the principal mode of entry into Canada for GloFish® Sharks will be through importation from the US, the CFIA will play a critical role in regulating disease agents of *E. frenatum* that are imported into Canada. Anyone importing aquatic animals on the Susceptible Species list require an Aquatic Animal Health Import Permit, while all other imports require a 'declaration' at the border.

Whether GloFish® Sharks have altered ability to act as a vector of disease agents has not been directly 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 has been shown to decrease T-cell activation (Koelsch et al. 2013), induce cytokine IL-6 secretion (Mak et al. 2007), inhibit immune-related signalling pathways (Baens et al. 2006), and alter expression of genes involved in immune function (Coumans et al. 2014) and response to stress (Badrian and Bogoyevitch 2007). As well, Chou et al. (2015) reported mice transgenic for DsRed had alterations in some white blood cell numbers (lymphocytes and monocytes) but not others. Numerous other transgenic fluorescent aquarium species and lines have been grown on a commercial scale in the US starting in 2003. Consequently, there is **negligible** potential for GloFish® Sharks to have altered capacity as a vector for disease relative to non-transgenic *E. frenatum*. As this has not been directly examined in GloFish® Sharks, there are limited data on a surrogate, and reliance on expert opinion, the uncertainty level for this rating is **moderate.** This concurs with assessment rankings for previously notified GloFish® Tetras, Danios, Bettas, Tiger Barbs, and Pristella Tetras (DFO 2018, 2019, 2020a, 2020b, 2021a, 2023, 2024).

5.2.6. Potential to Impact Biogeochemical Cycling

GloFish® Sharks are expected to contribute to nutrient cycles within habitats through ingestion of food items and release of waste (ammonia and feces). The potential effects of fluorescent protein in GloFish® Sharks 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 GloFish® Sharks have similar influences from fluorescent transgenic gene expression are not known, however, the small size of *E. frenatum* and potential low numbers of individuals anticipated to enter an ecosystem indicates a **negligible** potential for GloFish® Sharks to impact biogeochemical cycling in natural environments, even with altered metabolic pathways. Uncertainty is **moderate** due to a lack of studies directly examining this hazard. This concurs with assessment rankings for previously notified lines of GloFish® Tetras, Danios, Bettas, Tiger Barb, and Pristella Tetras (DFO 2018, 2019, 2020a, 2020b, 2021a, 2023, 2024).

5.2.7. Potential to Affect Habitat

Rainbow Sharks are a small species and are not known to build structures that are expected to impact habitats of other species. There have been no reports, anecdotal or otherwise, of GloFish® Sharks having altered behaviour, relative to domesticated *E. frenatum*, that may influence effects on habitat structure. Consequently, GloFish® Sharks are expected to have **negligible** effects on habitat with **moderate** uncertainty associated with this rating due to a lack of information regarding this hazard for GloFish® Sharks or the comparator species. This concurs with assessment rankings for previously notified GloFish® lines (DFO 2018, 2019, 2020a, 2020b, 2021a, 2023, 2024), though uncertainty has been increased from low to moderate, to account for the limited information available on *E. frenatum*, relative to the previously assessed species.This concurs with assessment rankings for previously notified GloFish® Tetras, Danios, Bettas, Tiger Barb, and Pristella Tetras (DFO 2018, 2019, 2020a, 2020b, 2021a, 2023, 2024).

5.2.8. Potential to Affect Biodiversity

Biodiversity can be negatively impacted by numerous drivers, including invasive species and the introduction of disease. Despite their long standing use in the ornamental aquarium trade, there have been no reports of *E. frenatum* becoming invasive or established anywhere outside of there natural range. As well, there is no evidence that GloFish® Shark lines have increased fitness that may increase invasiveness relative to non-transgenic Rainbow Sharks. Hill et al. (2017) estimated the invasiveness risk of *E. frenatum* within the continental USA to be low to moderate, using the Fish Invasiveness Screening Kit (FISK).

As elaborated above, GloFish® Sharks are not expected to negatively impact native species through trophic or hybrid interactions, act as a vector for disease agents of concern in Canada, impact biogeochemical cycling, or impact habitat. Addition of the transgenic construct and fluorescent protein in GloFish® Sharks is not expected to result in environmental toxicity, or cause hazards through HGT of the transgene. Taken together, there is a **negligible** hazard of GloFish® Sharks affecting biodiversity of Canadian ecosystems. However, the lack of data regarding the comparator species for invasiveness and biodiversity effects results in a **moderate** degree of uncertainty with this ranking.

5.2.9. Conclusions of the Hazard Assessment

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

The examined hazards have negligible to low rankings (Table 3), while uncertainty ranged from negligible to moderate, due to limited data specific to GloFish® Sharks, limited direct data on comparator species, variable data from surrogate models (e.g., RFP Zebrafish), and the reliance on expert opinion for the assessment of some hazards. Outside of its intended use as an ornamental fish in static aquaria, GloFish® Sharks are not expected to pose unique hazards beyond those of the intended use. Hazard ranking concurred with that previously assessed for GloFish® Tetras and GloFish® Danios, although uncertainty differed from that assessed in GloFish® Tetras in two hazard categories due to increased acknowledgement of data limitations (through HGT), or differences in family distributions (through hybridization, see Table 5).

Table 5. Summary of all ranks and uncertainty rating for environmental risk assessments of currently notified GloFish® Shark lines, as well as previously notified GloFish® Tetras, Danios, Bettas, Barbs, and Pristella (DFO 2018, 2019, 2020a, 2020b,2021a, 2023, 2024). Underlines indicate where previous assessments differ from the current assessment.

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

5.3. ASSESSMENT OF RISK

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

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

For each endpoint, hazard and exposure are ranked as: negligible, low, moderate, or high, and include an analysis of uncertainty for each. Overall Risk is estimated by plotting Hazard against Exposure, using a matrix or heat map, as illustrated in Figure 2. Though the matrix cannot be used as a tool for establishing a discreet conclusion or decision on risk, it can be used to facilitate communication and discussion. The uncertainty associated with the overall Risk rating is not estimated, rather uncertainty in the hazard and exposure assessments are discussed in the context of a final conclusion on risk.

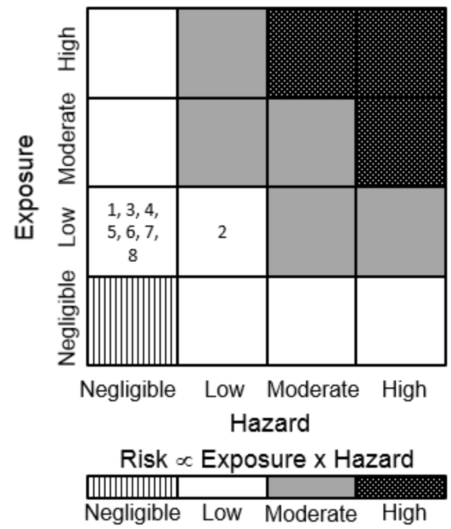

Figure 2. Risk matrix and pattern scale to illustrate how exposure and hazard are integrated to establish a level of risk in the environmental risk assessment. Risk assessments associated with assessed hazard components at the assessed exposure are identified by number: 1) through environmental toxicity; 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; and 8) to biodiversity.

5.3.1. Risk Assessment of the GloFish® Sharks

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

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

Using the risk matrix seen in Figure 2, GloFish® Sharks used in the ornamental aquarium trade or other uses in Canada pose **low risk** to Canadian environments. Individual hazards are expected to result in no harmful effects beyond natural fluctuations to Canadian environments under the assessed level of exposure. Sources of uncertainty in the environmental exposure and hazard assessments that may influence uncertainty in environmental risk assessment include a lack of data directly addressing hazards of the notified organism and comparator species, variability in data taken from surrogate organisms, and in some cases reliance on expert opinion.

Despite moderate uncertainty in some of the individual assessment components, there is no current evidence to suggest that overall risk ratings of GloFish® Sharks may be higher than the assessed low ranking for risk to Canadian environments. This concurs with low risk assessment rankings for previously notified GloFish® Tetras, Danios, Bettas, Tiger Barbs, and Pristella Tetras (DFO 2018, 2019, 2020a, 2020b, 2021a, 2023, 2024; see Table 5).

5.4. SUMMARY AND CONCLUSIONS

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

REFERENCES CITED

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

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

Blake, A., Crockett, R., Essner, J., Hackett, P., and Nasevicius, A. 2016. Recombinant constructs and transgenic fluorescent ornamental fish therefrom. United States Patent #9,271,478 B2.

Caldovic, L., Agalliu, D., and Hackett, P.B. 1999. Position-independent expression of transgenes in zebrafish. Transgen. Res. 8(5):321-334.

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

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

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

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

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

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

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

DFO. 2006. Proceedings of the expert panel meeting on the potential risks associated with horizontal gene transfer from novel aquatic organisms. DFO Can. Sci. Advis. Sec. Proceed. Ser. 2006/036.

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

DFO. 2018. Environmental and indirect human health risk assessment of the Glofish® Electric Green® Tetra and the Glofish® Long-Fin Electric Green® Tetra (Gymnocorymbus ternetzi): a transgenic ornamental fish. DFO Can. Sci. Advis. Sec. Sci. Advis. Rep. 2018/027.

DFO. 2019. Environmental and indirect human health risk assessment of the GloFish® Tetras (Gymnocorymbus ternetzi): five lines of transgenic ornamental fish. DFO Can. Sci. Advis. Sec. Sci. Advis. Rep. 2019/002.

DFO. 2020a. Environmental and indirect human health risk assessment of the GloFish® Sunburst Orange® Danio (Danio rerio): A transgenic ornamental fish. DFO Can. Sci. Advis. Sec. Scie.Advisory Report 2020/15.

DFO. 2020b. Environmental and Indirect Human Health Risk Assessment of GloFish® Cosmic Blue® and Galactic Purple® Danios (Danio rerio): Transgenic Ornamental Fish. DFO Can. Sci. Advis. Sec. Sci. Advis. Rep. 2020/016.

DFO. 2021a. Environmental and Indirect Human Health Risk Assessment of the GloFish® Bettas: three lines of transgenic ornamental fish. DFO Can. Sci. Advis. Sec. Sci. Advis. Rep. 2021/046.

DFO. 2021b. Science Advice on the Potential of Introducing Live Organisms by the Aquarium, Water Garden, and Live Food Trades in Canada. DFO Can. Sci. Advis. Sec. Sci. Advis. Rep. 2021/032.

DFO. 2023. Environmental and Indirect Human Health Risk Assessment of GloFish® Starfire Red®, Electric Green®, Sunburst Orange®, and Galactic Purple® Barbs (*PuntiGrus tetrazona*): Transgenic Ornamental Fishes. DFO Can. Sci. Advis. Sec. Sci. Advis. Rep. 2023/043.

DFO. 2024. Environmental and Indirect Human Health Risk Assessment of Glofish® Electric Green®, Starfire Red®, Sunburst Orange®, and Galactic Purple® Pristella Tetras (*Pristella maxillaris*): Transgenic Ornamental Fishes. DFO Can. Sci. Advis. Sec. Sci. Advis. Rep. 2024/017.

Dickey, J. W. E., Liu, C., Briski, E., Wolter, C., Moesch, S., & Jeschke, J. M. 2023. Identifying potential emerging invasive non-native species from the freshwater pet trade. People and Nature, 00, 1–14.

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

Gong, Z., Wan, H., Tay, T.L., Wang, H., Chen, M., and Yan, T. 2003. Development of transgenic fish for ornamental and bioreactor by strong expression of fluroescent proteins in the skeletal muscle. Biochem. Biophys. Res. Commun. 308:58-63.

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

Graham, L.A., and Davies, P.L. (2021) Horizontal Gene Transfer in Vertebrates: A Fishy Tale. TIGS 37(6):501-503.

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

Hill, J.E., Tuckett, Q.M., Hardin, S., Lawson, L.L., Lawson, K.M., Ritch, J.L., and Partridge, L. 2017. Risk screen of freshwater tropical ornamental fishes for the conterminous United States. Trans. Am. Fish. Soc. 146(5):927-938.

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

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

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

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

Jiang, P., Bai, J.J., Ye, X., Jian, Q., Chen, M., Chen, X.Q. 2011. Shoaling and mate choice of wild-type Tanichthys albonubes in the presence of the red fluorescent transgenic conspecifics. J Fish Biol. 78(1):127-37.

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

Ju, B., Chong, S.W., He, J., Wang, X., Xu, Y., Wan, H., Tong, Y., Yan, T., Korzh, V., and Gong, Z. 2003. Recapitulation of fast skeletal muscle development in zebrafish by transgenic expression of GFP under the mylz2 promoter. Dev. Dyn. 227(1):14-26.

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

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

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

Leggatt, R.A., Johnson, N., and McGowan, C. 2018a. Environmental risk assessment of the Glofish® Electric Green® Tetra and the Glofish® Long-Fin Electric Green® Tetra: transgenic ornamental fish, imported to Canada, for sale in the pet trade. DFO Can. Sci. Advis. Sec. Res. Doc. 2018/049: xii + 54 pp.

Leggatt, R.A. and Devlin, R.H. 2020. Fluorescent protein transgenesis has varied effects on behaviour and cold tolerance in a tropical fish (Gymnocorymbus ternetzi): implications for risk assessment. Fish Physiol. Biochem. 46:395-403.

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

Leggatt, R.A. 2019. Cold temperature tolerance of albino rainbow shark (Epalzeorhynchos frenatum), a tropical fish with transgenic application in the ornamental aquarium trade. Can. J. Zool. 97:376-378.

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

Liu, Z.J., Moav, B., Faras, A.J., Guise, K.S., Kapuscinski, A.R., and Hackett, P.B. 1990. Development of expression vectors for transgenic fish. Biotechnology 8(12):1268-1272.

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

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

Marson, D., Cudmore, B., Drake, D.A.R., and Mandrak, N.E. 2009. Summary of a survey of aquarium owners in Canada. Can. Manuscr. Rep. Fish. Aquat. Sci. 2905: iv + 20 p.

Matz, M.V., Fradkov, A.F., Labas, Y.A., Savitsky, A.P., Zaraisky, A.G., Markelov, M.L., and Lukyanov, S.A. 1999. Fluorescent proteins from nonbioluminescent Anthozoa species. Nat. Biotechnol. 17(10):969-973.

Merzlyak, E.M., Goedhart, J., Shcherbo, D., Bulina, M.E., Shcheglov, A.S., Fradkov, A.F., Gaintzeva, A., Lukyanov, K.A., Lukyanov, S., Gadella, T.W.J., and Chudakov, D.M. 2007. Bright monomeric red fluorescent protein with an extended fluorescence lifetime. Nat. Methods 4(7):555-557.

Nam, Y.K., Noh, C.H., and Kim, D.S. 1999. Transmission and expression of an integrated reporter construct in three generations of trasngenic mud loach (Misgurnus mizolepis). Aquaculture 172:229-245.

Owen, M.A., Rohrer, K., and Howard, R.D. 2012. Mate choice for a novel male phenotype in zebrafish, Danio rerio. Anim. Behav. 83(3):811-820.

Phimphan, S., Chaiyasan, P., Suwannapoom, C., Reungsing, M., Juntaree,, S., Tanomtong, A., Supiwong, W. 2020. Comparative karyotype study of three Cyprinids (Cyprinidae, Cyprininae) in Thailand by classical cytogenetic and FISH techniques. Comp Cytogenet. 14(4):597-612.

Ramirez-Santos, E., Rendon, P., Bourtzis, K., Schetelig, M.F., Caceres, C., Targovska, A., Rehling, T., Guillen-Navarro, G.K., Ruiz-Montoya, L., Toledo, J., and Liedo, P. 2018. Evaluation of horizontal gene transfer risk between the Mediterranean fruit fly Ceratitis capitata (Tephritidae) and its parasitoid Fopius ceratitivorus (Braconidae). PloS ONE 13(12): e0207999.

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

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

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

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

Schek, N., Cooke, C., and Alwine, J.C. 1992. Definition of the upstream efficiency element of the simian virus 40 late polyadenylation signal by using in vitro analyses. Mol. Cell. Biol. 12(12):5386-5393.

Shagin, D.A., Barsova, E.V., Yanushevich, Y.G., Fradkov, A.F., Lukyanov, K.A., Labas, Y.A., Semenova, T.N., Ugalde, J.A., Meyers, A., Nunez, J.M., Widder, E.A., Lukyanov, S.A., Matz, M.V. 2004. GFP-like proteins as ubiquitous metazoan superfamily: evolution of functional features and structural complexity. Mol Biol Evol. 21(5):841-50.

Shcherbo, D., Merzlyak, E.M., Chepurnykh, T.V., Fradkov, A.F., Ermakova, G.V., Solovieva, E.A., Lukyanov, K.A., Bogdanova, E.A., Zaraisky, A.G., Lukyanov, S., and Chudakov, D.M. 2007. Bright far-red fluorescent protein for whole-body imaging. Nat. Methods. 4(9): 741-746.

Shkrob, M.A., Yanushevich, Y.G., Chudakov, D.M., Gurskaya, N.G., Labas, Y.A., Poponov, S.Y., Mudrik, N.N., Lukyanov, S., Lukyanov, K.A. 2005. Far-red fluorescent proteins evolved from a blue chromoprotein from Actinia equina. Biochem J. 392(3):649-54.

Sipos, M.J., Lipscomb, TN., Wood AL, Ramee, S.W., Watson, C.A., DiMaggio, M.A. 2020. Evaluation of cGnRHIIa for induction spawning of two ornamental Epalzeorhynchos species. Aquac Res. 51:232–241.

Snekser, J.L., McRobert, S.P., Murphy, C.E., and Clotfelter, E.D. 2006. Aggregation behavour in wildtype and transgenic zebrafish. Ethology 112:181-187.

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

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

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

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

Vidthayanon, C. 2012. Epalzeorhynchos frenatum. The IUCN Red List of Threatened Species 2012: e.T181093A1697683. doi:10.2305/IUCN.UK.2012-1.RLTS.T181093A1697683.en.

Walczak, N., Puk, K., and Guz, L. 2017. Bacterial flora associatedwith diseased freshwater ornamental fish. J. Vet. Res. 61:445-449.

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

Wu, C., and Alwine, J.C. 2004. Secondary structure as a functional feature in the downstream region of mammalian polyadenylation signals. Mol. Cell. Biol. 24(7):2789-2796.

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

Zhang, E., and Kottelat, M. 2006. Akrokolioplax, a new genus of Southeast Asian labeonine fishes (Teleostei: Cyprinidae). Zootaxa. 1225:21-30.