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REVIEW

REVIEWS IN Aquaculture

# Cultivation of fish ciliate parasites: Progress and prospects

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## Abstract

Ciliates are considered the most harmful parasites of fish, causing mass mortalities and thus substantial economic losses to the aquaculture and ornamental fish industries. To better elucidate their pathogenic mechanisms and facilitate the development of effective prevention and control strategies, it is essential to apply novel technologies to ensure the continuous and sufficient supply of parasite specimens. This review discusses the latest research progress in the artificial culture of six important parasitic ciliates affecting both freshwater and marine fish globally: *Ichthyophthirius multifiliis*, *Cryptocaryon irritans*, *Chilodonella* (*C. hexasticha*, *C. piscicola*and *C. uncinata*), *Balantidium ctenopharyngodoni*, Scuticociliatida gen. spp., and *Tetrahymena* spp. Methods for in vitro and in vivo culture as well as cryopreservation/preservation (depending on the specific species) of these organisms are described herein. Challenges and future applications of parasite culture are also discussed.

**KEYWORDS**
aquaculture, cryopreservation, fish ciliates, in vitro culture, in vivo culture, preservation

## 1 | INTRODUCTION

The recent rapid growth of aquaculture has been accompanied by harmful parasitic diseases. To better understand their pathogenic mechanisms and facilitate the development of effective prevention and control strategies, the availability of parasite specimens is critical. To meet that need, investigators rely on in vivo and in vitro methods of parasite culture to provide sufficient materials to investigate the basic biology of host–parasite interaction, including host recognition, parasite establishment, and anti-parasitic immunity in fish. On a more practical level, a continuous supply of parasites is also required for studies on control strategies involving chemical or

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drug treatments, biological control measures, as well as the breeding of host resistance.

Ciliates (Protista: Ciliophora) are single-celled organisms distinguished by three major features – motile cilia, nuclear dimorphism and the use of conjugation for sexual reproduction.[1] While most ciliates are free-living, numerous species have adopted a parasitic or commensal lifestyle in association with animal hosts. Ciliates are considered the most harmful parasites in pisciculture.[2] They can invade various fish species and cause severe lesions on skin or gills, and in some cases infect deeper tissues and organs leading to mass mortality and thus considerable economic losses to the aquaculture and ornamental fish industries. Ciliates that are pathogenic in fish include both obligate and facultative parasitic species.

Obligate parasites require a host to complete their life cycles. This group includes a range of pathogenic species typically associated with the skin, such as *Ichthyophthirius multifiliis* Fouquet, 1876 (commonly known as 'Ich'); *Cryptocaryon irritans* Brown, 1951 (also known as 'marine Ich'); *Chilodonella hexasticha* (Kiernik 1909) Kahl, 1931; *Chilodonella piscicola* Zacharias, 1894; *Brooklynella hostilis* Lom and Nigrelli, 1970, and others. Additionally, members of the genus *Balantidium* such as *B. ctenopharyngodoni* Chen, 1955 in grass carp and *B. polyvacuolum* Li, 1963 in *Xenocypris*, are emerging obligate intestinal parasites that can establish symbiotic relationships with other pathogens and aggravate lesions to cause balantidiasis.

Facultative parasites, on the other hand, can exist as either free-living organisms or as opportunistic pathogens depending on the environmental conditions. Ciliate species acting as facultative pathogens of fish include *Chilodonella uncinata* Ehrenberg, 1838; members of the order Scuticociliatida Small, 1967; Coleps Nitzsch, 1827; Tetrahymena Furgason, 1940; Trichodina Ehrenberg, 1830; and Colpodium colpoda Stein 1860, among others.

This paper reviews the most recent developments related to the culture of parasitic ciliates of fish. We focus on the methods for in vitro and in vivo culture, as well as the (cryo-)preservation (depending on the species) of several important species of ciliates affecting both freshwater and marine fish globally. Although there are some other harmful ciliates such as Trichodinids that are important for aquaculture, they are not included in this review as the methods for their in vitro or in vivo culture are currently lacking.

## 2 | CULTIVATION OF FISH CILIATES

### 2.1 | *Ichthyophthirius multifiliis*

#### 2.1.1 | General biology

*Ichthyophthirius multifiliis* Fouquet, 1876 is a ciliated protozoan parasite of freshwater fish that causes 'white-spot' disease or ichthyophthiriasis. Ich has a broad host range and infects many fish species with high economic value, such as rainbow trout,[3] channel catfish,[4] carp[5] and salmon,[6] among others.

The life cycle of *I. multifiliis* (Figure 1) is well established and consists of four principal stages: theront, trophont, protomont and tomont. Host-associated trophonts feed on tissue and become large enough to be seen with the naked eye within the skin of infected fish (typically, ~0.4–0.6 mm). Trophonts exit the fish and swim briefly before attaching to a solid substrate (as tomonts), at which point they secrete a clear, gelatinous cyst wall. Tomonts then divide rapidly to form tomites that eventually emerge from the cyst to form infectious theronts. Theronts scan the water column and invade the epidermis of susceptible fish, where they quickly transform into feeding trophonts to complete the life cycle.[7]

Infections are generally confined to the epidermal layers of the skin, gills and buccal cavity, although they have been occasionally reported within the peritoneal cavities of experimentally infected fish.[8] Genomic sequencing studies have detected the presence of at least one symbiotic bacterial species, as well as additional bacteria that may associate with the surface of the parasite. This has led to the idea that theronts may serve as vectors for some bacterial pathogens that infect fish.[9–13] Following host invasion, parasitic activity in the skin induces marked inflammation and epidermal thickening.[14] Following a period of extensive growth, trophonts leave the host and become protomonts that swim freely in the water before attaching to a lightly coloured substrate.[15] Protomonts then secrete a gelatinous cyst wall to become tomonts, which then undergo rapid division to produce a large number of daughter cells, called tomites, that eventually break through the cyst wall as free-swimming, infectious theronts.[16] The length of the Ich life cycle is influenced by environmental factors such as water hardness, temperature, pH and salinity,[17,18] but generally takes 7–14 days at 14–25°C.

Finally, while definitive evidence of a sexual stage is lacking, the macronuclear genome encodes numerous genes required for conjugation in other ciliates, and comparisons of single nucleotide polymorphisms among field isolates is consistent with sexual activity at some stage of the life cycle of *I. multifiliis*.[19]

#### 2.1.2 | In vivo culture

Because *Ichthyophthirius* cannot be cultured in vitro (see below), maintaining parasites in the laboratory requires continuous passaging on fish. This is accomplished by either cohabitation of infected and naïve fish or by infecting naïve animals with known numbers of infectious theronts on a periodic basis. In either case, establishing an initial infection requires identifying infected fish from either a pet store or wild-caught or farm-raised sources. Once a source is identified, parasites can be brought into the laboratory.

For cohabitation, infected and uninfected animals are generally mixed at ratios of 1:3 to 1:5, respectively, using appropriately sized aquaria, with small fish (10–50 g) being more conducive to laboratory-based study. Zebrafish is an obvious choice as a possible model for the study of host–parasite interactions; however, *Danio rerio* tends to be resistant to *I. multifiliis* infection. More susceptible juvenile channel catfish (*Ictalurus punctatus*)[20] and goldfish (*Carassius auratus*) are more common host species for parasite maintenance.

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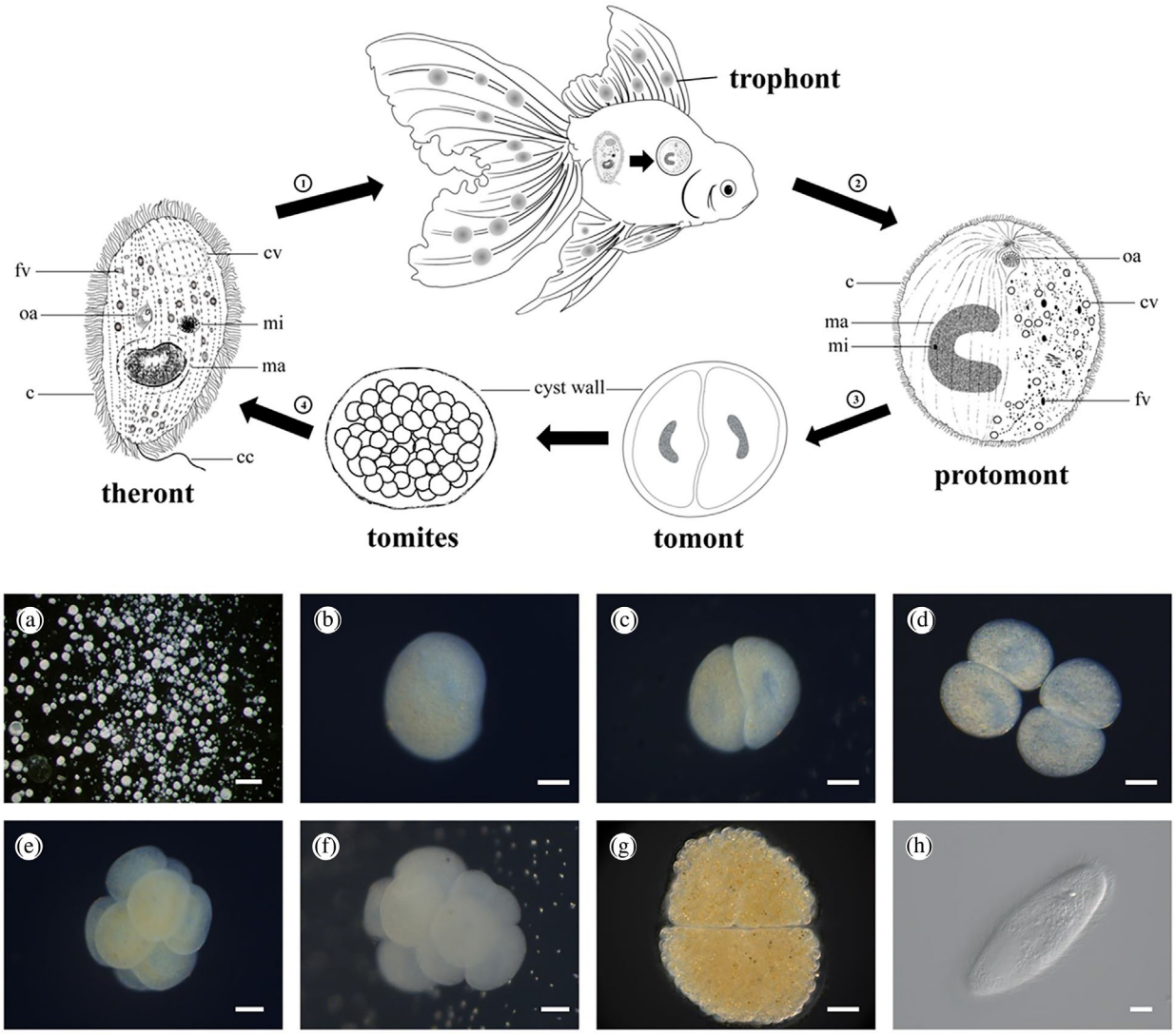


**FIGURE 1** Drawing of *Ichthyophthirius multifiliis*'s life cycle (top): (1) Theront invades host epidermis and grows into a trophont. (2) Trophont leaves host to become a protomont, which can swim freely. (3) Protomont secretes a cyst wall to become a tomont. Tomont undergoes binary fission, producing large numbers of tomites. (4) Tomites transform into theronts that break out from the cyst wall to find new host. c, cilia; cc, caudal cilium; cv, contractile vacuole; fv, food vacuole; ma, macronucleus; mi, micronucleus; oa, oral apparatus. This life cycle drawing shows the detailed processes of a tomont's division. (a) Tomonts. Scale bar = 500 μm. (b) One-cell period. Scale bar = 100 μm. (c) Two-cell period. Scale bar = 100 μm. (d) Four-cell period. Scale bar = 100 μm. (e) Eight-cell period. Scale bar = 100 μm. (f) Sixteen-cell period. Scale bar = 100 μm. (g) Multi-cell period. Scale bar = 100 μm. (h) Theront. Scale bar = 5 μm.

Although straightforward in principle, maintaining parasites by cohabitation requires some attention because their virulence can vary over time. Indeed, wild isolates are often highly pathogenic and can easily overwhelm fish, particularly when first introduced to the laboratory. Trophonts must reach a critical size to complete the life cycle, and in the worst-case scenario, animals succumb to hyperacute infection before parasites develop to the point that they can infect new fish. To prevent over-infection, the index animals (those used as the starting source of infection) can be removed from aquaria 12–24 h after exposing them to naïve fish. Additionally, water changes of up to 90% of the volume of the tank can be used to reduce the numbers of theronts present in the water. Once susceptible animals show signs of infection (white spots), new tanks are established with appropriate ratios of infected to uninfected fish in order to maintain continuous supplies of parasites.

More precise control of parasite levels can be achieved using standardised infection models in which known numbers of live theronts are used to infect fish. At the Key Laboratory of Aquaculture Disease Control, Institute of Hydrobiology, MARA & CAS, black goldfish with butterfly tails are used for in vivo cultivation of parasites. These fish are highly susceptible to Ich and have large fins that support parasite development and are conducive to experimental observation (unpublished). Important steps for in vivo culture of *Icthyophthirius* using a standardised infection model are summarised in Figure 2.

Both cohabitation and standardised infection models have the potential to use large numbers of experimental animals. To reduce the

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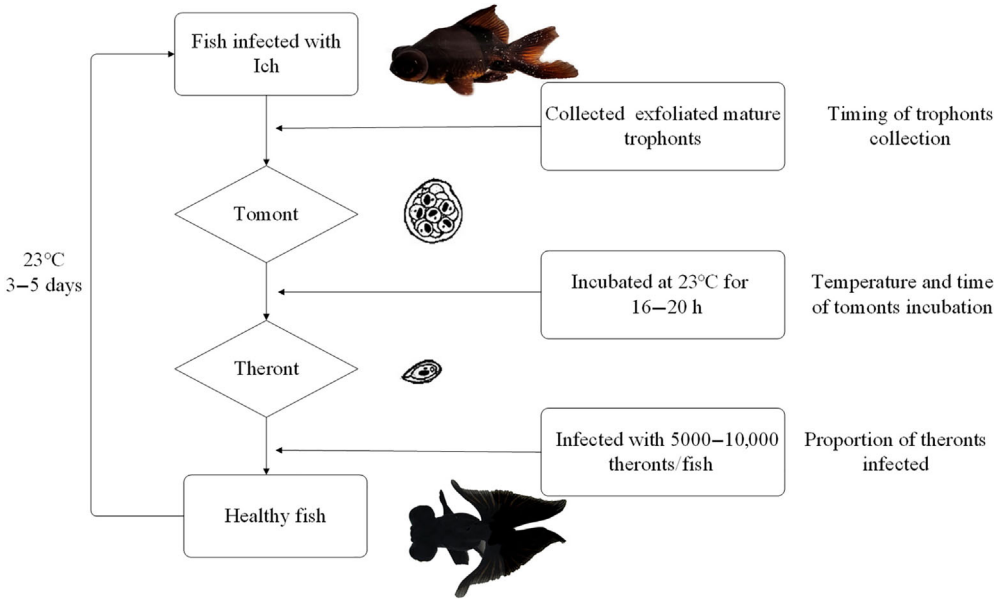


**FIGURE 2** In vivo culture of *Ichthyophthirius multifiliis*. The establishment of parasite cultures begins with the identification of infected fish from local markets or fish farms. Mature trophonts naturally shed from infected fish are then collected and incubated in chlorine-free water to generate infective theronts. Parasite development is generally complete within 16–20 h at 23°C. Theronts are either collected by low-speed centrifugation (300*g* for 2–3 min) or adjusted directly to known concentrations before infecting new fish. In this case, 3–5 naïve animals are incubated with 5000–10,000 parasites per fish in 1 L of water for 15–30 min with aeration before transferring the entire contents to a larger aquarium. Fish are then maintained at 23°C until parasites are visible and can be used to continue the infection.

number of fish required for parasite maintenance, Noe and Dickerson[21] used low water temperatures to lengthen the parasite life cycle. By maintaining juvenile channel catfish at 9°C, they were able to extend the life cycle from ~7 days (at 23°C) to >20 days. Clearly, this approach can lead to significant reductions in animal use and is highly recommended.

Even just a few of the infected fish（5-10） can generate sufficient material for analytical biochemical studies of *Ichthyophthirius*. Trophonts can be released from the skin by gentle rubbing, and then separated from host cells and tissue debris by filtering through a 250 μm (mesh-size) stainless steel screen. Using tandem screens, trophonts can then be collected on a 38 μm mesh before being washed into shallow dishes and harvested by low-speed centrifugation (300*g* for 2–3 min).[22] Alternatively, trophonts can be collected from the bottom of a conical tube after allowing them to gravity-settle for 15–20 min on ice. After harvesting, trophonts are used either directly for experimentation or placed into glass bowls (e.g., 8 inch Pyrex® dishes), where they attach to the walls of the container, encyst and then divide to form theronts. Theronts can be separated from undivided cells and other large debris by filtration (theronts pass through 38 μm screens) followed by low-speed centrifugation as above. Because theronts loose viability within 24 h of hatching, it is advisable to collect them on the day they emerge from cysts.

For genetic studies, as well as the analysis of defined *I. multifiliis* serotypes, new infections should be started using clonally derived parasites.[19,23,24] Single protomonts can be isolated by hand using a narrow-bore glass or plastic pipette and then placed into individual wells of a 96-well plate until they form theronts. Parasites from a single well are then used to infect individual fish in small containers (250–500 ml) before being expanded on new fish in separate aquaria (see above).

Finally, in addition to over-infection, a problem encountered in maintaining parasites by serial passage on naive fish is loss of infectivity. As noted earlier, parasite virulence can change over time. Furthermore, there may be strain differences regarding how long a given isolate can be maintained under controlled conditions in the laboratory.[25] While the reasons for this are unclear, it is not uncommon to lose an infection after only a few cycles on naïve fish.

### 2.1.3 | In vitro culture

To date, the life cycle of *I. multifiliis* cannot be completed in vitro. Ekless and Matthews[26] investigated the viability and development of theronts and trophonts using a variety of monophasic culture media. Theronts survived up to 120 h in media containing Eagle's minimal essential medium with Earle's salts (EMEM) supplemented with 10% fetal calf serum. Indeed, all media used in this study supported tomont survival longer than in freshwater alone, with a maximum period of 22 days in 50% EMEM-S (EMEM minus fetal calf serum). Regardless of the medium, however, parasites failed to develop to the trophont stage. Xu et al.[27] investigated the behaviour of theronts on freshly excised skin, fin and gill from channel catfish, as well as on the established fin-cell line, BF-2, grown in medium 119. Theronts attached to the fish tissues and transformed into trophonts, but did not recognise BF-2 monolayers. Pugovkin et al.[28] also used BF-2 cells to culture

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*I. multifiliis*; however, different from Xu et al.,[27] they cultured cells in EMEM medium rather than medium 119. In neither case did trophonts survive, form cysts or divide following transfer to freshwater.

Nielsen and Buchmann[29] tested the viability, survival and development of theronts in different cell culture media (EMEM or L-15), with or without EPC (epithelioma papulosum cyprini) fish cells. In addition, they attempted to use a 2-compartment system, with water on one side and a monolayer of EPC cells on an Anopore™ Tissue Culture Insert in contact with media on the other, to mimic the fish epidermis. Results showed that theronts transformed into trophonts in all treatments except in water controls. The presence of EPC cells improved the transformation and growth of trophonts from theronts, as did fish mucus and serum. Although the trophonts survived in culture media for up to 13 days at 15°C, they failed to develop into tomonts.

### 2.1.4 | (Cryo-)preservation

A method for the cryopreservation of this ciliate has the potential to save on labour and improve animal welfare. Based on successful efforts to cryopreserve the ciliate model *Tetrahymena thermophila*, Everett et al.[30] examined a wide range of vitrification media for freezing and thawing *Ichthyophthirius*. Surprisingly, media components that were well tolerated by *T. thermophila* (including dimethylsulfoxide [DMSO]) killed *I. multifiliis*. However, a vitrification medium containing 20%–30% Ficoll (70,000), 11% 1,2-propanediol, 3% N,N-dimethylacetamide and 2.5% Schneider's insect cell culture media (ICCM) or carbon filtered water, allowed recovery of theronts from a full cycle of freezing and thawing as generally intact and semi-motile cells.[30] In this case, theronts were starved for 3 days in carbon-filtered water, transferred to vitrification medium for 5 min at 23°C, and frozen at a controlled rate to −80°C before being placed in liquid nitrogen. Cells were thawed rapidly in 2.5% ICCM in carbon-filtered water, essentially as described for *T. thermophia* (for details, see below: Cryopreservation of *Tetrahymena*, Section 2.6.4). Still, while reasonable numbers of theronts appeared to survive this protocol, investigators found no evidence of infection following exposure of naïve fish.[30] Thus, methodological hurdles remain, and more research is needed before cryopreservation can be successfully applied to *Ichthyophthirius*.

## 2.2 | *Cryptocaryon irritans*

### 2.2.1 | General biology

The ciliate *Cryptocaryon irritans* Brown, 1951 is an obligate parasite of marine teleosts and causes Cryptocaryoniasis ('white spot' disease in marine fish). The life cycle of *C. irritans* consists of four developmental stages (Figure 3) – namely, theront, trophont, protomont and tomont.[31–33] Theronts, the free-swimming stage, invade the epithelial layers of the fish surface, including the skin, fins and gills, and then transform into trophonts. Theronts decrease their infectivity to fish after 2–3 h.[34,35] Trophonts grow, without cell division, in the epithelial layers of the fish surface. Fully developed trophonts leave the host as protomonts. Protomonts sink, settle on the substrate, and become encysted as tomonts, in which the parasite undergoes repeated cell divisions to produce theronts. Tomonts excyst theronts into the seawater. The theronts released from tomonts are alive longer than 24 h but remain infective to fish only for 3–4 h at 25–27°C.[34,35]

The development of each stage is affected by various factors: temperature, salinity, dissolved oxygen, light period and host fish. Trophonts can develop well in fish hosts between 18 and 32°C, and the time needed for trophonts to fully develop and leave host fish is temperature-dependent: 11 days at 18°C and 3–4 days at 24°C and higher.[36,37] Tomont development and subsequent theront excystation also occur between 18 and 32°C, and between 25 and 50 ppt in salinity.[31,37] The excystation is asynchronous even when tomonts are incubated under identical conditions.[36] Full development of tomonts are also temperature-dependent: the mean period needed is 11 days at 18°C, 5 days at 24–26°C and 4 days at 30–32°C.[37] The numbers of theronts released from a single tomont are estimated to be around 100–870 cells.[38] However, these biological characteristics also differ depending on the isolate and host species.[37,38] The action of protomonts leaving infected fish and the release of theronts from tomonts follow a clear circadian rhythm: when infected fish and tomonts are incubated in seawater and are exposed to 12L–12D light cycles, protomonts leave fish mainly during the dark period and early light period, and theronts exit cysts during the dark period or between 3 and 6 h before the start of the light period.[34,35,39,40] Development of tomonts is affected by dissolved oxygen concentrations in seawater; hypoxic conditions at 20%–24% air saturation stop the development of tomonts and make them dormant, while anoxic conditions kill them.[31,41,42]

### 2.2.2 | In vivo culture

Naturally infected fish have been used as a source of *C. irritans*. When heavily infected fish are available, protomonts and tomonts obtained from the fish can be used to initiate in vivo culture.[35,43] When the infection intensity is not high enough to collect tomonts, infected fish are cohabitated with naïve fish in an aquarium so that tomonts released from the infected fish can develop at the bottom of the aquarium and excyst theronts that can infect the naïve fish.[44]

In vivo culture can be achieved by exposing naïve fish in aquaria containing tomonts from infected fish, and by experimentally exposing naïve fish with theronts obtained from tomonts. For long periods of in vivo culture, the infection intensity should be controlled to some extent. Heavy infections can kill fish before trophonts fully develop and, when all experimental fish are killed, this will lead to failure of the culture. At low infection intensity, prevalence gradually decreases and finally ceases.

Burgess and Matthews[39] controlled the infection level with numbers of recipient naïve fish exposed to tomonts, in which two or more

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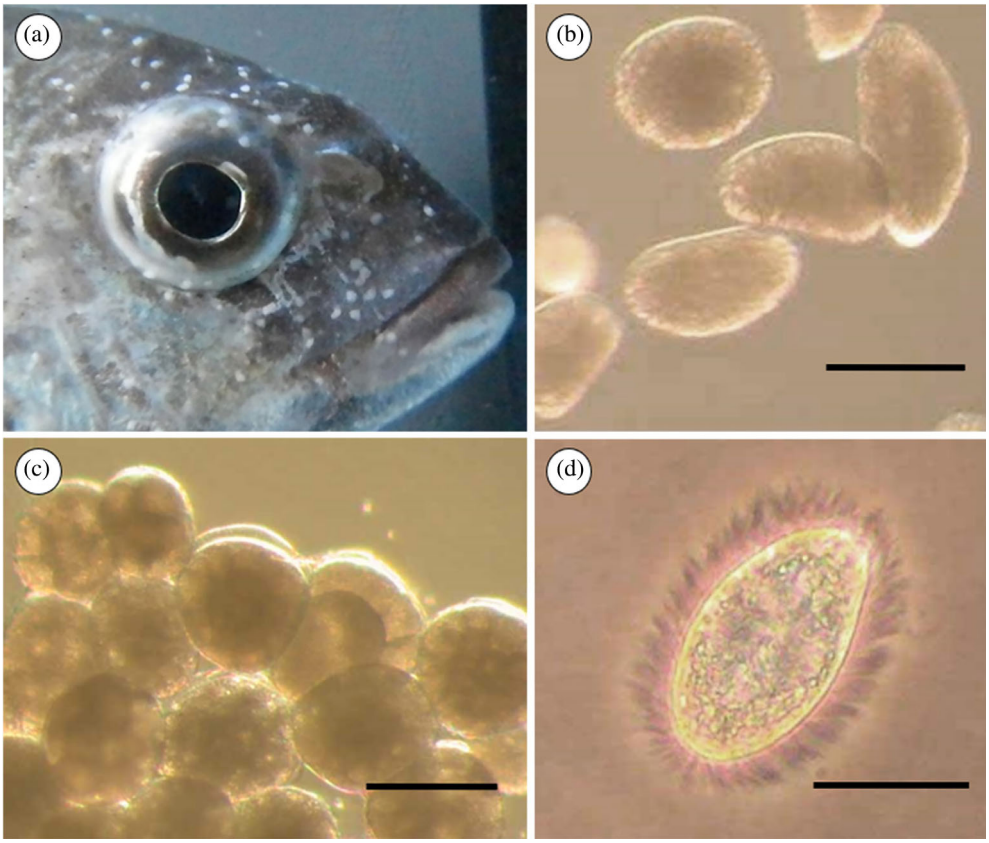


**FIGURE 3** Developmental stages of *Cryptocaryon irritans*. (a) Red seabream infected with trophonts in the epithelial layer of the skin. (b) Protomonts just after leaving hosts. (c) Encysted tomonts containing tomites. (d) Theront released from tomont. The bars represent 200 μm in (b) and (c), and 25 μm in (d).

individuals of grey mullet *Chelon labrosus* (3–6 g) were placed in a 10 L aquarium where donor fish were kept previously for 5 days, by which time all trophonts had left the donor fish and stayed at the bottom of the aquarium as tomonts. When white spots were visible on the recipient fish, they were placed in a new aquarium as donor fish for 5 days, which developed trophonts. By repeating this cycle, six out of seven isolates of *C. irritans* obtained from different fish species were kept for at least 7 cycles. Among the seven isolates, one isolate was maintained for as many as 30 cycles, although two isolates showed a gradual loss of viability and infectivity over less than three passages. Yoshinaga and Dickerson[34] cultured the parasite using saltwater-adapted black mollies (*Poecilia* hybrid) (2–4 cm in body length) as hosts in culture aquaria with an under-gravel filter, by leaving three infected fish and adding 10 naïve black mollies to the aquaria, in a similar method to that described by Burgess and Mattews.[39] By using black mollies, the infection of *C. irritans* was maintained for 2 years. When infection levels decreased, the culture was repeated from the very beginning by cleaning the gravel. However, most of the isolates maintained by this method lost their infectivity within 1–2 years and the culture was lost (Yoshinaga, personal communication). Ageing and lack of mating between different strains might be attributable to the loss of infectivity in both methods. In addition, as the number of tomonts or theronts could not be controlled in the aquaria, the infection level could not be precisely maintained in either method. Controlled challenges were also achieved by exposing a specific number of naïve fish to a certain number of tomonts (which varied with fish size and the desired infection level)[43,45] using the methods describe above.

Long-term culture has been achieved by repetition of experimentally challenging fish with controlled numbers of theronts using pompano (*Trachinotus ovatus*), a marine fish very susceptible to *C. irritans*.[35] Fish considerably larger than those used in Yoshinaga and Dickerson[34] and Burgess and Matthews[39] were used in the study. Ten fish were exposed to theronts in 100 L of still seawater in a bucket for 3 h, and then the seawater was circulated. Three and a half days after exposure, when white spots appeared on the fish, they were moved into a funnel-like special collection unit, in which seawater and air was continuously provided (Figure 4) for harvesting protomonts and tomonts. Moving protomonts and attached tomonts were recovered in a beaker or petri dish set on the bottom on the funnel, and incubated in seawater in a beaker with continuous aeration and daily replacement of seawater. Theronts released from the tomonts were used for the next round of fish challenge. In other in vivo culture systems, naïve fish have been used as recipient hosts, as infected fish obtained immunity against the parasite. However, as pompano are susceptible to *C. irritans*, they were repeatedly challenged by increasing the number of theronts in each infection round, with 8000, 10,000, 12,000 and 15,000 theronts/fish in the first, second, third and fourth infection, respectively. As parasites yielded remarkably

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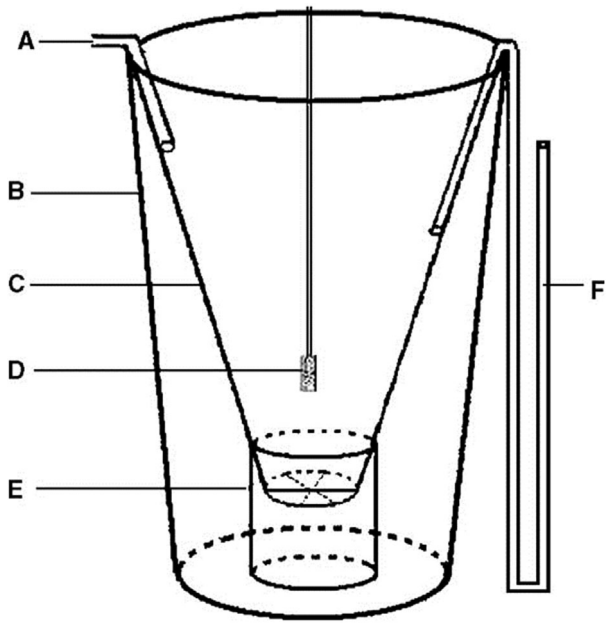


**FIGURE 4** Schematic diagram of the specially designed collection unit for harvesting the protomonts (cited from Dan et al.[35]): (A) incurrent tube; (B) 160 L plastic bucket; (C) funnel; (D) airstone; (E) 2 L beaker; (F) excurrent tube

decreased numbers after the fourth infection, naïve pompanos were used. *Cryptocaryon irritans* was successfully maintained for up to 40 successive cycles using this method.

The use of pompano appears to have advantages for laboratory culture. A large number of parasites can be produced, as this fish species is relatively larger than those used in previous studies.[34] The number of fish needed for culture can be decreased as repeated challenges are possible. Naïve fish are easily obtained without risk of contamination with different strains of *C. irritans* and acquiring immunity against the parasite, as the fish species is euryhaline and can be obtained from fish farms in low-salinity regions without infection. A controlled challenge has other advantages, such as the infection level can be monitored by the yields of protomonts and tomonts (parasite numbers).

Due to the circadian rhythm in the leaving of protomonts from hosts and the excystation of theronts from tomonts, experiments can be labour intensive. When protomonts are required before cyst formation, they must be collected in the very early morning as they leave their host during night or early morning and attach to the wall and bottom of containers within a few hours. In the same manner, as theronts are excysted during night or early morning and lose their infectivity within a few hours after excystation, the collection of theronts and challenging experiments should be carried out in the early morning. Therefore, in vivo culture of this parasite requires detailed planning in terms of the best time for experiments. However, recently, Watanabe et al.[40] found that the circadian rhythm of the parasite can be controlled by changing the daily light rhythm, and developed a method to obtain protomonts and theronts at any desired time of the day by changing the light rhythm, especially in laboratory settings.

A stable and quantitative small-scale laboratory culture method was developed by controlled exposure of fish to theronts, and by a specific light period,[46] and summarised in Figure 5. Seawater-adapted black mollies were exposed to fresh theront suspension in a small (2 L) aquarium in the dark for 6 h, before then being kept in a new aquarium until white spot appeared, and finally being moved into another new aquarium. Protomonts were allowed to leave from fish in the aquarium and to settle and attach to the bottom. Tomonts attached to the bottom were rinsed with seawater and incubated in 50 ml of seawater in the same aquarium in an incubator with 12L–12D light cycles. Theronts excysted from the tomonts were used to challenge another 10 naïve black mollies. Seven days were needed for 1 cycle between challenges at 25°C. Using this method, the parasite was maintained for more than 3 years.

Recently, Vaughan and Huston[47] proposed standard operating procedures for the in vivo culture of *C. irritans*, including methods to initiate the culture, to maintain the culture by challenging fish with tomonts, and to quantitively challenge fish with theronts.

In all of the above in vivo culture methods, challenged fish and tomonts were kept at 25–27°C in full-strength seawater (27‰–35‰), which are conditions suitable for host fish and the parasite. To prevent contamination and growth of unwanted micro-organisms, the seawater used for the incubation of tomonts was filtered and supplemented with some antibiotics.

### 2.2.3 | In vitro culture

Although the development from protomonts to theronts is easily achieved by keeping protomonts in seawater, no in vitro methods have been established to repeatedly complete the entire life cycle of *C. irritans*. Yambot and Song[43] reported that theronts grew to the size of trophonts in liquid media consisting of different concentrations of seawater, L-15 medium and fetal bovine serum (FBS), and strips of trypticase soy agar. However, the transformation ratio of theronts was as low as 0.28%–1.71%, and further development from trophonts to tomonts was not observed.

Yoshinaga et al.[48] developed a double-layered medium consisting of a layer of cultured fish cells and an agarose gel layer covering the cell layer. The agarose gel layer contained 0.22% ultra-low-melting-point agarose and 10% FBS in L-15 medium. When theronts were inserted between the layers with micro-pipette tips, theronts transformed to trophonts and grew by feeding on cultured fish cells (Figure 6). When trophonts were recycled from the layered medium and incubated in seawater, they developed to tomonts and finally excysted theronts that infected host fish. Due to the low recovery percentage of trophonts (5% and 11% in two trials), and asynchronous development and excystation of theronts, the propagation cycle could not be repeated. However, the double-layered medium has been used for in vitro assays to test various chemicals for the control of

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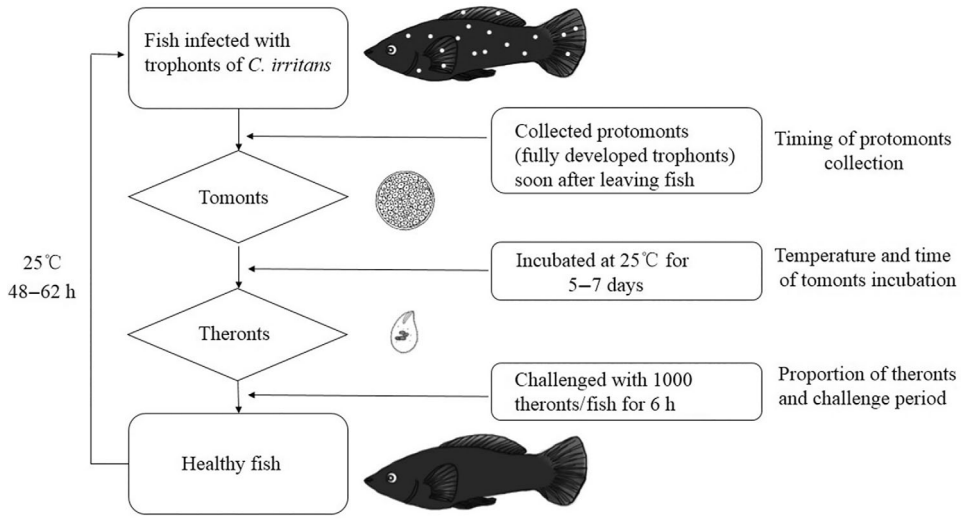


**FIGURE 5** In vivo culture of *Cryptocaryon irritans*. Seawater-adapted black mollies are used to establish the parasite cultures. Mature trophonts exit infected fish to complete the life cycle and form protomonts after they leave the host. Tomonts are rinsed with seawater and incubated at 25°C for 5–7 days with 12L–12D light cycles. Theronts excysted from the tomonts are then used to challenge naïve black mollies with 1000 parasites per fish in 1.5 L of filtered seawater in a 2 L plastic aquarium at 25 °C in the dark in an incubator for 6 h. Challenged fish are kept at 25°C in full-strength seawater (27‰–35‰) until new parasites develop and can be re-passaged. Using this method, parasite cultures were maintained for more than 3 years.

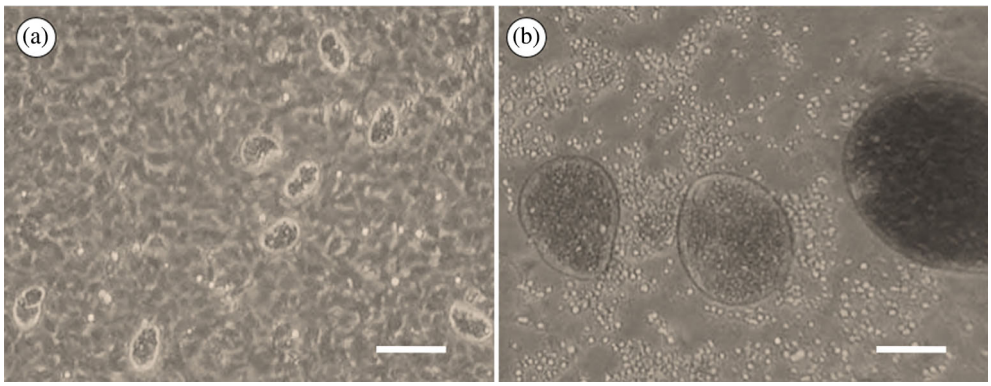


**FIGURE 6** In vitro development of *Cryptocaryon irritans* in a double-layered medium. (a) Theronts just after inoculation. (b) Trophonts after 3-day incubation. Scale bars represent 100 μm in (a) and (b).

*C. irritans*[49,50] and to determine the effects of protease inhibitors on the development of *C. irritans*.[50]

To secure a constant supply of *C. irritans* in experimental studies, the parasite still has to be maintained and cultured by being passaged on fish. This is not only labour-intensive but also undesirable from an animal welfare standpoint. Therefore, research on in vitro culture of *C. irritans* should continue to be pursued.

### 2.2.4 | (Cryo-)preservation

Cryopreservation of *C. irritans* has not been achieved at any developmental stage. Although, the parasite can be preserved as tomonts at low temperatures for some periods, and the tomonts can release theronts infective to fish.[51] When the parasites leaving hosts are transferred to 12°C before and after encystation as tomonts, they are able to release infective theronts even after 3–4 months. Tomonts can become and stay dormant, without any development, under hypoxic conditions.[36,42] This phenomenon could help the development of other preservation methods for this parasite in the future.

## 2.3 | *Chilodonella*

### 2.3.1 | General biology

Chilodonellosis outbreaks occur from time to time, which is a serious problem to freshwater aquafarms, causing high rates of mortality with little warning to farmers.[52–54] The disease is caused by *Chilodonella* Strand, 1928, a genus of ciliates mainly characterised by their

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free-living nature. This group of ciliates can graze on bacteria, diatoms, filamentous green algae, and cyanobacterial substrates of fish gills and skin.[55–57] This feeding behaviour has pathological consequences to affected fish, with high rates of mortality occurring rapidly within 24–72 h after parasites are observed in infected tissues.[2,58,59]

The life cycle of *Chilodonella* spp. is characterised by transverse binary fission or conjugation reproduction. Binary fission is the most common mode of propagation of this ciliate group when there are enough nutrients in the environment. In binary fission, the parent cells split asexually, generating two offspring cells of the same size. Conjugation is the sexual form of reproduction adopted by *Chilodonella* spp. during periods of limited starvation in the environment. Conjugation happens when adults of *Chilodonella* spp. find a complimentary individual mate. During the process of sexual reproduction, nuclear reactions occur, such as meiosis, exchange of gametic nuclei, and fertilisation, forming two exconjugant individuals.[2] Understanding the asexual and sexual cycles of *Chilodonella* spp. and their feeding strategies is an important aspect to consider when planning in vitro culture methods for this parasite, but also for future studies related to its control in fish farms.

Within this group, two main pathogenic species, *Chilodonella hexasticha* and *C. piscicola*, are considered quite dangerous as they can infect a wide range of wild and farmed freshwater fish species worldwide.[2,60–63] Besides, *C. uncinata* has also been reported as a facultative parasite of fish,[53,64] although it is commonly regarded as a free-living species and had been used as an experimental organism in many genetic studies.[57,65–68] It was confirmed to be infective to goldfish and can cause the same tissue damage as other *Chilodonella* species.[64]

### 2.3.2 | In vivo culture

Recently, a successful *C. uncinata* infection model was established.[64] In this model, goldfish (body length: 4.9 ± 0.4 cm) were used as the experimental fish. They were treated with three consecutive baths in 1:10,000 formalin solution for 12 h at 48 h intervals to remove all ectoparasites, and kept in aquaria for another 2 weeks following the formalin bath procedure. For each fish, 10 in vitro cultured *C. uncinata* cells were added to the gills using a micro-pipette. Treated goldfish were then kept in aquaria for 3 days and showed an infection rate of 100%. The infected fish showed the same clinical signs as *C. hexasticha* and *C. piscicola*, such as loss of appetite, abnormal swimming and excessive mucus production.[64] This model has the potential to be used in future diagnostic testing and drug screening to control chilodonellosis infections in aquaculture.

### 2.3.3 | In vitro culture

*Chilodonella* spp. (mainly *C. hexasticha* and *C. piscicola*) in vitro cultures have been reported before when these ciliates were isolated from gills and skin from infected barramundi (*Lates calcarifer*) from Australia.[53] Ciliate culture was maintained in the dark at room temperature using six-well plates (FalconTM) with 6 ml of filtered autoclaved pond water (from the fish farm where infected fish were diagnosed). To maintain bacterial growth in the culture as a source of nutrients for ciliates, a sterilised rice grain was added to each well (modified from Bellec et al.[69] and Bastos Gomes et al.[53]).

In many cases, it is not uncommon to find more than one species of *Chilodonella* when starting a culture in which the cells have been isolated from infected farmed fish.[53,58,59,61,70] Therefore, polycultures can be firstly established in vitro, and after 24 h the culture plates can be examined under a compound microscope (40 ×) to select single *Chilodonella* cells using a clean micro-pipette before being transferred into a new well, also with 6 ml of filtered, autoclaved pond water and a single sterilised rice grain. Bastos Gomes et al.[53] described repeating this step every third day for a period of 6 months.

Unlike the two obligate parasitic *Chilodonella* species (*C. hexasticha* and *C. piscicola*), *C. uncinata* is easier to cultivate under laboratory conditions with filtered and autoclaved pond water with an uncooked rice grain at room temperature and in a dark environment.[53,64,69,71] Ciliate cells of *C. uncinata* with high motility were selected from fish gills with micro-pipettes, washed three times with distilled water and then transferred to new Petri dishes filled with distilled water, and again allowed to swim freely for 10 min. Ten grammes of fresh wheat grains were added to 250 ml dd$H_2O$ and then sterilised to be prepared as the culture medium. Single ciliate cells in good condition (high motility and large in size) were placed into the medium to be cultured in the dark at 25°C.[64]

During in vitro culture, it was found that *C. uncinata* preferred to distribute densely on the surface of the medium and fed on the bacteria in the in vitro culture plate. The generation time was about 2.5 h during continuous 24 h microscopic observation and the optimal temperature for cultivation was 24–26°C.[64] Under extreme temperatures of 4 and 38°C, *C. uncinata* could survive for about 3 days and 2 weeks, respectively, but died within a day at 39°C (unpublished).

### 2.3.4 | (Cryo-)preservation

To date, no literature is available on the cryopreservation or preservation of *Chilodonella* species.

## 2.4 | *Balantidium ctenopharyngodoni*

### 2.4.1 | General biology

*Balantidium ctenopharyngodoni* Chen, 1955, is an obligate intestinal parasite that can inhabit the hindgut of grass carp (*Ctenopharyngodon idella*), particularly the intestinal epithelium.[72–74] There is disagreement about the ability of *B. ctenopharyngodoni* to infect different ages of grass carp. An early study reported that this parasite is capable of infecting all grass carp from fry and fingerling to adult fish,[72] but a later study reported that young grass carp (less than 2 years) cannot be infected by the balantidia.[73] The latter group of authors also

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researched the pathogenicity of *B. ctenopharyngodoni* in diseased fish and found that it may occasionally be pathogenic under the influence of certain predisposing factors, such as enteritis. Balantidia might interact with intestinal pathogenic bacteria, predisposing fish to infection; however, healthy grass carp at older ages also harboured numerous balantidia,[73] so balantidiosis in grass carp is a multifactorial disease. A large number of surveys conducted during the last 10 years by the Key Laboratory of Aquaculture Disease Control, Institute of Hydrobiology, MARA & CAS indicate that the prevalence of *B. ctenopharyngodoni* in healthy grass carp is even higher than those suffering from enteritis, and only grass carp older than two summer years are infected with *B. ctenopharyngodoni*.[75]

To the best of our knowledge, this species is believed to be the only ciliate parasite found in the digestive tract of grass carp (with strict host-specificity, i.e., it does not infect any other fish species) (Li M., personal observation; no reports in the literature). Besides, this notion is closely related to the feeding habits of the host (fry mainly feed on invertebrates and zooplankton, while juveniles mostly eat macrophytes and vegetation). Balantidia reproduce by transverse binary fission, and cysts might form when trophozoites are passed in the faeces.[76] Although cysts have been observed in the hindgut, the infection route of *B. ctenopharyngodoni* is still unclear. Li et al.[74] hypothesised that the infective stage of *B. ctenopharyngodoni* is when trophozoites exit the host body via the anus, and not with cysts been ingested through the mouth. Cysts could be considered, as in many other cases, a special survival form of parasite to resist challenging environments.

### 2.4.2 | In vivo culture

To date, no literature is available on the in vivo culture of *B. ctenopharyngodoni*.

### 2.4.3 | In vitro culture

A xenic medium (named BCM medium) has been used to culture *B. ctenopharyngodoni* in vitro, with the method for preparing the medium and the in vitro culture method both developed by the Key Laboratory of Aquaculture Disease Control, Institute of Hydrobiology, MARA & CAS.[75,77] The formulation of the culture medium includes proteose peptone (0.5% w/v), yeast extract (1% w/v), inorganic salts, serum (fetal bovine serum, 3% v/v; horse serum, 10% v/v), isolated fish intestinal bacteria (~2 × $10^9$ CFU/ml) and starches. The culture steps include inoculating the collected *B. ctenopharyngodoni* into a prepared medium, filling with nitrogen and sealing, culturing at 15°C for 48–72 h, then transferring to another fresh medium, cycling back and forth, proliferating the *B. ctenopharyngodoni* continuously.

Subsequent subcultures were maintained in BCM medium for intervals of 48 h with a generation time of 3.2 days at 15°C. Additionally, a single-cell clone of *B. ctenopharyngodoni* derived from one trophozoite was established in BCM medium successfully.[77] Different strains can be isolated and maintained in BCM medium for quite long periods, one of which was maintained for more than 4 years. Balantidia maintained in BCM medium for several months became larger than freshly isolated trophozoites (Figure 7).

Besides, there was no observation of *B. ctenopharyngodoni* cysts in the BCM medium during the period of cultivation. Some methods, such as nutrient deprivation (e.g., removal of starch or other components in the BCM medium) and extreme temperatures (e.g., cultivation at a lower temperature of 5°C) were adopted to induce encystment, but they did not seem to be effective and trophozoites ultimately died (unpublished).

BCM medium was the first in vitro cultivation medium successfully developed for the growth of an intestinal ciliate from freshwater fish.[75,77] This was also the first report, to the best of our knowledge, of in vitro cultivation of a fish ciliate using a single cell.[75] This in vitro medium provides good potential for studying the interactions between this protist and intracellular bacteria.[78]

### 2.4.4 | (Cryo-)preservation

To date, no literature is available on the cryopreservation or preservation of *B. ctenopharyngodoni*.

## 2.5 | Scuticociliatia

### 2.5.1 | General biology

Scuticociliates are basically free-living organisms. They proliferate by binary fission, feeding on micro-organisms such as bacteria or other protozoa, as well as organic matter in seawater and some species belonging to the subclass Scuticociliatia, which include *Uronema marinum*, *Miamiensis avidus*, *Pseudocohnilembus persalinus*, *Pseudocohnilembus longisetus* and *Philasterides dicentrarchi*, are known to opportunistically or facultatively infect the gills, skins, muscle tissues, visceral organs, ascite and brain of various fish species and can cause mortality of the infected hosts.[79–82]

*Uronema marinum* and *M. avidus* have previously been recognised as pathogens of marine ornamental fish[79,83]; however, more recently, there have been reports of scuticociliates infecting various food fish cultured in farms, including European sea bass (*Dicentrarchus labrax*), turbot (*Scophthalmus maximus*), olive flounder (*Paralicthys olivaceus*) and southern bluefin tuna (*Thunnus maccoyii*). While scuticocilatosis is still a major problem for ornamental marine fish (especially in public aquaria),[84] two commonly farmed flat fish species, olive flounder and turbot, are frequently infected with scuticociliates. In the late 1980s, mortality caused by an unidentified scuticociliate was reported in cultured olive flounder (*P. olivaceus*) in Japan.[80] Since then, mortality attributed to this group of ciliates has also been widely reported in olive flounder farms in South Korea and Japan. Although *P. persalinus* and *P. longisetus* have also been reported to infect olive flounder,[85] *M. avidus* is considered the most common species from this group found in olive flounder.[86]

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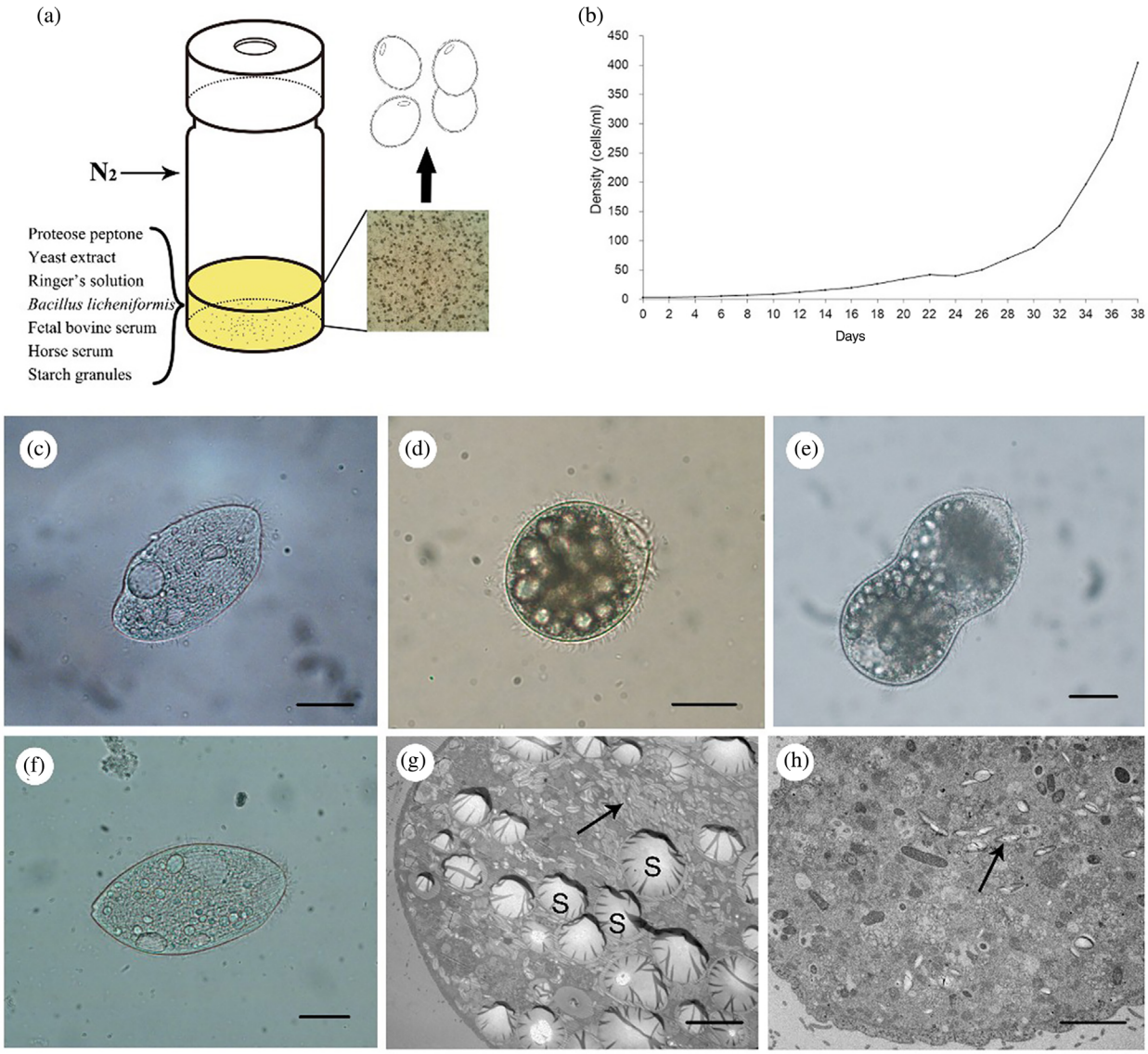


**FIGURE 7** General profiles of *Balantidium ctenopharyngodoni* cultured in BCM medium (cited from Zhao et al.[75]). (a) Illustration of in vitro culture system. (b) Growth curve of *B. ctenopharyngodoni* cultured in BCM medium. (c) General view of *B. ctenopharyngodoni* collected from hindgut. Scale bar = 30 μm. (d) General view of *B. ctenopharyngodoni* cultured in BCM medium, showing the round body. Scale bar = 30 μm. (e) Cultured *B. ctenopharyngodoni* in division process. Scale bar = 30 μm. (f) *B. ctenopharyngodoni* after starvation in BCM medium without starch added. Scale bar = 30 μm. (g) TEM image of *B. ctenopharyngodoni* cultured regularly, showing the starch granules (S) and glycogen granules (arrow) in the cytoplasm. Scale bar = 10 μm. (h) TEM image of *B. ctenopharyngodoni* after starvation, showing the reduced starch granules and glycogen granules (arrow). Scale bar = 5 μm.

In southern Europe, including France and Spain, outbreaks of scuticociliate infections were reported in cultured European sea bass (*Dicentrarchus labrax*) and turbot (*Scophthalmus maximus*) in the late 1990s and early 2000s, respectively, and the ciliate was identified as *Philasterides dicentrarchi*, which has been a major parasite affecting turbot.[81,82]

There is still confusion on the taxonomy of *M. avidus* and *P. dicentrarchi*. Some studies have indicated that the two species are synonymous, based on comparisons of the morphology and nucleotide sequences of ribosomal genes from two species isolated from fish.[86,87] This synonymy was once widely accepted,[86,88–90] but the taxonomic priority was left unclarified. Conventionally, scuticociliates found in olive founder in Asia and in turbot in Europe are often named *M. avidus* (syn. *P. dicentrarchi*). However, based on multigene analysis, De Felipe et al.[91] speculated *P. dicentrarchi* and *M. avidus* are distinct species. In the present review, based on these recent results reported by De Felipe et al.[91] that highlight the uncertainty in the taxonomic relation between these two scuticociliates, we tentatively recognise them as two different species.

### 2.5.2 | In vivo culture

To date, no literature is available on the in vivo culture of scuticociliates.

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### 2.5.3 | In vitro culture

Animal and plant tissues and bacterial mixtures in seawater, proteose peptone-based media, cell culture media, and cultured fish cells in culture media have all been used as the nutrient source for the in vitro culture of scuticociliates. These ciliates can be isolated mainly from the brain tissue and abdominal liquid originated from ascites from infected fish and used as original isolates for cultures.[81,82,85,86,92–96]

Some early studies of the in vitro culture of scuticociliates mainly used animal or algal tissues as the feeding source. *Uronema marinus* was isolated from ornamental fish in a public aquarium and cultured on fish tissue and green algae (*Ulva* spp.) by using the particles obtained by passing minced and boiled fish tissue and algae through a sieve.[79] When unidentified scuticociliates and *U. marinum* were isolated from the brain tissue of infected olive flounder, ciliates were proliferated by feeding on the brain tissue in seawater.[92,93] *Philasterides dicentrarchi* isolated from European sea bass and turbot was cultured in seawater using fish blood and ox spleen, and boiled wheat grains together with a bacterial mixture as nutrient sources.[81,82]

Proteose peptone-based media, which are conventionally used for the culture of free-living ciliates such as *Tetrahymena*, can also support the growth of some scuticociliates in axenic conditions (culture free from other living organisms; only the target species is present). Yoshinaga and Nakazoe[92] propagated unidentified scuticociliates from olive flounder in an autoclaved solution of 2% proteose-peptone and 1% yeast extract dissolved in three-times-diluted artificial seawater, in which the highest cell density of the ciliate was $10^4$ cells/ml. When 10% FBS was added to the medium, the cell density reached approximately $5 \times 10^5$ cells/ml (Yoshinaga, personal communication). Soldo and Merlin[97] developed two proteose-peptone based media for marine ciliates including *M. avidus*. One proteose-peptone medium (named medium C) consisted of 1% proteose peptone, 1% trypticase, 0.1% yeast nucleic acid and a vitamin mixture dissolved in seawater containing 0.5% extract of Cerophyl® (a commercial nutritional supplement produced from dried grass) (Pines, Lawrence, Kansas). In the other proteose-peptone based medium (named medium M), Cereopyl was replaced with asolectin (mixture of soy phospholipids), cephalin (phosphatidyl-ethanolamine) and Tween 80 (polyoxyethylene sorbitan monooleate). The cell density of *M. avidus* reached $5 \times 10^5$ to $10^6$ cells/ml in the two proteose-peptone based media. A medium based on medium C is registered as ATCC 1651 MA Medium in the American Type Culture Collection. ATCC 1651 MA Medium supported a good proliferation of *P. dicentarchi* isolated from turbot, in which the cell density reached $5 \times 10^5$ cells/ml.[98,99] 'PGY medium', consisting of 1.5% proteose peptone, 0.5% glucose and 0.5% yeast extract powder in seawater, supported the growth of a strain of *U. marinum* obtained from natural seawater, in which the cell density of the strain reached $4.2 \times 10^5$ cells/ml.[100] The proteose-peptone media can be sterilised by autoclaving, followed by addition of sterile vitamin solutions and FBS, when needed, and can be used for axenic cultures.

Chemically defined media are needed for nutritional and biochemical studies of these ciliates. S medium (chemically defined medium) was developed by Soldo and Merlin[97] and consists of 18 amino acids, nucleotides, vitamins, fatty acids and stigmasterol, metal cations, and salts. In the medium, a *Miamiensis* sp. strain and *U. nigricans*, both isolated from natural seawater, grew well in the medium. Successful use of chemically defined media, however, has not been achieved to culture scuticociliates isolated from fish.

Leibovitz L-15 medium is conventionally used for the culture of fish cell lines. To avoid complications related to creating new media, L-15 medium was supplemented with fetal bovine serum (10%), lecithin (a glycerophospholipid) emulsified with Tween 80, nucleotides and glucose (standard complete L-15 medium), and was used to develop an axenic culture of *P. dicentrarchi*.[82] In the supplemented medium, *P. dicentrarchi* reached $10^5$–$2 \times 10^5$ cells/ml. This culture medium has been frequently used for studies of these ciliates isolated from turbot. A strain of *Uronema marinum* isolated from the brain of olive flounder was cultured for approximately 2 years in MEM (Minimum Essential Medium) supplemented with 10% FBS.[94]

Yoshimizu et al.[101] isolated unidentified scuticociliates obtained from olive flounder and cultured the isolate using fish cell lines as food. Ciliates were cultured in cell culture flasks containing confluent CHSE-214 cells (a cell line derived from common bluegill *Lepomis macrochirus*) cultured in MEM supplemented with 10% FBS – a culture system conventionally used for the isolation and cultivation of many fish viruses.[102,103] In this culture system, the cell density of ciliates can reach up to $10^6$ cells/ml. When CHSE-214 was replaced with 42 different cultured cell lines, ciliates grew well on any of them. Although the culture medium was sterile but not axenic (as live fish cells are present in the culture), this method successfully supported the proliferation of *M. avidus* isolated from olive flounder and has been frequently used for that species.

Microbial contamination, along with the salinity (or osmolarity) and temperature of the medium, are the most important factors other than its chemical composition for in vitro axenic culture of scuticociliates. Antibiotics have been conventionally used for sterilisation of scuticociliates and suppression of microbial growth in the medium. However, once axenic cultures have been established using antibiotics, they are no longer necessary.[101] An axenic culture was also obtained by repeatedly washing ciliates in sterile seawater using capillary pipettes under a microscope.[92]

A strain of unidentified scuticociliate from olive flounder grew in a wide range of osmolarity (between 200 and 940 Osm), but grew the most at 438 mOsm/L,[92] which roughly corresponds to the osmolarity of half-strength seawater. Although the final seawater density of M medium developed for *M. avidus* by Soldo and Merlin[97] was 1.015, this roughly corresponds to 2/3-strength seawater. Components of the proteose-peptone-based ATCC 1651 modified from M medium were diluted in half-strength artificial seawater. The osmolarities of cell culture media such as L-15 and MEM were adjusted to roughly 285 mOsm/L, equivalent to 1/3-strength seawater. The salinity of the media used for scuticociliates suggests that they have an affinity for osmolarities equivalent to 1/3- to 2/3-strength seawater. Given that they can proliferate in the body cavity and tissues of fish, and *M. avidus* shows high pathogenicity to fish reared in diluted seawater,[104] their preference to low osmolarities agrees with their nature when they behave as pathogens. Although differences in

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growth have not been examined well at different temperatures, scuticociliates have mostly been cultured at a wide range of temperatures from 15 to 27°C. Optimal temperatures and tolerance to high temperatures may differ among species and strains. Optimal temperatures for in vitro growth should be examined for each specific strain of interest.

Through the establishment of in vitro culture methods for scuticociliates, the development of inactivated vaccines against them can potentially be explored.[105,106]

### 2.5.4 | (Cryo-)preservation

Yoshimizu et al.[101] stated that a strain of an unidentified scuticociliate isolated from olive flounder and cultured on CHSE-214 was successfully cryopreserved with a method conventionally used for fish cell lines, which consisted of slow preliminary freezing in FBS supplemented with 10% DMSO (dimethyl sulfoxide) and storage in liquid nitrogen, but no detailed data on the results are available. Anderson et al.[107] described successful cryopreservation of *U. marinum* isolated from New Zealand grouper (*Polyprion oxygeneios*) and cultured in standard L-15 complete medium.[82] Folgueira et al.[98] developed an optimised cryopreservation protocol for *P. dicentrarchi*, in which the ciliate in the stationary phase in 2-fold diluted 1651 MA medium (MM medium) culture was suspended in MM medium supplemented with 11% DMSO and 5% glycerol, and frozen at approximately −1°C/min in a commercial freezing container before being transferred to a freezer (−80°C) for 2 h and then stored in liquid nitrogen. The protocol was effective for a strain of *M. avidus* (ATCC 50180) and a strain of *Miamiensis* sp. (ATC50179) of undescribed origin, as well as for another eight strains of *P. discentrarchi* from turbot.[98]

## 2.6 | *Tetrahymena*

### 2.6.1 | General biology

The genus *Tetrahymena* Furgason, 1940, is comprised of a large number of species inhabiting primarily freshwater ecosystems throughout the world.[108,109] Although most species are free-living (consuming bacteria and decaying organic matter for their nutritional needs), their dietary requirements are complex[108,110,111] and histophagous species have been isolated from a variety of animals including adult and larval insects,[112–115] flatworms,[116] molluscs,[117,118] amphibians,[119,120] and freshwater teleosts where they can be highly pathogenic, particularly in guppies (*Poecilia reticulata* Peters), one of the most popular species of ornamental fish.

While evidence suggesting an association between *Tetrahymena* spp. and fish disease can be traced back to early work by Epstein,[121] Corliss made the first definitive identification of a parasitic form of *Tetrahymena* in the cerebrospinal fluid and tail musculature of an embryonic trout in 1953.[122] This was followed by cursory reports identifying *Tetrahymena*-like ciliates in infected tissues of other types of fish including *P. reticulata*. Hoffman et al.[123] made similar observations and ascribed disease outbreaks in guppies to *Tetrahymena corlissi*, a species described by Thompson[120,124,125] as a facultative parasite of amphibian embryos and later shown to be capable of infecting guppies through superficial wounds in the skin. Numerous reports have since made clear that *Tetrahymena* spp. can infect a variety of ornamental and edible fish,[126–128] with an apparent predilection for guppies leading to significant losses in fish farms, primarily in Southeast Asia.[129–132]

Initial reports on guppies and other fish suggested that *Tetrahymena* spp. invade the host through superficial wounds or abrasions of the skin. Consistent with this, infected animals often present with diffuse white lesions on the skin from which *Tetrahymena* spp. can easily be recovered. Nevertheless, it remains unclear whether *Tetrahymena* is saprophytic (an organism that obtains nourishment from the products of organic breakdown and decay) or truly parasitic on fish. On the one hand, *Tetrahymena* identified on live fish can also be recovered from dead animals of both susceptible and resistant species such as tilapia,[128] suggesting that it has more of an opportunistic, saprophytic lifestyle. This is reinforced by evidence that environmental stress seems to predispose animals to infection.[128,133] Nevertheless, some isolates are clearly capable of invading deep into tissues, including the musculature, blood vessels of the gills, sub-mucosa of the gut, peri-orbital region of the eye, cranial nerves and spinal cord, and in some cases, visceral organs such as the liver.[123,129,134] While more indicative of parasitic behaviour, the true nature of the association of *Tetrahymena* spp. with fish may ultimately depend on both the host and ciliate species involved.[134,135]

Irrespective of their status as parasites, *Tetrahymena* strains (including those that are histophagous on animals) vary in other aspects of their life cycles.[109] For example, some species can alternate between mitotically dividing and non-dividing dispersal forms, as well as resting cysts depending on their environment. Additionally, some species can form reproductive cysts, much like *Ichthyophthirius* and *Cryptocaryon*.[109] Finally, *Tetrahymena* species containing micronuclei have the potential to undergo sexual reproduction (via conjugation), an activity that has been extensively studied in the laboratory model, *Tetrahymena thermophila*.[136,137]

In parallel with clinical studies on fish, developments in molecular phylogeny have resulted in major advances in our understanding of ciliate taxonomy.[111,138,139] These advances allow discrimination of parasite isolates based on molecular markers and have led to the identification of formerly cryptic species and a wholesale revamping of the *Tetrahymena* phylogenetic tree. For example, based on Cox1 barcoding, four *Tetrahymena* isolates obtained originally from infected guppies in Singapore and elsewhere (designated SIN, NI, RA and CO) have recently been separated into two distinct groups.[111] The first, comprising SIN alone, is a new species that has formally been assigned the name *Tetrahymena alphapoecilia*.[111] The second, comprising NI, RA and CO, forms a separate group awaiting species designation.

### 2.6.2 | In vivo culture

Despite their association with animals, most histophagous *Tetrahymena* appear to be facultative parasites (or transitional free-living

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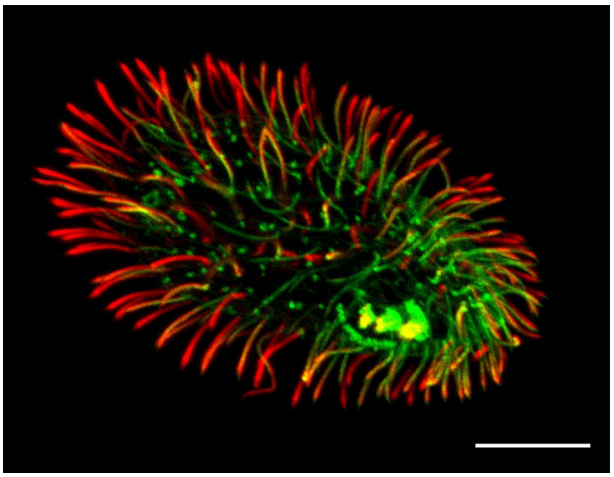

**FIGURE 8** A fluorescence confocal light micrograph showing a single cell of the species *Tetrahymena thermophila*. The cell is expressing GFP-tagged beta-tubulin (green) and is labelled with anti-polyglycylated tubulin antibodies (red). Note the extensive ciliature surrounding the cell. By permission of Mayukh Guha, Muthugapatti Kandasamy and Jacek Gaertig (Department of Cellular Biology, University of Georgia). Scale bar = 10 μm.

forms) allowing them to be cultivated for laboratory use without the need to maintain them on host species (see the next section below, entitled 'In vitro culture'). This includes *Tetrahymena* spp. that infect guppies, such as the SIN, NI, RA and CO isolates described above, with the caveat that in order to maintain virulence, periodic sub-culture in host species (namely, fish) may be necessary.[134] Instances in which histophagous strains of *Tetrahymena* could not be cultured have been reported,[111] although it is not clear whether these failures were due to inappropriate growth conditions or the isolation of true obligate parasitic species.

### 2.6.3 | In vitro culture

In addition to species that behave as facultative parasites, free-living *Tetrahymena* spp. (especially *T. thermophila* and *T. pyriformis*) have served as important models in eukaryotic cell biology and genetics since the 1960s (Figure 8).[140]

Methods for the cultivation and preservation of laboratory species have been described in several excellent reviews and are applicable to in vitro culture of pathogenic strains of *Tetrahymena* as well.[141–143] The media and conditions for culturing cells can be divided into two broad categories: those favouring long-term maintenance, and those permitting rapid, high cell density growth. As might be expected, robust cell growth occurs in well-oxygenated, nutrient-rich (generally, proteose peptone-based) broth, whereas long-term maintenance is better achieved in more minimal media. Field isolates are generally maintained at 18–23°C, although some laboratory species (e.g., *Tetrahymena thermophila*) can be grown at temperatures between 30 and 38°C.

Proteose peptone-based media are inexpensive, easy to prepare, and generally sustain rapid cell growth of most *Tetrahymena* isolates provided they are supplemented with iron, most often in the form of iron salts.[142] Dissolved oxygen concentrations necessary to support growth can generally be achieved by using containers with high surface area-to-volume ratios. For moderate-scale cultures (100–1000 ml), Fernbach or wide-bottom Erlenmeyer flasks filled to a shallow depth work well, while for smaller cultures (0.1–10 ml), petri dishes or plastic multi-well plates are more appropriate. For very large-scale cultures (≥10 L), achieving high cell densities may require agitation or the use of air stones for bubbling to maintain optimal concentrations of dissolved oxygen during log-phase growth.[144] In the latter case, a few drops of a silicone anti-foam agent may be added during media preparation and prior to autoclaving to prevent excess foaming.[142,145] Lastly, cells can be maintained in loosely capped, 15 ml glass culture tubes with serial transfer every 2–4 weeks as stock cultures for up to 6 months (see below).[142]

Commonly used proteose peptone-based media for growth of laboratory strains include SSP, SP210, PPY, PPYG and NEFF, which vary with respect to the proteose peptone concentration and levels of additives such as yeast extract, glucose, iron, antibiotics, etc. (see Cassidy-Hanley[142] for formulations). While bacterial contamination is rarely a problem when culturing *Tetrahymena* spp., sterile media and growth containers should always be used, and cells should be manipulated under aseptic conditions to prevent overgrowth of adventitious microbes should they enter the culture. Along the same lines, antibiotics (penicillin, streptomycin and amphotericin B) are generally added to growth media, except when bacterized media are purposely used.[141,142]

In addition to the media described above, *Tetrahymena* can be maintained on a variety of other nutrient sources, such as skim-milk,[145,146] axenic or bacterized rye grass infusions (marketed originally as Cerophyl®),[147,148] and axenic media supplemented with mammalian liver hydrolysates,[142] as well as fully chemically defined media.[149,150]

While there have been relatively few studies comparing in vitro growth of pathogenic strains of *Tetrahymena* on different media, Leibowitz and Zilberg[134] found that the *Tetrahymena* isolate NI, which is pathogenic in guppies, grew best in the proteose peptone-based media RM-9 (containing liver extract) and ATCC 375 supplemented with fresh excised gill tissue, as compared with either PPY1 or PPY2 media alone. Neither PPY1 nor PPY2 used in this study appeared to contain iron,[134] and it is possible that the liver extract and gill tissue in the RM-9 and ATTC 375 media, respectively, provided essential factors (e.g., iron, sterols, phospholipids) required for optimal cell growth. However, not all *Tetrahymena* spp. isolated from infected fish appear to grow well in supplemented RM-9 or ATCC 375 media,[128,134] and growth requirements may vary depending on the ability of cells to invade deep tissues (as parasites) versus grazing on the surfaces of live fish (as saprophytes).[134]

Importantly, in the case of *Tetrahymena* spp. isolated from guppies, maintaining virulence of cultured cells has been reported to require periodic re-infection of fish.[134] This can be achieved by serial

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passaging of cells in liquid culture for 3–4 weeks, re-infecting fish by immersion, cohabitation or intraperitoneal injection, and then re-isolating cells from infected tissues for continued maintenance in liquid culture.[133,134] For infection via immersion or cohabitation, maintenance of infected animals under sub-optimal conditions of ammonia and nitrates (4–6 and 1–2 ppm, respectively, for guppy fry) may favour re-infection.[134] Additionally, as for laboratory strains, allowing cells to reach stationary phase for brief periods during serial transfer in rich media may be useful in avoiding the emergence of slow-growing cells (so-called long telomere phenotypes).[142]

Aside from potential loss of virulence, long-term culture in rich media can also result in loss of fertility in laboratory strains, and while fertility is only a concern for genetic work in *Tetrahymena*, phenotypic alterations to cells (owing principally to random assortment of chromosomes in the macronucleus) can be minimised either by slowing growth (i.e., by reducing the total number of divisions within a given culture) or by freezing cells. For long-term maintenance of stock cultures, laboratory strains are often kept in low-nutrient bean media prepared by autoclaving a single garbanzo or soybean in 10 ml of distilled water in a standard glass test tube.[151,152] Tubes are then inoculated with 50–100 µl of cells from log-phase cultures, topped with sterile paraffin oil to prevent evaporation, and loosely capped.[142] Cultures can be maintained at 15–20°C for up to 6–8 months before transfer to a fresh tube (preferably with short-term growth in rich medium, or potentially on fish, in between). As an alternative to bean media, species that are not easily frozen have been maintained over long periods in crude cultures of rat intestine.[151] As in the case of bean cultures, these are prepared using 1 cm long, thoroughly cleaned sections of rat intestine in 15 ml glass tubes layered with paraffin oil and autoclaved. Such preparations can be maintained for up to 1 year and then re-invigorated by limited serial passaging in enriched media (and/or live fish) before re-transfer to new rat culture tubes.

Additional options for in vitro cultivation of *Tetrahymena* include the use of scrupulously clean glassware with no detergent residue, and gentle handling of cells.[142] Although a true microbe, unlike bacteria and yeast, *Tetrahymena* spp. are large cells with no rigid wall structures and are consequently susceptible to lysis by excess mechanical shear or high-speed centrifugation. When culturing cells, low-speed centrifugation (300–1000*g*) for concentrating samples is preferable, as is gentle pipetting to resuspend cell pellets (as opposed to vortexing). Subjecting cells to temperature extremes and/or anoxic conditions (including maintaining cells in tight pellets for prolonged periods of time) should also be avoided. Finally, a wide range of laboratory strains and natural isolates of *Tetrahymena* spp., along with protocols for maintaining cells and recipes for growth media are available through the Tetrahymena Stock Center at Washington University, St. Louis (https://tetrahymena.vet.cornell.edu/index.php).[142]

### 2.6.4 | (Cryo-)preservation

For cell lines that are amenable to freezing, *Tetrahymena* can be maintained, more or less indefinitely, in liquid nitrogen.[142,153] Briefly, healthy cells from log-phase cultures are starved for 2–3 days at 30°C in 10 mM Tris–HCl, pH 7.5, concentrated by centrifugation at 300*g*, and resuspended in 1 ml of starvation buffer. Following addition of four volumes of 10% dimethylsulfoxide (DMSO), 300 µl aliquots are dispensed to cryovials (e.g., Nalgene® #5000-0020 or #5000-0012) and incubated for 30 min at room temperature. Vials are then transferred to a controlled-rate freezing container (Nalgene® Cryo 1°C 'Mr. Frosty' Freezing Container Rack) and incubated at −80°C overnight. Finally, tubes are placed into a liquid nitrogen dewar or insulated bucket with dry ice pellets and immediately transferred to a liquid nitrogen container for long-term storage.[142,153] Recovery of cells from liquid nitrogen is relatively inefficient; however, enough cells survive thawing that strain recovery is generally successful following addition of growth media. In the case of *T. thermophila*, frozen aliquots are thawed rapidly by placing cryovials directly into a water bath at 42°C and adding 1 ml of culture medium prewarmed to the same temperature with occasional shaking until the frozen samples are just thawed. Cells are then transferred immediately to petri dishes with 10 ml of culture medium at the desired growth temperature. Further details of freezing and thawing laboratory strains of *Tetrahymena* are described by Cassidy-Hanley[142,153] (see also, https://tetrahymena.vet.cornell.edu/recipes.php).

## 3 | APPLICATION OF FISH CILIATE CULTURES IN AQUACULTURE: CONCLUDING REMARKS AND PROSPECTS

The rapid expansion of the global aquaculture industry has been followed by a disease crisis epidemic. While aquacultural enterprises are adapting more modern technologies than ever before, mortality rates and economic losses due to infectious diseases are still a serious aspect of this industry.[154] There are multiple factors that can contribute to the current trends in outbreak events within fish farming. The nature of aquaculture production where animals are confined to limited spaces under high densities, the type and quality of feed provided to animals, poor water quality conditions, and certainly climate change, are just a few (and simplistic) examples of the reasons behind the high prevalence of diseases within aquaculture.[155] Manifestation of diseases in aquatic animals is complex, and this topic by itself is deserving of an entire paper or book for its clarification (which is outside the scope of the present review).

Within the myriad reasons and causes of infectious diseases currently experienced by aquatic animals, the emerging number of pathogens, including parasites, is certainly an important one. Among these, ciliate parasites occupy a significant place as they are generally underestimated and unresearched within the aquaculture production sector. As stated before, ciliate parasites can be the most harmful creatures affecting fish but also the most neglected ones. Their microscopic and pervasive nature certainly contributes to the difficulty in better understanding fish ciliate parasites.[2] More research is critically needed to elucidate the challenges and ways to control these important aquatic pathogens. However, the lack of knowledge on how to culture them

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under laboratory conditions is one of the challenges faced by researchers and pharmaceutical companies.

There are multiple applications of fish ciliate parasite cultivation, as follows: (1) improvement of current diagnostic approaches (e.g., morphology-based methods) and the development of new diagnostics, including genetic and immunological tests; (2) development of novel environmental screening methods for ciliate parasite surveillance (e.g., environmental DNA-based methods, molecular epidemiology); (3) identification of antibodies and other protective methods adopted by fish together with more cost-effective vaccine development; (4) determination of specific proteins and mechanisms of invasion/infection used by ciliate parasites to infect the host; (5) vaccine development and its efficacy testing using intact parasites; (6) discovery and in vitro testing of new drugs and therapeutic agents before exposing animals to potentially toxic compounds (animal welfare); (7) identification of different parasite strains and isolates that can be resistant or susceptible to specific fish species and specific drugs/chemicals; (8) understanding the role of ciliate parasites and specific microbiomes in clinical fish disease manifestation; and (9) providing a constant supply of parasite isolates/strains for experimental fish disease models.

Current knowledge regarding in vitro and in vivo cultivation of fish ciliate parasites is still not well disseminated. While a good body of research exists on the artificial culture of ciliates for different purposes (e.g., understanding of human genetic and epigenetic mechanisms using *Tetrahymena* spp. and *Chilodonella* spp.),[57,156] a detailed explanation of how to culture parasitic ciliates for aquaculture purposes is not well communicated in the literature. Understanding the detailed processes and protocols on how to cultivate these micro-organisms could not only clarify their development, behaviour, metabolism, physiology and propagation from host to host, but also illuminate the path to new and innovative control methods and treatments for mitigation and eradication (when possible) of fish ciliate parasites.[157]

## AUTHOR CONTRIBUTIONS

**Ming Li:** Funding acquisition; methodology; writing – original draft; writing – review and editing. **Giana Bastos Gomes:** Methodology; writing – original draft, Writing – review & editing. **Weishan Zhao:** Data curation; formal analysis; writing – original draft. **Guangran Hu:** Data curation; formal analysis; writing – original draft. **Ke Huang:** Data curation; formal analysis; writing – original draft. **Tomoyoshi Yoshinaga:** Data curation; writing – original draft; writing – review and editing. **Theodore G. Clark:** Formal analysis; writing – original draft; writing – review and editing. **Wenxiang Li:** Data curation; formal analysis. **Hong Zou:** Data curation; formal analysis. **Shangong Wu:** Data curation; formal analysis. **Guitang Wang:** Funding acquisition; writing – review and editing.

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## CONFLICT OF INTEREST

The authors declare no conflict of interests.

## DATA AVAILABILITY STATEMENT

Data available on request from the authors.

## PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

We have got permission of Prof. Dan XM to cite his schematic diagram (Figure 4) of the specially designed collection unit for harvesting the protomonts of *Cryptocaryon irritans* from Dan et al.[35]:

Dan XM, Li AX, Lin XT, Teng N, Zhu XQ. A standardized method to propagate *Cryptocaryon irritans* on a susceptible host pompano *Trachinotus ovatus*. *Aquaculture*. 2006;258 (1–4):127–133. doi:10.1016/j.aquaculture.2006.04.026

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