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Indirect Human Health Risk Assessment of GloFish® Electric Green® (GPM2021), GloFish® Starfire Red® (RPM2022), GloFish® Sunburst Orange® (OPM2021), and GloFish® Galactic Purple® (PPM2021) Pristella Tetras (*Pristella maxillaris*): Transgenic Ornamental Fishes

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Canada

Foreword

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

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ABSTRACT

An indirect human health risk assessment was conducted on four lines of genetically modified Pristellas (*Pristella maxillaris*) known as the GloFish® Electric Green® Pristella (GPM2021), GloFish® Starfire Red® Pristella (RPM2022), GloFish® Sunburst Orange® Pristella (OPM2021), and the GloFish® Galactic Purple® Pristella (PPM2021), that were notified under the *Canadian Environmental Protection Act* (CEPA). GPM2021, RPM2022, OPM2021, and PPM2021 are modified lines of diploid, hemizygous or homozygous, Pristellas, containing genes encoding for different fluorescent proteins. GPM2021, RPM2022, OPM2021, and PPM2021 appear green, red, orange, or purple, respectively under ambient and blue light (including sunlight). The four lines are proposed for import from the United States for use as ornamental fish in home aquaria. This risk assessment examined the potential for the four lines to cause harmful effects to humans in Canada relative to wild-type Pristella Tetras as a consequence of environmental exposure including under its intended use in home aquaria. The parental strain, *P. maxillaris*, has been available as a home aquarium fish since the 1950s without any reported adverse human health effects. There is no evidence to suggest a risk of adverse human health effects for the general Canadian population from use of GPM2021, RPM2022, OPM2021, or PPM2021 as ornamental aquarium fish or other identified potential uses. As such, there is no expectation that GPM2021, RPM2022, OPM2021, and PPM2021 will pose any more risks to human health than wild-type *P. maxillaris.*

INTRODUCTION

The following indirect human health risk assessment was conducted on *Pristella maxillaris* GPM2021, RPM2022, OPM2021, and PPM2021, four genetically modified lines of diploid, hemizygous or homozygous, Pristellas, containing genes coding for recombinant fluorescent green, red, yellow, or purple proteins, respectively. Wild type *P. maxillaris* have wide spread use in Canada and other parts of the world as tropical ornamental fish. This risk assessment examines the potential for GPM2021, RPM2022, OPM2021, and PPM2021 to cause harmful effects to humans in Canada, relative to wild-type *P. maxillaris*, as a consequence of environmental exposure, including exposure in natural environments and environments under its intended use (i.e., home aquaria). GPM2021, RPM2022, OPM2021, and PPM2021 are green, red, orange, and purple in colour, respectively, when displayed in ambient and blue light, including sunlight, and are proposed to be imported from the United States for use as ornamental fish in home aquaria. The risk assessment was conducted under the *Canadian Environmental Protection Act* (CEPA) and *New Substances Notification Regulations (Organisms)* (NSNR[O]).

HAZARD ASSESSMENT

IDENTIFICATION AND CHARACTERIZATION OF *PRISTELLA MAXILLARIS* GPM2021, RPM2022, OPM2021, AND PPM2021

Binomial name

Pristella maxillaris (Ulrey 1894) GPM2021, RPM2022, OPM2021, and PPM2021

Taxonomy

Kingdom	Animalia
Phylum	Chordata
Subphylum	Vertebrata
Superclass	Actinopterygii
Class	Teleostei
Order	Characiformes
Family	Characidae
Genus	*Pristella*
Species	*maxillaris*
Strains	GPM2021, RPM2022, OPM2021, and PPM2021

Synonyms, common, and superseded names

Synonym/common names: X-ray Tetra, X-ray fish, *Aphyocharax maxillaris* (Ulrey 1894), Golden Tetra

Trade names: GPM2021 - GloFish® Electric Green® Pristella

RPM2022 - GloFish® Starfire Red® Pristella

OPM2021 - GloFish® Sunburst Orange® Pristella

PPM2021 - GloFish® Galactic Purple® Pristella

Characterization and substantiation of the taxonomic identification

Pristella maxillaris GPM2021, RPM2022, OPM2021, and PPM2021 are genetically modified lines of diploid, hemizygous or homozygous, Pristellas containing genetic constructs which makes them appear green (GPM2021), red (RPM2022), orange (OPM2021), or purple (PPM2021) under ambient and blue light, including sunlight. All four lines were derived from a domestic strain of Pristella Tetra.

Pristella Tetras are also known as the X-ray Tetra due to the translucent body and considered to be valuable ornamental fish with a large market. Morphological diversification has resulted in three primary phenotypes: wild-type with a black-and-gray body colour with black spots on the trailing edge and fin of the operculum; mutant I has a silvery-white body colour; and mutant II has a fully transparent body with clearly observed visceral tissues (Bian et al. 2019).

Some identifying features of Pristella Tetras include the presence of one irregular row of mostly unicuspid premaxillary teeth, the first infraorbital extended to the anterior margin of the antorbital, the pseudotympanum extended anterior to the first pleural rib, bony hooks on the first pelvic-fin ray of males, and a dark blotch on the dorsal fin. A second species was recently added to the *Pristella* genus and named *P. ariporo*, from the Río Orinoco basin in Colombia, which differs from *P. maxillaris* in lacking maxillary teeth, possessing all teeth of premaxilla and dentary conical, the absence of a dark blotch on the pelvic fin, and the absence of a humeral blotch (Conde-Saldaña et al. 2019). More recently, a third species from the middle rio Tocantins and middle rio São Francisco basins in Brazil was added to the genus. The new species *P. crinogi* can be distinguished from the other two species by a combination of colour pattern and teeth morphology. In addition, *P. crinogi* shows a reversed sexual dimorphism such that females possess a more developed colour pattern than males (Lima et al. 2021).

Strain History

The notified lines GPM2021, RPM2022, OPM2021, and PPM2021 were produced by microinjection of expression cassettes containing respective transgenes into blastomeres of *P. maxillaris* eggs. Greater detail regarding strain development and history of the notified lines has been provided by the company for the expressed purpose of the current risk assessment and review but is identified as confidential business information and is not included in this report. Broodstocks for GPM2021, RPM2022, OPM2021, and PPM2021 are maintained separately, and the same breeding protocol is used for all four lines. Furthermore, to maintain line integrity, non-transgenic fish produced during development of the lines were humanely euthanized and disposed according to the notifier's protocols.

Genetic modifications: purpose, method, genetic and phenotypic changes

The notified lines which have been modified to appear green (GPM2021), red (RPM2022), orange (OPM2021), or purple (PPM2021) under ambient and blue light, including sunlight, are

intended for use by the general public for home aquarium display purposes only. Just like the wild-type *P. maxillaris*, which is a non-food species that has been used safely in aquaria worldwide for approximately 70 years (Innes 1950), GPM2021, RPM2022, OPM2021, and PPM2021 are not intended for food use.

According to the information provided by the notifier, in addition to GPM2021, RPM2022, OPM2021, and PPM2021 appearing green, red, orange, and purple, respectively, under ambient and blue light, the four lines have a lower reproductive success rate compared with their non-transgenic Pristella siblings. The reproductive success of OPM2021, RPM2022, and PPM2021 was significantly lower, and more variable among batches, than that of their non-transgenic counterparts. The reproductive success of GPM2021 was lower than their non-transgenic siblings, but not significantly different. The notifier also provided results from a temperature tolerance test that demonstrate variable sensitivity to low temperatures across the four lines. RPM2022 and PPM2021 appear slightly more sensitive to low temperatures compared to their non-transgenic siblings. There was no significant difference in temperature sensitivity between OPM2021 and GPM2021 compared to their non-transgenic siblings.

Biological and ecological properties

Pristella Tetras are small tropical characid fish found in the Amazon and Orinoco basins as well as in coastal river drainages (Bian et at. 2019; Conde-Saldaña et al. 2019; Laidlaw 2020). They differ from other Tetras in that they have an ability to tolerate the brackish waters of the region. Similar to other Tetras, Pristella Tetras are also able to thrive in freshwater environments, such as streams and tributaries in the dry season and flooded marshlands during the rainy season (Laidlaw 2020). Their diet primarily consists of small species of worms, aquatic insects and their larvae, and small crustaceans, but they will also feed on some plant and algal material (Laidlaw 2020; Froese and Pauly 2022). Due to their small size, they are susceptible to predation by a wide variety of other species such as larger predatory fish, amphibians, as well as various bird and snake species. Pristella Tetras will often be found near the bottom of the water column to avoid predation (Laidlaw 2020).

The maximum length of Pristella Tetras is approximately 5 cm, and females are on average slightly larger than males (Laidlaw 2020). They are a gregarious and non-aggressive schooling fish that is best kept in groups of five or more individuals (Froese and Pauly 2022).

Pristella Tetras reach sexual maturity at approximately five to eight months of age and breed when the grasslands and marshes are flooded, producing approximately 300 to 400 eggs scattered among the vegetation. Eggs hatch in as little as 24 hours and fry become free-swimming within a few days. Life expectancy in the wild is approximately three to four years but can reach seven to eight years in an aquarium (Laidlaw 2020).

HUMAN HEALTH EFFECTS

Zoonotic potential

In-house literature searches found no reports of zoonoses or other adverse effects attributed to wild-type *P. maxillaris*, or to other commercially available GloFish® lines. Compared to other common types of Tetras, Pristellas are described in the hobbyist community as being relatively resilient against disease, but infections are still possible (Sheppard 2021). Aquarium pet fish can carry pathogenic agents, of bacterial, viral, fungal, or parasitic etiology, that may have a zoonotic feature endangering the persons handling the animals (Cardoso et al. 2019). Bacteria are the primary etiological agents of zoonotic infection from aquatic animals, however, there have also been reports of zoonoses by parasitic, fungal, and viral pathogens (Iqbal et al. 2018).

These infections have largely been spread through contact with tropical ornamental fish or through ingestion of food or drinking water that has been contaminated with pathogens and parasites associated with ornamental or aquarium fish.

Contact is the main route of transmission leading to bacterial infections in humans that develop from handling of aquatic organisms (Lowry and Smith 2007). Young children, pregnant women, and immunocompromised individuals are at higher risk (Dinç et al. 2015). Children are also more susceptible to severe disease outcomes compared to adults, and often have less stringent hygienic practices (Dunn et al. 2015). While most infections are self-limiting, more serious cases are often associated with immune deficiency, infection with highly virulent strains, contact with a large inoculum, deeper skin penetration, or a combination of these factors (Haenen et al. 2020).

Bacterial disease is extremely common in ornamental fish and is most frequently associated with bacteria that are ubiquitous in the aquatic environment acting as opportunistic pathogens secondary to stress (Roberts et al. 2009). The most common bacterial species associated with tropical fish capable of causing human illness include *Aeromonas* spp.*, Mycobacterium marinum, Salmonella* spp.*,* and *Streptococcus iniae* (CDC 2015). The most commonly reported infections are associated with *M. marinum* (Weir et al. 2012).

In humans, *M. marinum* is the causative agent for the disease “fish tank granuloma” which results in ulcerative skin lesions or raised granulomatous nodules. These lesions are typically limited to the distal extremities such as the hands, legs, and feet because *M. marinum* has an optimum growth temperature range of 26°C to 32°C (Mutoji and Ennis 2012; Gauthier 2015). However, these nodular cutaneous lesions can progress to tenosynovitis, arthritis and osteomyelitis (Hashish et al. 2018). In addition, rare cases of systemic mycobacteriosis have been reported in immunocompromised individuals (Lowry and Smith 2007). Infections are generally contracted from exposure of wounds and skin abrasions to contaminated water (Gauthier 2015). In humans, mycobacteriosis is classified into four types (I – IV). Type I is found in immunocompetent patients with clinical signs including superficial lesions with crusted and ulcerates nodules or verrucuous plaques. The lesions are small, painless, bluish-red papules approximately 1 to 2 cm in diameter. Signs develop over the course of weeks to months. Type II occurs in immunosuppressed individuals and involves lesions with abscesses, inflammatory nodules, and granulomas. The lesions may be single or multiple subcutaneous granulomas, with or without ulceration. In Type III, infections occur in deep tissues with or without skin lesions with clinical signs including arthritis, tenosynovitis, osteomyelitis, and bursitis. Type IV is very rare, but can occur in patients with lung disease (Delghandi et al. 2020). Virulence determinants for *M. marinum* have not been fully elucidated (Narendrakumar et al. 2022).

Almost all species of fish are thought to be susceptible to *Mycobacterium spp*., with mortality ranging from 10% to 100% (Delghandi et al. 2020). *M. marinum*, *M. chelonae*, and *M. fortuitum* are most commonly reported as the species causing piscine mycobacteriosis (Phillips Savage et al. 2022), and *M. marinum* has been reported to cause infection in more than 200 species of freshwater, brackish and marine fishes (Narendrakumar et al. 2022). Other examples of species of *Mycobacterium* known to cause infections in fish include *M. abscessus*, *M. flavescens*, *M. gordonae, M. haemophilum*, *M. kansasii*, and *M. peregrinum* (Cardoso et al. 2019; Pate et al. 2019; Puk and Guz 2020).

Although most cases of fish-related infections in humans are caused by *M. marinum*, home aquarists should also be aware of the zoonotic potential of other species of *Mycobacterium* (Puk and Guz 2020). In immunosuppressed humans and children, *M. haemophilum* has been associated with subcutaneous infections, lymphadenitis, septic arthritis, osteomyelitis, pneumonitis and disseminated disease (Emmerich et al. 2019; Franco-Paredes et al. 2019). Cameselle-Martínez et al. (2007) reported a cutaneous infection by *M. haemophilum* in a

severely immunosuppressed AIDS patient following a bite from an aquarium fish. The infection was successfully treated following a combined therapy of six antibiotics. *M. abscessus*, *M. chelonae*, *M. fortuitum*, and *M. peregrinum* are also associated with cutaneous infections in humans (Kamijo et al. 2012; Franco-Paredes et al. 2019). Li et al. (2014) reported successful treatment with antibiotics of a cutaneous *M. chelonae* infection on the left arm of an 82-year old woman with a hobby of rearing tropical fish. While cutaneaous mycobacterial infections may be successfully resolved with antibiotics, the choice of antibacterial combinations and length of therapy is species-specific (Franco-Paredes et al. 2018). Guz and Puk (2022) examined the antibiotic susceptibility of 99 isolates of nontuberculous mycobacteria (13 species of *Mycobacteria*) from diseased ornamental fish. The authors found the majority of isolates were susceptible to kanamycin, amikacin, clarithromycin, sulfamethoxazole, ciprofloxacin, and doxycycline, with most being resistant to isoniazid and rifampicin. An in-house literature search found no reports of human mycobacterial infections attributed to Pristella Tetras from home aquarium exposure.

Zoonotic infections from *S. iniae* are opportunistic and have most often been associated with puncture wounds from the handling and preparation of infected fish by persons with underlying medical conditions such as diabetes mellitus, chronic rheumatic heart disease, or cirrhosis (Baiano and Barnes, 2009; Haenen et al. 2020). From the handling of live or recently killed infected fish, *S. iniae* may cause severe disease including septicaemia, endocarditis, arthritis, meningitis, fever, abdominal distension, and pneumonia (Lowry and Smith 2007; Boylan 2011; Gauthier 2015; Haenen et al. 2020). People with weakened immune systems or open skin wounds could get infected by *S. iniae* while handling fish or cleaning aquaria (CDC 2015). Other *Streptococcus* species capable of causing infections in fish include *S. agalactiae*, *S. difficile*, *S. difficilis*, *S. dysgalactiae*, and *S. shiloi* (Ziarati et al. 2022). However, there are no reports in the scientific literature of human streptococcal infections attributed to Pristella Tetras from home aquarium exposure.

Aeromonas spp. are opportunistic pathogens that are associated with a number of diseases in ornamental fish (Hossain et al. 2018). *Aeromonas hydrophila* is the most commonly reported Aeromonad that possesses zoonotic potential; *A. caviae, A. jandaei, A. sobria, A. salmonidae*, and *A. veronii* have also been reported (Boylan 2011; Zariati et al. 2022). Water with high nutrient levels can cause bacterial blooms capable of being infectious to humans through wounds or ingestion; however, infections are rare and typically involve immune suppression (Boylan 2011). The risk of *Aeromonas* infection can be reduced by maintaining good water quality, promptly removing dead fish, and washing hands (CDC 2015). In humans, *A. hydrophila* exposure may result in local skin infections and occasionally, diarrheal disease (Haenen et al. 2020). *A. hydrophila* was one of the species of bacteria isolated from cough swabs of an 11-month old boy with cystic fibrosis (Cremonesini and Thomson 2008). The authors believe the infection was the result of aerosol spread of the bacterium due to the aeration process of fish tanks in the home because isolations of *A. hydrophila* only ceased following removal of the tanks. While the report by Cremonesini and Thomson (2008) did not identify the species of fish, there are no reported cases of *A. hydrophila* zoonotic infections attributed to *P. maxillaris* exposure. Among the pathogenic *Aeromonas* spp., *A. veronii* appears to exhibit the broadest host range; species ranging from invertebrates to mammals, including humans, have shown susceptibility to this pathogen (Lazado and Zilberg 2018). *A. veronii* (26.3%) and *A. hydrophilla* (16.2%) were the most commonly isolated bacterial species from 112 fish found positive from a total of 126 ornamental fish collected from a wholesaler in São Paulo, Brazil (Cardoso et al. 2021). However, an in-house literature search found no reported cases of zoonotic infections of *A. veronii* from ornamental fish exposure.

Salmonella infection can occur through contact with an animal's habitat such as an aquarium (CDC 2015). While *Salmonella* is not a known pathogen for tropical fish, they may act as bacterial reservoirs and excrete *Salmonella* in their feces during periods of stress (Gaulin et al. 2005). Musto et al. (2006) reported 78 cases in which *Salmonella* Paratyphi B biovar Java infections were reported in people with aquaria containing tropical fish in Australia. Infections were mostly seen in children (median age of cases was three years old) following exposure to aquarium water and resulted in diarrhea, fever, abdominal cramps, vomiting, bloody stool, headaches, and myalgia. Types of tropical fish reported in this study included Tetras, Guppies and Angel Fish. Similarly, out of 53 reported cases of *Salmonella* Paratyphi B, var. Java infections reported in the province of Quebec from January 2000 to June 2003, 33 infected individuals owned an aquarium and 21 of the aquaria tested positive for *Salmonella* (Gaulin et al. 2005). However, the authors did not identify any of the tropical fish species owned by the infected individuals. An in-house literature search found no reports of *Salmonella* zoonotic infections attributed to *P. maxillaris* exposure.

Zoonotic infections primarily occur through puncture, cuts, scrapes, abrasions, or sores in the skin (Boylan 2011). Infections may be prevented by wearing gloves when handling fish or cleaning fish tanks, and avoiding contact with potentially contaminated water if open skin wounds are present. Washing hands and skin with soap and water after contact with aquarium water and fish is also highly recommended. In addition, people with compromised immune systems or underlying medical conditions, as well as children, should avoid cleaning tanks or handling fish (Haenen et al. 2013; 2020).

There are no reports that specifically associate either the notified organisms or wild-type *P. maxillaris* with any parasites of human health significance. During line development, no major health issues were observed by the notifier for any of the four colours. There were no significant differences from the wild-type in relation to growth, age, deformities, behavior, and general health. Routine health evaluations (necropsy, microbiology) were conducted on limited sample sizes of six fish of each colour plus wild-type (non-transgenic). Histology was conducted on an additional six fish of each colour, plus wild-type, at a fish disease diagnostic laboratory at the University of Florida in 2021 (GPM2021), (OPM2021), (PPM2021), wild-type, and 2022 (RPM2022).

The reports stated that the findings described above were unrelated to the transgenic nature of the fish and that there were no significant anatomical or microscopic variations between the wild-type and transgenic groups. The veterinary pathologist stated there was no evidence that transgenic fish differ from wild-type with regard to susceptibility to, or transmission of, parasites. While many species of ornamental fish have been reported to be susceptible to parasites (Florindo et al. 2017a,b; Iqbal et al. 2018; Trujillo-González et al. 2018), there are no reports specifically associating the notified organisms or the wild-type *P. maxillaris* with any parasites of human health significance. In addition, no bacterial growth was observed after 48 hours (at 28°C) in brain and posterior kidney samples plated onto blood agar plates (TSA + 5% sheep's blood) for all four notified lines or for the wild-type Pristella Tetra.

Allergenicity/Toxigenicity

In-house amino acid sequence analyses of all the expressed fluorescent proteins were done using the AllergenOnline Database (v21; 14 February, 2021). Similar to previous analyses on these fluorescent proteins performed on previously notified GloFish® lines, no matches with greater than 35% identity, nor exact matches for 80 and 8 sliding window amino acid segments, respectively, were found for any of the fluorescent proteins. Similarly, results provided by the notifier from analyses using the Allermatch™ website found no matches for 80 amino acid sliding window alignments using the 35% cutoff or exact matches using segments 8 amino acid in

length. The 35% identity for 80 amino acid segments is a suggested guideline proposed by the Codex Alimentarius Commission for evaluating newly expressed proteins produced by recombinant-DNA plants (WHO/FAO 2009). According to the AllergenOnline Database website, the 8 amino acid exact match is used as a precautionary search, but there has been no evidence seen that a cross-reactive protein will be identified that was not found using the 80 amino acid 35% match.

As seen with GB2011 (NSN 21071) and PB2019 (NSN 21073), analyses conducted for all the other reading frames found a positive result using the 80mer sliding window for a putative open reading frame (ORF) in the 5'3' Frame 3 direction in the expression cassette sequences for GPM2021, and in both the 3'5' Frames 1 and 3 directions for PPM2021. The ORF in GPM2021 was found to have 35.03% identity with a predicted collagen alpha-1(I) chain-like isoform X1 from the Barramundi (*Lates calcarifer*). However, the full-length alignment resulted in only 35.4% identity and there was a high E-value (expectation value) of 99. The ORFs in PPM2021 were each found to have a 35.03% identity with serine protease from the fungus *Aspergillus niger*. Full length alignments resulted in 33.0% identities with high E-values of 1800 and 630. Cross-reactivity typically requires the matches to be 40% identical over 80 amino acids with an E-value score of 1e-15 or less (Dr. Richard Goodman, University of Lincoln-Nebraska, personal communication). Thus, allergic cross reactivity is not likely for any of the three putative ORFs. In addition, Basic Local Alignment Search Tool (BLAST) analyses on the amino acid sequences with BLASTP found no significant similarity to a known protein for GPM2021 while 57.2% identities to the same synthetic construct were found for the sequences seen in PPM2021. Analyses on the inserted nucleotide sequences for predicting translation initiation sites using an online program only found sites with a high reliability associated for the expected fluorescent proteins. Therefore, these putative ORFs would most likely not result in expressed proteins in either GPM2021 or PPM2021.

BLAST analyses of the inserted fluorescent protein sequences do not indicate any homologies to sequences of potential toxins or allergens. No adverse effects were observed in male rats fed pure green fluorescent protein (GFP) or canola expressing GFP for 26 days (Richards et al. 2003). Furthermore, there is no evidence indicating any potential for GPM2021, RPM2022, OPM2021, and PPM2021 or wild-type *P. maxillaris* to produce toxic or other hazardous materials that may accumulate in the environment or be consumed by humans or other organisms in the environment.

History of Use

GPM2021, OPM2021, and PPM2021 received their Enforcement Discretion decisions by the U.S. Food and Drug Administration (USFDA) in early 2022, while RPM2022 received its decision in November 2022. GPM2021, OPM2021, and PPM2021 have been commercially available in the U.S. since August 2022. The fluorescent proteins used in GPM2021, RPM2022, and OPM2021 have been used in other GloFish® lines since as early as 2006, while the fluorescent protein in PPM2021 has been used since at least 2011. Wild-type Pristella Tetras have been sold worldwide as aquarium fish since the 1950s (Innes 1950).

HAZARD CHARACTERIZATION

The human health hazard potential of GPM2021, RPM2022, OPM2021, and PPM2021 is assessed to be low (Table 1) because:

1. GPM2021, RPM2022, OPM2021, and PPM2021 are genetically modified tropical fish containing transgene constructs at a single site of insertion (although alternate insert

patterns may exist in the population) that appear phenotypically stable based on line maintenance protocols;

2. The methods used to produce GPM2021, RPM2022, OPM2021, and PPM2021 do not raise any indirect human health concerns. While some of the source organisms from which the inserted genetic material was derived appear to produce toxins, there is no indication that any of the inserted genetic material or expressed proteins in these lines are associated with any toxicity, allergenicity, or pathogenicity in humans;
3. While there are reported cases of zoonotic infections associated with tropical aquarium fish, particularly for immunocompromised individuals and children, there are no reported cases attributed to any of the commercially available lines of GloFish® or to wild-type Pristella Tetras. The zoonotic potential of GPM2021, RPM2022, OPM2021, and PPM2021 is not expected to be any different than for wild-type Pristella Tetras currently commercially available;
4. Sequence identities of the inserted transgenes do not match any known allergens. Amino acid sequences of the four fluorescent proteins are identical to those used in previously assessed GloFish® lines. While analyses conducted on the other potential reading frames found potential matches in both GPM2021 and PPM2021, the results suggest there is little evidence for cross-reactivity; and
5. While there is no history of safe use for the notified lines, there is for other commercially available lines of GloFish® and the wild-type Pristella Tetra has been safely used globally as an ornamental aquarium fish since the 1950s.

Table 1: Considerations for hazard severity (human health)

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

UNCERTAINTY RELATED TO INDIRECT HUMAN HEALTH HAZARD ASSESSMENT

The ranking of uncertainty associated with the indirect human health hazard assessment is presented in Table 2. Adequate information was either provided by the notifier or retrieved from other sources that confirmed the identification of the notified organisms. Adequate information was also provided describing in good detail the methods used to genetically modify the wild-type *P. maxillaris* including the sources of the genetic materials and the stability of the resulting genotypes and phenotypes. Sequence analyses of the inserted transgene constructs for the four notified lines did not match any toxins or allergens and no reports were found of adverse effects attributed to the inserted proteins in humans.

While there were no reports of adverse human health effects directly associated with the notified organisms or the other commercially available lines of GloFish®, surrogate information from the literature on other ornamental fish appear to indicate the potential for transmission of human pathogens. However, such cases of infections are common to all ornamental aquarium fish and are not unique to Pristella Tetras. The inserted fluorescent proteins have been used in other lines of GloFish® for several years and there are no reports of adverse human health effects. Consequently, combining both empirical data on the notified organisms, surrogate information from the literature on other ornamental aquarium fish and the lack of adverse effects supported by the history of safe use for other lines of GloFish®, the indirect human health hazard assessment of GPM2021, RPM2022, OPM2021, and PPM2021 is considered to be **low** with **low uncertainty**. The uncertainty is considered low because much of the information on human health effects are based on reports from other ornamental aquarium fish as there are a limited number of studies in the scientific literature on *P. maxillaris*. In addition, there is a limited history of safe use in the United States for three of the notified lines (GPM2021, OPM2021, and PPM2021) while RPM2022 is not yet commercially available. Finally, there are no particular studies that have investigated human health effects associated with fluorescent transgenic ornamental fish.

Table 2. Categorization of uncertainty related indirect human health hazard.

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

EXPOSURE ASSESSMENT

OVERVIEW

Figure 1 shows the generalized human exposure pathways for GPM2021, RPM2022, OPM2021, and PPM2021 assuming potential exposure through:

1. Import from the United States and distribution to retailers in Canada;

2. Introduction in Canada through the intended use as ornamental fish in home aquaria;
3. The environment and environmental fate following accidental, deliberate, or unintended environmental releases; and
4. Other potential uses.

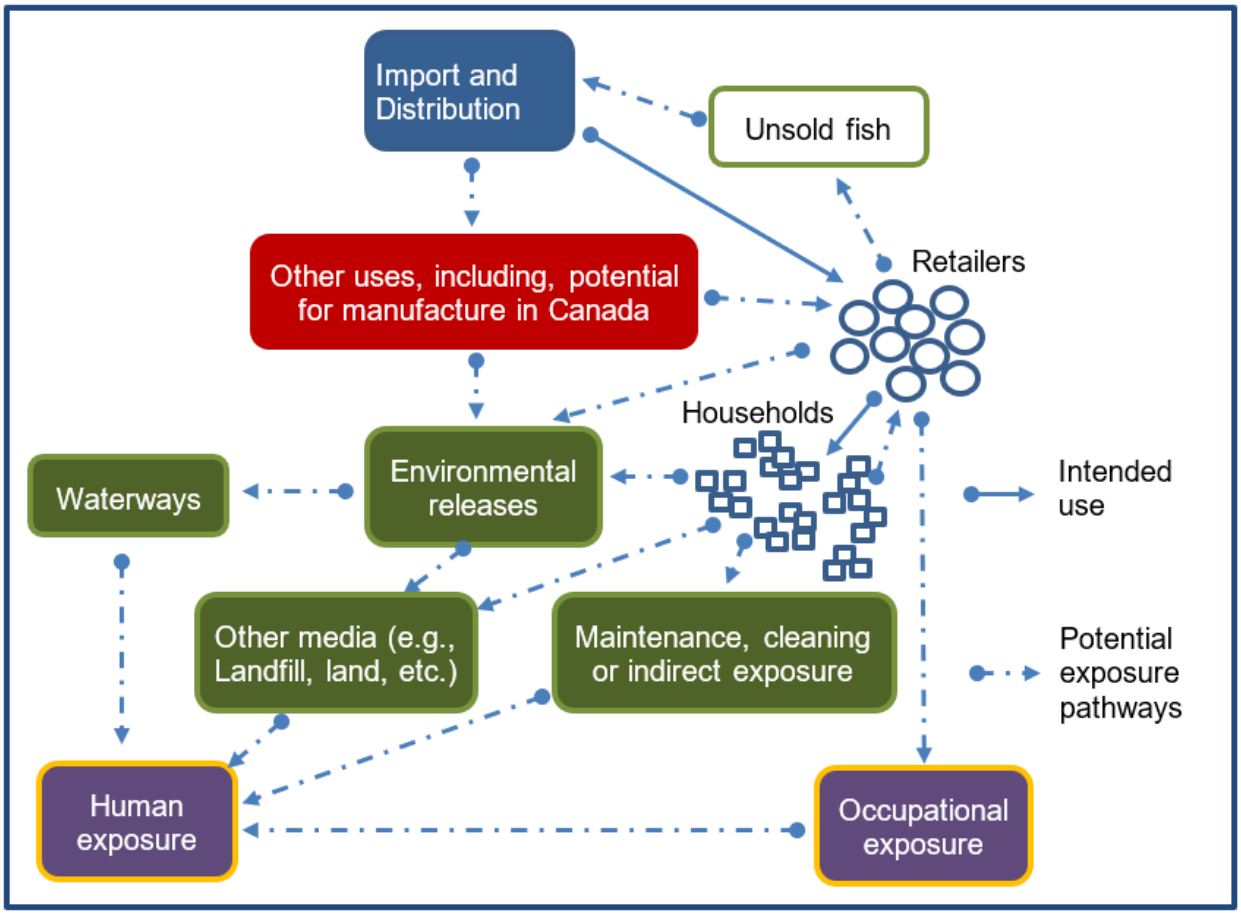

Figure 1: Generalized human exposure pathways for GPM2021, RPM2022, OPM2021, and PPM2021.

IMPORT

Imported fish will enter Canada through authorized distributors and their points of entry. Broodstock are maintained using the same breeding protocol for all types of F_2 fish that become the lines identified as GPM2021, RPM2022, OPM2021, and PPM2021. In the production locations in United States, the Division of Aquaculture of the Florida Department of Agriculture and Consumer Services regulates the production of the notified lines to ensure the use of best management practices and help protect the environment. The notifier intends to ship adult fish to distributors and eventually to pet stores in quantities ordered and held until when sold to the public.

The notifier plans to market GPM2021, RPM2022, OPM2021, and PPM2021 fish in Canada using approximately 500 retail outlets based on market size relative to United States. The exact number and locations where the notified organisms will be available for sale are not currently known. As ornamental fish intended for sale to the public, it is anticipated that they will be confined inside aquaria in homes and retail outlets. For the intended use, human exposure could happen during distribution involving the transportation of fish by the importer as well as

during storage, handling, and sale by the retailer. Based on a survey of store owners in Montreal, Quebec, fish are either kept and put on sale by retailers until sold or returned to the distributor and less likely to be released into the environment by retailers (Gertzen et al. 2008). Since retailers are not expected to be the final users of GPM2021, RPM2022, OPM2021, and PPM2021, human exposure during importation and distribution to retailers is expected to be largely occupational.

INTRODUCTION OF THE ORGANISM

Human exposure by home aquarists that purchase the notified lines directly from retailers or receive them from other aquarists will most likely occur through contact with the notified fish during maintenance activities such as water changes and tank cleanings. Stocking rate per household and the number of households planning to purchase the notified lines would be helpful in estimating human exposure through the intended use, as ornamental fish in home aquaria. Typical stocking of wild type Pristella Tetras in home aquaria was not provided. The recommendation is to keep Pristella Tetras in aquaria that have a minimum tank size of 57 to 76 L (15 - 20 gallons) and in groups of 5 or more individuals (Herzog 2021; Froese and Pauly 2022).

While the proportion of home aquarists planning to purchase GPM2021, RPM2022, OPM2021, and PPM2021 is not known, a 2009 survey estimated 12% of Canadian households owned fish (Perrin 2009; Whitfield and Smith 2014), and another survey (Marson et al. 2009) reported that approximately 45% of respondents (190 out of 418 aquarium owners) had species of Tetras in their aquaria. In another survey done in Montreal, Quebec (Gertzen et al. 2008), about 12.9% of the fish sold by the pet stores were various species of Tetra. According to the 2021 census, Canada has 16 million households (Statistics Canada 2021). Assuming the same proportion of Canadians practice some form of fishkeeping at present, approximately 2 million households could come in contact with GloFish® fluorescent *P. maxillaris* (i.e., 12% of the 16 million households have fish as pets).

The recommended temperatures for home aquaria established for *P. maxillaris* is between 24°C and 28°C (Aqua-Fish 2014). These temperatures, and conditions in aquariums, favour the growth of opportunistic pathogens like *M. marinum* (Kent et al. 2006; Mutoji and Ennis 2012; Gauthier 2015) or parasites like *Cryptosporidium* species (Ryan et al. 2015; Golomazou and Karanis 2020). Due to the risk of infection with non-tuberculosis mycobacteria (Kušar et al. 2017) or diarrhea associated with *Edwardsiella tarda* (Vandepitte et al. 1983), caution is advised in handling fish in pet-shops and home aquariums. While we have no knowledge of the health status of people that may be exposed, it is expected that the households intending to purchase the notified lines could include immunocompromised individuals, children, and those with underlying medical conditions.

ENVIRONMENTAL FATE

According to the notifier, the intended use of GPM2021, RPM2022, OPM2021, and PPM2021 is not for environmental release but rather for use in home aquaria. However, one cannot rule out environmental release since there are reports of this happening for fish kept in home aquaria (Duggan et al. 2006; Gertzen et al. 2008). In many regions of the world, ornamental fish are often deliberately released as a ‘humane’ method of disposal of unwanted pets (Chan et al. 2019). According to Gertzen et al. (2008), aquarists could potentially release unwanted aquarium fish into the environment when they become bored with the fish or when fish become aggressive, sick, large in size, or reproduce rapidly. While there is no data to substantiate the release of Pristella Tetras, this species is not known to exhibit aggressive behavior towards same or different species.

In an event of environmental releases of live GPM2021, RPM2022, OPM2021, and PPM2021 in Canada, future establishment will depend on environmental conditions at the point of release and the ability of the released fish to survive, grow, reproduce, disperse, and establish self-sustaining populations (Duggan et al. 2006; Strecker et al. 2011; Leggatt et al. 2018). Temperature tolerance is a key criterion for determining the ability of aquarium fish to survive, establish and overwinter in the Great Lakes and in Canadian waters as a whole (Rixon et al. 2005; DFO 2018; Leggatt et al. 2018). All transgenic genotypes showed slightly lower sensitivity to cold-water temperatures compared to their non-transgenic siblings but still within the lethal water temperature ranges for *P. maxillaris*. However, the temperature tolerance of RPM2022 and PPM2021 was significantly lower than their non-transgenic siblings.

P. maxillaris lost equilibrium in temperatures ranging from 14.6°C – 11.8°C with a mean critical minimum temperature of 13.14 ± 0.07 °C (Leggatt 2022). In another study, Yanar et al. (2019) reported that the critical minimum temperature of thirteen popular ornamental fish species range from 11.66°C to 13.94°C. Even the most cold tolerant *P. maxillaris* lost equilibrium at temperatures several degrees above typical winter water temperatures in Canada (4°C or less) and temperatures in the warmest recorded lakes of 6°C or less in winter (Leggatt et al. 2018; Leggatt 2022). It is therefore less likely that GPM2021, RPM2022, OPM2021, and PPM2021 could survive and disperse in typical winter water temperatures (both fresh and brackish water environments) in Canada. There is potential for persistence of *P. maxillaris* in isolated warm pockets of water (e.g., hot springs, thermal effluent from industrial sites), although temperatures would need to remain above 13°C for the fish to persist in these areas (Leggatt 2022). Della Venezia et al. (2018) examined the potential establishment of freshwater ornamental fish in North America under a climate change scenario forecast for the year 2050. According to the authors, while the model forecasted a two-fold average invasion risk in Quebec, the establishment risk remained extremely low as the minimum temperature for the coldest month would not likely be high enough in 2050 to make Quebec suitable to potential invasions from species currently in the ornamental trade. Furthermore, if live or dead GPM2021, RPM2022, OPM2021, and PPM2021 are released into the environment, it is expected that both fish and the inserted fluorescent protein would biodegrade normally and not accumulate or be involved in biogeochemical cycling in a manner different from other living organisms. Therefore, the likelihood of GPM2021, RPM2022, OPM2021, and PPM2021 establishing self-sustaining populations in Canada is very low due to their inability to survive water temperatures lower than 10°C based on temperature tolerance studies. Therefore, the likelihood of human exposure to the notified organisms in the environment is low.

In the event a fish dies before sale to or while in the care of a home aquarist, the notifier suggests a disposal procedure similar to all other domestic waste and there are no special handling or disposal procedures required. The notifier has indicated that no specific procedures or treatments are required for disposal of the notified organisms (GPM2021, RPM2022, OPM2021, and PPM2021) compared to the wild-type species as the only difference (for each line) is the addition of a fluorescent protein derived from species of coral or sea anemones. Additionally, sale of these lines can be halted at any time if it is determined necessary to terminate the introduction of GPM2021, RPM2022, OPM2021, and PPM2021 in Canada.

OTHER POTENTIAL USES

The sole intended use for GPM2021, RPM 2022, OPM2021, and PPM2021 is as ornamental fish for interior home aquaria. According to the notifier, the four notified lines are not suitable for use in outdoor ponds, as bait fish, for human consumption, or as environmental sentinels. While unlikely, their potential use in insect control is possible as Pristella Tetras will seek out and prey on insect larvae and small bugs found on the water surface in the wild (Sheppard 2021). In

research, Pristellas have been a model organism for the study of shoaling behavior (Schaerf et al. 2017; Ward et al. 2018; Wilson et al. 2019) and parasitic infestations (Ponpornpisit et al. 2000).

Manufacture of the notified organisms is not anticipated to occur in Canada as GPM2021, RPM2022, OPM2021, and PPM2021 are only produced in Florida. However, should manufacture or other potential uses occur in Canada, no additional risks are foreseen that are different from any other typical aquarium fish. The notifier recommends that individuals that no longer wish to maintain the organisms after purchase either return them to the retailer, give them to another aquarium hobbyist, or humanely euthanize them.

EXPOSURE CHARACTERIZATION

Risks from workplace exposure to the notified strain are not considered in this assessment[1]

The human exposure potential of GPM2021, RPM2022, OPM2021, and PPM2021 is assessed to be low to medium (Table 3) because:

1. The primary sources of human exposures would stem from the proposed import of adult fish for the four lines (GPM2021, RPM2022, OPM2021, and PPM2021) through unidentified points of entry in Canada and distribution through about 500 retail outlets;
2. The sole intended use of GPM2021, RPM2022, OPM2021, and PPM2021 is as ornamental aquarium fish, thus limiting potential exposure primarily to those possessing a home aquarium;
3. Like other aquarium fish, human exposure may include immunosuppressed individuals, children, those with underlying medical conditions or other vulnerable individuals;
4. Typical human exposure to live or dead fish in the home is most often related to maintenance activities such as tank cleanings and water changes. Low winter water temperatures in Canadian waters and low cold tolerance of notified fish limits human exposure through the environment; and
5. No significant increase in human exposure is expected from other potential uses of GPM2021, RPM2022, OPM2021, and PPM2021, such as for insect control and for research purposes.

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

Table 3: Exposure considerations (human health)

Exposure	Considerations
High	• The release quantity, duration and/or frequency are high. • The organism is likely to survive, persist, disperse proliferate and become established in the environment. • Dispersal or transport to other environmental compartments is likely. • The nature of release makes it likely that susceptible populations or ecosystems will be exposed and/or that releases will extend beyond a region or single ecosystem. • In relation to exposed humans, routes of exposure are permissive of toxic, zoonotic or other adverse effects in susceptible organisms.
Medium	• The organism is released into the environment, but quantity, duration and/or frequency of release is moderate. • The organism may persist in the environment, but in low numbers. • The potential for dispersal/transport is limited. • The nature of release is such that some susceptible populations may be exposed. • In relation to exposed humans, routes of exposure are not expected to favour toxic, zoonotic or other adverse effects.
Low	• The organism is used in containment (no intentional release). • The nature of release and/or the biology of the organism are expected to contain the organism such that susceptible populations or ecosystems are not exposed. • Low quantity, duration and frequency of release of organisms that are not expected to survive, persist, disperse or proliferate in the environment where released.

UNCERTAINTY RELATED TO INDIRECT HUMAN HEALTH EXPOSURE ASSESSMENT

Uncertainty ranking associated with the information used to assess indirect human health exposure for GPMB2021, RPM2022, OPM2021, and PPM2021 is presented in Table 4. As indicated, the notified organisms will not be manufactured in Canada and the source of exposure will be restricted to the import of adult fish for the four lines. In the environment, empirical data supports the conclusion that the survival of these fish is expected to be limited by their poor tolerance to temperatures below 10°C. However, this does not preclude the potential for human exposure (general public and vulnerable individuals [i.e., immunocompromised, children, medical conditions, etc.]) in Canada through home aquaria mainly from maintenance and cleaning activities. This exposure assessment is limited by the lack of information on actual number of notified organisms to be imported in subsequent years and poor survey data on household ownership of ornamental fish. It is therefore difficult to gauge public uptake and popularity beyond the import number in the first year. Furthermore, household surveys looking into aquarium fish ownership in Canada are based on reports from more than 10 years ago (Duggan et al. 2006; Gertzen et al. 2008; Marson et al. 2009; Perrin 2009). These reports are not specific to GPMB2021, RPM2022, OPM2021, or PPM2021 and do not investigate factors influencing human exposure to aquarium fish. Therefore, because of limited information on the specific exposure scenarios in the Canadian market, the human exposure to the notified organisms is considered low to medium with moderate uncertainty.

Table 4: Uncertainty ranking associated with the indirect human health exposure.

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

RISK CHARACTERIZATION

NOTIFIED USE

In this assessment, risk is characterized according to a paradigm: Risk ∝ Hazard x Exposure. The two components ("hazard" and "exposure") are considered embedded in the definition of "toxic" under section 64 of CEPA 1999 and hence, there is no risk in absence of either. The risk assessment conclusion is based on the hazard, and on what we can predict about exposure from the notified use.

GPM2021, RPM2022, OPM2021, and PPM2021 are genetically modified lines of diploid, hemizygous or homozygous, Pristellas containing fluorescent protein genetic constructs derived from species of sea anemones or soft corals which makes them appear green (GPM2021), red (RPM2022), orange (OPM2021), and purple (PPPM2021) under ambient and blue light, including sunlight. Each line of the GloFish® Pristellas was derived from a line of domesticated Pristella Tetra.

The notified organisms will be marketed throughout Canada for use as ornamental fish in home aquaria.

Although there are reported cases of zoonotic infections from exposure to aquarium fish, wild type Pristella Tetras are popular in home aquaria with a long history of safe use having been sold worldwide as aquarium fish since the 1950s (Innes 1950). GPM2021, OPM2021, and PPM2021 received Enforcement Discretion decisions by the USFDA in early 2022 and have been commercially available in the United States since August 2022. The Enforcement Discretion decision for RPM2022 was received in November 2022. The fluorescent proteins used in the four notified lines have been used in other GloFish® lines that are now commercially available in Canada. There are no reported adverse human health effects associated with wild type Pristella Tetras in general, the inserted fluorescent protein genes, and the methods used to modify the notified lines, leading to the conclusion that the notified lines do not present any pathogenic or toxic potential towards humans that differ from the risks associated with the wild-type counterparts.

While there was moderate uncertainty regarding some components, this did not affect the overall indirect human health risk ratings. Owing to the low potential hazard and the low to

medium potential exposure, the human health risk associated with the use of *P. maxillaris* GPM2021, RPM2022, OPM2021, or PPM2021 as ornamental aquarium fish is assessed to be low.

OTHER POTENTIAL USES

Other uses that have been identified include the use of the notified organisms for insect control and for research purposes. Regardless of the use, the available information does not indicate a potential human health implication from any of these uses. No additional risks to human health are foreseen that are different from those of any other typical aquarium fish.

RISK ASSESSMENT CONCLUSION

There is no evidence to suggest a risk of adverse human health effects at the exposure levels predicted for the general Canadian population from the use of GPM2021, RPM2022, OPM2021, or PPM2021 as ornamental aquarium fish or any other potential uses. This risk to human health associated with GPM2021, RPM2022, OPM2021, or PPM2021 is not suspected to meet criteria in paragraph 64(c) of CEPA 1999. No further action is recommended.

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