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Review

Current status of parasitic ciliates *Chilodonella* spp. (Phyllopharyngea: Chilodonellidae) in freshwater fish aquaculture

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Abstract

Freshwater fish farming contributes to more than two-thirds of global aquaculture production. Parasitic ciliates are one of the largest causes of production loss in freshwater farmed fishes, with species from the genus *Chilodonella* being particularly problematic. While *Chilodonella* spp. include 'free-living' fauna, some species are involved in mortality events of fish, particularly in high-density aquaculture. Indeed, chilodonellosis causes major productivity losses in over 16 species of farmed freshwater fishes in more than 14 countries. Traditionally, *Chilodonella* species are identified based on morphological features; however, the genus comprises yet uncharacterized cryptic species, which indicates the necessity for molecular diagnostic methods. This review synthesizes current knowledge on the biology, ecology and geographic distribution of harmful *Chilodonella* spp. and examines pathological signs, diagnostic methods and treatments. Recent advances in molecular diagnostics and the ability to culture *Chilodonella* spp. *in vitro* will enable the development of preventative management practices and sustained freshwater fish aquaculture production.

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Introduction

Most of the world's aquaculture production takes place in freshwater. Freshwater fish culture represents two-thirds (44.2 million tonnes) of global aquaculture production (FAO 2014). Parasitic disease can seriously compromise the sustainability of this industry as they cause mortality, slow fish growth, lower food conversion rates and decreased marketability (see Nowak 2007; Buchmann 2013 and Shinn *et al.* 2015 for reviews). Ciliates are considered some of the most harmful parasites of cultured fish (Lom & Dyková 1992) and can facilitate secondary infections (e.g. bacterial infections) in farmed fishes (Lom & Dyková 1992; Hossain *et al.* 2013; Padua *et al.* 2013). Ciliates of the genus *Chilodonella* (Phyllopharyngea: Chilodonellidae) are primarily free-living, although at least two species, *Chilodonella hexasticha* (Kiernik 1909) and *C. piscicola* (Zacharias 1894; syn. *C. cyprini* Moroff 1902), can infect animal hosts (Lom & Dyková 1992) and cause severe epizootic outbreaks in wild and farmed freshwater fishes. Persistent fish infections are a constant threat to aquaculture production with direct and indirect economic impacts to producers

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(e.g. Lom & Dyková 1992; Rintamaki, Torpstrom & Bloigu 1994; Jee, Kim & Park 1996; Nikolic & Simonovic 1996; Rintamäki-Kinnunen & Valtonen 1997; Schisler *et al.* 1999; Evans & Lester 2001; Nikolic, Simonovic & Maric 2006; Mitra, Bandyopadhyay & Gong 2013; Padua *et al.* 2013; Bowater & O'Donoghue 2014). Infections caused by *Chilodonella* spp. have been documented for more than a century ((Leibovitz 1980; Langdon *et al.* 1985; Lom & Dyková 1992), and in the last four decades, it has attracted increasing research interest as the freshwater fish production industry expands (Fig. 1).

Chilodonella spp. have a voracious appetite for living cells. A specialized mouth organ, the cytostome, is used to graze on bacteria, diatoms, filamentous green algae and cyanobacteria present on biofilm substrates of fish gills and skin (Foissner 1988; Blatterer & Foissner 1992; Dopheide *et al.* 2011). The cytostome directly penetrates fish epithelial cells, allowing uptake of the contents (Paperna & Van As 1983; Wiles, Cone & Odense 1985; Noga 2010). The pathological consequences of feeding can be severe and mortalities can occur within 24 h following detection of infection (Mitra *et al.* 2013; Padua *et al.* 2013), with loss of 50−95% of fish stock (e.g. Bowater & O'Donoghue 2014). Rapid epizootic events are particularly difficult for farm health managers to predict or preventatively treat, because parasites may not necessarily be observed during routine fish health screening (i.e. microscopic examination of gill clip and skin scraping samples). Outbreaks cause considerable negative economic impact to farms, including costs from direct mortalities and loss of product, chemical treatments and labour. Indeed, loss of stock and treatment due to *Chilodonella* spp. infections have been estimated to cost around 10% per grow-out cycle in Australian freshwater barramundi, *Lates calcarifer* (Private Industry manager, pers. comm. 2015).

Chilodonella spp. infections cause major losses in freshwater production in at least 16 species of freshwater fishes cultured in 14 countries (see Table 1) (Mitra & Haldar 2004; Mitra *et al.* 2013; Padua *et al.* 2013; Bowater & O'Donoghue 2014; Bradley *et al.* 2014). This review outlines new *in vitro* culture methods and molecular strategies (such as environmental DNA and LAMP) to examine aspects of infection dynamics and diagnosis which could facilitate rapid treatment for these ciliates in fish aquaculture. Specifically, we explore current knowledge on the biology, ecology, behaviour and genetic characteristics of *Chilodonella* spp., and the impacts of infection on cultured fish.

Chilodonella spp. morphology and genetic characteristics

Species of *Chilodonella* are distinguished morphologically based on the number of ciliary rows present in the left or right kinetic bands (Fig. 2a,b), the morphology of the cytostome (cell mouth)

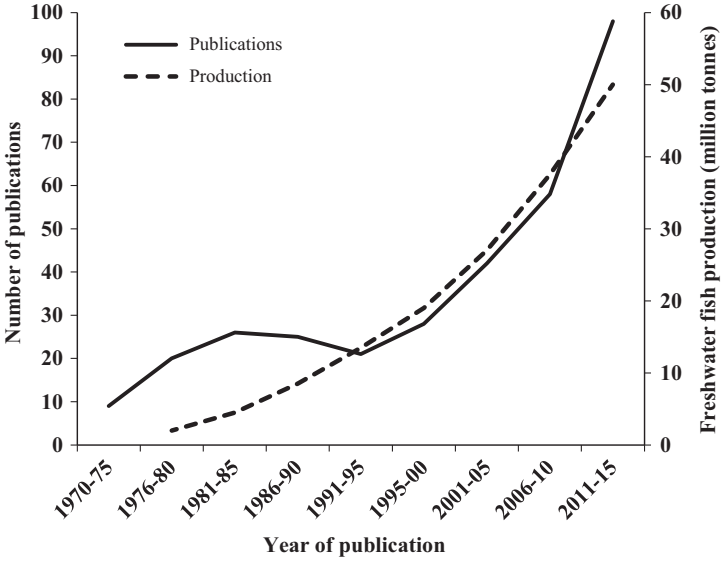

Figure 1 Number of publications on parasitic *Chilodonella* and global freshwater fish production (FAO 2014) by year. Data generated through visual screening and count of articles based on search criteria '*Chilodonella* AND fish' using Web of Science and Google Scholar (search conducted in January 2016).

Table 1 Parasitic *Chilodonella* species reported from wild and farmed fishes

Chilodonella species	Water temperature range (°C)	Fish origin	Parasite species identification by	Fish species	Location	Reference
Chilodonella hexasticha	19–22	Farmed	Morphology	*Ictalurus punctatus* and *Carassius auratus*	USA	Hoffman *et al.* (1979)
	8–13	Wild	Morphology	*Nernatalosa erebi*, *Neosilurus* sp., *Amniataba percoides*, *Leiopotherapon unicolor, Melanotaenia splendidi tatei*	Australia (Finke River)	Langdon *et al.* (1985)
	–	Wild	Morphology	*Carassius auratus*	China	Hu (2012)
	25–27	Farmed	Morphology	*Lates calcarifer*	Australia	Bowater & O'Donoghue (2014)
	20–30	Wild	Morphology	*Nandus nandus*	India	Mitra & Haldar (2004)
	10–20	Farmed	Morphology	*Oncorhynchus mykiss*	Serbia	Nikolic *et al.* (2006)
	26	Farmed	Morphology	*Oreochromis niloticus, Gymnotus aff. inaequilabiatus*	Brazil	Padua *et al.* (2013)
	10–30	Farmed	Morphology	*Bidyanus bidyanus*	Australia	Read (2007)
	13–24	Farmed	Morphology	*Tilapia rendalli and Pseudocrenilabris Philander*	Israel and South Africa	Paperna & Van As (1983)
	–	Wild	Morphology	*Labeo rohita* and *Cyprinus carpio*	India	Mitra *et al.* (2013)
	25–30	Ornamental	Morphology	*Symphysodon discus*	Japan	Imai, Hatai & Ogawa (1985)
	22	Wild	Morphology	*Carassius carassius*	Korea	Jee *et al.* (1996)
	–	Wild	Morphology	*Aristichthys nobilis*	Malaysia	Shariff (1984)
Chilodonella piscicola	5–10	Farmed	Morphology	*Ictalurus punctatus* and *Carassius auratus*	Poland	Kazubski & Migala (1974)
	17–27	Farmed	Morphology	*Maccullochella peelii*	Australia	Bradley *et al.* (2014)
	9–11	Farmed	Morphology	*Oncorhynchus masou*	Japan	Urawa & Yamao (1992)
	5–18	Farmed	Morphology	*Oncorhynchus mykiss*	Denmark	Jorgensen *et al.* (2009)
	–	Ornamental	Morphology	*Carassius auratus*	Turkey	Kayış *et al.* (2013)
	–	Farmed	Morphology	*Salmo trutta*	Finland	Valtonen & Koskivaara (1994)
	–	Ornamental	Morphology	*Paracheirodon innesi*	Australia	Evans & Lester (2001)
	–	Wild/Farmed	Morphology and genetics	*Schizothorax o'connori* and *Oxygymnocypris stewartii*	Tibet	Deng *et al.* (2015)
Chilodonella hexasticha and *C. piscicola*	20	Farmed	Morphology	*Ictalurus punctatus* and *Carassius auratus*	USA	Wiles *et al.* (1985)
	13	Farmed	Morphology	*Salmo salar, Salmo trutta* m. *Trutta*	Finland	Rintamaki *et al.* (1994)
	10–17	Farmed	Morphology	*Salmo salar, Salmo trutta* m. *trutta, Salmo trutta* m. *Lacustris*	Finland	Rintamäki-Kinnunen & Valtonen (1997)
	5–22	Wild	Morphology	*Perca fluviatilis* and *Carassius auratus gibelio*	Yugoslavia	Nikolic and Simonovic (1996)
	–	Farmed	Morphology	*Oreochromis niloticus*	Saudi Arabia	Abdel-Baki, Gewik & Al-Quraishy (2014)
Chilodonella spp.	23	Farmed	Unidentified specific species	*Odontesthes bonariensis*	Brazil	Fernandes *et al.* (2011)
	–	Ornamental	Unidentified specific species	*Xiphophorus maculatus*	Brazil	Piazza *et al.* (2006)
	–	Wild	Unidentified specific species	*Oncorhynchus mykiss*	USA	Schisler *et al.* (1999)

and the size dimensions of mature cells (Ashburner & Ehl 1973; Nikolic *et al.* 2006; Mitra *et al.* 2013). *Chilodonella* spp. generally exhibit a flattened body (ventrally) and a long right ventral ciliary band that is arched and longer than the band on the left side of the cell (Fig. 2a,b). Their specialized mouth structure (cytostome or cytopharynx) can attach strongly to surfaces by producing a vacuum on the ventral side or by specialized cilia which function as an adhesive (Risse-Buhl *et al.* 2009). Parasitic *Chilodonella* use the cytostome to feed on fish skin mucus and gill substrate (bacteria and organic material) which causes an inflammatory response by the host (Lom & Dyková 1992; Urawa & Yamao 1992; Padua *et al.* 2013).

Most *Chilodonella* species have been characterized on potentially plastic morphological features. Recent genetic analyses of nuclear small subunit ribosomal DNA (rDNA) and mitochondrial SSU (mtSSU) rDNA of *C. uncinata* collected from the environment have shown that significant cryptic species diversity exists in North America (Riley & Katz 2001; Bellec & Katz 2012; Zufall, Sturm & Mahon 2012). This genetic variation appears to be a common feature within other *Chilodonella* species, suggesting there is significant cryptic diversity yet to be characterized (Katz *et al.* 2011).

Chilodonella spp., like most ciliates, have nuclear dualism. Each chilodonellid cell contains an inactive micronucleus and a macronucleus responsible for gene expression (generative diploid micronuclei and vegetative polyploidy macronuclei) (Riley & Katz 2001; Bellec & Katz 2012). The macronucleus contains gene-sized chromosomes and has similar function to somatic nuclei of animals (Riley & Katz 2001; Bellec & Katz 2012; Zufall *et al.* 2012). The macronucleus formation occurs through a comprehensive rearrangement, including genome fragmentation, amplifi cation and elimination of micronuclear-limited

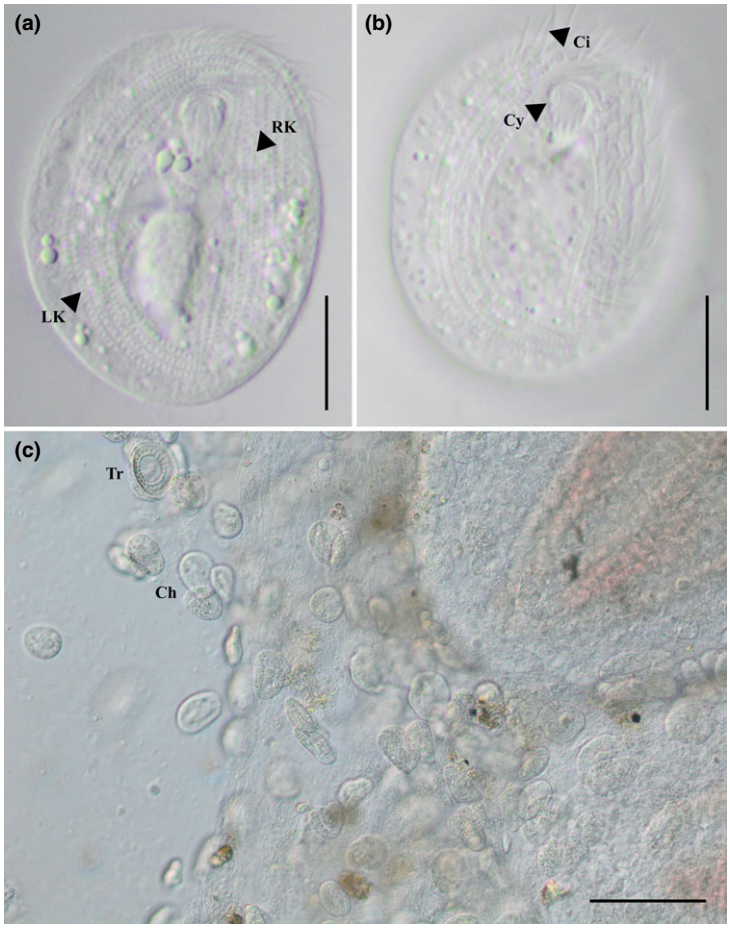

Figure 2 *Chilodonella hexasticha* from the gills of farmed barramundi, *Lates calcarifer*, in tropical north Queensland, Australia, showing (a) LK, left kinetic bands; RK, right kinetic bands; (b) Ci, cilia and Cy, morphology of the cytostome; (c) infection on gills by *Chilodonella* sp. cells (Ch). Co-infection by *Trichodina* sp. (Tr). Scale bars = 100 μm.

sequences (Prescott 1994; Juranek & Lipps 2007; Bellec & Katz 2012). This pervasive rearrangement in chilodonellid cells yields a macronucleus with gene-sized chromosomes which divide through amitosis (cell division that happens without common features of mitosis) (Riley & Katz 2001; Katz & Kovner 2010; Bellec & Katz 2012). Research examining patterns of molecular evolution among ciliates and epigenetic mechanisms has used *C. uncinata* species as a model organism because of this unique nuclear dualism (Bellec & Katz 2012).

Ciliates have many unique features related to their genome structure, including variation in gene copy number (CNV) between individuals within a population and gene expression levels (Spring, Pham & Zufall 2013). This modification can be explained by innate gene duplication in eukaryotes, presence of gene clusters and a comprehensive existence of extrachromosomal circular DNA, which may be involved in genome plasticity (Walsh 1987; Cohen & Segal 2009; Bellec & Katz 2012). The study of changes in CNV and expression level can explain genomic structural variations observed when comparing individuals within a population. CNV can be related to adaptive evolution of species and also can be associated with some human diseases (Spring *et al.* 2013). Therefore, *Chilodonella* spp. represents a perfect model to study CNV due to the presence of individual genes on unlinked chromosomes (Riley & Katz 2001; Bellec & Katz 2012).

Life cycle

Chilodonella spp. primarily reproduce by transverse binary fission (Lynn 2008; Bellec, Maurer-Alcala & Katz 2014). During binary fission, the zygotic nucleus divides, generating a new nucleus. One nucleus will turn into a micronucleus and the other will develop into a macronucleus (Riley & Katz 2001; Bellec & Katz 2012). The parent cell then divides by binary fission (asexual and mitotic process) producing two 'daughter' cells (offspring) which have the same size (Lynn 2008). Asexual reproduction occurs continually if there is sufficient food in the environment (Lynn 2008).

Chilodonella spp. can also reproduce by a sexual process known as conjugation (Lynn 2008; Bellec *et al.* 2014). Conjugation starts when mature *Chilodonella* spp. cells find other complimentary mating individuals. A mating reaction occurs between the cells and during this process many nuclear events occur such as meiosis, exchange of gametic nuclei and fertilization (Lynn 2008). Two exconjugant cells are formed. If these cells cannot conjugate, they undergo a period of senescence with death temporarily delayed by autogamy or self-fertilization (Hausmann & Bradbury 1996; Sugiura *et al.* 2005; Lynn 2008). *Chilodonella uncinata* particularly starts its sexual cycle by meiosis of the micronucleus (MIC), and then conjugation with exchange of haploid MIC between cells takes place, followed by nuclear fusion forming a zygotic nucleus (Bellec *et al.* 2014). *Chilodonella* cells with similar morphology form a pair and are connected to each other by their cytostome. This sexual process can be stimulated in *Chilodonella* cells *in vitro* through limited starvation (Lynn 2008).

Understanding the asexual division and sexual cycle of *Chilodonella* is important for morphological characterization of species. Generally, species are identified using measurements of the macronucleus (MAC) and MIC (Fan *et al.* 2014; Qu *et al.* 2014). However, considering *Chilodonella* cells undergo complex processes in their different life cycle stages, mischaracterization of species could occur using MAC and MIC measurements.

Chilodonella spp. in freshwater fish aquaculture: ecology, known hosts and geographic distribution

Four *Chilodonella* species have been isolated from the gills and skin of bony fish, including *Chilodonella uncinata*, *C. cucullulus, C. hexasticha* and *C. piscicola* (see Migala & Kazubski 1972; Rintamaki *et al.* 1994). The most serious mortalities in fish aquaculture appear to have been associated with infections caused by *C. hexasticha* and *C. piscicola* (see Hoffman *et al.* 1979; Mitra & Haldar 2004; Mitra *et al.* 2013; Padua *et al.* 2013), while most reports of *C. uncinata* and *C. cucullulus* (Muller, 1976) are from collection made directly from the environment (i.e. not associated with a fish host). *Chilodonella* spp. primarily feed on bacteria and algae in the environment and on organic material and bacteria when infecting fish (Noga 2010; Padua *et al.* 2013). High number of *Chilodonella* cells (Fig. 2c) feeding on fish can cause severe pathological signs, including hyperplasia of gill epithelium and necrosis in the gill and skin of fishes (Ashburner & Ehl 1973; Mitra & Haldar 2004; Karvonen *et al.* 2010; Padua *et al.* 2013).

Parasitic *Chilodonella* spp. exhibit a wide temperature tolerance (Table 1). Rapid changes in environmental parameters, such as temperature and oxygen, or increases in organic material in ponds, can contribute to the proliferation of *Chilodonella* spp. on fish (Bowater & O'Donoghue 2014; Bradley *et al.* 2014). Additionally, extreme temperatures promote host stress, which can compromise the host fish's immune system and affect the capacity to combat infection (Hossain *et al.* 2008, 2013; Macnab & Barber 2012). *Chilodonella hexasticha* is associated with warmer climates or seasons between 26 and 31 °C, while *Chilodonella piscicola* exhibits a wide thermal tolerance between 4 and 20 °C (Table 1). The ideal environmental conditions associated with chilodonellosis (infections caused by *Chilodonella* spp.) are unidentified (Kepner, Wharton & Coats 1999; Hossain *et al.* 2008, 2013) and it is not clear whether specific temperatures impact the reproduction and proliferation of *Chilodonella* spp. Clinical signs and detection of infections caused by *Chilodonella* spp. have been generally found during drastic changes in the weather (transition from summer to autumn; dry to wet season; Bradley *et al.* 2014; Hossain *et al.* 2008, 2013). This indicates that stress may predispose fish to infections as it can compromise their immune system (Oidtmann *et al.* 2011, 2013).

Many ciliates are considered microaerophilic (optimal growth at relatively low levels of oxygen) (Lynn 2008; Fenchel 2014). Ciliates also have a chemosensory behaviour which orientates the cells in the direction of their preferable O_2 levels (Fenchel & Bernard 1996; Fenchel 2014). *Chilodonella* spp. epizootics rapidly develop in the presence of low levels of dissolved oxygen (Langdon *et al.* 1985; Garcia *et al.* 2009; Fenchel 2014). Indeed, Dopheide *et al.* (2011) proposed that anaerobic bacteria present in anoxic conditions may be some of the most preferable food items for *Chilodonella* spp. High stocking densities in ponds may predispose fish to stress, as low levels of DO (dissolved oxygen) are common when ponds are overpopulated (Mitra & Haldar 2004; Padua *et al.* 2013; Bowater & O'Donoghue 2014). Although there is some indication of what conditions might be associated with chilodonellosis (low oxygen levels, constant temperature fluctuation and high levels of organic material in water/soil) (Fenchel & Bernard 1996; Bowater & O'Donoghue 2014; Fenchel 2014), it is clear that research is necessary to elucidate the combination of environmental parameters that facilitate rapid multiplication of *Chilodonella* spp..

Clinical presentations and pathology associated with *Chilodonella* spp. infections

Clinical signs associated with *Chilodonella* spp. infections are not unique, which makes initial diagnosis of chilodonellosis challenging (Noga 2010). Fish infected with *Chilodonella* spp. can exhibit gasping behaviour, anorexia, skin depigmentation, ulceration, scale loss, excessive mucus excretion and gill lesions (Padua *et al.* 2013). The clinical signs most commonly observed are a mottled/grey appearance on the skin (caused by excessive mucus production), lethargy, gill viscous mucus production, swimming slowly near the surface and edges, slim appearance (caused by appetite loss) and sometimes gill lesions and scale loss (Read 2007; Padua *et al.* 2013; Bradley *et al.* 2014). Examination of moribund fish is necessary to avoid misdiagnosis. In general, intense parasite infections are accompanied by acute gill lesions sufficient to kill affected fish (Langdon *et al.* 1985; Padua *et al.* 2013; Bowater & O'Donoghue 2014). Fish infected with few cells usually do not exhibit clinical signs of illness (Read 2007). However, *Chilodonella* cells can rapidly multiply (population numbers can double in only a few hours; pers. obs.; Read 2007; Padua *et al.* 2013).

Infestations of *Chilodonella* spp. cause gill epithelial hypertrophy, hyperplasia and are generally observed with complete or partial lamellar fusion followed by lymphocytic infiltration. Necrosis and some mild oedema can also be observed (Paperna & Van As 1983; Padua *et al.* 2013; Bowater & O'Donoghue 2014; Bradley *et al.* 2014). Fish skin usually demonstrates a non-specific lymphocytic dermatitis (Padua *et al.* 2013; Bowater & O'Donoghue 2014; Bradley *et al.* 2014).

Diagnostic methods for detecting *Chilodonella* spp. in wild and aquaculture fishes

The lack of host specificity, cosmopolitan distribution and pervasiveness of species in *Chilodonella* compromises accurate diagnosis of species that cause severe disease (Urawa & Yamao 1992; Urawa 1996; Mitra & Haldar 2004; Nikolic *et al.*

2006; Jorgensen, Larsen & Buchmann 2009; Gao *et al.* 2012). Generally, *Chilodonella* species are considered free-living ciliate protozoans. However, it is unknown whether species considered 'free-living' can also occupy a parasitic lifestyle given optimal conditions and opportunity (Noga 2010). Moreover, it is poorly understood how many *Chilodonella* species elicit harmful pathology to their fish hosts in favourable conditions (Lom & Dyková 1992; Noga 2010).

Morphological identification of *Chilodonella* spp. is largely based on cell shape and oral ciliature. Species are largely characterized or distinguished by the number of kinetic bands (cilia rows; Fig. 1) along each side of the cell (Kazubski & Migala 1974; Padua *et al.* 2013; Bowater & O'Donoghue 2014; Bradley *et al.* 2014). Gill and mucus smears are typically impregnated with Klein's silver stain (Padua *et al.* 2013; Bowater & O'Donoghue 2014; Bradley *et al.* 2014) or Giemsa which stains micro- and macronuclei (Padua *et al.* 2013). Morphological characters for identification of *Chilodonella piscicola* and *C. hexasticha* are shown in Table 2.

Chilodonella is considered to comprise a cryptic species complex, as discordance between morphology and genetic identification is common (Lahr *et al.* 2014). Many strains of the morphospecies (species distinguished based on morphology alone) of *C. uncinata* have genetic dissimilarity ranging from 2.2% to 13.5% in mitochondrial SSU rDNA and protein-coding loci (e.g. β-tubulin P3 locus) (Katz *et al.* 2011). Cryptic species can also be characterized based on discordance between morphology and mating behaviour of ciliates as a result of their reproductive style (conjugation) (Hall & Katz 2011; Katz *et al.* 2011).

Nucleic acid detection techniques have been implemented in the last decade to identify important pathogens affecting farmed fish (Gasser 1999; Cunningham 2002); however, DNA diagnosis of parasitic fish ciliates has not been implemented as a routine diagnostic method in the aquaculture industry. Nucleic acid detection has the advantage of determining specific *Chilodonella* species infecting fish, even when fish do not exhibit clinical signs of disease (Cunningham 2002; Stead & Laird 2002). Indeed, DNA-based diagnostics have great potential to replace protein-based detection methods in the future (McKeever & Rege 1999; Cunningham 2002).

Combining traditional methods with sensitive DNA-specific genetic tools could enable early identification of opportunistic parasitic ciliate species such as *Chilodonella* spp. Scuticociliates (subclass of ciliates from Oligohymenophorea class) such as *Pseudocohnilembus persalinus* had been considered free-living ciliates found in marine environments and traditionally identified based on morphological characters (Zhan *et al.* 2014). Recent research has demonstrated these ciliates are emerging opportunistic pathogens for cultured aquatic animals (Jones, Prosperi-Porta & LaPatra 2010; Zhan *et al.* 2014). Although morphological techniques are routinely used to identify scuticociliates, this methodology alone may have limitations as many scuticociliates species can exhibit similar morphology. Using silver staining techniques to identify species can be time-consuming and there are a limited number of experts in protistan taxonomy. Ideally, species identification should combine morphological and molecular techniques (Zhan *et al.* 2014).

Table 2 Morphological characters for differentiation of parasitic *Chilodonella* species

Morphometric characteristics	*Chilodonella hexasticha*[a]	*Chilodonella piscicola*[a] (syn. *C. cyprini*)
Cell body shape	Round	Heart-shaped; posterior notch
Cell body length	30–65 μm	30–80 μm
Cell body Width	20–50 μm	20–60 μm
No. of kinetic bands on short (left) row	6–8	9–15
No. of kinetic bands on long curved (right) row	5–7	8–13

[a]Lom & Dyková (1992), Mitra & Haldar (2004), Noga (2010), Padua *et al.* (2013).

Management and treatment methods

Treatment for chilodonellosis is still based on limited evidence (Read 2007; Noga 2010; Loh & Landos 2011; Bowater & O'Donoghue 2014; Bradley *et al.* 2014). Treatment largely involves using drugs to kill parasites attached to fish (Table 3); however, implementation of efficient biosecurity measures demonstrates better results than chemical treatments (Bowater & O'Donoghue 2014). Traditionally, infected fish are treated separately in tanks containing chemicals (e.g. formalin, NaCl; Ashburner & Ehl 1973; Read 2007;

Bradley *et al.* 2014). Even though these treatments can be used temporarily to reduce infection, they are not highly efficacious (Noga 2010; Bowater & O'Donoghue 2014; Bradley *et al.* 2014). Treating sick fish using only osmotic saltwater baths and replacing them into ponds or cages is not effective (Read 2007; Bradley *et al.* 2014). Once fish are returned to untreated pond water, which contains *Chilodonella* cells, re-infection is unavoidable. Moreover, fish are likely to be immunocompromised by being sick and stressed from manual handling.

Most common chemicals used to treat fish infected with *Chilodonella* spp. include formalin (formaldehyde solution) and potassium permanganate. Formalin has bactericidal properties, but its efficacy has never been studied specifically against chilodonellosis. Limited information exists about formalin bioaccumulation in fish and the potential implications for human health are unknown (Boyd & Tucker 1998; Boyd & Massaut 1999; Wooster *et al.* 2005).

Potassium permanganate ($KMnO_4$) is another chemical also used to combat *Chilodonella* spp. infections. $KMnO_4$ can cause serious corrosion to fish when in contact with gills and skin and it is highly explosive when in direct contact with organic substances (Schlenk *et al.* 2000). This chemical oxidizes organic and inorganic substances and kills bacteria. Permanganate oxidizes existing organic material and other reduced substances transforming it into relatively non-toxic manganese dioxide. Considering most chilodonellosis events occur when organic material accumulates in ponds, $KMnO_4$ use in aquaculture farms must be well monitored (Boyd & Massaut 1999; Schlenk *et al.* 2000). The chemical can be toxic to phytoplankton and will reduce the production of dissolved oxygen by photosynthesis. Therefore, mortality of aquatic organisms is possible, including beneficial bacteria and ciliates but also parasitic ciliates (Tucker & Boyd 1977; Tucker 1989; Boyd & Massaut 1999; Schlenk *et al.* 2000).

The drug 35% PEROX-AID® has been the only form of hydrogen peroxide (H_2O_2) approved by the US Food and Drug Administration (FDA) to manage some diseases (e.g. saprolegniasis, bacterial gill disease and columnaris) of freshwater-reared fish (Yanong 2008). There have been limited clinical trials examining the efficacy of

Table 3 Common chemicals and dosages used by aquaculture industry[a] to treat *Chilodonella* spp. and other ciliate infections in ornamental and farmed fish

Chemical	Dosage rate for ornamental tanks	Duration/frequency of treatment	Dosage rate for ponds/cages	Duration/frequency of treatment	References
Hydrogen peroxide (H_2O_2) animals	200 ppm	30 min	250–500 ppm	1 day 24-h constant aeration	Yanong (2008)
Hydrogen peroxide (H_2O_2) 10% (extra oxygen supply for soil/water)	–	–	General dose 250–350 g $acre^{-1}$	Constant aeration and monitoring	Mostafa & Kumar (2012)
Formalin (CH_2O)	150 ppt	1 h Not recommended for young animals	20–30 ppm	4–5 days 24-h constant aeration	Read (2007), Noga (2010), Loh & Landos (2011)
Potassium permanganate ($KMnO_4$)	20 ppm	60 min	2–3 ppm	2–3 days 24-h constant aeration	Schlenk *et al.* (2000), Read (2007), Noga (2010), Loh & Landos (2011)
Copper sulphate[b] ($CuSO_4$)	0.15–0.20 ppm	Gradually over 2–3 days	0.3 ppm	1 day 24-h constant aeration	Read (2007), Noga (2010), Loh & Landos (2011)
Salt[c] (NaCl)	10 ppt	60 min Repeat next day	Non-applicable	Non-applicable	Read (2007), Noga (2010), Loh & Landos (2011)

[a]Most are unapproved and non-tested in food fish species.
[b]Pond alkalinity must be tested before using copper sulphate.
[c]Salt in ponds is not practical.

hydrogen peroxide against chilodonellosis. Most tests using H_2O_2 against ciliates focus on the damage to host species (e.g. fish). Nevertheless, hydrogen peroxide has been successfully used to treat *Chilodonella* spp. infections in Murray cod, *Maccullochella peelii* (see Bradley *et al.* 2014). Adding hydrogen peroxide to ponds leads to an increase in oxygen levels and could mitigate *Chilodonella* spp. growth and proliferation as chilodonellids grow well under low levels of oxygen (Langdon *et al.* 1985; Garcia *et al.* 2009; Fenchel 2014).

Copper sulphate ($CuSO_4$) is known in aquaculture for its antiparasitic and algaecide properties, although some countries have banned its use (Watson & Yanong 1989; Yanong 2008). Some Murray cod farms in Australia still use this chemical to combat chilodonellosis (Bradly *et al.* 2014). Treatment concentration needs to be calculated carefully to prevent toxicity to fish (Watson & Yanong 1989). When used for treating ponds containing large quantities of algae, copper sulphate can cause a drop in oxygen levels which could facilitate *Chilodonella* spp. replication. It has showed that prolonged use of $CuSO_4$ is not effective against chilodonellosis and may cause serious damage to fish gills and skin. Copper can also be toxic to the zooplankton (e.g. rotifers) and invertebrates (e.g. snails). Toxicity is even more problematic in low alkalinity waters. When pond water alkalinity is unknown, or if relying on zooplankton as a food source for young fish, this drug may not be the best option (Watson & Yanong 1989). Ideally, *Chilodonella* spp. infections should be prevented through reducing stocking density in ponds, frequent water quality monitoring, water exchange, weekly fish health examination and adequate feed rates (Read 2007; Noga 2010; Bowater & O'Donoghue 2014).

Future insights into *Chilodonella* spp. biology and epidemiology

Chilodonella spp. can be artificially cultured *in vitro* in controlled laboratory conditions, which enables experimental research examining parasite life cycles and new treatment options. Artificial cultures of some protozoan parasites *in vivo* and/or *in vitro* are exceptionally challenging (Crosbie *et al.* 2012; Pinheiro & Bols 2013) and most parasitic ciliates need the presence of their host to grow under artificial conditions (e.g. *Cryptocaryon irritans*). However, eliminating the necessity for maintaining infected animals greatly increases the feasibility of culturing parasitic ciliates *in vitro* and contributes to improved animal welfare (Crosbie *et al.* 2010, 2012). *Chilodonella uncinata* and other species in the genus can be cultured *in vitro* (Bellec & Katz 2012; Bellec *et al.* 2014). *Chilodonella* spp. grow well in the dark (room temperature, RT) using filtered and sterilised pond water containing a grain of rice for prolonged periods (Bellec *et al.* 2014). *Chilodonella uncinata* can also be maintained in a cereal wheat grass media inoculated with *Klebsiella* sp. with optimal growth between 25 and 30 °C (Lynn 2008). The ability to culture *Chilodonella* spp. can enable targeted research on biology and epidemiology which will advance scientific knowledge on environmental and host triggers that facilitate harmful outbreaks.

The future of *Chilodonella* spp. detection in aquaculture

The ability to pre-empt outbreaks of chilodonellosis would be a considerable advantage to industry. New technologies using sensitive DNA-specific genetic tools can enable early detection of ciliate parasites species affecting freshwater farmed fishes. Environmental DNA (eDNA) is a potential new monitoring tool of small amounts of genetic material present in water or soil (Ficetola *et al.* 2008). Novel eDNA techniques and technologies have the power to identify and quantify species of ciliate protozoans (using qPCR) present in a pond or tank aquaculture system (Bass *et al.* 2015). Commonly, fish do not present signs of disease unless stressed, even when parasites are present in the environment (Noga 2010). Monitoring water from ponds using eDNA would enable quantification of protozoan parasites, even when fish are not presenting clinical signs of infection. Quantifying the number of ciliate protozoans in water and correlating parasite species abundance with levels of oxygen and temperature could reveal important ecological patterns associated with *Chilodonella* spp. outbreaks. Determining the ecological parameters associated with epizootic events together with high numbers of ciliates in the water may facilitate early preventative parasite management (e.g. new feed strategies, treatment of the water before stocking ponds).

Another potential new diagnostic tool for aquaculture is LAMP (loop-mediated isothermal

amplification). LAMP is a novel nucleic acid detection method that can amplify the target species DNA in isothermal conditions (Notomi *et al.* 2000). This means simple equipment such as a heat block or a water bath can be used to diagnose presence/absence of pathogen DNA. This technique is ideal for rapid and practical field or 'on-farm' detection for potential pathogens in a system. Combining this detection technology with eDNA to monitor infections in aquaculture may facilitate the early detection and prophylactic treatment of parasites before epizootic outbreaks occur. LAMP also has the potential to assist aquaculture managers to monitor protozoan parasite fauna in the environment as a part of ongoing health surveillance programme.

The future of a sustainable fish aquaculture industry relies on the ability to mitigate the impact of disease on fish production. Research on the complex interaction between *Chilodonella* spp., the environment and fish host is necessary to better understand infection dynamics. Molecular characterization for the identification of *Chilodonella* species present in fish farms, new diagnostic techniques, treatments and monitoring tools must be explored to promote cost-effective management of parasitic *Chilodonella* spp. on freshwater fish aquaculture farms.

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