Ecology and detection of harmful freshwater fish ciliate parasites *Chilodonella* spp. in aquaculture

Thesis submitted by

Giana Bastos Gomes

August, 2017

For the degree of Doctor of Philosophy

in the School of Marine and Aquaculture Sciences

James Cook University

Statement of animal ethics:

Experiments within this research were performed in conformity to JCU Animal Ethics Approval Number A2065.

Statement on the contributions of others:

At the time of the submission of this thesis Chapters 2, 3 and 4 were published in peer-reviewed international journals. Chapter 5 is in preparation for publication in peer-reviewed international journal. I am the lead author of all three articles and the unpublished Chapter derived from this PhD thesis. I acknowledge the specific contribution of co-authorship to these articles:

Chapter	Details of publication(s) on which chapter is based	Nature and extent of the intellectual input of each author and their affiliations
2	**Bastos Gomes, G.[1]**, Miller, T.L. [1,2], Jerry, D.R.[1], Hutson K.S.[1], **2016**. Impacts and current status of parasitic *Chilodonella* spp. (Phyllopharyngea: Chilodonellidae) in freshwater fish aquaculture. Journal of Fish Diseases, 40(5): 703-715. DOI: 10.1111/jfd.12523	**Bastos Gomes, G.[1]** Project conception, writing and editing. **Miller, T.L.[1,2], Jerry, D.R.[1], Hutson K.S.[1]** Research supervision, editing and funding.
3	**Bastos Gomes, G.[1]**, Miller, T.L.[1,2], Vaughan, D.B. [1], Jerry, D.R.[1], McCowan, C.[3], Bradley, T.L.[4], Hutson K.S.[1], **2017**. Evidence of multiple species of *Chilodonella* (Protozoa, Ciliophora) infecting Australian farmed freshwater fishes. Veterinary Parasitology, 237, 8-16. DOI: http://doi.org/10.1016/j.vetpar.2017.03.004	**Bastos Gomes, G.[1]** Execution of project, writing and editing. **Miller, T.L.[1,2]** Research supervision, data analysis, editing and funding. **Vaughan, D.B.[1]** Execution of project, editing. **Jerry, D.R.[1]** Research supervision and editing. **McCowan, C. [3], Bradley, T.L. [4]** Assistance providing samples and editing. **Hutson K.S.[1]** Project conception, research supervision, editing and funding.
4	**Bastos Gomes, G.[1]**, Hutson K.S.[1], Domingos, J.A.[1,5], Chung, C.[6], Hayward, S.[6], Miller, T.L.[1,2], Jerry, D.R.[1], **2017**. Use of environmental DNA (eDNA) and water quality data as a management tool to predict protozoan parasites outbreaks in fish farms. Aquaculture. DOI: https://doi.org/10.1016/j.aquaculture.2017.06.021	**Bastos Gomes, G.[1]** Project conception, execution of project, writing and editing. **Hutson K.S.[1]** Research supervision, editing and funding. **Domingos, J.A.[1]** Data analysis, editing. **Chung, C.[6], Hayward, S.[6]** Assistance providing samples and editing. **Miller, T.L.[1,2]** Research supervision, editing and funding. **Jerry D.R.[1]** Research supervision, editing.

5	**Bastos Gomes, G.[1]**, Hutson K.S.[1], Domingos, J.A.[1,5], Huerlimann, R.[1], Miller, T.L.[1,2], Jerry, D.R.[1]. Interactions between parasitic protozoan *Chilodonella* spp. and bacterial communities within a tropical aquaculture system. (*In preparation*) Aquaculture Environment Interactions.	**Bastos Gomes, G.[1]** Project conception, execution of project, writing and editing. **Hutson K.S.[1]** Research supervision, editing and funding. **Domingos, J.A.[1,5]** Data analysis, editing. **Huerlimann, R.[1]** Data analysis, editing. **Miller, T.L.[1,2]** Research supervision, editing and funding. **Jerry D.R.[1]** Research supervision, editing.

Author's affiliations:

1 Marine and Aquaculture Sciences, College of Science and Engineering and Centre for Sustainable Tropical Fisheries and Aquaculture, James Cook University, Townsville, 4811, QLD, Australia

2 Fish Health Laboratory, Department of Fisheries Western Australia, South Perth 6151, WA, Australia

3 Department of Economic Development, Jobs, Transport and Resources, 5 Ring Road Bundoora, 3083, VIC Australia

4 Department of Economic Development, Jobs, Transport and Resources, Chief Veterinary Officer's Unit, 475 Mickleham Road, Attwood, 3049, VIC Australia

5 Department of Agriculture and Fisheries, Agri-Science Queensland, Bribie Island Research Centre, Woorim 4507, QLD, Australia.

6 Sealord King Reef, 188 Cowley Creek Road Cowley, 4871, QLD, Australia.

Acknowledgements

I am sincerely thankful to my supervisor Dr Kate Hutson for the immense support and encouragement during my candidature. Each and every piece of advice given to me by Kate during my struggles were an incentive to continue my journey. Kate provided me scientific guidance and writing skills that were crucial for the completion of this thesis. I also learned with Kate the importance of professional ethics in the workplace and the skills necessary to be a great team leader. Kate has been an important role model for my professional career. But most importantly, thank you Kate for being a good friend.

I am also grateful to my co-supervisor Prof. Dean Jerry for his teachings on career development and the importance of having an open mind to innovation. Dean gave me many work opportunities before my PhD which were essential to develop my self-confidence to embark into this journey. Thank you Dean for every single opportunity you offered me and for the friendship we developed during the last eight years. I am also very grateful to my co-supervisor Dr Terry Miller for his scientific guidance, field trip support and friendship during my PhD.

I thank all my colleagues in the Marine Parasitology Laboratory, especially David B. Vaughan, Alejandro Trujillo Gonzalez and Alex Brazenor for their help and support in different aspects of my PhD. My gratitude goes also to my colleagues in the Molecular Ecology and Evolution Laboratory, especially Tansyn Noble and Julie Goldsbury for their support in different phases of my PhD. I'm very grateful to Scott Hayward and Catherine Chung from Sealord King Reef barramundi farm for the research partnership which enabled the experiments of this thesis to be performed using real data from a large-scale commercial fish farm. I am very thankful to James Cook University for offering me all the resources necessary to develop my

research. I am eternally grateful to James Cook University for supporting my PhD stipend scholarship and my candidature.

I am immensely grateful to my dog Maya and my cat Baby, who stood by my side, without judgement, during each difficulty I encountered. Finally, I would like to express my deepest gratitude to my husband, Jose, who always believed in me more than myself. Jose, thank you for your immense patience and respect. Ze, thank you, with all my love, for giving me unconditional emotional, financial and technical support.

Abstract

Freshwater fish farming contributes to more than two thirds of global aquaculture production. This rapidly growing agribusiness can be seriously compromised by diseases which can constrain industry development and profitability. In fact, disease problems account for forty percent of global aquaculture production loss. Parasitic ciliates are one of the largest causes of production loss in aquaculture and evidence suggests that they serve as the first portal of entry for many secondary infections in farmed fishes. Ciliate protozoan species of the genus *Chilodonella* are problematic and ubiquitous in global freshwater aquaculture. While *Chilodonella* spp. are important components of the 'free-living' fauna of freshwater ecosystems, some species have been implicated in outbreaks resulting in fish mortalities, particularly in high density aquaculture. A comprehensive review on the impacts and current status of parasitic *Chilodonella* spp. in freshwater fish aquaculture is presented in Chapter 2.

Limited information exists on the ecology, infection dynamics and potential triggers leading to outbreaks of *Chilodonella* spp. in fish production. To assist with preventative management practices and sustainability of freshwater fish aquaculture, this research addressed key knowledge gaps related to the biology, ecology, geographic distribution, and identity of harmful *Chilodonella* spp. in Australia and examined new non-invasive diagnostic methods. Collectively, the research reported in this thesis provides fundamental baseline understanding of *Chilodonella* spp. ecology and their detection in fish aquaculture.

While *Chilodonella piscicola* ((Zacharias, 1894; syn. *C. cyprini* (see Moroff, 1902)) and *Chilodonella hexasticha* (Kiernik, 1909) have been reported to globally infect wild and farmed fishes, the diversity of *Chilodonella* spp. parasitising Australian freshwater farmed fishes was

unknown prior to this study. *Chilodonella* cells were isolated from infected barramundi, *Lates calcarifer* (Bloch, 1790) and Murray cod, *Maccullochella peelii* (Mitchell, 1838) from fish farms in tropical north Queensland (QLD), temperate Victoria (VIC) and New South Wales (NSW) for genetic and morphological analysis (Chapter 3). Parasites were stained and measured for morphological description and comparative phylogenetic analyses were performed using three markers: ITS1-5.8S-ITS2, small subunit (SSU) rRNA and the mitochondrial small subunit (mtSSU) rDNA marker. However, only mtSSU rDNA revealed significant genetic variation between *Chilodonella* species. Morphological analyses revealed four distinct morphotypes of *Chilodonella* infecting farmed barramundi and Murray cod. However, phylogenetic analyses detected only three distinct genotypes, with the morphotypes putatively identified as *C. hexasticha* and *C. piscicola* sharing 100% sequence identity. This suggests that Australian isolates identified as *C. hexasticha* or *C. piscicola* likely represent the same species and exhibit marked phenotypic plasticity.

Ciliate protozoans cause rapid epidemic events with little or no warning, resulting in mortalities and considerable economic losses in affected farms. Changes in environmental conditions (temperature, sunlight, oxygen levels and pH) have been associated with changes in ciliate population numbers as these conditions may affect life cycles, reproduction, and their ability to obtain food from the environment. To identify and treat protozoan infections prior to harmful outbreaks, novel methods to determine which combination of environmental factors can trigger outbreaks were applied. Environmental DNA (eDNA) is a technique suited to rapidly assess the background presence of pathogens in water in fish farms, thereby providing managers with critical information on pathogens' threshold, which can be used to mitigate disease threats. In Chapter 4, the ciliate protozoan *Chilodonella hexasticha* was used as a model to examine the relationship between environmental DNA of *C. hexasticha*, critical water

parameters and the occurrence of disease outbreaks on a commercial barramundi farm. Production pond water was sampled monthly over a one-year timeframe and a qPCR assay based on the SSU-rDNA gene was used to monitor the abundance (SSU-rDNA copies/µl) of *C. hexasticha*. Increased *C. hexasticha* eDNA levels correlated with occurrence of subsequent fish mortality events ($r = 0.402$; $P < 0.001$), with smaller fish more prone to epidemics ($r = -0.189$; $P < 0.05$). However, there was no correlation between water quality parameters (rainfall, water temperature and dissolved oxygen) and parasite abundance, although there were significantly more fish mortalities observed during the warmer, wetter monsoonal season compared to the cooler, dry season (1,280 vs. 135 total fish mortalities, respectively; $P < 0.05$). These findings demonstrate that eDNA based techniques have the potential to be used on farms as a management tool to rapidly assess parasite loads in water and minimize the risk of disease outbreaks in aquaculture systems.

The management of ciliate protozoans in aquaculture ponds is complex. Ciliates naturally consume bacteria, algae and other organic or cellular material (e.g. decomposing eukaryote cells) and constantly interact with other microorganisms in the aquatic environment. The association and interaction among different microorganisms in finfish aquaculture systems are rarely explored. In Chapter 5 the microbiome profile from ponds and barramundi gills were characterised and the relationship of this fauna with *C. hexasticha* abundance and fish mortalities were explored. Water samples from four barramundi ponds with a history of *C. hexasticha* fish infections and twenty infected fish were collected from October 2013 to September 2014. Presence of *C. hexasticha* infecting fish gills was confirmed by microscopic and morphological analyses. The abundance of *C. hexasticha* in water samples was estimated by SSU-rDNA qPCR and the relative abundance of bacterial communities from water and gills was estimated by deep amplicon sequencing of the V3 region of the 16S rRNA gene.

Additionally, fish mortalities were also monitored daily on the farm. *Flavobacterium columnare* was significantly ($P < 0.05$) associated with *C. hexasticha* abundance in water (eDNA) and fish mortalities. However, *Bdellovibrio bacteriovorus* was the most significant bacteria correlated ($P < 0.05$) to eDNA. This study demonstrated, for the first time, a possible link between infections caused by the ciliate *C. hexasticha*, fish mortalities and the pathogenic *F. columnare* in an aquaculture environment.

In summary, this research advanced knowledge on the morphology and genetic characteristics of *Chilodonella* spp. in Australian freshwater fish farming, revealed the applicability of eDNA techniques to predict fish mortalities in aquaculture and indicated the potential association of *Chilodonella* spp. outbreaks with bacterial communities on fish farms.

Table of Contents

List of Figures

List of Tables

Chapter 1. General Introduction

1.1 Food security and aquaculture

Food security for the expanding global human population is one of the biggest challenges of humanity. Aquaculture, the farming of aquatic plants and animals, is considered the fastest growing food-production sector in the world (FAO, 2014; FAO, 2016). This industry supplies one third of global fish production for human consumption which represents 73.8 million tonnes of nutritional protein with an estimated value of US$ 160.2 billion (FAO, 2016). Among the species farmed globally, farming of finfish accounts for 67.5 % (US$ 99.2 billion) of value, molluscs 21.8% (US$ 19 billion), crustaceans 9.3% (US$ 36.2 billion), while other aquatic animals account for 9.9% (US$ 3.7 billion). In 2014, the world consumption of fish was an average of 20 kg per person (FAO, 2016).

Freshwater finfish produced in earthen ponds offer an important contribution for food security in developing countries, accounting for 87.4% of global fish production for human consumption (FAO, 2016). Therefore, aquaculture has the potential to supply high quality protein for billions of people, especially with rapid advances in technology and knowledge within this sector (Lehane, 2013; FAO, 2014). However, to ensure sustainable growth, this industry has to address important production issues including disease characterisation and management.

1.2 Diseases in aquaculture

Diseases are recognised as the most limiting factor for both social and economic development of aquaculture (Subasinghe et al., 2001; FAO, 2014). It is estimated that 40% of aquaculture production (64 million tonnes with estimated value of US$ 102 billion) is lost due

to diseases (FAO, 2012). Economic loss due to diseases constrains the continued growth and profitability of the sector. The impacts of disease to the aquaculture sector can be catastrophic. Economic losses caused by diseases are consequence of a series of direct and indirect factors including cost of pharmaceutical and chemical treatments, poor food conversion, slow growth, additional labour and low marketability (Shinn et al., 2015). Furthermore, infectious diseases cause serious pathological tissue changes in infected animals, which impact animal welfare, promote mortality and threaten the sustainability of the aquaculture industry (Buchmann, 2013; Shinn et al., 2015, FAO, 2016).

Infectious disease ecology in aquaculture systems involves a complex interaction between the host, the pathogen and the environment. Whenever an unbalance in this interaction occurs, illness is manifested (Oidtmann et al., 2011b; Oidtmann et al., 2013). Isolating one of these factors and trying to identify cause and consequence of problems in aquaculture farms is usually not efficient (Peeler and Taylor, 2011; Oidtmann et al., 2013). For example, aquatic animals are poikilothermic ("cold-blooded") so their body temperature is similar to the water temperature. Thus immune system activity changes depending on water temperatures; low temperatures may slow down host immune response to pathogens and may be negatively affected when animals are stressed due to high temperatures (Bowden et al., 2007). In addition, an aquatic animal's immune system is compromised when dramatic fluctuations in water temperature occur (e.g. during the wet season in the tropics (Watts et al., 2001; Bowden et al., 2007; Oidtmann et al., 2013). Therefore, regular monitoring of the health of fish and water quality parameters on aquaculture farms are essential for proactive disease surveillance. However, despite technological advances and increases in aquaculture production, data management interpretation, diagnosis and control of disease remain important research areas for aquaculture (Rimmer, 2006; Peeler and Taylor, 2011; FAO, 2012; Oidtmann et al., 2013).

1.3 Disease surveillance and sources of infection

The success of aquaculture industries rely greatly on strict biosecurity measures. Aquaculture biosecurity refers to all integrated activities and measures to prevent the entrance and spread of pathogens in the culture system. While aquaculture has rapidly expanded worldwide, appropriate measures to avoid introduction and spread of pathogens into culture systems has become more difficult. The movement of aquatic species from one region to another has promoted aquaculture growth. However, the movement of live aquatic species without appropiate biosecurity and quarantine has accelerated the spread of pathogens within the aquaculture industry (Subasinghe and Bondad-Reantaso, 2006).

Disease prevention and management requires adequate surveillance systems in place (Groner et al., 2016). Disease surveillance in aquaculture involves a systematic collection, collation, and analysis of data related to animal health and culture parameters to recommend prevention and management of disease issues when they arise (Häsler et al., 2011; Peeler and Taylor, 2011). Pathogen surveillance is essential for successful and sustainable production as it provides information for decision making by aquaculture farmers. Population size, rearing system adopted and poor visibility (i.e. the fact that animals live under the water) are common problems which can affect the success of surveys (Oidtmann et al., 2011b; Oidtmann et al., 2013). However, accurate and rapid detection of pathogens affecting aquatic animals is fundamental to ensure biosecurity, trade, management, animal health and welfare. Furthermore, a better understanding of environmental data related to outbreaks is essential for implementing pre-emptive management strategies (Peeler and Taylor, 2011; Groner et al., 2016). When environmental triggers (e.g. low dissolved oxygen and high or low water temperatures) that may favour the outbreak of a specific pathogen are identified early, appropriate biosecurity and integrated management strategies can be implemented to reduce the impact of an event.

Introduction of new pathogens can be avoided (e.g. through quarantine for new animals introduced in the farm; restricting movement of animals between ponds) and control of pathogenic species already present in the culture system by managing environmental conditions appropriately (Oidtmann et al., 2011a; Groner et al., 2016). When disease surveillance is integrated with detailed monitoring of environmental conditions it is possible to accelerate the response of farmers to disease risks (Darling and Mahon, 2011; Häsler et al. 2011; Oidtmann et al. 2013; Groner et al., 2016).

1.4 Finfish aquaculture and susceptibility to diseases

Farmed freshwater fishes comprise the largest section of the global aquaculture sector (FAO, 2016). With the increase of freshwater fish farms worldwide there is increased risk of emergent disease within these aquaculture systems (Oidtmann et al., 2011b; Oidtmann et al., 2013; FAO, 2016). One of the most common pathways for pathogen introduction in freshwater fish farms is through sub-clinically (no signs of infection) infected animals being bought into the farming system. The aquaculture environment and farming conditions (e.g. high fish density in ponds) then further contribute to the spread of pathogens and increase stress on animals, providing ideal conditions for pathogen establishment. Furthermore, inland farms generally use untreated water from rivers or lakes adjacent to farm locations to stock ponds. This water source may contain harmful pathogens that occur naturally in wild fish populations adjacent to farms. Water treatment before stocking ponds can be expensive on a commercial scale and in many cases it is not practical (Oidtmann et al., 2011b). However, farms could take additional steps to monitor the presence of pathogenic species in pond systems to avoid outbreaks. However, there are currently no reliable methods to accurately detect and quantify specific pathogens, in particular protozoan parasites, present in farm ponds before they infect fish.

1.5 Ciliate protozoans and their interactions in aquaculture

Ciliates (Phylum Ciliophora; Doflein, 1901) are unicellular organisms characterised by the presence (at least in one stage of their life) of cilia (hair-like organelles) on the pellicle covering the cell. The cilia are used for swimming, crawling, attachment, feeding, and sensation (variable with species). Feeding occurs using elaborated buccal structures. Ciliates have nuclear dualism (generative diploid micronuclei and vegetative polyploid macronuclei; Lom and Dyková, 1992). They use conjugation as the sexual process and division (in most cases) by transverse binary fission. Most ciliates are free-living organisms found in water and soil, but they can develop parasitic or symbiotic relationships with animal hosts (Lom and Dyková, 1992).

Under ideal conditions, harmful ciliate populations may rapidly increase and result in considerable disease epidemics with mortalities and economic losses in affected farms. Changes in environmental conditions (temperature, sunlight, dissolved oxygen and pH) have been associated with changes in ciliate population numbers as these conditions may affect their life cycles, reproduction and ability to obtain food from the environment (Shukla and Gupta, 2001). However, limited information exists on which environmental parameters may trigger epizootic events of ciliates in aquaculture systems. Different fish aquaculture systems and variable environmental conditions are relevant to fish infections caused by ciliates (Rintamäki et al., 1994; Shukla and Gupta, 2001). Some studies suggest pathogenic ciliates are opportunistic and only cause disease and mortalities when fish are affected by stressors (Lom and Nigrelli, 1970; Egusa, 1983; Urawa and Yamao, 1992). Nevertheless, diseased fish impact aquaculture by increasing costs of production and causing propagation of pathogens in the farm and associated wild environment (Segner et al., 2012).

Some of the challenges in detecting potentially harmful ciliate species includes their lack of host specificity, cosmopolitan distribution and in their ability to alternate between parasitic and free-living stages (Jorgensen et al., 2009; Mitra and Haldar, 2004; Nikolic et al., 2006; Urawa, 1996; Urawa and Yamao, 1992). Difficulty in detecting and treating ciliates prior to infections occurring inspired the need to explore novel ways to detect and prevent outbreaks. Parasitic and free-living freshwater ciliates present in farm ponds are traditionally differentiated by morphological characteristics. However, the use of morphology alone for species identification can be problematic (Mitra et al., 2013; Warren, 2013). Combining traditional methods with sensitive DNA-specific genetic tools would enable early identification of ciliate parasites species affecting farmed fish. This information could allow preemptive management of parasitic infections before causing economic loss to fish farmers.

Among ciliate protozoans impacting freshwater fishes, *Chilodonella* spp. in particular, are of particular concern. Mortalities often occur with little warning in pond-reared fishes as there are few initial symptoms of infection prior to an outbreak (Lom and Dyková, 1992; Bowater and O'Donoghue, 2014). Most *Chilodonella* species are free-living; however, some species can promote disease in farmed fishes and cause economic loss (Padua et al., 2013; Bowater and O'Donoghue, 2014). Two *Chilodonella* species are considered to be pathogenic to freshwater fishes: *Chilodonella piscicola* [syn. *C. cyprini* (Moroff, 1902)] and *C. hexasticha* (Kiernik, 1909). *Chilodonella* spp. outbreaks occur globally and negatively affect fish aquaculture including the Australian finfish industry.

Ciliates and their association with other microorganisms present in freshwater aquaculture systems is not well understood. The biological complexity within aquaculture systems enables constant interactions between different microorganisms (Zhang et al., 2014). Considering *Chilodonella* spp. feeding behavior and survival strategies (opportunism), their

interaction with the bacterial community in the aquatic environment is crucial for understanding potential triggers of outbreaks. The interaction between bacteria and protozoans that feed on them are known to influence bacterial virulence (positively or negatively) and directly affect disease dynamics particularly in aquatic environments (Matz et al., 2004; Adiba et al., 2010; Zhang et al., 2014). Understanding which bacteria species are associated with *Chilodonella* spp. outbreaks may reveal unknown dynamics underpinning chilodonellosis (infections caused by *Chilodonella* spp.) in freshwater fish farms.

1.6 Parasite diagnosis in aquaculture

Accurate species-level diagnostics is critical for the development of effective management strategies targeting pathogens. Accurate diagnostics are particularly important when dealing with protozoan, myxozoan, or platyhelminth parasites, which can be morphologically similar among species, but have distinctive pathological impacts on their host (Miller et al., 2010a). Successful management of parasite threats in any animal production system is based on correct recognition and quantification of parasite species present in the culture system to understand the potential risk and contribution of each species into reducing production (Subasinghe and Bondad-Reantaso, 2006; Oidtmann et al., 2013). Early identification of parasites using reliable analytical and non-lethal techniques is essential to ensure the sustainability of fish aquaculture (Scholz, 1999).

DNA-based technologies have emerged to minimise the impacts of diseases in aquaculture. Many of these technologies can help to control the spread of pathogens in the culture system as they offer quick results with high specificity and sensitivity (Walker and Subasinghe, 2000). Advances in DNA technologies have improved the quality of assays and consequently reduced costs associated with diagnostics (Cunningham, 2002; Bott et al., 2010). While post-mortem necropsy and histopathology are primary techniques used for diagnostics

of diseases in aquaculture, these methods are time consuming and generally require follow-up DNA sequencing to confirm pathogen identity. Many aquaculture farms are usually located in remote regions, thus diagnostic tests need to be fast, reliable and highly sensitive. Appropriately designed DNA-based diagnostic tools may be able to aid in on-farm detection and surveillance. Speed in obtaining accurate results and sensitivity of assays are especially important when dealing with massive mortalities, or when monitoring animals without clinical sign of infections, respectively (Bott et al., 2010). Independent of the kind of DNA-based technology used, on-farm diagnostic tools aim to advance the ability of the aquaculture industry to prevent, rather than react, to pathogens.

1.7 Thesis aims and structure

This research aims to address fundamental knowledge gaps related to the genetic characteristics, environmental and biological triggers of *Chilodonella* spp. impacting freshwater farmed barramundi in tropical Queensland. Ultimately, it was intended that these results could be used to improve the management and control of parasites in fish farms through the development of new molecular monitoring techniques for early detection and quantification of specific pathogenic *Chilodonella* spp. from aquaculture systems (research in progress as part of a Science and Innovation Award for Young People in Agriculture, Fisheries and Forestry awarded to the PhD candidate). Thesis structure of Chapters 2 to 5 is as follows:

Chapter 2 provides a comprehensive review of the current status of parasitic ciliates *Chilodonella* spp. (Phyllopharyngea: Chilodonellidae) in freshwater fish aquaculture globally. Specifically, the review examines the biology, ecology, host and geographic distribution of *Chilodonella* spp. infecting fish. Emphasis is placed on the impact of these ciliates in fish aquaculture including pathology, diagnostic methods and treatments. Chapter two was

published in the Journal of Fish Diseases (Bastos Gomes et al., 2016) and is presented herein with minor modifications (doi:10.1111/jfd.12523).

Chapter 3 reveals the existence of multiple species of *Chilodonella* infecting Australian farmed freshwater fishes. A combined morphology and molecular approach was used to characterise parasitic *Chilodonella* in Australia. This is the first empirical evidence that the two most notorious species in global fish culture, *Chilodonella hexasticha* (Kiernik, 1909) and *Chilodonella piscicola* (Zacharias 1894; syn. *C. cyprini* Moroff, 1902), are genetically similar. Morphological plasticity was demonstrated among *C. hexasticha* and *C. piscicola* and it was suggested that *C. hexasticha* and *C. piscicola* might be the same species. *Chilodonella acuta* was isolated from fish for the first time. Chapter 3 reinforced the importance of adopting a combined morphological and molecular approach for characterisation of protozoan species. Chapter 3 was published in Veterinary Parasitology (Bastos Gomes et al., 2017a) and is presented herein with minor modifications (doi:10.1016/j.vetpar.2017.03.004).

Chapter 4 evaluated the use of environmental DNA (eDNA) as a novel monitoring tool for detection of harmful ciliates in farmed freshwater fishes. A combined approach using abundance of parasite in water (eDNA), along with the environmental and stocking conditions on fish farms was used to predict fish mortalities and inform disease management strategies. Chapter 4 *de*monstrated that the incorporation of eDNA in ciliate surveys with routine water quality monitoring may contribute to the development of new preventive parasite management and control strategies in fish farms (e.g. increase in oxygen levels, stocking ponds with larger fish, increase water excahnge). *Chapter 4* was published in Aquaculture (Bastos Gomes et al., 2017b) and is presented herein with minor modifications (doi:10.1016/j.aquaculture.2017.06.021).

Chapter 5 characterised the bacterial community from a freshwater barramundi (*Lates calcarifer*) farm in tropical Australia and associated the microbial community with abundance of the *Chilodonella* spp. (assessed using eDNA). This chapter highlights that an increased understanding of the bacterial community linked to abundance of *C. hexasticha* in water, fish gills and mortality data is crucial for comprehension of disease dynamics in freshwater fish farms. Chapter 5 will be submitted to *Aquaculture Environment Interactions* shortly.

Chapter 2. Current status of parasitic ciliates *Chilodonella* spp. (Phyllopharyngea: Chilodonellidae) in freshwater fish aquaculture

---

2.1. 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 diseases 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 and Dyková, 1992) and can facilitate secondary infections (e.g. bacterial infections) in farmed fishes (Lom and 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 and 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 (e.g. Lom and Dyková, 1992; Rintamaki et al., 1994; Jee, Kim and Park, 1996; Nikolic and Simonovic, 1996; Rintam€aki-Kinnunen and Valtonen, 1997; Schisler et al., 1999; Evans and Lester, 2001; Nikolic et al., 2006; Mitra et al., 2013; Padua et al., 2013; Bowater and O'Donoghue, 2014). Infections caused by *Chilodonella* spp. have been documented for more than a century (Leibovitz 1980; Langdon et al. 1985; Lom and Dyková 1992), and in the last four decades, it

has attracted increasing research interest as the freshwater fish production industry expands (Fig. 1).

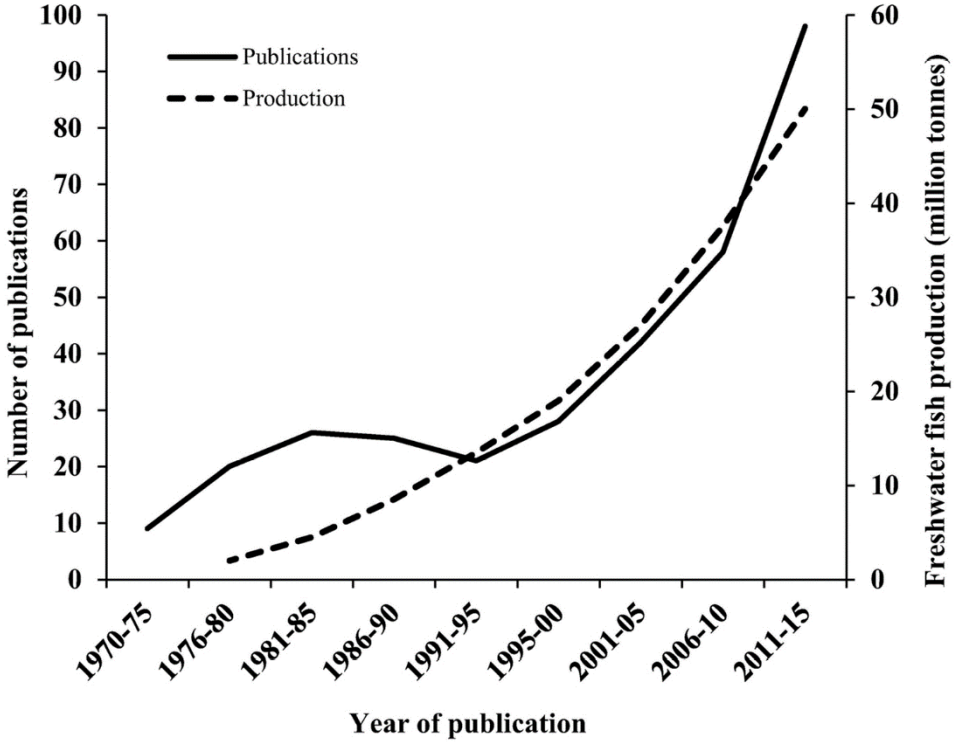

Figure 1 Number of publications on parasitic Chilodonella and global freshwater fish production (FAO 2014) by year. Data generated through Web of Science and Google Scholar based on search criteria 'Chilodonella AND fish' (search conducted in January 2016).

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 and Foissner, 1992; Dopheide et al., 2011). The cytostome directly penetrates fish epithelial cells, allowing uptake of the contents (Paperna and Van As, 1983; Wiles, Cone and 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 and 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 and Haldar, 2004; Mitra et al., 2013; Padua et al., 2013; Bowater and 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.

Table 1 Parasitic *Chilodonella* species found on 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 and O'Donoghue (2014)
	20-30	Wild	Morphology	*Nandus nandus*	India	Mitra and 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 et al. (2007)
	13-24	Farmed	Morphology	*Tilapia rendalli and Pseudocrenilabris*	Israel and South Africa	Paperna and Van As (1983)

***Chilodonella* species**	**Water temperature range (°C)**	**Fish origin**	**Parasite species identification by**	**Fish species**	**Location**	**Reference**
				Philander	India	Mitra et al. (2013)
	-	Wild	Morphology	*Labeo rohita* and		
				Cyprinus carpio	Japan	Imai et al. (1985)
	25-30	Ornamental	Morphology	*Symphysodon discus*	Korea	Jee et al. (1996)
	22	Wild	Morphology	*Carassius carassius*	Malaysia	Shariff (1984)
	-	Wild	Morphology	*Aristichthys nobilis*		
Chilodonella piscicola	5-10	Farmed	Morphology	*Ictalurus punctatus and*	Poland	Kazubski and Migala, (1974)
				Carassius auratus		
	17-27	Farmed	Morphology	*Maccullochella peelii*	Australia	Bradley et al. (2014)
	9-11	Farmed	Morphology	*Oncorhynchus masou*	Japan	Urawa and Yamao (1992)
	5-18	Farmed	Morphology	*Oncorhynchus mykiss*	Denmark	
	-	Ornamental	Morphology	*Carassius auratus*	Turkey	Jorgensen et al. (2009)
	-	Farmed	Morphology	*Salmo trutta*	Finland	Kayış et al. (2013)
	-	Ornamental	Morphology	*Paracheirodon innesi*	Australia	Valtonen and Koskivaara (1994)
						Evans and Lester (2001)

***Chilodonella* species**	**Water temperature range (°C)**	**Fish origin**	**Parasite species identification by**	**Fish species**	**Location**	**Reference**
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*	Finland	Rintamaki-Kinnunen and Valtonen (1997)
	5-22	Wild	Morphology	*Salmo trutta* m. *Lacustris*	Yugoslavia	Nikolic and Simonovic (1996)
	-	Farmed	Morphology	*Perca fluviatilis* and *Carassius auratus gibelio* *Oreochromis niloticus*	Saudi Arabia	Abdel-Baki et al. (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)

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 and Katz, 2001; Bellec and Katz, 2012). The macronucleus contains gene-sized chromosomes and has similar function to somatic nuclei of animals (Riley and Katz, 2001; Bellec and Katz, 2012; Zufall et al., 2012). The macronucleus formation occurs through a comprehensive rearrangement, including genome fragmentation, amplification and elimination of micronuclear limited sequences (Prescott, 1994; Juranek and Lipps, 2007; Bellec and 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 and Katz, 2001; Katz and Kovner, 2010; Bellec and 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 and 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 (Srping et al., 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 and Segal, 2009; Bellec and 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 and Katz, 2001; Bellec and Katz, 2012).

2.2. Life cycle

Chilodonella spp. primarily reproduce by transverse binary fission (Lynn, 2008; Bellec et al., 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 and Katz, 2001; Bellec and 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 ex-conjugant cells are formed. If these cells cannot conjugate, they undergo a period of senescence with death temporarily delayed by autogamy or self-fertilization (Hausmann and 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).

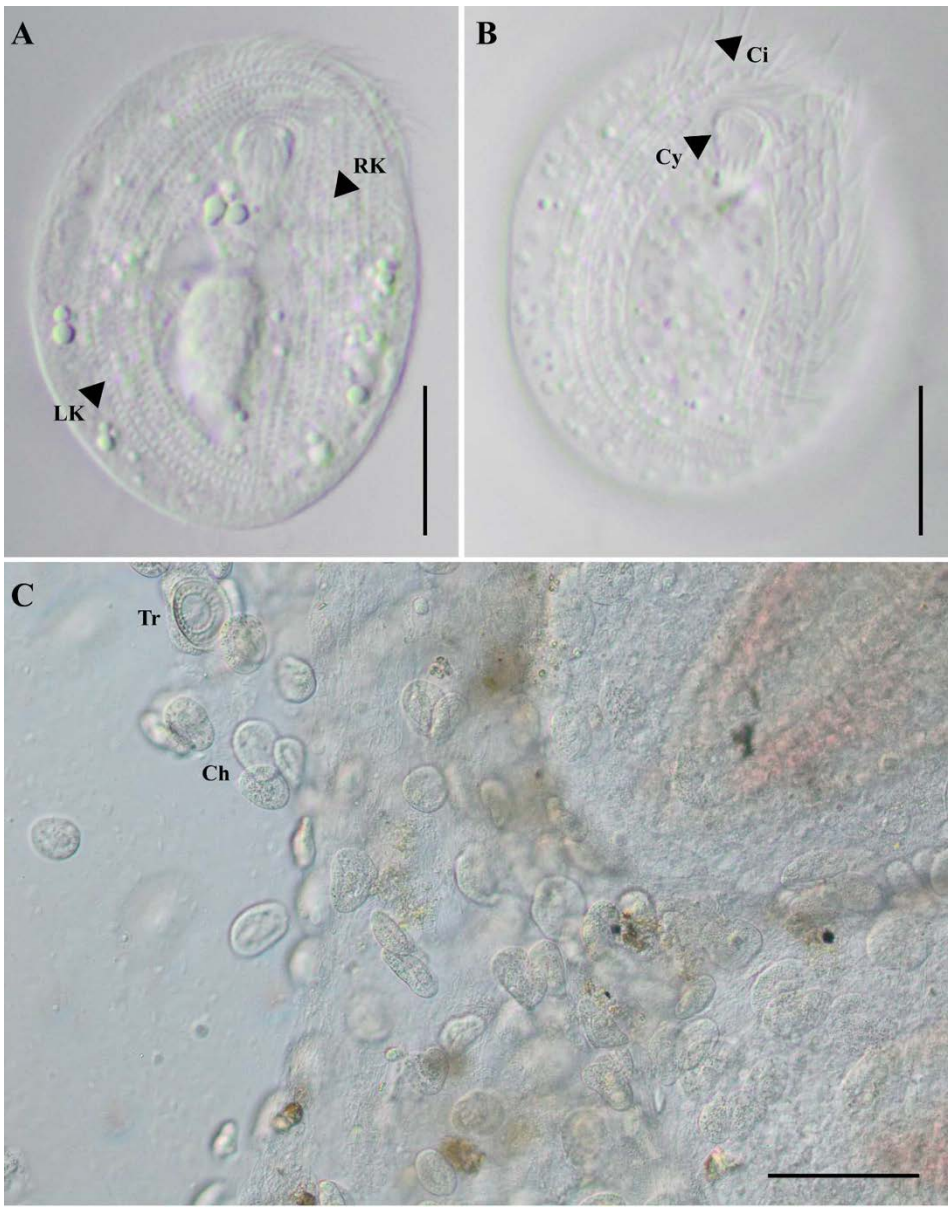

Figure 2 *Chilodonella hexasticha* from the gills of farmed barramundi, *Lates calcarifer*, in tropical north Queensland, Australia. A) LK: left kineties bands; RK: right kineties bands; B) Ci: cilia and Cy: morphology of the cytostome; A and B scale bars = 10µm; C) Infection on gills by Chilodonella sp. cells (Ch). Co-infection by Trichodina sp. (Tr); C scale bars = 100µm.

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.

2.3. *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 and 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 and Haldar, 2004; Mitra et al., 2013; Padua et al., 2013), while most reports of *C. uncinata* and *C. cucullulus* (Muller, 1976) are from collections 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 and Ehl, 1973; Mitra and 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 and 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 and 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 et al., 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., 2011a,b, 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 and Bernard, 1996; Fenchel, 2014). *Chilodonella* spp. epidemics 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 dissolved oxygen (DO) are common when ponds are overpopulated (Mitra and Haldar, 2004; Padua et al., 2013; Bowater and 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 and Bernard, 1996; Bowater and 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..

2.4. 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 and 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 and Van As, 1983; Padua et al., 2013; Bowater and O'Donoghue, 2014; Bradley et al., 2014). Fish skin usually demonstrates a non-specific lymphocytic dermatitis (Padua et al., 2013; Bowater and O'Donoghue, 2014; Bradley et al., 2014).

2.5. 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 and Yamao, 1992; Urawa, 1996; Mitra and Haldar, 2004; Nikolic et al., 2006; Jorgensen et al., 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 and 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 and Migala, 1974; Padua et al., 2013; Bowater and O'Donoghue, 2014; Bradley et al., 2014). Gill and mucus smears are typically impregnated with Klein's silver stain (Padua et al., 2013; Bowater and 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.

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

Morphometric characteristics	***Chilodonella hexasticha*†**	***Chilodonella piscicola*† (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
N° kinetic bands on short (left) row	6−8	9−15
N° kinetic bands on long curved (right) row	5−7	8−13

† (Lom and Dyková 1992; Mitra *et al.* 2004; Noga 2010; Padua *et al.* 2013).

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 and 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 and Laird, 2002). Indeed, DNA-based diagnostics have great potential to replace protein-based detection methods in the future (McKeever and 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 et al., 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).

2.6. Management and treatment methods

Treatment for chilodonellosis is still based on limited evidence (Read et al., 2007; Noga, 2010; Loh and Landos, 2011; Bowater and O'Donoghue, 2014; Bradley et al., 2014). Treatment for *Chilodonella* spp. infections 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 and O'Donoghue 2014). Traditionally infected fish are treated separately in tanks containing chemicals (e.g. formalin, NaCl; Ashburner and Ehl, 1973; Read et al., 2007; Bradley et al., 2014). Even though these treatments can be used temporarily to reduce infection, they are not highly efficacious (Noga, 2010; Bowater and 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 and Tucker, 1998; Boyd and 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 and 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 and Boyd, 1977; Tucker 1989; Boyd and Massaut, 1999; Schlenk et al., 2000).

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	200ppm	30min	250-500ppm	1 day 24hr constant aeration	Yanong (2008)
Hydrogen peroxide (H_2O_2) 10% (extra oxygen supply for soil / water)	-	-	General dose 250–350 gm/acre	Constant aeration and monitoring	Mostafa Shamsuzzaman and Kumar Biswas (2012)
Formalin (CH_2O)	150ppt	1hr Not recommended for young animals	20-30ppm	4-5 days 24h constant aeration	Read et al. (2007); Noga (2010); Loh and Landos (2011)
Potassium permanganate ($KMnO_4$)	20ppm	60min	2-3 ppm	2-3days 24hr constant aeration	Schlenk et al. (2000); Read et al. (2007); Noga (2010); Loh and Landos (2011)
Copper sulphate [b] ($CuSO_4$)	0.15-0.20ppm	Gradually over 2-3days	0.3 ppm	1 day 24hr constant aeration	Read et al. (2007); Noga (2010); Loh and Landos (2011)
Salt [c] (NaCl)	10ppt	60min Repeat next day	Non applicable	Non applicable	Read et al.*(*2007); Noga (2010); Loh and 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.

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 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 anti-parasitic and algaecide properties, although some countries have banned its use (Watson and 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 and 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 been shown that prolonged use of CuSO4 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 and 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 and O'Donoghue, 2014).

2.7. 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 and 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 and 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.

2.8. 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 program.

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.

Chapter 3. Evidence of multiple species of *Chilodonella* (Protozoa, Ciliophora) infecting Australian farmed freshwater fishes

---

3.1. Introduction

Parasitic ciliates are considered some of the most harmful parasites of cultured fishes (Lom and Dyková, 1992; Padua et al., 2013; Pinheiro and Bols, 2013). The genus *Chilodonella* (Phyllopharyngea: Chilodonellidae) contains many free-living ciliate species, but only two *Chilodonella piscicola* ((Zacharias 1894; syn. *C. cyprini* (see Moroff, 1902), and *Chilodonella hexasticha* (Kiernik, 1909) are reported to be opportunistic parasites on freshwater fishes (see Chapter 2; Bastos Gomes et al., 2016). Chilodonellosis (infections caused by *Chilodonella* spp.) may result in rapid epidemics on fish farms with mortalities within two or three days of infection and losses of 50−95% of fish stocks (Paperna and Van As, 1983; Karvonen et al., 2010). It is hypothesised that parasitic *Chilodonella* spp. are generally opportunistic and only cause disease and mortalities when fishes are affected by stressors (Lom and Nigrelli, 1970; Egusa, 1983; Urawa and Yamao, 1992). Changes in environmental conditions (temperature, sunlight, oxygen levels and pH) have been associated with changes in *Chilodonella* spp. numbers as these conditions impact reproduction, generation time and the ability to obtain food from the environment (Rintamäki et al., 1994; Shukla and Gupta, 2001).

Recently, it was proposed that some *Chilodonella* species may comprise cryptic species complexes given discordance between morphological descriptions and genetic analyses (Mitra et al., 2013; Warren, 2013). Cryptic species can be grouped by shared morphology, but advances in molecular studies have revealed divergence in genetic loci characterising multiple genetic and/or biological varieties (Blaxter, 2004; Finlay et al., 2006; Katz et al., 2011).

Chilodonella species have been traditionally distinguished based on the number of ciliary rows present in the left (longer cilia bands), or right (shorter cilia bands) kinety bands (cilia arranged closely in longitudinal rows), the morphology of the cytostome and the size of mature cells (Ashburner and Ehl, 1973; Nikolic et al., 2006; Mitra et al., 2013; Warren, 2013). However, genetic analysis of *C. uncinata* (Ehrenberg 1838) has shown that considerable cryptic species diversity exists within North America *Chilodonella* strains (Dunthorn et al., 2011). Therefore, Australian *Chilodonella* species could also represent a cryptic species complex.

Fish infections caused by *Chilodonella* spp. have attracted increased research interest as the farming of freshwater fish for food production expands globally. In Australia, freshwater fish farms are affected by frequent parasitic *Chilodonella* spp. outbreaks that can cause losses of up to 10% of revenue for each production cycle (Chapter 2; Bastos Gomes et al., 2016). However, until now only two species (*C. hexasticha* and *C. piscicola*) have been associated with infections in Australian fishes. Furthermore, limited investigation into the taxonomy of Australian parasitic *Chilodonella* has been performed to confirm that these infections are caused by only two species. The aim of this research was to use a combined morphological and molecular approach to resolve the taxonomic status of *Chilodonella* impacting freshwater farmed fish in Australia.

3.2. Materials and methods

3.2.1. *Chilodonella* spp. collection

Chilodonella spp. were collected for species characterisation from two commercially farmed fishes, Murray cod, *Maccullochella peelii* (Mitchell 1838), and barramundi, *Lates calcarifer* (Bloch 1790), from eastern Australia between March 2013 and November 2014. Fish hosts of both species ranged from 100 g to 3 kg. For *M. peelii*, parasites were scraped from gills and skin mucus of fish showing behavioural signs of infection (i.e. not feeding and gasping at

tank edges) from three Victorian farms (27 samples) and one farm in New South Wales (3 samples). These parasites were preserved in RNAlater® (Life Technologies Australia Pty Ltd.30-32 Compark Circuit, Mulgrave Victoria 3170) and 80% ethanol. In the case of *L. calcarifer*, fish showing behavioural signs of infection were selected for sampling from a single farm from tropical north Queensland. Five to 10 fish per pond (126 fish over a period of 20 months) were sedated using the anaesthetic AQUI-S® (Aqui-S New Zealand Ltd; AQNZ) according to the manufacturer's instructions. Mucus from the skin and gills was carefully removed using the blunt edge of a scalpel blade to prevent excess blood in the sample. Mucus was spread on a glass slide using scalpel blades and the fresh mounts examined under a light microscope (40X objective) to confirm infection. Sixty slides (from *L. calcarifer* samples) containing *Chilodonella* cells were selected for silver staining and morphological description. An additional 60 samples containing part of the gill or mucus with *Chilodonella* spp. were also preserved in 80% ethanol for genetic analysis. A detailed workflow for the putative *Chilodonella* spp. isolated from Australian freshwater farmed fishes is found in Table 4.

Table 4 Workflow of putative Chilodonella spp. isolated from Australian freshwater farmed fishes. NA = Not applicable as live specimens were not available.

***Chilodonella* species**	**Origin within Australia**	**Sample size for morphological identification**	**Sample size for genetic analysis**	***In vitro* culture (duration)**
Chilodonella hexasticha	*Lates calcarifer* farm from North QLD	30	60	Yes (one week)
Chilodonella '*acuta* '	*Lates calcarifer* farm from North QLD	30	20	Yes (three months)
Chilodonella '*uncinata*'	*Lates calcarifer* farm from North QLD	30	20	Yes (twelve months)
Chilodonella '*piscicola*' 1	*Maccullochella peelii* farm from Victoria	0	27	NA
Chilodonella '*piscicola*' 2	*Maccullochella peelii* farm from New South Wales	3	3	NA

3.2.2. *Chilodonella* spp. in *vitro* culture

Chilodonella spp. cells isolated from the gills and skin of infected freshwater *Lates calcarifer* from various epizootic events between October and November 2013 were maintained *in vitro* to enable a surplus of specimens for morphological characterisation and molecular analyses. Ciliates were maintained in six-well plates (FalconTM) in the dark at room temperature. Each well contained 6 mL of filtered, autoclaved pond water obtained from the fish farm of origin. A sterile rice grain was added to each well to support bacterial growth (modified from Bellec et al., 2014).

Initially, polycultures were established from all ciliates present on *L. calcarifer* gills and skin. After a period of 24 hr in culture plates in the dark, ciliate cultures were examined under a compound microscope (20X). A single *Chilodonella* cell from mixed cultures was collected

using a clean micropipette and placed into the well of a new culture plate (containing 6 mL of filtered, autoclaved pond water and a single sterile rice grain) for specific species isolation (i.e. monoculture). This process was repeated every third day for six months. *Chilodonella* cells were transferred into new plates for continuing *in vitro* culture growth. For fresh preparations, single cells were collected from culture plates and placed on glass slides with two drops of 4% formalin (to stop cell movement).

3.2.3. Morphological diagnosis of *Chilodonella* spp.

The *Chilodonella* spp. ex *L. calcarifer* were isolated from infected farmed fish from tropical north Queensland and freshly prepared by placing parasite cells directly onto glass slides. *Chilodonella* sp. ex *M. peelii* were isolated from infected fish from New South Wales and Victorian farms and stained using Klein's dry silver impregnation (Lom and Dyková, 1992) to observe informative morphological characters for species level identification. For the silver impregnation method, slides were covered with 2% aqueous solution of silver nitrate ($AgNO_3$) for approximately 8 min and rinsed in distilled water. Slides were placed under shortwave UV light for 1 hr. Dried slides were mounted with Canada balsam using a coverslip and analysed using compound microscopy (100X objective). Images were taken using a UC50 camera fitted to an Olympus BX53 compound light microscope. Line drawings were produced by examining specimens and photomicrographs. Morphological measurements were performed using LabSens software (Olympus Soft Imaging Solutions). Cell measurements (width and length) are represented in micrometres (μm).

3.2.4. Molecular identification of *Chilodonella* spp.

3.2.4.1. DNA extraction and amplification

DNA was extracted from *Chilodonella* cells obtained from farmed fish and from monocultures maintained *in vitro*. Before DNA extraction *Chilodonella* cells collected from fish farms and preserved in 80% ethanol were concentrated to facilitate cell manipulation. Tubes containing cells were pelleted by spinning at 16,000 g for 15 min, whereas *Chilodonella* obtained from monocultures were pelleted by spinning at 6,000 g for 20 min. DNA was extracted using a CTAB (Cetyl Trimethyl Ammonium Bromide) protocol modified from Edwards et al. (1991).

PCR reactions were carried out in a final volume of 10 µL, containing 2 µL of each DNA sample. When PCR inhibitors were present (from fish mucus) DNA samples were diluted 1 in 10 and 2 µL of diluted DNA added to the PCR reaction. A negative (H_2O) and a positive control (*Chilodonella* spp. DNA sourced from infected farmed fish) were used in each extraction. Q5 hot start high fidelity DNA polymerase (New England BioLabs, MA) was used (volumes according manufacturer's instructions). Three markers, SSU rRNA, mtSSU rDNA and ITS1-5.8S-ITS2 were amplified for species identification and for phylogenetic analyses. These markers were chosen based on other studies trying to understand the morphological and molecular phylogenetic relationships among ciliates (Dunthorn and Katz, 2008; Lynn, 2008; Dunthorn et al., 2011; Deng et al., 2015). Each marker was amplified using 10 µM of each gene specific set of primers; mtSSU rDNA primers were mtSSUF and mtSSUR (Dunthorn et al., 2011), with PCR cycling conditions of 1 min at 98 °C; 35 cycles of 5 s at 98 °C, 10 s at 60 °C, 20 s at 72 °C; 1 min of final extension at 72 °C; SSU rRNA was amplified using universal eukaryotic primers Euk A and Euk B (Medlin et al. 1988); ITS-5.8S-ITS2 was amplified using ITS-F and ITS-R (Yi et al., 2009). Amplification for these two later genes was performed

according to Yi et al. (2009) and Dang et al. (2015). Cycling conditions were: 5 min at 98 °C; 35 cycles of 30 s at 98 °C, 90 s at 67 °C, 30 s at 72 °C; 10 min of final extension at 72 °C. Results were observed under UV light, using 1.5% agarose gel electrophoresis. Samples with positive bands on agarose gel electrophoresis were purified before sending for DNA sequencing. The final PCR product volume was increased to 20 µL by adding 12 µL of ultrapure water (Gibco®). This procedure was performed to facilitate the movement of PCR product through Sephadex G-50 spin columns. DNA purification was performed using Sephadex G-50 spin columns (modified from Miller et al., 1999). In brief, 5 g of dry Sephadex G-50 was hydrated adding 75 mL of RO water and autoclaved. Each spin column received 350 µL of the Sephadex G-50 in suspension (at room temperature) and centrifugation was performed at 1075 g for 6 min. On the centre of the remaining hydrated Sephadex G-50 in the spin column, 20 µL of PCR product was added and again centrifuged at 1075 g for 6 min. The purified product collected was sent to the Australian Genome Research Facility for Sanger sequencing.

3.2.4.2. Phylogenetic analyses

Samples were sequenced for accurate species-level identification and to determine potential intra- and inter-specific genetic variation within *Chilodonella* species. Phylogenetic analyses based on nucleotide alignments were performed as described previously (Miller and Cribb, 2007; Miller and Cribb, 2008; Miller et al., 2010a, 2010b). Contigs were aligned, assembled and annotated using Geneious v.9.1 software (http://www.geneious.com, Kearse et al., 2012). Alignments for each gene were edited and trimmed to match the shortest sequence in the alignment. The final alignments included 478 sites for the mtSSU-rDNA marker, 993 sites for the SSU rRNA marker and 304 sites for the ITS1-5.8S-ITS2 marker. Sequences obtained from the NCBI GenBank database from other closely related ciliate taxa were used as outgroups for comparative phylogenetic analyses of the SSU rRNA and ITS1-5.8S-ITS2

markers. The mtSSU-rDNA sequences for the *Chilodonella* species sequenced in this study and *C. uncinata* sequences from North America (NCBI GenBank database) are unique within the GenBank database and BLAST searches do not reveal any suitable closely-related outgroup sequence data for inclusion in subsequent phylogenetic analyses. Therefore, no outgroup was used in analyses of the mtSSU rDNA dataset. The software jModelTest version 2.1.7 (Posada, 2008) was used to estimate the best nucleotide substitution model based on Akaike Information Criterion (AIC) for the three genes analysed. Bayesian inference was performed using MrBayes on the CIPRES portal following jModelTest results. The Bayesian inference (BI) analyses were conducted using the TVM + G (0.1810) model for mtSSU-rDNA, TrN + G (0.850) for ITS1-5.8S-ITS2 and TIM2 + I (0.5700) + G (0.6540) for SSU rRNA. The length of chains was 10,000,000 generations with sampling each 1000 generations and the first 3000 were discarded as burn-in. Trees were edited and annotated in Figtree (http://tree.bio.ed.ac.uk/software/figtree/). Maximum likelihood (ML) analyses were performed using the RAxML algorithm (Stamatakis et al. 2008) for all three genes based on jModelTest results above. Bootstrap percentages were obtained after 1000 replicates. Distance matrices were constructed with the absolute pairwise character difference and the percentage of uncorrected "p" pairwise character differences using MEGA5 (Tamura et al., 2011). For all taxa the pairwise comparison of absolute sequence divergence was calculated with gaps treated as missing data.

3.3. Results

3.3.1. Morphometric diagnosis of *Chilodonella* species

Morphological examination revealed the presence of four putative *Chilodonella* isolates from the two Australian farmed fishes. Three different *Chilodonella* species (or morphotypes based on microscopic observations) were obtained from *Lates calcarifer* (*C. hexasticha*,

putative *C. acuta* and *C. uncinata*) and an additional species from *Maccullochella peelii* (*C. piscicola*). A combination of morphological characters was used to provide putative species-level diagnosis including the cell size and shape, number of oral nematodesmata (bundles of microtubules which support the cytopharynx (see Laybourn-Parry, 1984), and the number of kinety bands on both sides of the ventral surface of each cell. Morphometric details of the *Chilodonella* species from *L. calcarifer and M. peelii* are presented in Table 4. Below are the descriptions of *Chilodonella* species identified in the present study.

Chilodonella hexasticha (Kiernik, 1909) (Table 5; Fig. 3A and B)

Host: *Lates calcarifer* (freshwater)

Locality: Tropical North Queensland

Site: External skin surface and gills

Mature cells ventrally flattened, dorsally vaulted, oval to round, 42 ± 6.6 μm (30–56, n = 30) long and 25±7.4 μm (16–43, n = 30) wide. Posterior notch absent. Ventral surface containing 7 ± 0.6 (6–8, n = 30) short-row kinety bands running the length of the right side, and 6 ± 0.6 (5–7, n = 30) long-row kinety bands running the length of the left side, extending anteriorly and curving right to meet anterior part of right kinety bands (Fig. 3A). Cytoplasm with digestive vacuoles and single macronucleus positioned centro-posteriorly. Transverse elliptical cytostome present with 11 oral nematodesmal rods (Fig. 3).

Remarks on *Chilodonella hexasticha*

Morphological identification is in agreement with morphometric measurements provided for this taxon by Noga (2010) and other reports as reviewed at Chapter 2 (Bastos Gomes et al., 2016). However, cell size varies according to life stage and availability of feed

such as bacteria and algae in the environment (GBG personal observation). This taxon could not be cultured long-term *in vitro*, but was maintained in culture for one week to facilitate identification.

Table 5 Morphometric characterization of the putative Chilodonella spp. isolated from Australian freshwater farmed fishes. Mean counts and measurements presented in µm.

Morphometric characteristics	***Chilodonella hexasticha*** **Australia N = 30**	***Chilodonella acuta*** **Australia N = 30**	***Chilodonella uncinata*** **Australia N = 30**	***Chilodonella piscicola*** **Australia N = 3**
Cell body shape	Oval to round	Elongate; tail-like appendage	Elongate ellipsoid	Round
Cell body length	42 ±6.6	41 ±4.9	37 ±3.7	45 ±1.0
Cell body width	25 ±7.4	21 ±3.2	21 ±3.6	56 ±1.5
No. kinety bands on short row	7 ± 0.6	5 ± 2.5	4	10 ±0.6
No. kinety bands on long curved row	6 ± 0.6	4 ± 1.9	3	8 ±1.2
No. oral nematodesmal rods*	11	9	11	-

N = Number of individual cells examined; ± = standard deviation; no± = morphometric parameters not observed in all cells; - data not available; * morphometric parameter not visible in all individuals.

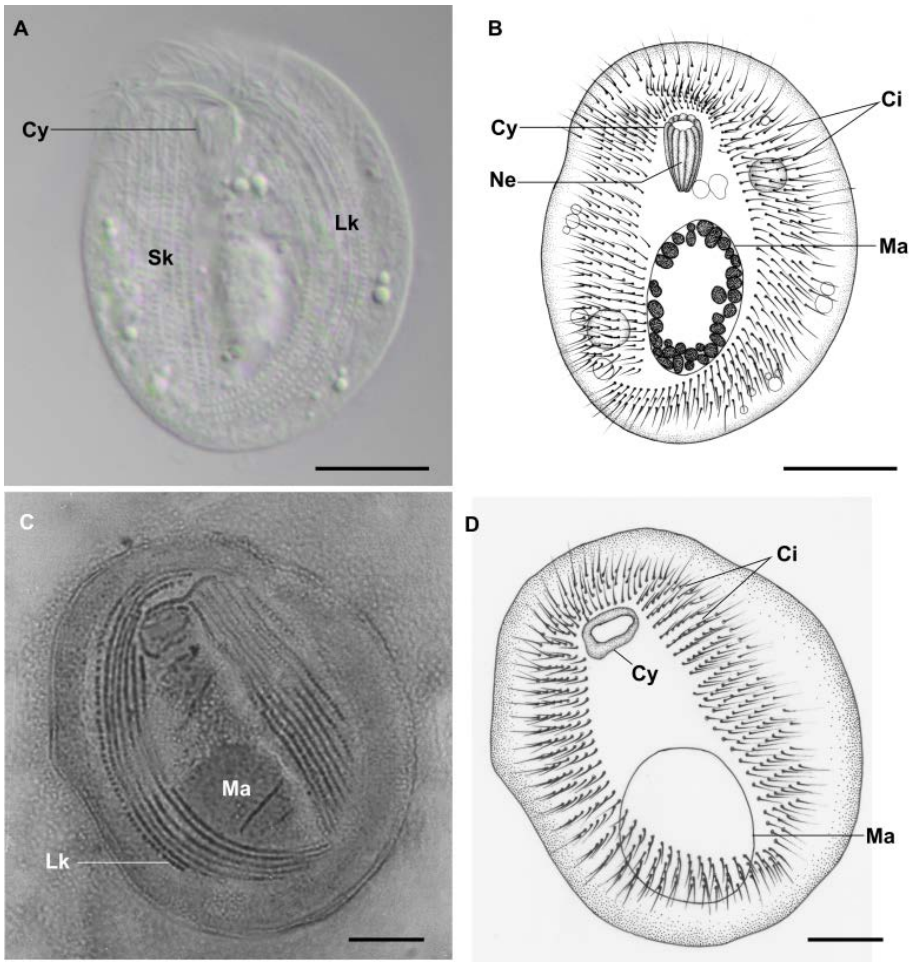

Figure 3 Putative Chilodonella hexasticha (A, B) from the gills and skin of farmed barramundi, Lates calcarifer in tropical north Queensland and putatively identified Chilodonella piscicola (C, D) obtained from gills and skin of farmed Murray cod, Maccullochella peelii from New South Wales, Australia. A) Fresh cell preparation showing short (Sk) and long (Lk) kinety bands and Cytostome = Cy. B) Ink drawing of the cell showing Cilia = Ci; Cytostome = Cy; Oral nematodesmata = Ne and Macronucleus = Ma. C) Silver stained cell (image courtesy of Brett Ingram); D) Ink drawing of cell. Other abbreviations as for A and B. Scale bars: 10µm.

Chilodonella 'acuta' (Kahl, 1931) (Table 5; Fig. 4A and B)

Host: *Lates calcarifer* (freshwater)

Locality: Tropical North Queensland

Site: External skin surface and gills

In vitro cell culture 1. Mature cells ventrally flattened, dorsally vaulted, elongate, 41 ± 4.9 μm (30–49, n = 30) long, 21 ± 3.2 μm (16–30, n = 30) wide. Posterior tail-like appendage present (Fig. 4B). Ventral surface containing 5 ± 2.5 (n = 30) short-row kinety bands running the length of the left side, and 4 ± 1.9 (n = 30) long-row kinety bands running the length of the right side curving left anteriorly. Kinety bands non-confluent posteriorly. Cytoplasm with numerous digestive vacuoles and single macronucleus positioned centro-posteriorly. Transverse elliptical cytostome present (not depicted in Fig. 4B) with 9 oral nematodesmal rods (Fig. 4B).

Remarks on *Chilodonella* '*acuta*'

Morphological identification is in agreement with morphometric measurements provided for this taxon in previous reports as reviewed by Fan et al. (2014). *In vitro* culture was maintained for up to 3 months.

Chilodonella 'uncinata' (Ehrenberg, 1838) (Table 5; Fig. 4C and D)

Host: *Lates calcarifer* (freshwater)

Locality: Tropical North Queensland

Site: External skin surface and gills

In vitro culture 2. Mature cells ventrally flattened, dorsally vaulted, elongate, 37 ± 3.7 μm (27–42, n = 30) long, 21 ± 3.6 μm (13–29, n = 30) wide. Posterior notch absent; posterior end rounded. Ventral surface containing 4 (n = 30) short-row kinety bands running the length of the right side, and 3 (n = 30) long-row kinety bands running the length of the left side curving right anteriorly. Kinety bands non-confluent posteriorly. Cytoplasm with numerous digestive

vacuoles and single central macronucleus. Elliptical cytostome present with 11 oral nematodesmal rods (Fig. 4D).

Remarks on *Chilodonella* '*uncinata*'

Morphological identification is in agreement with measures provided for this taxon by Kazubski and Migala (1974). *In vitro* culture was maintained for up to 12 months.

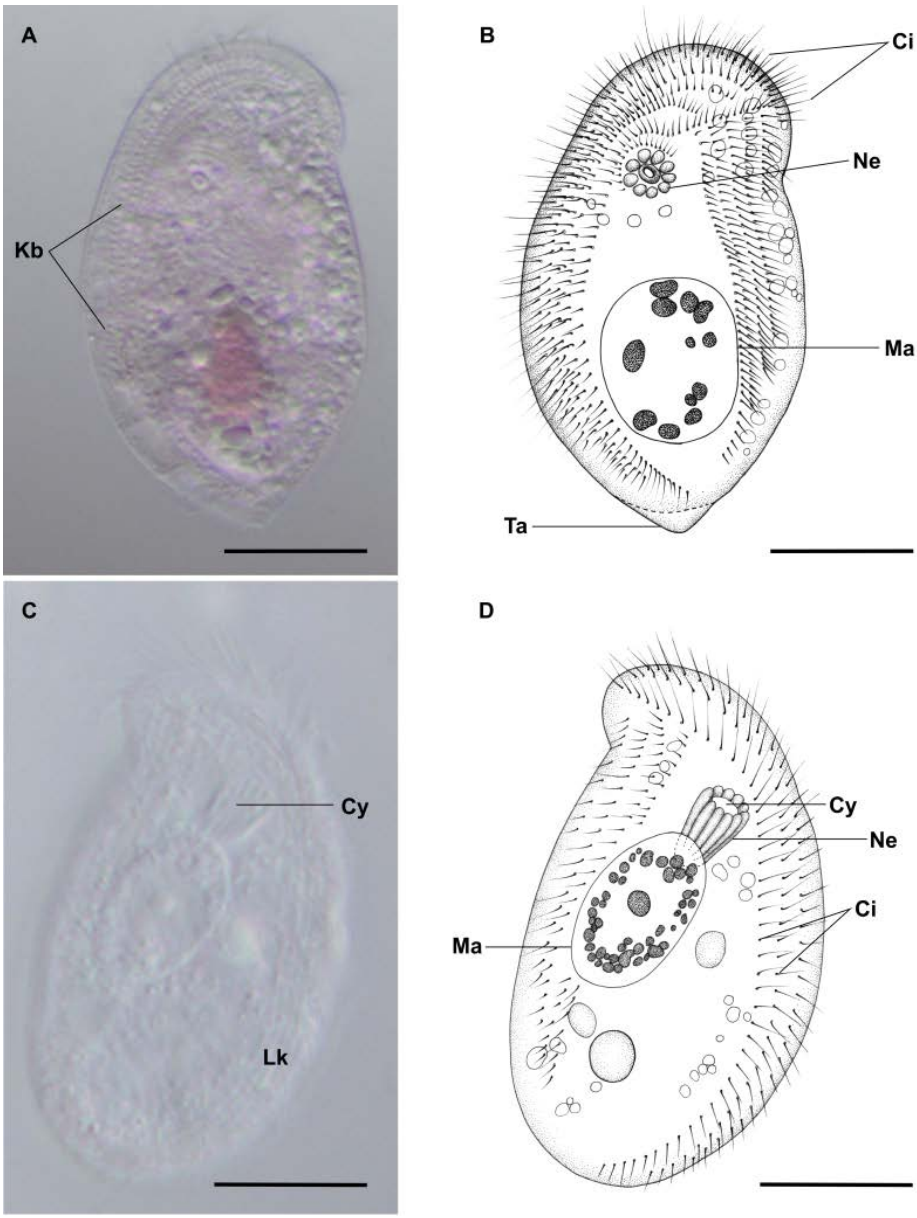

Figure 4 Putative Chilodonella acuta (A, B) and Chilodonella uncinata (C, D) originally isolated from gills and skin of farmed barramundi, Lates calcarifer, from tropical north Queensland, Australia and maintained in vitro. A)

Fresh cell preparation slightly stained with Haematoxylin to improve contrast: Kb = Kinety bands; B) Ink drawing of cell showing tail- like appendage = Ta. C) Fresh cell preparation; D) Ink drawing of cell. Other abbreviations as for A and B. Scale bars: 10μm.

Chilodonella 'piscicola' (Ehrenberg, 1838) (Table 5; Fig. 3C and D)

Host: *Maccullochella peelii* (freshwater)

Locality: Victoria and New South Wales

Site: External skin surface and gills

Mature cells ventrally flattened, dorsally vaulted, round, 56 ± 1.5 μm (55-58, n = 3) long, 45 ± 1.0 μm (44-46, n = 3) wide. Ventral surface containing 10 ± 0.6 (9-10, n = 3) short-row kinety bands running the length of the left side (Fig. 3C), and 8 ± 1.2 (7-9, n = 3) long-row kinety bands running the length of the right side curving left anteriorly (Fig. 1C). Cytoplasm with single posteriorly positioned macronucleus (Fig. 3C and D); digestive vacuoles not observed. Cytostome present; number of oral nematodesmal rods not observed.

Remarks on *Chilodonella* '*piscicola*'

Morphological identification is in agreement with measures provided for this taxon by Noga (2010) and other reports as reviewed at Chapter 2 (Bastos Gomes et al., 2016). This species was not maintained in culture.

3.3.2. Molecular phylogenetic analysis

Chilodonella spp. used for phylogenetic analyses represented the four putative species characterized by morphology isolated in this study. Phylogenetic analyses were conducted on all three of the markers examined (mtSSU rDNA, SSU rRNA and ITS1-5.8S-ITS2); however only mtSSU rDNA sequences demonstrated significant sequence variation between

Chilodonella species and is discussed further herein. Phylograms resulting from analyses of the SSU rRNA and ITS regions are presented in Figure 5. Assembled mtSSU rDNA, SSU rRNA and ITS1-5.8S-ITS2 sequences for each species were deposited in GenBank (Table 6).

Table 6 GenBank accession numbers for the mtSSU rDNA, SSU rRNA and ITS1-5.8S-ITS2 genes sequenced from the putative *Chilodonella* species isolated from Australian freshwater farmed fishes.

***Chilodonella* species ID**	**mtSSU rDNA**	**SSU rRNA**	**ITS1-5.8S-ITS2**
Chilodonella '*hexasticha*', Australia	KY508243	KY508252	KY508248
Chilodonella '*uncinata*' ex culture 2, Australia	KY508246	N/A	KY508251
Chilodonella '*acuta*' ex culture 1, Australia	KY508247	N/A	N/A
Chilodonella '*piscicola*' 1, Australia*	KY508244	N/A	KY508249
Chilodonella '*piscicola*' 2, Australia**	KY508245	N/A	KY508250

**Chilodonella 'piscicola'* 1 Australia refers to isolate originated from Victoria;
***Chilodonella 'piscicola'* 2 Australia refers to isolate originated from New South Wales (NSW).

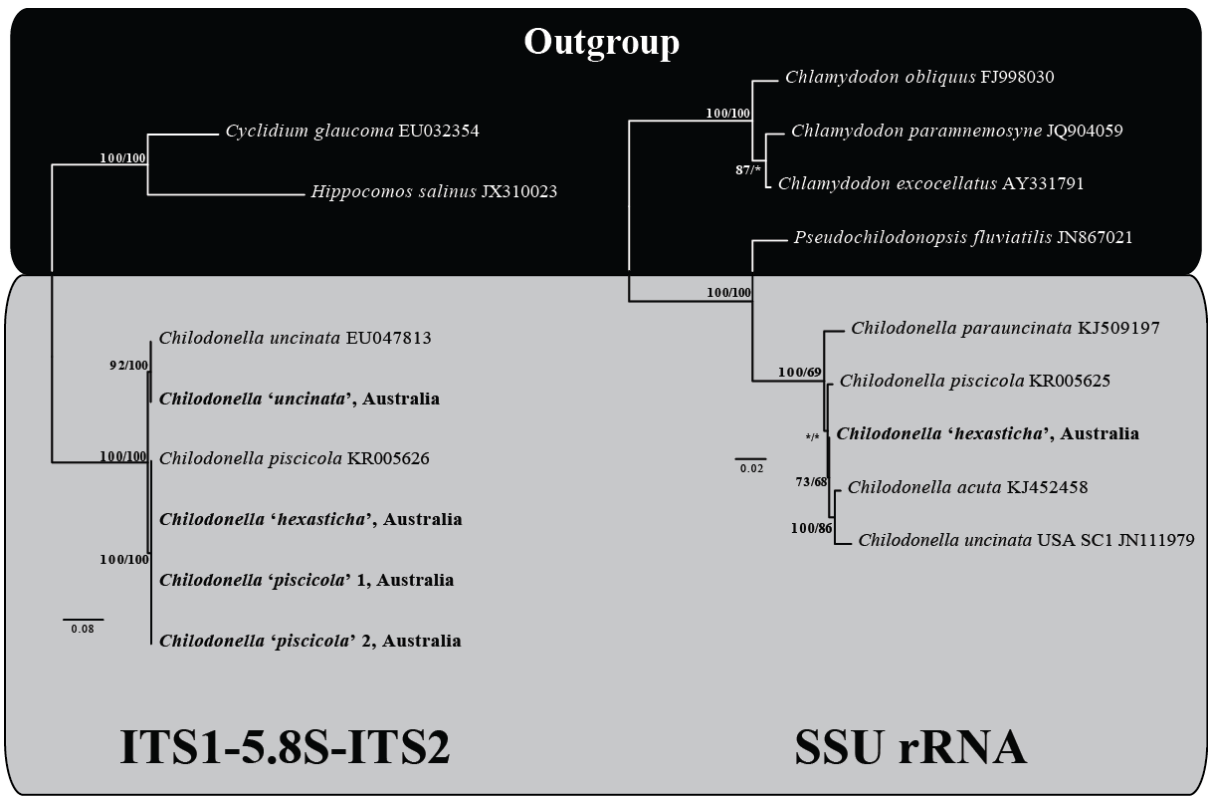

Figure 5 ITS1-5.8S-ITS2 and small subunit SSU-rRNA (SSU rRNA) topologies of Chilodonella spp. analysis. Bootstrap and posterior probability values are indicated at nodes (ML/BI). */* represent bootstrap and posterior probability below 50%. The Bayesian and the ML tree are similar for both topologies. Phylograms are midpoint rooted. Chilodonella 'piscicola' 1 and 2 represent isolates from Victoria and NSW respectively. Sequences of this study are showed in bold.

Post alignment and trimming mtSSU-rDNA sequences represented a total of 478 basepairs, with 117 sites showing polymorphism and thus phylogenetically informative. Phylogenetic trees constructed with BI and ML methods were combined into a single consensus tree due to their similar topology (Fig. 6). The consensus tree showed that the putative *C. hexasticha* and *C. piscicola* clustered in the same clade with high bootstrap support (100% ML, 1.0 BI). The *C. uncinata* isolate from Australia clustered in the same clade as *C. uncinata* strains from North America, with strong support of 100% (ML) and 0.97 (BI). Similarities between *Chilodonella* species over this region and *P*-distance inferred from mtSSU-rDNA sequence data for ten *Chilodonella* sequences is shown in Table 7. Among the three markers analysed in this study, mtSSU-rDNA was the only one to demonstrate significant distances between *Chilodonella* species.

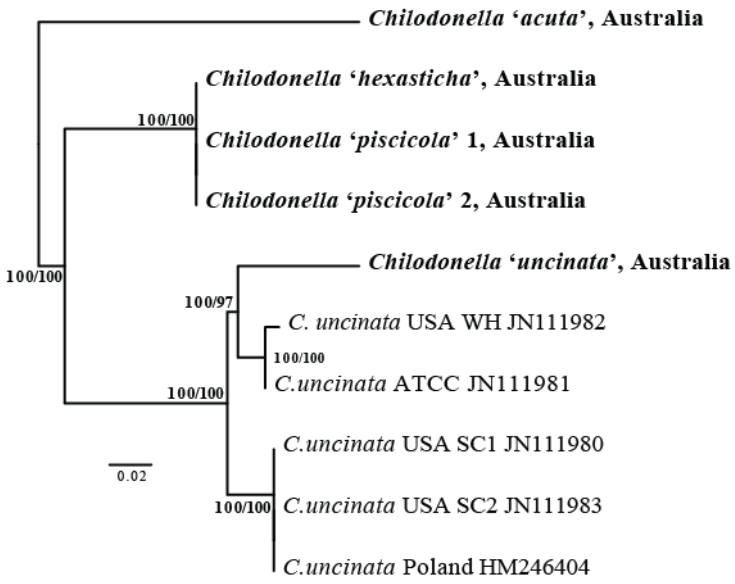

Figure 6 Relationships between the species of Chilodonella examined here based on Bayesian inference and Maximum likelihood analyses of mitochondrial SSU-rDNA (mtSSU rDNA). Posterior probability and bootstrap values are indicated at nodes (BI/ML). Phylogram is midpoint rooted. Chilodonella 'piscicola' 1 and 2 represent isolates from Victoria and NSW respectively. Sequences generated in this study are showed in bold.

Table 7 Mitochondrial SSU rDNA pairwise character differences per site for ten *Chilodonella* species (including species identified in this study). Values above the diagonal are the total number of base differences and below the diagonal is the percentage of uncorrected "p" pairwise differences.

Species	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1. *Chilodonella* '*hexasticha*' Australia		0	0	15	15	15	14	18	25	16
2. *Chilodonella* '*piscicola*' 1 Australia*	0		0	15	15	15	14	18	25	16
3. *Chilodonella* '*piscicola*' 2 Australia**	0	0		15	15	15	14	18	25	16
4. *C. uncinata* USA SC1 1 JN111980	11.4	11.4	11.4		0	0	1	1	8	4
5. *C. uncinata* USA SC1 2 JN111983	11.4	11.4	11.4	0		0	1	1	8	4
6. *C. uncinata* Poland HM246404	11.4	11.4	11.4	0	0		1	1	8	4
7. *C. uncinata* ATCC 50194 JN111981	10.3	10.3	10.3	3.6	3.6	3.6		1	7	5
8. *C. uncinata* USA WH JN111982	10.5	10.5	10.5	4.1	4.1	4.1	0.6		4	4
9. *Chilodonella* '*acuta*' Australia	13.3	0.1	13.4	14.5	14.5	14.5	13.8	14.0		16
10. *Chilodonella* '*uncinata*' Australia	12.1	12.1	12.1	6.7	6.7	6.7	5.6	5.8	16.2	

**Chilodonella 'piscicola'* 1 Australia refers to isolate originated from Victoria;
***Chilodonella 'piscicola'* 2 Australia refers to isolate originated from New South Wales (NSW).

3.4. Discussion

3.4.1. *Chilodonella* species identification

Our combined morphological and comparative molecular analyses approach revealed four morphological *Chilodonella* types, but only three distinct genotypes from freshwater farmed fish from Australia. The four morphologically distinct isolates were putatively identified as *C. hexasticha*, *C. uncinata*, *C. acuta* and *C. piscicola*. Morphometric results (see Table 2) for the four species agreed with previous studies (Kazubski and Migala, 1974; Noga, 2010; Padua et al., 2013; Bowater and O`Donoghue, 2014; Fan et al., 2014). However, only

three distinct genotypes were identified, with two taxa, *C. hexasticha* (isolated from *L. calcarifer*) and *C. piscicola* (isolated from *M. peelii*) having identical mtSSU rDNA sequences. This is the first time that isolates of putative *C. hexasticha* and *C. piscicola* have been compared using genetics, as most previous reports characterized these two *Chilodonella* species using only morphological features (Kazubski and Migala, 1974; Lom and Dyková, 1992; Noga, 2010; Padua et al., 2013; Bowater and O`Donoghue, 2014; Lahr et al., 2014) nodes in many ciliates according to Dunthorn et al. (2011).

Recent comparative genetic studies between ciliate taxa have demonstrated that there is often discordance between genetic and morphological characters (Lahr et al., 2014). *Chilodonella uncinata* is considered a cryptic species complex as it demonstrates dissimilarity at mitochondrial SSU rDNA (mtSSU-rDNA) and protein-coding loci (divergences from 2.2 to 13.5%; Hall and Katz, 2011; Lahr et al., 2014). Genetic analyses of mtSSU rDNA of *C. uncinata* collected from the environment has shown that significant cryptic species diversity exists in North America (Riley and Katz, 2001; Bellec and Katz, 2012; Zufall et al., 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).

In contrast, the putative Australian *C. hexasticha* and *C. piscicola* were genetically similar based on the mtSSU-rDNA marker. The topology of our mtSSU-rDNA phylogenetic tree demonstrated intra genetic variation with strong support (92-100% ML, 1.0 BI; 100% ML, 0.97-1.0 BI, respectively). The consensus tree for mtSSU-rDNA supported the presence of up to three distinct genotypes, even though based on morphology four species exist within Australia. The markers ITS1-5.8S-ITS2 and SSU-rDNA did not show significant characters for species differentiation. These results confirmed that mtSSU-rDNA is the best marker for inferring genetic variation between *Chilodonella* species as it helps to infer well-supported

3.4.2. Morphological plasticity within *Chilodonella*

Chilodonella spp. have the potential to demonstrate morphological plasticity (Hall and Katz, 2011; Lahr et al., 2014) where organisms present distinct morphologies within the same species. This can be caused by developmental and/or environmental differences, or the morphological differences represent different life cycle stages (West-Eberhard, 1989; Lahr et al., 2014). Isolates of *Chilodonella* from *L. calcarifer* and *M. peelii* present morphological dissimilarities (Fig.1A-D), but were all genetically identical. As the *C. hexasticha* isolate did not survive more than a week *in vitro* we cannot confirm that life stage differences contributed to the observed morphological plasticity. However, considering the morphological dissimilarities and the genetic similarities between Australian isolates of *C. hexasticha, C. piscicola* 1 and 2 and *C. piscicola* ex *Schizothorax o'connori* and *Oxygymnocypris stewartii* from Tibet (Deng et al., 2015) suggests that *C. hexasticha* (Kiernik, 1909) and *C. piscicola* (Zacharias, 1894; syn. *C. cyprini* (see Moroff, 1902) may be the same species. Further analysis of *C. piscicola* (pending molecular and morphological re-examination of material from their type-localities) may confirm similarities between *C. piscicola* and *C. hexasticha*.

3.4.3. Opportunistic parasitism within *Chilodonella*

As *Chilodonella* spp. are generally free-living, but have the potential to become parasitic, it is surprising that only two species *C. hexasticha* and *C. piscicola* have been implicated in freshwater fish epidemics (Noga, 2010; Padua et al., 2013; Bowater and O`Donoghue, 2014). While *C. uncinata* and *C. cucullus* species had been found in fishes

previously, our putative *C. acuta* isolate had never been isolated from fish (Hoffman et al., 1979; Mitra and Haldar, 2004; Mitra et al., 2013, Padua et al., 2013).

This study demonstrates that Australian freshwater farmed fishes have the potential to be infected by at least three different *Chilodonella* species (*C. piscicola* (proposed syn. *C. hexasticha*), putative *C. acuta* and *C. uncinata*) and that limiting ciliate characterization to morphological analysis alone may be an ineffective approach to accurately identify ciliate species. A systematic analysis of *Chilodonella* spp. including morphological and molecular techniques can lead to a better understanding of discordance and their true biodiversity. Furthermore, these findings can assist future treatment and control strategies for *Chilodonella* species in farm conditions.

Chapter 4. Use of environmental DNA (eDNA) and water quality data to predict protozoan parasites outbreaks in fish farms

4.1. Introduction

Food security is predicted to be a global challenge as the human population reaches nine billion people (FAO, 2014). Aquaculture, which is currently the fastest growing agribusiness, will be a major supplier of future animal protein requirements for this expanding population (FAO, 2016). However, disease currently results in approximately 40% of lost production potential (~USD$102 billion), from direct (e.g. mortalities) and indirect (e.g. additions of chemicals, waste of feed) factors (FAO, 2012). Consequently, early disease detection and management is critically important for future food production from aquatic farm environments.

Environmental DNA, also known as eDNA, is a novel front-line molecular tool that has the potential to change the way detection and monitoring of disease occurs in aquaculture (Bass et al., 2015). The technique enables non-invasive sampling and detection based purely on the collection of water samples when there is no visible presence of the target organism (Robson et al., 2016). The eDNA approach was first applied by Ogram et al. (1987) to understand microbial communities in sediments. However, it was not until Ficetola et al. (2008), who used eDNA on the American bullfrog, *Rana catesbeiana*, as a model for invasive species studies, that this technique started to grow in popularity as a tool to detect biodiversity in aquatic systems. Due to its detection power and relative ease application, environmental DNA has now been applied to address questions related to microbial community diversity, evolution, ecology and even interactions between hosts and pathogens (Goldberg et al., 2014; Taberlet et al., 2012; Bass et al., 2015).

Environmental DNA methodologies offer potential to improve animal health monitoring systems in aquaculture, as many pathogens are microscopic, have waterborne life-stages, are hard to directly detect, and clinical signs of diseases are often only observed in advanced stages of infection where subsequent treatment options become ineffective (Bass et al., 2015). Traditional diagnostics, such as histopathology and morphological identification, are time consuming and often lack detection sensitivity unless large numbers of samples from animals are processed, or only detect the presence of a parasite on the species under culture once a widespread epizootic outbreak is in progress (Adrian-Kalchhauser and Burkhardt-Holm, 2016; Chapter 2; Bastos Gomes et al., 2016). Through monitoring levels of parasites in water, however, eDNA methodologies may offer a simple early detection and disease risk assessment technique before animals became clinically symptomatic, merely through the sampling of water from ponds or the surrounding aquatic ecosystem. Furthermore, coupling routine eDNA sampling, quantitative PCR (qPCR), fish production data and environmental water parameters may lead to new understandings of the key environmental drivers of disease outbreaks and quantitative prediction of the likelihood of fish mortalities.

Ciliate protozoans are considered among the most economically important parasites for finfish aquaculture (Lom and Dyková, 1992; Chapter 2; Bastos Gomes et al., 2016), with *Chilodonella* spp. (Phyllopharyngea: Chilodonellidae) being particularly harmful to farmed freshwater fishes (Padua et al., 2013; Chapter 2; Bastos Gomes et al., 2016). The presence of large numbers of *Chilodonella* cells in water predispose their attachment to fish gills and skin epithelial cells, causing severe pathology (Noga, 2010; Mitra et al., 2013; Padua et al., 2013). Chilodonellosis (disease caused by *Chilodonella* spp.) progresses rapidly, causing mortalities within two to three days of infection and losses of 50−95% in fish stocks (Paperna and Van As, 1983; Karvonen et al., 2010). Rapid detection of *Chilodonella* spp. can be challenging as

epidemics often occur without warning (Chapter 2; Bastos Gomes et al., 2016). The use of eDNA-based technologies offer a novel approach for the early detection of increasing numbers of *Chilodonella* spp. in water and can alert farmers to implement pre-emptive, rather than reactive management to minimise production losses.

The aim of this study was to demonstrate the applicability of integrating eDNA-based techniques and farm water quality parameters as a management tool for predicting future parasite epizootics in commercial fish farms. Here quantitative real-time PCR (qPCR) was used to determine the presence and abundance of *C. hexasticha* in aquaculture ponds and relationships between parasite loads in water, environmental conditions and large-scale fish mortality events were examined to highlight how eDNA methodology may help understand environmental drivers of disease outbreaks.

4.2. Material and Methods

4.2.1. Pond selection and collection of water samples

Water samples were collected from ~1.4 ha earthen ponds (~20 Megalitres; ML) within a commercial freshwater barramundi, *Lates calcarifer* (Bloch), farm near Innisfail, north Queensland, Australia, monthly for one year (from October 2013 to September 2014, except March 2014) (Fig. 1). Four ponds were selected for regular sampling which had a history of *Chilodonella* infections and another four ponds were opportunistically chosen based on the farm's health reports on the presence of stressed fish and no considerable reduction of ciliate numbers post chemical treatment (CC, personal communication). Triplicate 15 mL pond water samples were collected approximately 1 m from the edge of ponds (30 cm below the water surface) using individual plastic cups attached to a 1 m long pole in three different sites within each pond (i.e. total of nine water samples per pond) and carefully poured into 50 mL centrifuge tubes containing 1.5 mL of sodium acetate (3 M) and 33.5 mL of absolute ethanol for DNA

preservation (modified from Ficetola et al., 2008). To ensure there was no cross contamination among tubes during sampling, or DNA extraction, negative control samples (i.e. distilled water transported to the field from our laboratory) were also taken at each sampling site. Immediately following collection, tubes were stored on ice until transportation to the laboratory for processing.

4.2.2. Collection of *Chilodonella hexasticha* cells, environmental parameters and mortality data

Parasite cells were collected between October 2013 and September 2014 for species identification and validation of a quantitative PCR (qPCR) assay. Barramundi showing behavioural signs of infection (ranging from loss of appetite to gasping at pond edges) were selected for sampling. Animals were sedated using AQUI-S (AQUI-S, New Zealand Ltd) and samples from the skin and gills were scraped gently using the blunt edge of a scalpel blade to prevent bleeding. Mucus was spread on a glass slide and the fresh gill mount examined under a light microscope (200X) to confirm infection by *C. hexasticha*. Sixty slides containing *C. hexasticha* cells were selected for silver staining and morphological description. *Chilodonella hexasticha* was identified on the slides using comparative morphology techniques (Chapter 3; Bastos Gomes et al., 2017a). Sixty samples containing part of the gill or mucus with *Chilodonella* spp. were preserved in 80% ethanol for species characterisation through genetic tests as outlined below.

Environmental and biological parameters were monitored on the farm throughout the study. Dissolved oxygen and water temperature (maximum and minimum) measurements were taken three times a day with an YSI Pro20 meter with a galvanic dissolved oxygen sensor (YSI Inc). Daily rainfall data was obtained from the Australian Government Bureau of Meteorology website (http://www.bom.gov.au/climate/data/index.shtml?bookmark=136), based on readings from Mena Creek Post Office station (8.6 km away from farm). Mean fish weight was obtained

by measurements of 200 animals/pond three times over the year and also during fish manipulation (e.g. fish transfer from one pond to another). Mean fish weight in each pond was based on phenotypic records and growth curve algorithms based on feed consumption and water temperature (Glencross, 2008). Fish mortalities were recorded by farm technicians who visually surveyed and removed dead fish from ponds three times per day.

The relationship between water quality parameters and the abundance of *C. hexasticha* SSU-rDNA copies/µl (eDNA) was assessed using the mean maximum water temperature, minimum dissolved oxygen levels and rainfall over a consecutive 5 day period prior to water sampling for each pond. Likewise, the relationship between *C. hexasticha* SSU-rDNA copies/µl in pond water and fish mortality was examined using the mean number of dead fish recorded over the 5 day period following water sampling in each pond.

4.2.3. DNA extraction

A direct eDNA precipitation and extraction method was adopted, whereby above mentioned sample tubes containing pond water and preservative were centrifuged at 3,200 *g* for 60 min at 6 ºC and the resulting supernatant discarded. The genetic material then present in the resulting pellet was extracted using a CTAB (cetyl trimethyl ammonium bromide) DNA extraction protocol (modified from Edward et al., 1991) and resuspended in 60 µl of 1x TE buffer. DNA quality was checked using a 0.8% agarose gel and DNA quantified with a Nanodrop (ND-1000 Spectrophotometer, Thermofisher Scientific). Pond water samples yielded relatively high concentrations of genetic material (mean ± S.D. = 28.88 ± 17.63 µg DNA/µl). Environmental DNA extracted from the three 15 ml water samples taken at each collection site were pooled together, resulting in three eDNA samples analysed per pond.

CTAB (cetyl trimethyl ammonium bromide) extracted DNA from *C. hexasticha* cells isolated with the aid of a light microscope from gills and mucus of infected fish was used as template for validation and positive controls of the qPCR assay described in the following section. To determine the absolute concentration of *Chilodonella hexasticha* (SSU-rDNA copies/µl) of the DNA template, digital PCR reactions were performed using QuantStudio™ 3D Digital PCR Master Mix v1, forward and reverse primers and a TaqMan probe (specified in Table 1) and a QuantStudio™ 3D Digital PCR Chip v1, in a ProFlex™ 2x Flat PCR System, following the manufacturer's instructions (Thermofisher Scientific). Thermal cycling conditions were: initial 10 min step at 96 °C, followed by 39 cycles of 60 °C for 2 min and 98 °C for 30 sec and final step of 60 °C for 2 min.

4.2.4. Quantitative PCR (qPCR) assay design

A qPCR assay targeting *C. hexasticha* small subunit ribosomal DNA (SSU-rDNA) gene region was developed to quantify cell copy abundance in water samples. To avoid contamination, dedicated pipettes, tips, plasticware and gloves were UV sterilized in biosafety cabinet for 30 minutes before use. Five different brands of master mixes (Power SYBR green master mix - Thermofisher Scientific; QuantiFast Probe PCR Master Mix – QIAGEN; TaqMan universal master mix - Thermofisher Scientific; Kapa probe fast universal master mix - Kapabiosystems; SsoFast EvaGreen master mix -Bio-Rad) were tested against our positive control and random eDNA samples at different dilutions to evaluate qPCR reaction efficiencies and potential inhibitory effects of eDNA. A 1:10 dilution of eDNA extracts in ultra-pure distilled water (Invitrogen) was sufficient to overcome potential qPCR inhibitory effects (Bott et al., 2010). Quantitative PCR reactions and cycling conditions were validated using six serial dilutions of the *C. hexasticha* DNA template, run in triplicates at concentrations ranging from 1.1×10^{-1} to 1.1×10^{3} SSU-rDNA copies/µl, as a standard curve with the following parameters

was obtained: Efficiency = 1.01 (assessed as $E = (10^{-1/slope}) - 1$; Bellec and Katz, 2012), slope = 3.31 and $R^2 = 0.9977$. Quantitative PCR reactions comprised of 7.5 µl of SsoFast EvaGreen master mix (Bio-Rad), 0.6 µl of each primer (600 nM of 985F and 1147R; Table 8), 1.3 µl of ultra-pure distilled water (Invitrogen) and 5 µl of 1:10 diluted eDNA on a Corbett Rotor-Gene 6000 system (QIAGEN). Quantitative PCR runs were performed with each sample in triplicate with the addition of two positive control samples of known concentrations derived from the standard curve and a non-template control (ultrapure water). Thermal cycling conditions comprised of an initial 2 min step at 50 °C and 10 min 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60°C for 1 min and a final melt curve analysis with the temperature increasing from 50 °C to 95 °C at a rate of 0.5 °C every 10 sec to ensure product specificity (Tm amplicon 82.75 °C). In addition, from a total of 147 water samples used for qPCR amplification and where eDNA was detected, 30% were Sanger sequenced and all confirmed to be within *C. hexasticha* SSU-rDNA gene region with 100% NCBI BLASTN sequence alignment identity to GenBank KY508243.1 (Chapter 3; Bastos Gomes et al., 2017a). Quantification cycle (Cq) values of each technical triplicate serial dilution were averaged (mean) and multiplied by 10 (dilution factor). Samples with Cq values outside of the linear range of the standard curve and samples with melting profiles different than the expected (Tm amplicon 82.75 °C) were not considered for further analysis. Non-template controls of all qPCR runs were negative. The relationship between the qPCR Cq values and the number of *C. hexasticha* was established by linear regression of the standard curve (SSU-rDNA copies/µl).

Table 8 Specific primers and probes to target SSU-rDNA gene from *C. hexasticha* for Quantitative PCR.

Primer/probe	**Primer/probe sequence 5' → 3'**	**Length (bp)**	**Tm of amplicon (° C)**	**Reference**
FSSU Chilo Primer	CGCAAGGCTGAAACTTAAAGGA	85	N/R*	This study
RSSU Chilo Primer	CCCCGTGTTGAATCAAATTGA			
SSU Chilo Probe	CTCCTGGTGGTGCCCT- FAM			
985F	GAGTATGGTCGCAAGGCTGAAAC	162	82.75	Bellec and Katz, 2012
1147R	GCACCACCATCCCTCAAATCAAG			

***** Not relevant for TaqMan chemistry.

4.2.5 Statistical Analyses

Linear regression was used to assess the ability of measured variables (i.e. abundance of *C. hexasticha* (eDNA), rainfall, dissolved oxygen and water temperature and fish weight), to predict fish mortalities observed in the 5 days post water sampling. Furthermore, linear regression was also used to evaluate whether any of the observed environmental variables could predict the abundance of *C. hexasticha* in the water samples. Preliminary analyses were conducted to ensure no violation of the assumptions of normality, linearity, multicollinearity and homoscedasticity. Fish weight, fish mortalities and eDNA (*C. hexasticha* abundance in water samples) data were *Ln* transformed to conform to a normal distribution prior to analysis (Kolmogorov–Smirnov test, $P > 0.05$).

The association between the observed variables, and more importantly, their association with the abundance of parasite cells in pond water, was examined using a principal component analysis (PCA). Here, the original set of six variables of interest were reduced into two principal

components for which eigenvalues were greater than 1 and represented as a two-dimensional plot. To explore the strength, direction and significance of relationships between variables, a correlation matrix based on Spearman rho (*r*) bivariate analyses was constructed.

4.3. Results

Environmental parameter data from eight farm ponds sampled between October 2013 and August 2014 were analysed. Table 9 shows the statistical summary for all environmental parameters tested in this study. Quantification of *C. hexasticha* in barramundi farm water varied between ponds, sampling sites (within ponds) and from month to month. Although the abundance of *C. hexasticha* in water samples (SSU-rDNA copies /µl) was extremely variable, the parasite was constantly present in farm water (Fig. 7). The abundance of *C. hexasticha* (SSU-rDNA copies /µl) in pond water was found to be positively correlated with fish mortalities ($r = 0.402$; $P < 0.001$), and negatively correlated with fish size ($r = -0.189$; $P < 0.05$) (Table 10). Fish mortalities were also strongly and positively correlated with warmer water temperatures ($r = 0.448$; $P < 0.001$). As expected in the wet tropics, warmer water temperatures were also strongly and positively correlated with rainfall ($r = 0.415$; $P < 0.001$) and negatively correlated with dissolved oxygen levels ($r = -0.526$; $P < 0.001$) (Table 10). Results from linear regression indicated that the abundance of the ciliate parasite ($\beta = 0.272$, $P = 0.005$) and fish weight ($\beta = -0.450$, $P < 0.001$) explained 41% of the variance in observed fish mortality ($F_{5, 68} = 11.008$, $P < 0.001$), whereas rainfall, water temperature and dissolved oxygen were not significantly associated with parasite abundance ($P > 0.05$). Decreased rainfall ($\beta = -0.200$, $P = 0.026$) and fish weight ($\beta = -0.192$, $P = 0.038$) were also associated with *C. hexasticha* abundance ($F_{DF\ 4, 139} = 3.385$, $P = 0.011$), although these parameters explained only 6.3% of the variance observed.

Table 9 Summary statistics for environmental and biological parameters tested from eight ponds from a freshwater barramundi, Lates calcarifer, farm from tropical Australia in 2013/14. n = number of observations.

Variables	n	mean	minimum	maximum	SD	SE
***Chilodonella hexasticha* in water (SSU-rDNA copies/µl)**	147	10.97	0.00	412.90	40.01	0.19
Water temperature (^{0}C)	52*	26.63	21.3	30.34	2.72	0.22
Dissolved oxygen (mg/L)	52*	3.86	1.95	6.73	1.26	0.10
Mortality*(fish/day)**	53**	41.19	0.00	1,631.00	230.77	19.03
Rainfall (mm)	55*	7.53	0.00	85.00	14.81	1.99
Fish weight (g)	53	1,213.4	25.5	4,147.6	1,108.10	91.4

*Mean data five days prior to eDNA water sampling; **Mean data five days following eDNA water sampling; *** Mortality recorded five days following eDNA sampling; SD = standard deviation; SE = standard error.

Table 10 Spearman's rho correlation coefficient values among variables monitored in the study (below diagonal). Significance (P) values are represented in bold above diagonal ($P < 0.05$). Spearman's Rho value r = 1 means a perfect positive correlation and the value r = -1 means a perfect negative correlation.

Variables	*Chilodonella* (eDNA)	Fish mortality	Fish weight	Water temperature	Dissolved oxygen	Rainfall
***C. hexasticha* (eDNA)**	-	**0.000**	**0.022**	0.585	0.422	0.341
Fish mortality (fish/day)	0.402	-	**0.000**	**0.000**	0.071	0.370
Fish weight (g)	-0.189	-0.561	-	**0.000**	0.501	0.782
Water temperature (˚C)	0.046	0.448	-0.386	-	**0.000**	**0.000**
Dissolved oxygen (mg/l)	0.067	-0.211	0.057	-0.526	-	**0.011**
Rainfall (mm)	-0.079	0.106	0.023	0.415	-0.212	-

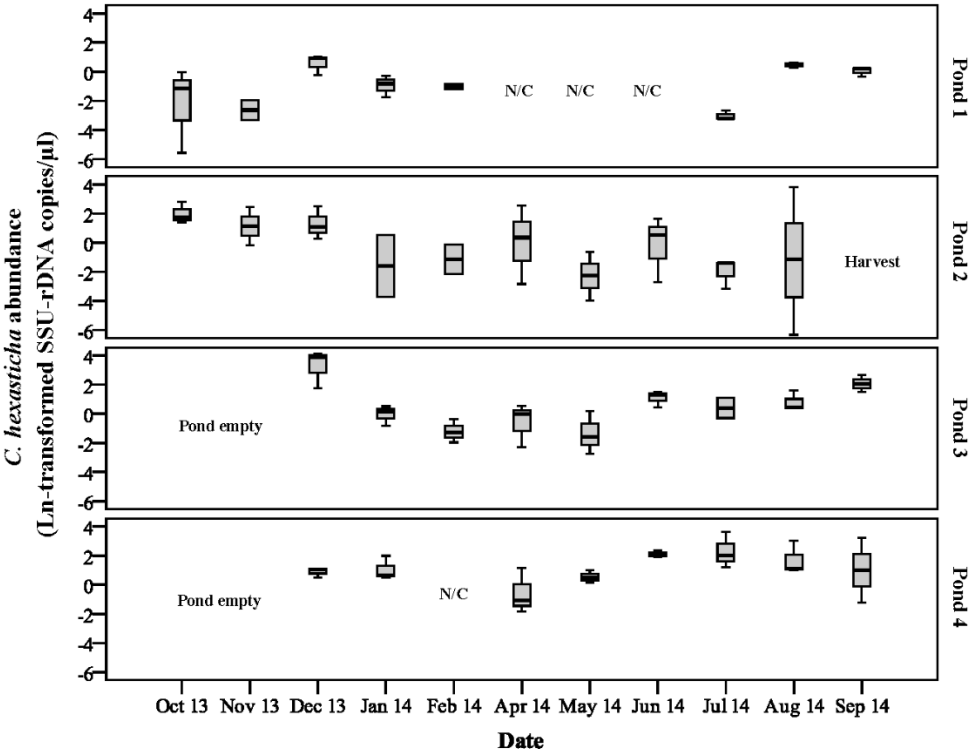

Figure 7 Variation of *Chilodonella hexasticha* abundance (Ln-transformed cell copies/µl) in pond water between Oct 2013 and September 2014, as assessed by the eDNA technique. Ponds 1 to 4 were selected for regular sampling due to history of *Chilodonella* outbreaks. N/C, sample not collected due to chemical treatment applied to ponds.

During the tropical wet season (December to February) the warmest water temperatures were observed, along with the highest monthly rainfall and the lowest dissolved oxygen (Fig. 8A, B). Between December and April, when water temperatures were above 27 °C and daily rainfall were higher than 40 mm, mean fish mortality followed similar trends to the mean abundance of *C. hexasticha* in the water (Fig. 8A, B). During this period (December to April), fish mortality (n=1280) was significantly higher than between May to November (n=135) (Mann-Whitney U test, $P < 0.05$).

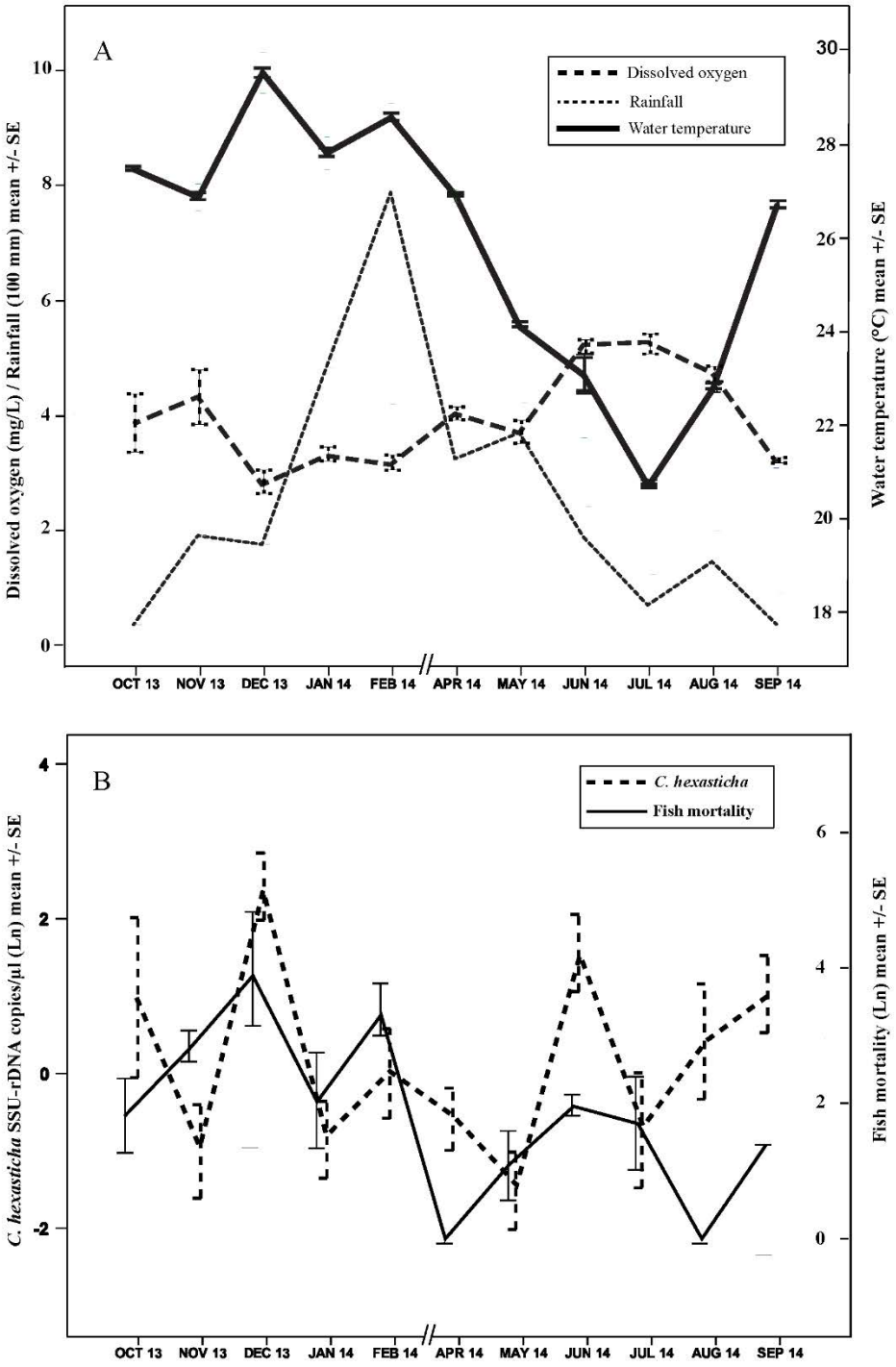

Figure 8 Environmental and biological parameters from a freshwater barramundi *Lates calcarifer* farm located in North Queensland, Australia in 2013/14. A: Mean (SE) dissolved oxygen, water temperature and rainfall; B: Mean (SE) abundance of *C. hexasticha* in water (eDNA) and mean fish mortality.

Two principal components derived from PCA explained 66% of the variance among monitored farm parameters (Fig. 9). A positive association was observed between rainfall and water temperature (quadrant II; Fig 9), which were inversely related to dissolved oxygen levels (quadrant I; Fig 9). Abundance of *C. hexasticha* in pond water (eDNA) and fish mortalities were directly associated (quadrant II; Fig 9) and inversely related to fish weight.

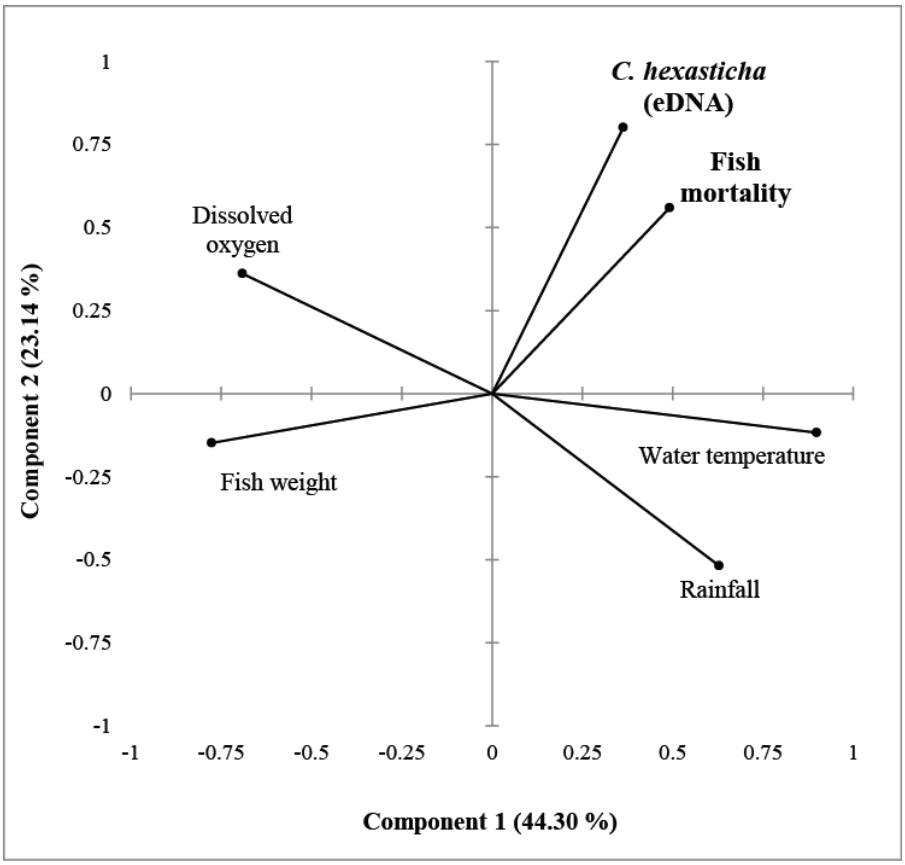

Figure 9 First and second components of principal component analysis (PCA), showing relationship among variables: abundance of *Chilodonella hexasticha* in Lates calcarifer pond water (eDNA), fish mortalities, fish weight, rainfall, dissolved oxygen and average temperature for nine ponds sampled between 2013/14. Mean dissolved oxygen and water temperature were recorded five days prior eDNA collection. Mean fish mortality were recorded five days following eDNA collection.

4.4 Discussion

Environmental DNA (eDNA) analyses are revolutionising how aquatic biodiversity surveys are conducted and also have an application in the detection of pathogens impacting on

aquaculture. In the present study, an eDNA approach was used to quantify the abundance of an important parasitic protozoan that causes chilodonellosis in commercial freshwater finfish aquaculture. We show that eDNA is a powerful approach to detect and quantify levels of *C. hexasticha* in commercial barramundi aquaculture ponds and that there were significant correlations between abundance of this parasite and subsequent fish mortality events. Given that severe fish mortalities can occur with no warning and there are practical difficulties in directly identifying ciliate parasites in pond water and/or on cultured fish, the direct quantification of parasite DNA from water samples can provide farm managers with an efficient tool to assess the risk of future disease outbreaks.

As shown in the present study, environmental DNA and qPCR can be used to monitor abundance of *C. hexasticha* in fish farms and to pre-empt the likelihood of mortalities, particularly during early stages of fish culture. High numbers of *C. hexasticha* in pond water in this study predisposed smaller fish to high mortalities (Table 2, mean = 41.2 fish/day), whereas increased rainfall and decreased dissolved oxygen levels were not directly linked to fish mortalities, or to parasite abundance. Water temperature, however, was shown to be positively correlated with fish mortality events. Other environmental and biological factors, in addition to those measured and reported in this study, may be promoting increased abundance of *C. hexasticha* in fish ponds. Parameters such as ammonia, pH, the availability of organic matter and the association with other microbial (e.g. bacterial, protozoan, microalgae) communities, can impact ciliate protozoan demographics (Lynn, 2008) and could provide further insights into the population dynamics of *C. hexasticha* in farmed barramundi water ponds.

While the relationship between abundance of *C. hexasticha* and fish mortality was evident, the correlation between fish mortality and high rainfall was not significant. Furthermore, low levels of dissolved oxygen had a negative relationship with higher water

temperatures and rainfall. These results are expected within aquaculture ponds, as both dissolved oxygen solubility and saturation decreases with increased water temperatures and reduced algal photosynthetic capacity on overcast rainy days (Boyd and Tucker, 1998). Smaller fish may have a developing immune system which predispose them to be more affected by sub-optimal environmental conditions, such as low dissolved oxygen and drastic environmental changes. These changes can cause stress and impair a fish's immune response to pathogens (Alvarez-Pellitero, 2008). Although generally environmental parameters observed alone cannot clearly indicate early problems, when used in an integrated approach with ways to estimate parasite abundance, such as eDNA, environmental variables can help farmers to make the best management decisions to avoid disease outbreaks.

Abundance of *Chilodonella* in farm water from the fish farm monitored in the present study varied within and between ponds over the year. The specific reasons for variation on *Chilodonella* levels in pond water are difficult to determine based only on our results (Fig. 7). However, parasite density variation may be due to specific pond environmental conditions beyond those monitored in this study (e.g. organic matter, algal blooms, interaction with bacterial community, etc). Furthermore, *Chilodonella* abundance might be influenced by transmission dynamics between parasite and fish, patterns of water exchange in the farm, and physical, chemical and biological characteristics of the water column. It has been reported that in high-stressed aquatic environments (e.g. aquaculture farms) ciliates' population richness tends to be reduced while an increase in resistant species (e.g. potential pathogenic species) is observed (Li et al., 2009).

This study used eDNA to monitor ciliate parasites in a semi-intensive barramundi farm, with focus on the association of environmental parameters and abundance of parasites in water. Molecular genetic quantification of fish parasites such as *Parvicapsula minibicornis,*

Ceratomyxa shasta, *Neoparamoeba perurans*, *Kudoa yasunagai* and *Ichthyophthirius multifiliis* have also been used previously to detect parasites in rivers, the open ocean and aquariums (Jousson et al., 2005; Hallett and Bartholomew, 2006; Atkinson and Bartholomew, 2010; Bridle et al., 2010; Hallett et al. 2012; Ishimaru et al., 2014); however, most of these studies primarily focused either on the qPCR assay development itself, or on the seasonal, geographic and temporal distribution of parasites and their association with fish infections, rather than as a predictive tool for day-to-day farm management. If, however, frequent (weekly) water sampling can be performed on farm, potentially stronger correlations between parasite abundance and fish mortalities can be established, allowing fish farmers to promptly adopt preventative health management and early intervention strategies (e.g. such as water exchange, improvement of oxygen levels, stocking timing and size of fish stocked). In our study we collected water samples on a monthly basis with a clear trend evident between parasite abundance and subsequent fish mortality events. More regular sampling than this, however, would be recommended, as water quality parameters can rapidly change over shorter time frames and these may be what was ultimately influencing parasite community structure and downstream fish health.

Chapter 5. Interactions between parasitic protozoan *Chilodonella* spp. and bacterial communities within a tropical aquaculture system

---

5.1. Introduction

Fish farm ponds are complex and interactive systems where millions of microbes exponentially grow and co-exist with cultured animals (Blancheton et al., 2013; Salipante et al., 2013). The abundance and diversity of these microorganisms are tightly associated to the levels of dissolved and particulate organic matter in the culture systems, influencing water quality parameters and disease risk to culture animals (Blancheton et al., 2013). Interactions between microorganisms and cultured fish species can increase stress, compromise immune capacity and reduce the ability to combat infections (Kotob et al., 2016).

The association between bacteria and ciliate protozoan communities in freshwater pond aquaculture systems is poorly understood. Secondary infections caused by bacteria may increase when parasitic infections physically damage fish epithielium or compromise the immune response (Bowden et al., 2007; Kotob et al., 2016). Furthermore, ciliate parasites can carry bacteria and deliver it to the host while feeding (Kotob et al., 2016). Many studies have reported increased mortality rates in fishes co-infected by parasites and bacteria (Sun et al., 2009; Xu et al., 2014; Xu et al., 2015; Kotob et al., 2016). For instance, co-infections caused by the protozoan *Trichodina* spp. and the bacteria *F. columnare* can cause serious mortalities in the hybrid tilapia, *Oreochromis niloticus* x *Oreochromis aureus* (Xu et al., 2015).

Freshwater fish farms commonly source water from rivers or lakes adjacent to the farm location. This water source contains a diverse range of microorganisms, including potential harmful pathogens which naturally occur in infected wild fish populations (Oidtmann et al.,

2011a,b, 2013). Water treatment such as filtration and sterilization before pond stocking can be expensive and is not practical in many cases. Additionally, water treatments can significantly affect the balance between autotrophic (e.g. bacteria responsible for oxidation of ammonia to nitrate) and heterotrophic (bacteria responsible for degradation of organic material) bacteria (Hagopian and Riley, 1998). Water treatment may also eliminate desirable pond microorganisms (e.g. phytoplankton, zooplankton) and thus impact fish performance (Blancheton et al., 2013).

Diseased fish impact aquaculture operations by propagation of pathogens within farms and adjacent natural environments (Segner et al. 2012). Proliferation and epidemiology of pathogenic ciliate protozoans is particularly complex, because ciliates may change their behaviour (e.g. reproduction and feeding) according to the diversity of bacteria and changes in the environmental parameters of the culture systems (e.g. dissolved oxygen; Rintamäki et al., 1994; Shukla and Gupta, 2001; Blancheton et al., 2013). Ciliates, notably, *Chilodonella* spp., can significantly impact farmed freshwater fishes as they can rapidly proliferate in responses to changes in environmental parameters (e.g. temperature and rainfall) and the bacterial community present in the water (Lom and Dyková, 1992; Keeling et al. 2014; Chapter 2; Bastos Gomes et al., 2016). To date, limited information exists in relation to microbiome composition, community dynamics and their relationship with parasite induced mortalities in freshwater fish farms.

Monitoring the parasite and bacterial community which co-habit the water column could generate a better understanding about the ecology of these microorganisms, improve operational design and promote new ways to prevent disease outbreaks (Rutvisuttinunt et al. 2013). Through co-surveillance (investigation of multiple microorganisms), interactions between microbial communities (e.g. bacteria, viruses, fungi and protozoans) in fish farms can

deepen our understanding of the culture environment and consequently improve animal health, ensuring appropriate biosecurity management and promoting innovative and sustainable production (Rutvisuttinunt et al. 2013; Kotob et al., 2016; Rud et al., 2016; Stentiford et al., 2017).

Next generation DNA sequencing (e.g. high throughput 16S sequencing) now permits a deep understanding of microbiome composition and diversity from aquaculture systems at increasingly lower costs (Qin et al., 2016). Furthermore, microbial investigation from infected animals through non-invasive and environmentally friendly techniques (e.g. eDNA sampling) presents an alternative approach to conventional techniques (e.g. microbiology culture; histopathology) for preventive health management in fish farms. This study presented the first report on the interactions between bacterial communities and the ciliate protozoan *Chilodonella hexasticha* (Kiernik, 1909) in a freshwater barramundi (*Lates calcarifer*) farm from tropical Queensland, Australia.

5.2. Material and Methods

5.2.1. Water collection, mortality records and gills sampling

Water samples were collected from a commercial semi-intensive freshwater barramundi (*Lates calcarifer*) farm operated with limited flow-through water exchange, situated in north Queensland, Australia. Samples were collected from October 2013 to September 2014 (except March 2014), from four production ponds (~1.4 ha earthen ponds ~20 ML) with a history of fish infections caused by *Chilodonella hexasticha*. The collection of water samples and extraction of eDNA followed the protocols previously described (Chapter 4). This eDNA material was used to quantify the abundance of *C. hexasticha* using qPCR (Chapter 4) and for the analysis of the bacterial communities using a 16S metagenomics approach (described below). Additionally, fish mortalities from these ponds were monitored by farm technicians

who visually surveyed and removed dead fish from ponds three times per day. The association among fish mortalities, the abundance of *C. hexasticha* (SSU-rDNA copies/µl), and the composition of bacteria (16S metagenomics) in pond water, was assessed using the mean number of dead fish recorded over the 5 day period immediately following water sampling in each pond.

Lates calcarifer (ranging from 100 g to 3 kg) showing behavioural and clinical signs of *Chilodonella* infection (i.e. not feeding and gasping at pond edges) were also sampled between October 2013 and September 2014. Specifically, infected fish were sedated with AQUI-S (AQUI-S, New Zealand Ltd) and gills scraped gently using the blunt edge of a scalpel blade to prevent bleeding. Over ten months, twenty gills of highly infected fish were collected by the farm's health manager and preserved in 80% ethanol for subsequent metagenomics analyses. Of note, 'low' (< 5 *Chilodonella* cells/per field of view) and 'high' (> 5 *Chilodonella* cells/per field of view) infection intensity reflects the standard maximum used by the farm prior to pond treatment to manage *Chilodonella* outbreaks. Presence of *Chilodonella* cells in the gill samples of all twenty fish was further confirmed by Sanger sequencing (see Chapter 3; section 3.2.4.). The specific bacterial community found on barramundi gills infected by *C. hexasticha* was also analysed (see section 5.2.5).

5.2.2. DNA extraction, qPCR and library preparation

DNA extraction from fish gills and water samples followed the protocol described in Chapter 3 and 4 (Bastos Gomes et al. 2017a and b). Abundance of *C. hexasticha* in pond water samples was quantified using an eDNA sampling approach (see Chapter 4; sections 4.2.1., 4.2.3 and 4.2.4). DNA extracted from water samples (eDNA) and fish gills was quantified using the Qubit dsDNA HS Assay Kit (Invitrogen, Eugene, OR, USA) and diluted to obtain 1 ng in 5 mL of ultra-pure water. Amplicon PCR was performed following the Illumina Nextera XT kit

protocol, and targeted the V3 region of the bacterial 16S gene using the 16S Amplicon PCR Forward Primer (5'-AGAGTTTGATCCTGGCTCAG-3') and 16S Amplicon PCR Reverse Primer (5'-ACGGCTACCTTGTTACGACTT-3') with Illumina overhang adapters for sequencing (IDT, Iowa, USA). PCR reactions were carried out in a final volume of 25 µL using 10 µM of each 16S amplicon primers and Q5 hot start high fidelity DNA polymerase (New England BioLabs, MA, USA) following manufacturer's instructions. PCR cycling conditions were as follows: 1 min at 98 °C, 35 cycles of 5 s at 98 °C, 10 s at 60 °C, 20 s at 72 °C; 1 min of final extension at 72 °C. Amplicons were purified using Sera-Mag Speedbeads Carboxilate - Modified Magnetic Particles (Thermo Scientific, CA, USA) according to Faircloth and Glenn (2014). A second PCR was performed using Illumina indexing adapters with the Nextera XT Index Kit D (Illumina, CA, USA) with PCR cycling conditions as above except for using only 15 cycles, followed by a second amplicon cleaning step. Final libraries were quantified as described earlier using Qubit dsDNA HS Assay Kit and 1 µL of the undiluted library was analysed on a 2100 Bioanalyzer using a High Sensitivity DNAchip (MAKER). The library was normalised, pooled and denatured to be sequenced on a MiSeq sequencing platform, using a 2 X 300 cycle v3 flow cell (Illumina, CA, USA).

5.2.3. Quality control of sequence reads

A total of 1,726,619 paired-end reads were obtained from the microbial sequences, with an average of 28,300 high quality reads per sample post-quality filtering. The resulting reads permitted the identification of a total of 6,157 Operational Taxonomic Units (OTUs) and 139 genera with a 97% identity threshold. The identity of each OTU represents a singular 16S rRNA marker sequence that refers to a specific taxonomic rank (e.g. family, genus, species; Rud et al., 2016). Individual rarefaction curves of mean phylum (pond water) and species (fish gills) richness per sample reached a plateau at about 30,000 and 20,000 sequence reads, respectively

(Fig. 10A and B, respectively), indicating an acceptable depth of sequencing that represents the entire bacterial community within each pond and fish gills sampled throughout the year.

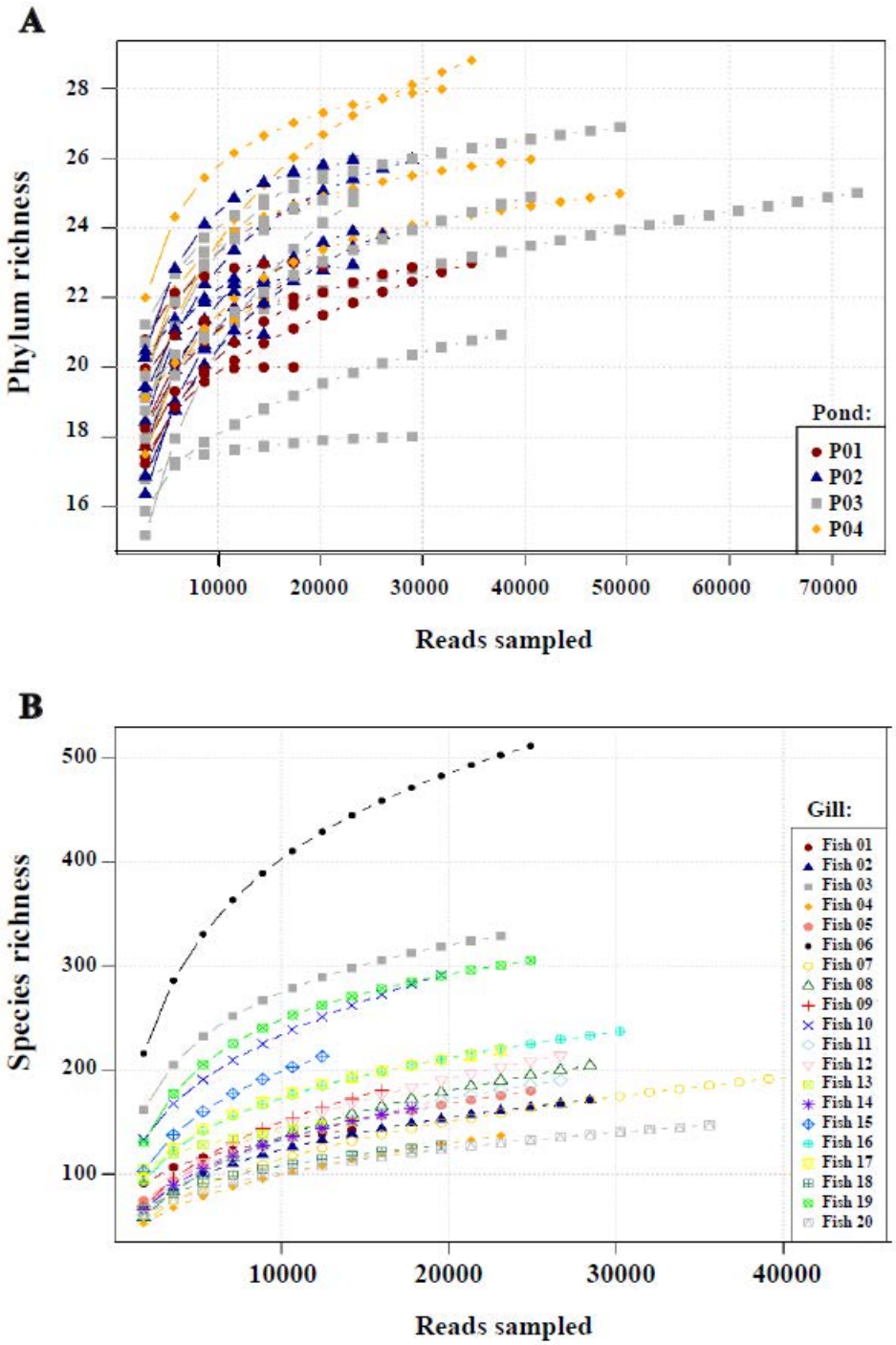

Figure 10 A) Rarefaction analysis of mean phylum richness of ponds 1, 2, 3 and 4 over 11 months. Number of observations = 83 per month. B) Rarefaction analysis of mean species richness from fish gills. Number of observations = 20.

5.2.4. Data treatment and analysis

The Illumina MiSeq sequencing platform control software (MCR/RTA) was used for read multiplexing and initial quality filtering. The primer sequence was removed by trimming 16 bp from the 5" ends of sequences using fastx_trimmer (FASTX-Toolkit). Quality trimming for Q20 was then carried out in Sickle (Joshi and Fass, 2011) and only the forward reads were used for analysis. The remaining data processing was carried out in QIIME (Caporaso et al., 2010). Here split_libraries_fastq.py was used to merge the individual samples into one file. Chimeras were identified using identify_chimeric_seqs.py with usearch (v 6.1, Edgar et al., 2011) and removed using filter_fasta.py. Clustering and operational taxonomic units (OTUs) identification was then carried out using pick_de_novo_otus.py. Finally, non-prokaryotic OTUs were removed using fitler_taxa_from_otu_table.py, low abundance OTUs were removed with filter_otus_from_otu_table.py at 0.01% of the data, and normalization was carried out with normalize_table.py using CSS. The resulting taxonomic classification tables were analysed using the online tool Calypso (v5, http://bioinfo.qimr.edu.au/calypso; Zakrzewski et al., 2017).

5.2.5. Statistical analysis

Taxonomic analysis of the data was performed from phylum to species level. To understand the microbial diversity within water and gill samples of infected fish, data was submitted to alpha diversity analysis in Calypso (v5, http://bioinfo.qimr.edu.au/calypso) using Shannon diversity index to examine the microbiome diversity within ponds (Fig. 12), along the year. Shannon index was calculated as $\Sigma pisi{=}1 lnpi$ where s equals the number of bacteria species, p represents the proportion of one species divided by the total of species found, and ln is the natural log (Shannon, 1948).

Relative abundance of bacteria in the pond microbiome and the relationship with the abundance of *C. hexasticha* in water and fish mortalities was performed based on the Bray-

Curtis distance metric through a Pearson's correlation represented by a heatmap diagram generated in Calypso (v5, http://bioinfo.qimr.edu.au/calypso). Colour intensity on the heatmap diagram represents the strength and direction of Pearson's Correlation as similarity measures. Pearson's Correlation was calculated on the relative number of reads assigned to each family. Pearson's correlations < - 0.4 were visualised as dark blue and > 0.4 as yellow to dark red.

For comparative analysis, cut off values between low and high fish mortalities, and low and high abundance of *C. hexasticha* in water, from all sampled ponds were arbitrary determined by the median value of 7.4 dead fish/pond/day and 1.5 SSU-rDNA copies/µl, respectively. To determine whether significant differences exist within bacterial species counts and abundance of *C. hexasticha* in water within the farm, pair-wise comparisons were performed by t-tests using Calypso (v5, http://bioinfo.qimr.edu.au/calypso). Likewise, to investigate significant differences within bacterial species counts and observed fish mortalities t-tests were also performed using the online tool Calypso. Furthermore, differences in relative abundance of bacteria (species level) from fish gills were analysed by a Kruskal-Wallis test (P <0.05) in SPSS (version 23.0; IBM, USA).

5.3. Results

Chilodonella hexasticha was ubiquitously present in every pond all year around, but in different concentrations throughout the year (Chapter 4; section 4.3.; Fig. 7). Fish mortalities were also variable, following a similar trend as the abundance of *Chilodonella* in water. Months with higher abundance of *Chilodonella* in water were also months with higher mortalities (Chapter 4; section 4.3.; Fig. 8B). Specifically, abundance of parasite in water and fish mortalities were higher between December 2013 and April 2014. Fish mortalities were highly variable within ponds, with a maximum of 1,631 dead animals reported in a single day (Table

11). Summary statistics of fish mortality and *C. hexasticha* abundance data obtained from four farm ponds sampled between October 2013 and September 2014 are presented in Table 11.

Table 11 Statistical summary of abundance of *Chilodonella hexasticha* in water and fish mortality data recorded from four ponds from a freshwater barramundi Lates calcarifer farm from tropical Australia for a 11 month period. n = number of observations.

Variable	**n**	**mean**	**minimum**	**maximum**	**S.D.**	**S.E**
eDNA (*C. hexasticha* cell copies/µl)	83	4.8	0.0	63.3	10.3	1.02
Mortality * (fish/day)	29**	56.5	0.0	1631.1	278.7	27.88

* Mortality recorded five days following eDNA sampling; **Average data from 5 days post eDNA water sampling over 11 month period; S.D. = standard deviation; S.E. = standard error.

5.3.1. Microbial composition in *Lates calcarifer* ponds

Thirty-one bacterial phyla were identified in the freshwater barramundi farm pond water over the 10 month period. The four most abundant phyla were Actinobacteria (42%), Proteobacteria (28%), Bacteroidetes (10%) and Cyanobacteria (6%), followed by Planctomycetes, Verrucomicrobia, Fusobacteria, Chloroflexi, Chlorobi and OD1 (Fig. 11). While minor changes were observed within and between ponds over the sampling period, the microbial community diversity among ponds were not significantly different (as assessed by Shannon diversity index) at the phylum level; Shannon, $P > 0.05$; (Fig. 12). Given the absence of any significant difference in bacterial diversity among the four sampled ponds, pond origin was not included as an effect in downstream analyses.

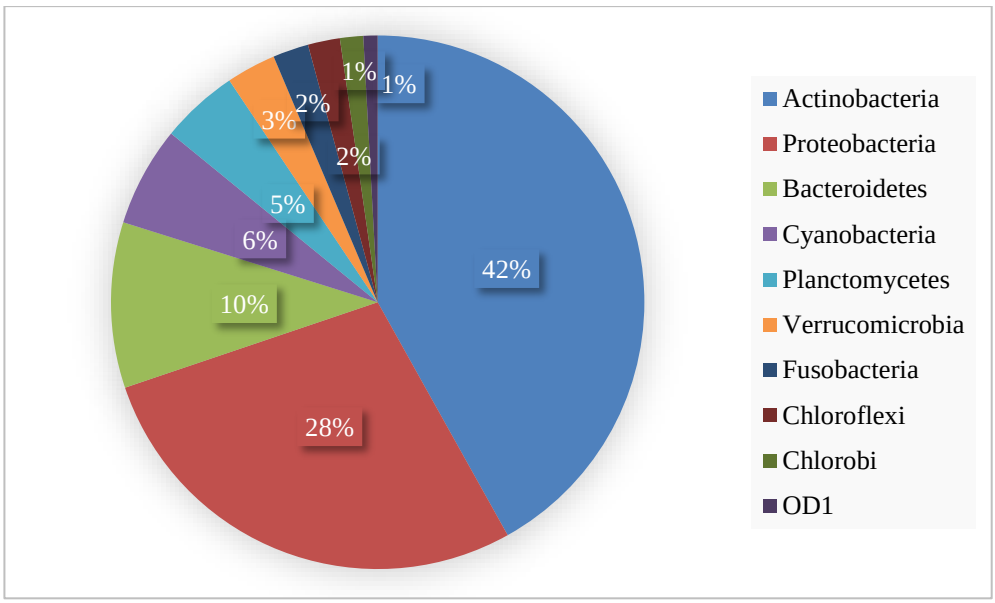

Figure 11 Ten most abundant bacterial phyla present in ponds in a freshwater barramundi *Lates calcarifer* farm from tropical Australia.

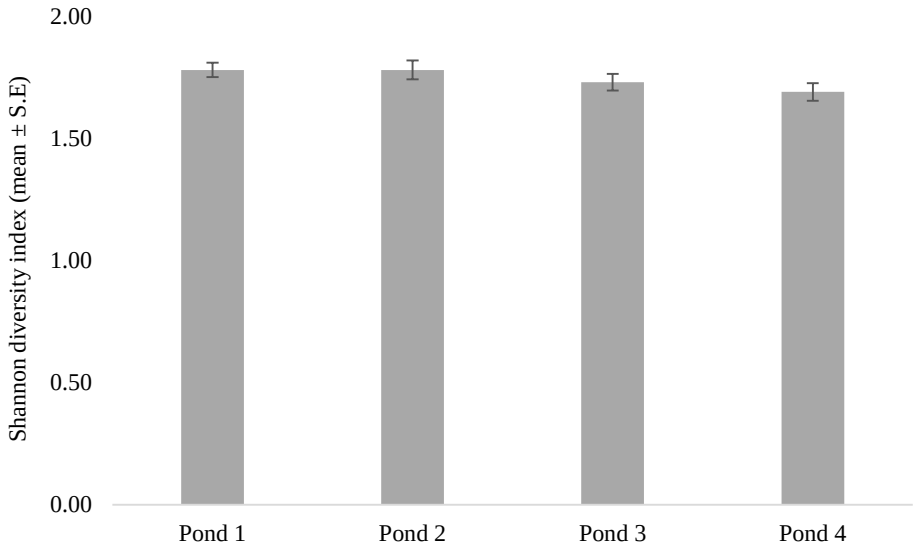

Figure 12 Bacterial diversity for each pond (1, 2, 3 and 4) sampled over a 11 month period.

5.3.2. Abundance of bacterial communities, *Chilodonella* spp in pond water and observed fish mortalities.

The relative abundance of the pond bacterial profile at species level was correlated with the abundance of *C. hexasticha* in pond water and fish mortalities. Bacterial profiles associated to each variable were primarily divided into two main clusters (1 and 2), as observed in the hierarchical clustering dendogram and heatmap (Fig. 13). Positive correlations were found between *C. hexasticha* abundance and *Leptospira biflexa* and the unclassified (unknown bacteria) group (cluster 1) and *Veillonella dispar, Flavobacterium columnare*, *Bdellovibrio bacteriovorus, Aquirestis caiciphila, Cryobacterium psychrophilum, Agrococcus jenensis* and *Blastomonas natatoria* (cluster 2), whereas positive correlations were found for fish mortalities and *F. columnare, V. dispar,* and *B. bacteriovorus* (Fig. 13). Further, the association between the abundance of each bacterial species against high and low levels of *C. hexasticha* abundance and fish mortalities was also investigated. Here, high *Bdellovibrio bacteriovorus* abundance was significantly associated with high levels of *C. hexasticha* in water ($P < 0.05$, see Fig. 14), whereas high *F. columnare* abundance was significantly associated with high fish mortalities ($P < 0.05$, see Fig. 15). Conversely, high abundance of *Limnohabitants curvus, F. succinicans* and *Cetobacterium somerae* were associated with low rates of fish mortalities ($P < 0.05$, see Fig.15).

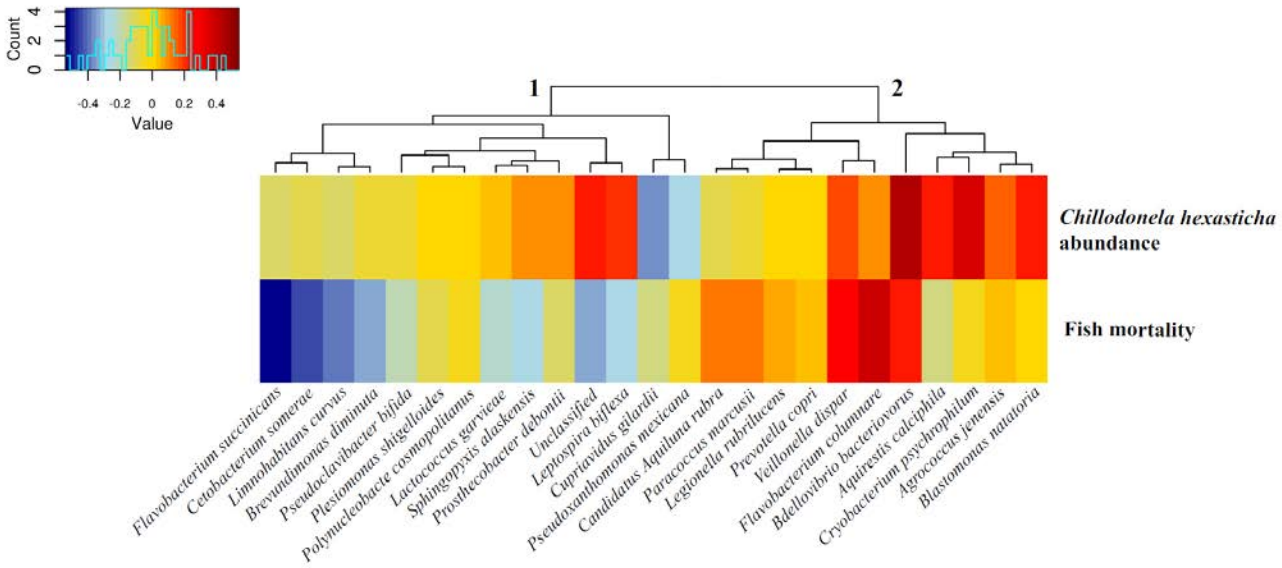

Figure 13 Relative abundance of barramundi *Lates calcarifer* pond bacterial microbiome linked to *Chilodonella hexasticha* SSU-rDNA copies/µl and fish mortalities tested on farm over 10 months (family-level diversity). Heatmaps were hierarchically organised by coloured dendogram on top left of figure where darker blue and red represent a strong negative and positive correlation, respectively; bacterial clusters 1 and 2 are indicated. Microbial communities are represented at the X axis and variables on right Y axis.

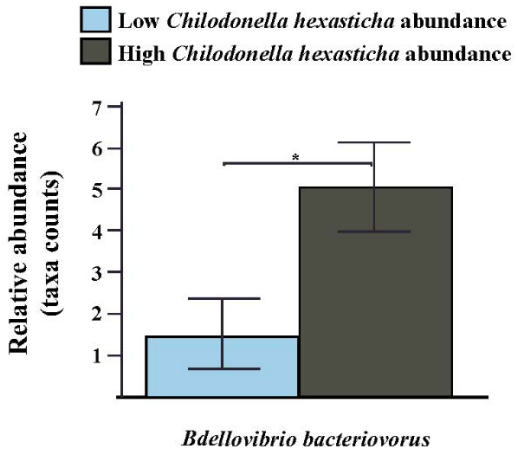

Figure 14 Relative abundance of *Bdellovibrio bacteriovorus* taxa counts associated with high or low abundance of *Chilodonella* (SSU-rDNA copies/µl) in water (significantly different; * $P < 0.05$). Cut off levels for *Chilodonella hexasticha* abundance (high vs. low) was based on the median value of 1.53 SSU-rDNA copies/µl. Mean taxa counts ± S.E.

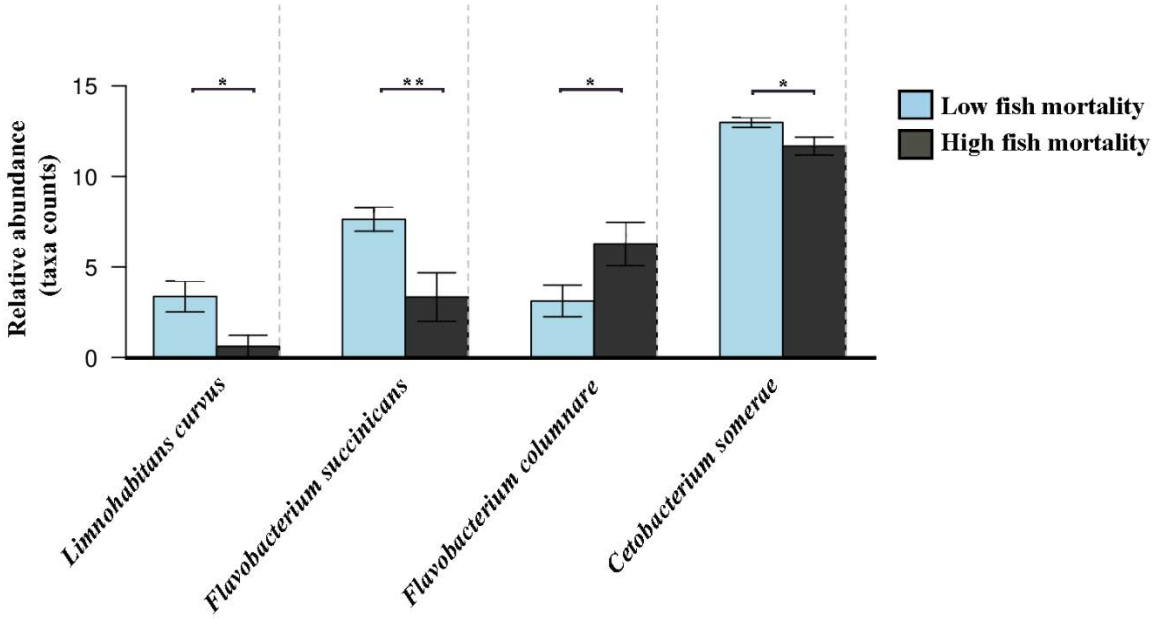

Figure 15 Relative abundance of bacterial species taxa counts associated with high or low fish mortality (* $P < 0.05$; ** $P < 0.001$). Cut off levels for fish mortality was based on the median value of 7.4 dead animals per day. Mean taxa counts ± S.E.

5.3.3. Bacterial communities from barramundi gills with high abundance of *C. hexasticha*

Flavobacterium colmnare was the most abundant bacteria that was identified to species on the gills of fish infected with *C. hexasticha,* followed by *Limnohabitans curvus* and *Sphingobacterium multivorum*. However, ninety percent of bacteria found on barramundi gills were unclassified (or yet unknown) species. *Flavobacterium colmnare* was also positively correlated with abundance of *C. hexasticha* in pond water and significantly correlated with fish mortalities on farm (Fig. 13).

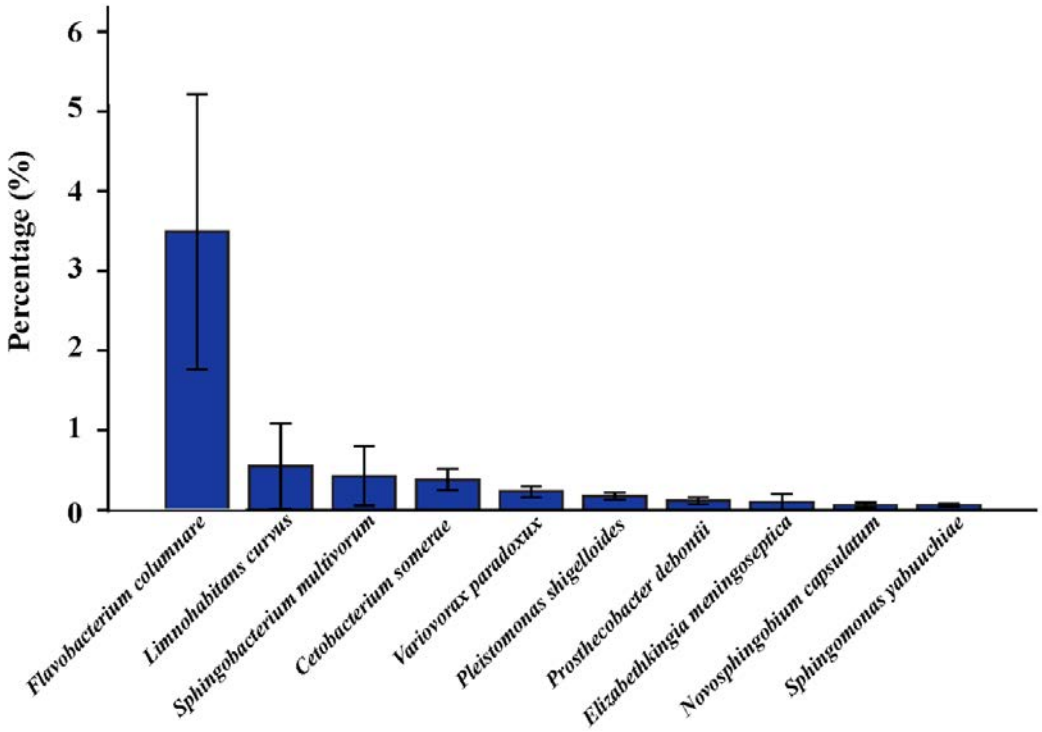

Figure 16 Ten most abundant bacterial species found on gills with high abundance of *Chilodonella hexasticha* (n = 20; number of gills confirmed infected by *C. hexasticha*; mean percentage ± S.E.) in freshwater barramundi (*Lates calcarifer*).

5.4. Discussion

The complexity of disease outbreaks is intrinsically linked to the abundance, diversity and interaction of microbial communities present within the culture environment (Rud et al., 2016). To manage disease events and sustainably expand aquaculture production, novel systematic approaches are necessary to understand the role played by different microbial communities. Understanding the role of specific microbiome found in aquaculture systems can highlight the best pond/cage management strategies to minimise the impacts of outbreaks in fish farms (Rutvisuttinunt et al., 2013; Rud et al., 2016; Stentiford et al., 2017). This study provided a combined metagenomics and eDNA methodology as a novel strategy to better understand the demographics of bacterial communities and their association with the parasitic

protozoan *C. hexasticha* present in pond water and on the gills of farmed barramundi. Although bacteria and ciliates are the most abundant microorganisms found in aquatic environments (Lom and Dykova, 1992), Hahn and Höfle (2001) have suggested that the interaction between these two groups are potentially more common than has been reported. *Chilodonella hexasticha and C. piscicola* (see Chapter 3; Bastos Gomes et al. 2017a for likely synonymy with *C. piscicola*) are opportunistic ciliate parasites reportedly able to infect more than 32 fish species worldwide (Chapter 2; Bastos Gomes et al., 2016). *Chilodonella* spp. feed on the epithelium tissues of fish, but also on attached cells such as bacteria and algae present in the aquatic environment (Lynn, 2008). Thus the abundance of certain bacterial and algal prey species may be an integral factor in driving the population dynamics of this ciliate species. Nevertheless, despite this possibility, interactions between *C. hexasticha* and particular bacterial species have never been previously reported.

High throughput next generation sequencing of the V3 region of the 16S rRNA gene allowed for the first time for a greater understanding of the microbial composition present in tropical freshwater ponds where barramundi is farmed all year round. Actinobacteria was the most abundant bacterial phylum present in tested ponds, followed by Proteobacteria, Bacteroidetes and Cyanobacteria, respectively. It was also demonstrated that similar levels of bacteria diversity existed within the four tested fish production ponds. A previous study also demonstrated similar bacterial composition in freshwater fish ponds in China, although in this case, Proteobacteria was the most abundant phylum followed by Bacteroidetes, Actinobacteria, and Cyanobacteria (Qin et al., 2016). However, the characterisation of bacteria from freshwater shrimp *Litopenaeus vannamei* farming using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and 16S rDNA pyrosequencing technology demonstrated that the most abundant phyla (Actinobacteria, Proteobacteria, Bacteroidetes and Cyanobacteria) in

their aquaculture system (Tang et al., 2014) was similar to the present study and corroborated the fact that Actinobacteria represent some of the most abundant bacteria found in the water column of freshwater environments (Klausen et al., 2004).

Distribution and abundance of Actinobacteria taxa in freshwater culture systems can be influenced by different water parameters (e.g. pH and dissolved organic carbon). But Actinobacteria abundance in the environment can be especially affected by the concentration of humic substances (major components of the natural organic material) in water (Wei et al., 2015), as these bacteria consume humic substances and their presence in aquaculture systems can promote degradation of organic material and water quality improvement (Klausen et al., 2004). Furthermore, high amounts of Actinobacteria members in freshwater systems is also associated with the production of geosmin and 2-methylisoborneol (MIB) odours which are associated with flavour tainting of freshwater farmed fishes (Summerfelt and Hochheimer, 1997).

The second most common bacterial phylum in freshwater barramundi ponds from tropical Australia was Proteobacteria which have high metabolic diversity and are extremely important for the carbon, sulfur and nitrogen cycles in the environment (Kersters et al., 2006; Qin et al., 2016). This phylum has important significance for medical, veterinary, industrial and agricultural industries, as it comprises many Gram-negative pathogenic bacteria (Kersters et al., 2006). Considering that the bacterial community composition of fish digestive traits is similar to aquaculture water and sediment (Wu et al., 2012), fish in the studied ponds were constantly exposed to potentially pathogenic bacteria. However, the presence of pathogenic bacteria in the environment is not always translated into disease events. Other factors such as stocking density, water quality parameters and feed quality play an important role in fish illness

(Oidtmann et al., 2011b): hence it is critical to maintain best management practices on farm to minimise fish stress and improve the culture conditions.

Importantly in this study, the eDNA sampling methodology of pond water coupled with qPCR and a high throughput 16S metagenomics approach was able to reveal novel interactions among the abundance of *C. hexasticha* and the bacterial species *V. dispar, F. columnare* and *B. bacteriovorus* in the context of barramundi mortality events (Fig. 14; cluster 2). When samples were further categorised by high and low barramundi mortality events post sampling, *Flavobacterium columnare* was the only bacterial species identified for which higher concentrations in pond water were associated with fish mortalities (Fig. 15). In gills of barramundi infected by *C. hexasticha, F. columnare* was by far the most abundant bacterial species, outnumbering the second most abundant species (*Limnohabitants curvus*) 6-fold (see Fig. 16), suggesting an intrinsic harmful interaction between them. *Flavobacterium columnare* is a well-known opportunistic pathogenic bacteria responsible for columnaris disease in freshwater fishes (Declercq et al., 2013). This globally distributed bacteria has been extensively isolated from various commercial fishes such as common carp (*Cyprinus carpio*), channel catfish (*Ictalurus punctatus*), goldfish (*Carassius auratus*), eel (Anguilliformes), perch (*Perca* spp.), salmonids (*Salmo* spp.; *Oncorhynchus* spp.) and tilapia (*Oreochromis* spp.; *Sarotherodon* spp.; *Tilapia* spp.) (see Decostere et al., 1998; Figueiredo et al., 2005; Bernardet and Bowman, 2006; Řehulka and Minařík, 2007; Soto et al., 2008; Suomalainen et al., 2009; Morley and Lewis, 2010; Declercq et al., 2013).

Interactions between important fish parasites and *F. columnare* have been previously reported. For example, the myxosporean *Myxobolus tilapiae* and *F. columnare* co-infected earthen pond reared Nile tilapia (*Oreochromis niloticus*) and Nile catfish (*Clarias gariepinus*) during drastic water quality changes in the culture environment (Eissa et al., 2010). It has been

shown that *F. columnare* can be transmitted by *Ichthyophthirius multifiliis* cilia, promoting co-infections to the channel catfish *Ictalurus punctatus* (see Sun et al., 2009). Furthermore, treatment of concurrent infections by the protozoan *Trichodina* spp. and *F. columnare* has a demonstrated reduction on *F. columnare* loads on gills and mortalities of the hybrid tilapia, *Oreochromis niloticus* x *Oreochromis aureus* (see Xu et al., 2015).

In the barramundi farm monitored in this study, environmental conditions within ponds may have favored a synergistic interaction between *C. hexasticha* and *F. columnare. Chilodonella* exhibit a special feeding mechanism (Chapter 2: Bastos Gomes et al., 2016) which could provide a portal of entry for pathogenic bacteria such as *F. columnare*. Gill and skin damage (e.g. lesions, ulcers and abrasions) caused by the protozoan *Ichthyophthirius multifiliis* have been demonstrated to impact fishes' first line of protection against infections, therefore promoting opportunities for bacterial entrance (Xu et al., 2014). Potential disease outbreaks caused by these two microorganisms are intrinsically linked to particular environmental conditions present in fish farms (Pulkkinen et al., 2010; Kunttu et al., 2012; Declercq et al., 2013). *Chilodonella hexasticha* abundance in freshwater fish ponds has been linked with warm temperatures and the presence of high organic matter in the environment (see Chapter 2; Bastos Gomes et al., 2016; Bowater and O'Donoghue, 2014). Likewise, high organic load, temperatures around 25 °C and alkaline water are considered favorable environmental conditions to maintain *F. columnare* in the water column for extensive periods (Declercq et al., 2013).

Chilodonella uncinata uses chemical signals (quorum sensing) sent by bacterial cells to get access to them for feeding purposes (Dopheine et al., 2011). Therefore, the association found in this study between *C. hexasticha* and *F. columnare* could follow a similar pattern in fish farming conditions, where *C. hexasticha* obtains chemical cues from *F. columnare* for

feeding and reproduction purposes. Considering aquaculture farms are complex systems where many microorganisms interact constantly with each other and different environmental parameters have specific roles in maintaining good water quality and fish welfare, rarely will a single agent or factor will be responsible for disease outbreaks (Oidtmann et al., 2011b and 2013).

Bdellovibrio bacteriovorus was also observed to have a strong correlation with the abundance of *C. hexasticha* in pond water and *Lates calcarifer* mortalities (Fig. 13). *Bdellovibrio* bacterial species are motile, obligate aerobic Gram negative Deltaproteobacteria with strong predatory lifestyle as they kill larger pathogenic Gram negative bacteria and algae (Sockett, 2009). The abundance of *B. bacteriovorus* in water during elevated loads of *C. hexasticha* in ponds may be linked to the action of this bacterium against other harmful bacterial species (e.g. *F. columnare*) present in the farm water. Burnham et al. (1968) demonstrated that *B. bacteriovorus* can invade *Escherichia coli* causing its death. *Bdellovibrio bacteriovorus* was also able to cause complete lysis of green algae (*Phormidium luridum*) and cyanobacteria (*Microcystis aeruginosa*) cells (Burnham et al., 1976; Caiola and Pellegrini, 1984). Furthermore, *B. bacteriovorus* has been isolated from cultured fish ponds previously and used to control experimental *Aeromonas hydrophila* infections (Chu and Zhu, 2010). However, further research is needed to confirm that the abundance of *B. bacteriovorus* in water was caused by the presence of pathogenic bacteria (e.g. *F. columnare*), and harmful parasites (e.g. *C. hexasticha*), or the concurrent presence of all these microorganisms under farm conditions due to other population drivers.

While high rates of freshwater barramundi mortalities were significantly associated with pathogenic *F. columnare*, low mortality rates were significantly associated with the presence of *Limnohabitants curvus, F. succinicans* and *Cetobacterium somerae* in pond water samples.

The direct link between *L. curvus*, *F. succinicans* and *C. somerae* and low rates of barramundi mortalities is difficult to identify. *Limnohabitants curvus* is an aerobic and non-motile bacterium recently isolated for the first time from freshwater lakes (Hahn, et al., 2010) and *C. somerae* is an obligated anaerobic bacterium naturally found in the intestines of freshwater fishes (Tsuchiya et al., 2008; Roeselers et al., 2011). However, *F. succinicans* is considered an opportunistic bacterium, normally found in healthy fish, but sometimes acting as a facultative pathogenic bacteria to fishes (Loch and Faisal, 2015). Thus, *Limnohabitants curvus, F. succinicans* and *Cetobacterium somerae* may be worthy of further investigation in case these bacterial species provide a probiotic effect on infection-likelihood of fish.

In conclusion, this study provided the first report on the potential interaction between parasite abundance with the bacteria microbiome (in the environment and on fish) and associated fish mortalities. While significant advances had been made on the use of metagenomics approach to understand the microbiome community of environmental samples this technology has its limitations. There is a number of bacterial species within the aquatic environment that are still unknown and therefore limits our true understanding on the interactions between parasites and bacterial communities within aquaculture systems. Future research could elucidate other microorganism interactions within aquaculture systems and the drivers of pathogen population dynamics. Better understanding of the microbiome dynamics in fish farms may lead to development of vaccines or other prophylactic therapies against diseases. The complex aquatic environment in fish farms, where micro- and macroorganisms are constantly interacting, demands novel ways to monitor, prevent and control complex diseases in aquaculture. A shift from the model which uses single pathogen identification protocols to predict the occurrence of disease towards one that monitors the interaction between the

microbial community and host/host environment may be a useful strategy to guarantee the continuous growth of the aquaculture industry.

Chapter 6. General discussion

Diseases and their associated impacts are considered among the most significant obstruction factors for the exponential growth of the aquaculture sector (FAO, 2016). Successfully reducing the burden caused by parasitic agents may arise from changing the principle of 'complete pathogen exclusion' to 'system management' in aquaculture systems, where the constant presence of pathogens is often a reality (De Schryver et al., 2015; Peeler and Otte, 2016; Stentiford et al., 2017). This is particularly important as the efforts to eradicate established pathogens within culture systems, in many cases, may be ineffective and/or economically unpractical (Peeler and Otte, 2016). Even when strong biosecurity measurements are implemented, the idea of 'complete freedom from disease' may not be realistic (Bentzon-Tilia et al., 2016). Integrating technological and research advances to monitor and strategically manage complex aquaculture systems, should be the primary focus to achieve the best outcomes with minimum economic impact (Peeler and Otte, 2016; Stentiford et al., 2017).

Worldwide, considerable efforts have been made by government and industry to exclude pathogens from terrestrial and aquaculture production systems (Oidtmann et al., 2013; Peeler and Otte, 2016). While elimination of disease, and prevention of further incursions are important methods to protect animals, they may not be economically viable. For example, in Europe the elimination of harmful pathogens from culture systems such as white spot syndrome virus (WSSV) of prawns and the mollusc parasite *Bonamia ostreae* hasn't being pursued, as no evidence exists to balance the benefits (reduced risk of disease in wild populations) against the high costs of managing a disease freedom status (e.g. restriction on imported animals; De Schryver et al., 2015; Peeler and Otte, 2016). Therefore, considerable focus on innovative

prevention techniques, including aquatic animal health surveillance and environmental monitoring methods, is a more practical approach to complete elimination of pathogens and should be pursued. The rapid development of new molecular techniques (Chapter 3) such as high-throughput (HTS) and real-time sequencing should be integrated to traditional techniques (e.g. microbiology; *in vitro* culture; Chapter 2) to understand biological processes within aquatic environments (Chapter 4, Bastos Gomes et al., 2017b; Bentzon-Tilia et al., 2016). Furthermore, continually improved monitoring techniques that link HTS technologies to environmental monitoring methodologies (e.g. eDNA) and environmental data from aquaculture farms should be the focus of future research with the objective to deliver tangible recommendations for strategic aquatic animal health management (Bourlat et al., 2013; Ininbergs et al., 2015; Bentzon-Tilia et al., 2016).

6.1 Threat of parasitic ciliates for aquaculture

Research outlined in this thesis explored key knowledge gaps in regards to the prevailing status of parasitic ciliates *Chilodonella* spp. in the global freshwater aquaculture industry. Chapter 2 updated knowledge on the distribution of *Chilodonella* spp. that have been reported infecting fish worldwide. Furthermore, details about *Chilodonella* spp. reproduction and feeding strategies were described together with the current knowledge about pathological signs, diagnostic techniques and treatments used to manage infections in farmed fish. Chapter 2 provided the foundation for the development of new research insights generated in the posterior data chapters (Chapters 3-5). The review (Chapter 2), combined with new information on the identification of harmful *Chilodonella* species in Australia (Chapter 3) is a valuable tool for the future design and implementation of strategic parasite monitoring and management in fish farms.

Chapter 3 demonstrated the existence of multiple *Chilodonella* species affecting Australian freshwater fishes (barramundi and Murray cod), illustrating their morphological differences and genetic similarities. Results from Chapter 3 reinforced the importance of implementing basic knowledge research when dealing with economically important emerging parasites. Furthermore, the genetic similarity found between parasitic *C. hexasticha* and *C. piscicola* demonstrated the crucial importance of adopting multiple approaches and techniques (e.g. microbiology, PCR, sequencing) to characterise parasite species. Further research on the characterisation of the *Chilodonella* genome and its morphological plasticity can potentially reveal important information about this parasite's ecology. Chapter 3 confirmed that parasitic *Chilodonella* can exist naturally in the aquatic environment, without causing problems to fish. Similarly, the etiological agent of amoebic gill disease (AGD) in salmonids, *Neoparamoeba perurans* (Young et al., 2007) is also a free-living parasite with capacity to infect fish (Crosbie et al., 2012). Therefore, the potential of certain parasites to alternate between their free-living and parasitic behaviour should not be ignored, as this characteristic may be associated with unknown co-infections between pathogenic bacterial species and parasites (e.g. *C. hexasticha* and *F. columnare* in Chapter 5). Consequently, when monitoring parasites from complex aquatic systems, such as aquaculture farms, it is crucial to implement systematic approaches (e.g. morphological, molecular and histopathology techniques) to determine species biodiversity and identify disease-causing agents.

6.2 Innovative approaches to identify parasitic diseases in aquaculture

Aquaculture farms are generally located in remote areas. Despite all the technological advances within the aquaculture sector, diagnosis of pathogens in aquaculture facilities continue to rely on traditional techniques (such as histopathology, PCR, microbiology) which are time consuming. Generally when test results are returned to farmers, few reactive responses can be performed to recover moribund animals. Rapid response time and appropriate reaction

is the key for success when managing diseases in aquaculture. Therefore, the development of on-farm pathogen detection devices (e.g. PCR based or sequencing based) is a promising research area with significant potential to improve the response capability of farmers to diseases. However, farms still adopt traditional approaches related to environmental (e.g. dissolved oxygen, temperature) and biological (e.g. fish mortalities, gills health information) data collection and use. A small percentage of technologically advanced aquaculture farms know how to take advantage of environmental data daily collected on farms and integrate it in their health management programs. Collection of data on farms without specific purpose is not economically efficient (Stentiford et al., 2017). More awareness is needed within the aquaculture sector on how to efficiently use environmental data to empower this industry to improve its potential. The innovative parasite monitoring techniques applied in Chapters 4 and 5 integrated environmental and biological data that are valuable for the future of diagnosis and aquatic animal health programs in aquaculture.

Novel DNA-based detection technologies linked to environmental data have the ability to accelerate the response of farmers to parasitic diseases (Chapter 4). Environmental DNA (eDNA) is a cutting-edge detection tool that can rapidly identify and quantify pathogens in fish farms, thereby accelerating the process of diseases response by farmers (Bass et al., 2015; Stentiford et al., 2017). By identifying the abundance of parasites in water samples before outbreaks, eDNA can provide farm managers with critical information that can be used to mitigate parasitic diseases. Information on parasites abundance can help farmers to decide about the best time and season to stock ponds and ideal time to start treatments. A Quantitative real-time PCR was used in Chapter 4 to determine presence/absence and the quantification of *C. hexasticha* in aquaculture ponds, as a model parasite for future applications. For example, parasite abundance was found to correlate with fish mortality. Parasite abundance thresholds can now be

established which dictate routine management methods as a precursor for implementing the integration of eDNA technology and extrinsic environmental, biological data and management resources (e.g. water exchange protocols and feeding strategies). The use of parasite threshold levels and treatment has been implemented by Scottish and Norwegian authorities to control sea lice infestation within the salmon industry. In these countries, infected animals must be treated when 0.1 lice per fish is found (Bergheim, 2012).

Environmental DNA techniques have been extensively used in conservation and invasive species studies (Ficetola et al., 2008; Goldberg et al., 2015). The application of eDNA in aquaculture systems is very recent and still limited to open water systems (Ishimaru et al., 2014; Bass et al., 2015). However, eDNA techniques offers great potential for use within aquaculture sectors such as pond systems. Within limited water exchange systems, target DNA concentration is likely to be higher which increases the likelihood for detection of low levels of genetic material (Ficetola et al., 2008). Furthermore, it is crucial to determine the type of aquaculture system and pathogens to be detected by environmental DNA techniques, before developing specific protocols. The quantification of eDNA is particularly important when dealing with ciliate protozoans that naturally occur in aquatic environments. As discussed in Chapter 4, *Chilodonella* spp. were consistently present in farm water throughout the year. Therefore, future scope exists to explore the significance of assessing the abundance of parasites in water, on a regular basis (e.g. weekly) and linking this information with water quality data to empower farm managers with pre-emptive tools (e.g. decision-making charts for feeding, stocking, soil preparation and water quality parameters) to avoid disease outbreaks.

The comprehensive data set generated in Chapter 4, which combined farm parameters and parasite quantification in pond water, was important to assess the viability of adopting eDNA sampling methodology as a regular activity for early parasite detection in a commercial

farm production environment. Therefore, the use of eDNA methodology coupled with qPCR technology as a predictive tool for fish mortalities, received considerable interest from the commercial farm part of this research, with potential to develop into a regular activity adopted by the farm manager to improve their animal health monitoring program. Weekly monitoring of parasites abundance, as mentioned above, could potentially reduce current treatment time on farm, minimizing costs associated with parasites. The eDNA protocols developed in Chapter 4 allowed a range of farm management parameters to be assessed, from individual ponds but also from the production system on a tropical freshwater barramundi farm. These protocols were a fundamental pre-requisite for the assessment and understanding of the bacterial community associated with *Chilodonella* spp. epidemics explored in Chapter 5. Therefore, further studies should be performed to deepen our knowledge about the environmental triggers linked to the association between pathogenic bacterial and *Chilodonella* spp. in fish farms. Future studies could test the proliferation of *Chilodonella* spp. and what bacterial species are more significantly found under different environmental parameters (e.g. dissolved oxygen, temperature under controlled conditions). These studies could also include microbiological tests and compare the bacterial community found on Murray cod hosts (when fish is infected by *Chilodonella* spp.) with barramundi.

The limited information related to microorganisms' interaction in aquaculture systems is a concern for advances in microbial management. The specific relationship between the ciliate protozoan *C. hexasticha* and the bacterial community found in freshwater fish farms has never been explored previously. Thus, the study undertaken in Chapter 5 is the first of its kind, indicating a potential association between *C. hexasticha* epidemics and pathogenic *Flavobacterium columnare* in freshwater fish farms. While the economic importance of *F. columnare* for freshwater fish aquaculture industry has been extensively reported (Decostere et

al., 1998; Figueiredo et al., 2005; Bernardet and Bowman, 2006; Řehulka and Minařík, 2007; Soto et al., 2008; Suomalainen et al., 2009; Morley and Lewis, 2010; Declercq et al., 2013), its association with parasitic *C. hexasticha* has never been demonstrated. Potentially these two microorganisms have a symbiotic relationship where *C. hexasticha* feeding behavior (grazing on host's epithelial cells) may be the portal of entry for bacterial such as *F. columnare* or *C. hexasticha* carries bacteria within its cilia promoting its spread within the aquatic environment. These hypotheses need to be confirmed and highlight the limited knowledge existent about microorganism interactions, particularly within open environments such as aquaculture pond farms. Encouragingly, the *F. columnare* and *C. hexasticha* interaction may lead to future understanding of infections caused by *Chilodonella* and direct researchers into innovative solutions such as probiotics and prebiotics use to manage microorganisms in aquaculture.

6.3 Future prospects

Considerable advances have been achieved within the last ten years related to diseases in aquaculture (Peeler and Otte, 2016; Stentiford et al., 2017). The development of chemical treatments and vaccines against economic important pathogens has, certainly, helped this sector to move forward. While the aquaculture sector will continue to expand to meet the human demand for seafood, its development needs to occur in a sustainable way to minimise the impacts of diseases and reduce environmental impact of production systems. The focus must be on promoting a balanced environment where minimum stress is imposed to aquatic animals (e.g. well controlled water quality parameters, limited animal density and multi-species systems) and the continuous monitoring of the system (e.g. real-time data collection systems and decision-making charters), with special attention to early recognition of problems.

This study revealed important advances related to the use of environmental DNA methodology, qPCR and metagenomics as tools for improving pathogen detection under farm

conditions. However, this research also demonstrated how limited the information is related to the diversity and interactions of the microbial community that affect hosts in aquaculture systems. The applications for eDNA methodology and aquaculture metagenomics analysis are significantly broad. The integration of eDNA sampling methodology and metagenomics technologies may reveal new microbial biodiversity, along with the abundance and co-interactions of micro and macro-organisms. Advances in eDNA and metagenomics research may lead to changes in the current practices used for detecting pathogens such as bacteria, viruses, fungi and parasites and allow exploration of non-invasive strategies for pathogen sampling that minimise stress and improve animal wellbeing within aquaculture systems. The development of on-farm detection technologies based on eDNA sampling methodologies and the quantification of pathogens within the culture environment will accelerate the response capability of farmers to disease risks in aquaculture systems. Co-infections of pathogens and parasites and more detailed information about infections are also likely to benefit with the use of eDNA metabarcoding strategies, which could in one assay identify the presence and abundance of aquatic communities through the use of next-generation and high throughput sequencing methodologies (Bass et al., 2015). Furthermore, quarantine and biosecurity facilities can adapt eDNA-based techniques to rapidly and reliably screen the entry of exotic pathogens into countries.

The continuous and sustainable growth of the global aquaculture sector depends on the implementation of innovative methods to improve traditional practices used within this industry. The challenges aquaculture farms face due to the presence of diseases is a serious burden. Adopting a holistic approach when approaching disease management is paramount for the effective application of new management actions within aquaculture farms.

References

Abdel-Baki, A-AS., Gewik, M.M. and Al-Quraishy, S. (2014). First records of Ambiphrya and Vorticella spp. (Protozoa, Ciliophora) in cultured Nile tilapia (*Oreochromis niloticus*) in the central region of Saudi Arabia. Saudi Journal of Biological Sciences 21: 520-523.

Adiba, S., Nizak, C., van Baalen, M., Denamur, E., Depaulis, F. (2010). From grazing resistance to pathogenesis: the coincidental evolution of virulence factors. PLoS One 5: e11882.

Adrian-Kalchhauser, I., Burkhardt-Holm, P. (2016). An eDNA Assay to Monitor a Globally Invasive Fish Species from Flowing Freshwater. PLoS ONE. 11. e0147558.

Alvarez-Pellitero, P. (2008). Fish immunity and parasite infections: from innate immunity to immunoprophylactic prospects. Veterinary Immunology and Immunopathology. 126:171-198.

Ashburner L.D. and Ehl A.S. (1973) *Chilodonella cyprini* (Moroff), a parasite of freshwater fish and its treatment. Australian Society for Limnology Bulletin 5: 3-4.

Atkinson, S. D. and Bartholomew, J. L. (2010). Spatial, temporal and host factors structure the *Ceratomyxa shasta* (Myxozoa) population in the Klamath River Basin. Infection, Genetics and Evolution. 10: 1019-1026.

Bass, D., Stentiford, G.D., Littlewood, D.T.J., Hartikainen, H. (2015). Diverse Applications of Environmental DNA Methods in Parasitology. Trends in Parasitology. 31: 499-513.

Bastos Gomes, G., Jerry, D.R., Miller, T.L., Hutson, K.S. (2016). Current status of parasitic ciliates *Chilodonella* spp. (Phyllopharyngea: Chilodonellidae) in freshwater fish aquaculture. Journal of Fish Diseases. 40(5): 703-715.

Bastos Gomes, G., Miller, T.L., Vaughan D.B., Jerry, D.R., McCowan, C., Bradley, T.L., Hutson, K.S. (2017a). Evidence of multiple species of *Chilodonella* (Protozoa, Ciliophora) infecting Australian farmed freshwater fishes. Veterinary Parasitology. 237: 8-16.

Bastos Gomes, G., Hutson, K.S., Domingos, J.A., Miller, T.L., Chung, C., Hayward, S., Jerry, D.R. (2017b). Use of environmental DNA (eDNA) and water quality data to predict protozoan parasites outbreaks in fish farms. Aquaculture. 479: 467-473.

Bauer, O.N. and Nikol'skaya, N.P. (1957). *Chilodonella cyprini* (Moroff, 1902), ee biologija i epizootilogicheskoe znachenie. IZV. Vsesojuzn. nauchn.issled. Inst. Ozern. i. Rechn. Rybn. Loz., 42: 53-66.

Bellec, L., Maurer-Alcala, X.X. and Katz, L.A. (2014). Characterization of the life cycle and heteromeric nature of the macronucleus of the ciliate *Chilodonella uncinata* using fluorescence microscopy. Journal of Eukaryotic Microbiology. 61:313-316.

Bellec, L., Katz, L.A. (2012). Analyses of chromosome copy number and expression level of four genes in the ciliate *Chilodonella uncinata* reveal a complex pattern that suggests epigenetic regulation. Gene. 504: 303-308.

Bentzon-Tilia, M., Sonnenschein, E.C., Gram, L. (2016). Monitoring and managing microbes in aquaculture – Towards a sustainable industry. Microbial Biotechnology. 9: 576-584.

Bergheim, A. (2012). Recent growth trends and challenges in the Norwegian aquaculture industry. Latin American Journal of Aquatic Research. 40: 800-807.

Bernardet, J.F. and Bowman, J.P. (2006). The genus Flavobacterium. In: The Prokaryotes: A Handbook on the Biology of Bacteria, 3rd edn, vol. 7 (ed. by M. Dworkin, S. Falkow, E. Rosenberg, K.H. Schleifer, and E. Stackebrandt), pp. 481–531. Springer Press, New York, NY, USA.

Blancheton, J.P., Attramadal, K.J.K., Michaud, L., d'Orbcastel, E.R., Vadstein, O. (2013). Insight into bacterial population in aquaculture systems and its implication. Aquacultural Engineering. 53: 30-39.

Blatterer, H. and Foissner, W. (1992). Morphology and infraciliature of some cyrtophorid ciliates (protozoa, ciliophora) from fresh-water and soil. Archiv Für Protistenkunde 142:101-118.

Blaxter, M. L. (2004). The promise of a DNA taxonomy. Philosophical Transactions of the Royal Society of London. Series B Biological Sciences. 359(1444): 669–79.

Bott, N.J., Ophel-Keller, K.M., Sierp, M.T., Herdina, Rowling, K.P., McKay, A.C., Loo, M.G.K., Tanner, J.E., Deveney, M.R., 2010. Toward routine, DNA-based detection methods for marine pests. Biotechnology Advances. 28: 706-714.

Bourlat, S.J., Borja, A., Gilbert, J., Taylor, M.I., Davies, N., Weisberg, S.B., et al. (2013) Genomics in marine monitoring: new opportunities for assessing marine health status. Marine Pollution Bulletin. 74: 19–31.

Bowater, R.O. and O'Donoghue, P.J. (2014). An epizootic of chilodonelliasis in farmed barramundi *Lates calcarifer* (Bloch), a case report. Journal of Fish Diseases. 36: 1-6.

Bowden, T.J., Thompson, K.D., Morgan, A.L., Gratacap, R.M.L., Nikoskelainen, S. (2007). Seasonal variation and the immune response: A fish perspective. Fish and Shellfish Immunology. 22: 695-706.

Boyd, C.E. (1990). Water Quality in Ponds for Aquaculture. Birmingham Publishing Company, Birmingham, Alabama.

Boyd, C.E. and Massaut, L. (1999). Risks associated with the use of chemicals in pond aquaculture. Aquacultural Engineering. 20:113-132.

Boyd, C.E., Tucker, C.S., (1998). Pond Aquaculture Water Quality Management, Kluwer Academic Publishers, Boston, MA.

Bradley T., Mccowan C., Cohen S., Ingram B., Green C. and Mansell P., (2014). Improved fish health management for integrated inland aquaculture through better management practices. In Fisheries Research and Development Corporation, Department of Environment and Primary Industries, pp.51. Melbourne.

Bridle, A. R., Crosbie, P. B. B., Cadoret, K., and Nowak, B. F. (2010). Rapid detection and quantification of *Neoparamoeba perurans* in the marine environment. Aquaculture. 309: 56-61.

Buchmann, K. (2013). Impact and control of protozoan parasites in maricultured fishes. Parasitology. 142: 168-77.

Burnham, J.C., Hashimoto, T., Conti, S.F. (1968). Electron microscopic observations on the penetration of *Bdellovibrio bacteriovorus* into gram-negative bacterial hosts. Journal of Bacteriology. 96:1366–81.

Burnham, J.C., Stetak, T., Gregory, L. (1976). Extracellular lysis of the bluegreen alga *Phormidium luridum* by *Bdellovibrio bacteriovorus*. Journal of Phycology. 12: 306-313.

Caiola, M.G. and Pellegrini, S. (1984). Lysis of *microcystis aeruginosa* (kütz.) by *bdellovibrio*-like bacteria1. Journal of Phycology. 20: 471-475.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. Nature Methods. 7(5): 335-336.

Chu, W.H. and Zhu, W. (2010). Isolation of *Bdellovibrio* as biological therapeutic agents used for the treatment of *Aeromonas hydrophila* infection in fish. Zoonoses and Public Health. 57: 258-264.

Cohen, S. and Segal D. (2009). Extrachromosomal circular DNA in eukaryotes: possible involvement in the plasticity of tandem repeats. Cytogenet. Genome Research. 124:327-328.

Crosbie, P.B.B., Bridle, A.R., Leef, M.J., Nowak, B.F. (2010). Effects of different batches of *Neoparamoeba perurans* and fish stocking densities on the severity of amoebic gill disease in experimental infection of Atlantic salmon, *Salmo salar* L. Aquaculture Research. 41: e505-e516.

Crosbie, P.B.B., Bridle, A.R., Cadoret, K., Nowak, B.F. (2012). In vitro cultured *Neoparamoeba perurans* causes amoebic gill disease in Atlantic salmon and fulfils Koch's postulates. International Journal for Parasitology. 42: 511-515.

Cunningham, C.O. (2002). Molecular diagnosis of salmonid diseases. Springer, Dordrecht, The Netherlands.

Darling, J.A. and Mahon, A.R. (2011). From molecules to management: Adopting DNA-based methods for monitoring biological invasions in aquatic environments. Environmental Research. 111: 978-988.

De Schryver, P., Defoirdt, T., Sorgeloos, P. (2014). Early Mortality Syndrome Outbreaks: A Microbial Management Issue in Shrimp Farming? PLoS Pathogens. 10: e1003919.

Declercq, A.M., Haesebrouck, F., Van den Broeck, W., Bossier, P., Decostere, A. (2013). Columnaris disease in fish: a review with emphasis on bacterium-host interactions. Veterinary Research. 44: 27.

Decostere, A., Haesebrouck, F., Devriese, L. (1997). Shieh medium supplemented with tobramycin for selective isolation of *Flavobacterium columnare* (*Flexibacter columnaris*) from diseased fish. Journal of Clinical Microbiology. 35: 322– 324.

Deng, Q., Guo, Q., Zhai, Y., Wang, Z., Gu, Z. (2015). First record of *Chilodonella piscicola* (Ciliophora: Chilodonellidae) from two endangered fishes, *Schizothorax o'connori* and *Oxygymnocypris stewartii* in Tibet. Parasitology Research. 114: 3097–3103.

Dickerson, H.W, and Clark, T.G. (1996) Immune response of fishes to ciliates. Annual Review of Fish Diseases. 6:107-120.

Dopheide, A., Lear, G., Stott, R. and Lewis, G. (2011). Preferential Feeding by the Ciliates *Chilodonella* and *Tetrahymena* spp. and Effects of These Protozoa on Bacterial Biofilm Structure and Composition. Applied and Environmental Microbiology. 77: 4564-4572.

Dopheide, A., Lear, G., Stott, R., Lewis, G. (2008). Molecular Characterization of Ciliate Diversity in Stream Biofilms. Applied and Environmental Microbiology. 74: 1740-1747.

Dunthorn, M., Foissner, W., Katz, A.L. (2011). Expanding Character Sampling for Ciliate Phylogenetic Inference Using Mitochondrial SSU-rDNA as a Molecular Marker. Pro tist. 162(1): 85–99.

Dunthorn, M., Katz, L.A. (2008). Richness of morphological hypotheses in ciliate systematics allows for detailed assessment of homology and comparisons with gene trees. Denisia. 23: 389–394.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 27(16): 2194-2200.

Edwards, K., Johnstone, C., Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Research. 19:1349.

Egusa, S. (1983). Disease problems in Japanese yellowtail, *Seriola quinqueradiata*, culture: a review. Rapport and Process-Verbaux des Reunions. Conseil International pour la Exploration de la Mer. 182:10–18.

Eissa, A.E., Zaki, M.M., Aziz, A.A. (2010). *Flavobacterium columnare*/*Myxobolus tilapiae* concurrent infection in the earthen pond reared Nile tilapia (*Oreochromis niloticus*) during the early summer. Interdisciplinary Bio Central. 2: 1–10.

Evans, B.B. and Lester, R.J.G. (2001) Parasites of ornamental fish imported into Australia. Bulletin of the European Association of Fish Pathologists. 21:51–55.

Faircloth, B.C. and Glenn T.C. (2014). Protocol: Preparation of an AMPure XP substitute (AKA Serapure). doi: 10.6079/J9MW2F26.

Fan, X., Ma, R., Al-Farraj, S.A., Gu, F. (2014). Morphological and molecular characterization of *Parafurgasonia zhangi* spec. nov. and *Chilodonella acuta* Kahl, 1931 (Protozoa, Ciliophora), from a soil habitat of Saudi Arabia. International Journal for Systematic and Evolutionary Microbiology. 64: 2385–2394.

FAO (2012). The State of World Fisheries and Aquaculture.Rome. FAO.

FAO (2014). The State of World Fisheries and Aquaculture.Rome. FAO.

FAO (2016). The State of World Fisheries and Aquaculture.Rome. FAO.

FASTX Toolkit http:hannonlab.cshl.edu/fastx_toolkit/index.html by Hannon Lab

Fenchel, T. (2014). Protozoa and Oxygen. Acta Protozoologica. 53 (1): 3-12.

Fernandes, J.M., Portelinha, M.K., Rocha, C.B., Pouey, J.L.O.F. and Piedras, S.R.N. (2011). Occurrence and control of *Chilodonella* spp. in pejerrey *Odontesthes bonariensis*. Arquivo Brasileiro de Medicina Veterinária e Zootecnia. 63: 788-790.

Ficetola, G.F., Miaud, C., Pompanon, F., Taberlet, P. (2008). Species detection using environmental DNA from water samples. Biology Letters. 4: 423-425.

Figueiredo, H.C.P., Klesius, P.H., Arias, C.R., Evans, J., Shoemaker, C.A., Pereira, D.J. Jr., Peixoto, M.T.D. (2005). Isolation and characterization of strains of *Flavobacterium columnare* from Brazil. Journal of Fish Diseases. 28:199–204.

Finlay, B.J., Esteba, G.F., Brown, S., Fenchel, T., Hoef-Emden, K. (2006). Multiple cosmopolitan ecotypes within a microbial eukaryote morphospecies. Protist. 157(4): 377–390.

Foissner, W. (1988). Taxonomy and ecology of some saprobic ciliates (protozoa, ciliophora).2. family chilodonellidae. Hydrobiologia. 162: 21-45.

Gao, S., Huang, J., Li, J. and Song, W. (2012). Molecular Phylogeny of the Cyrtophorid Ciliates (Protozoa, Ciliophora, Phyllopharyngea). PLoS ONE. 7: e33198.

Garcia, F., Fujimoto, R.Y., Martins, M.L. and Moraes, F.R. (2009). Protozoan parasites of Xiphophorus spp. (Poeciliidae) and their relation with water characteristics. Arquivo Brasileiro de Medicina Veterinária e Zootecnia. 61:156-162.

Gasser, R.B. (1999). PCR-based technology in veterinary parasitology. Veterinary Parasitology. 84: 229-258.

Glencross, B.D. (2008). A factorial growth and feed utilization model for barramundi, *Lates calcarifer* based on Australian production conditions. Aquaculture Nutrition. 14(4): 360-73.

Goldberg, C. S., Strickler, K.M., Pilliod, D.S. (2015). Moving environmental DNA methods from concept to practice for monitoring aquatic macroorganisms. Biological Conservation. 183: 1-3.

Groner, M.L., Maynard, J., Breyta, R., Carnegie, R.B., Dobson, A., Friedman, C.S., Froelich, B. et al. (2016). Managing marine disease emergencies in an era of rapid change. Philosophical Transactions of the Royal Society B: Biological Sciences. 371: 20150364.

Hagopian, D.S. and Riley, J.G. (1998). A closer look at the bacteriology of nitrification. Aquacultural Engineering. 18, 223-244.

Hahn, M.W. and Höfle, M.G. (2001). Grazing of protozoa and its effect on populations of aquatic bacteria. FEMS Microbiology Ecology. 35: 113-121.

Hahn, M.W., Kasalický, V., Jezbera, J., Brandt, U., Jezberová, J., Šimek, K. (2010). *Limnohabitans curvus* gen. nov., sp. nov., a planktonic bacterium isolated from a freshwater lake. International Journal of Systematic and Evolutionary Microbiology. 60: 1358-1365.

Hall, M.S. and Katz, L,A. (2011). On the nature of species: insights from Paramecium and other ciliates. Genetica. 139 (5): 677-84.

Hallett, S.L., Bartholomew, J.L. (2006). Application of a real-time PCR assay to detect and quantify the myxozoan parasite *Ceratomyxa shasta* in river water samples. Diseases of Aquatic Organisms. 71: 109-118.

Hallett, S.L., Ray, R.A., Hurst, C.N., Holt, R.A., Buckles, G.R., Atkinson, S.D., Bartholomew, J.L. (2012). Density of the Waterborne Parasite *Ceratomyxa shasta* and Its Biological Effects on Salmon. Applied and Environmental Microbiology. 78: 3724-3731.

Häsler, B., Howe, K., Stark, K. (2011). Conceptualising the technical relationship of animal disease surveillance to intervention and mitigation as a basis for economic analysis. BMC Health Services Research. 11: 225.

Hoffman, G.L., Kazubski, S.L., Mitchell, A.J. and Smith, C.E. (1979). *Chilodonella hexasticha* (Kiernili, 1909) (Protozoa, Ciliata) from North American warm water fish. Journal of Fish Diseases. 2:153–157.

Hossain, M.D., Hossain, M.K., Rahaman, M.H., Aktr, A. and Khanom, D.A. (2008). Prevalence of ectoparasites of carp fingerlings at Santaher, Bogra. University Journal of Zoology. 27:17–19.

Hossain, M.D., Islam, K, Hossain, M.K. and Rahman, M.H. (2013). Environmental Impact Assessment of Fish Diseases on Fish Production. Journal of Fish Foundation. 9 (1and 2): 125-131.

IBM Corp. Released 2014. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.

Imai, S., Hatai, K. and Ogawa, M. (1985). *Chilodonella hexasticha* (Kiernili, 1909) found from the gills of a discus, Symphysodon discus Heckel, 1940. Japanese Journal of Veterinary Science. 47(2): 305–308.

Ininbergs, K., Bergman, B., Larsson, J., and Ekman, M. (2015). Microbial metagenomics in the Baltic Sea: recent advancements and prospects for environmental monitoring. Ambio. 44: 439–450.

Ishimaru, K., Matsuura, T., Tsunemoto, K., Shirakashi. S. (2014). Seasonal monitoring of Kudoa yasunagai from sea water and aquaculture water using quantitative PCR. Diseases of Aquatic Organims. 108: 45-52.

Jee, B-Y., Kim, K. and Park, S. (1996). *Chilodonella hexasticha* (Protozoa, Ciliata) from Korean freshwater fish. Journal of Fish Pathology. 9:113–118.

Jorgensen, T.R., Larsen, T.B. and Buchmann, K. (2009). Parasite infections in recirculated rainbow trout (*Oncorhynchus mykiss*) farms. Aquaculture. 289:91-94.

Joshi, N.A. and Fass, J.N. (2011). Sickle: A sliding-window, adaptative, quality-based trimming toll for FastQ files (version 1.33) (Software). https://github.com/najoshi/sickle

Jousson, O., Pretti, C., Bello, D.D., Cognetti-Varriale, A.M., 2005. Non-invasive detection and quantification of the parasitic ciliate *Ichthyophthirius multifiliis* by real-time PCR. Diseases of Aquatic Organims. 65, 251-255.

Juranek, S.A. and Lipps, H.J. (2007). New Insights into the Macronuclear Development in Ciliates. In: Kwang, W.J. (Ed.) International Review of Cytology. Academic Press. pp. 219-251.

Karvonen, A., Rintamaki, P., Jokela, J. and Valtonen, E.T. (2010). Increasing water temperature and disease risks in aquatic systems: Climate change increases the risk of some, but not all, diseases. International Journal for Parasitology. 40:1483–1488.

Katz, L. A., and Kovner, A. M. (2010). Alternative processing of scrambled genes generates protein diversity in the ciliate *Chilodonella uncinata*. Journal of Experimental Zoology Part B-Molecular and Developmental Evolution. 314B: 480-488.

Katz, L. A., De Berardinis, J., Hall, M. S., Kovner, A. M., Dunthorn, M., and Muse, S. V. (2011). Heterogeneous rates of molecular evolution among cryptic species of the ciliate morphospecies *Chilodonella uncinata*. Journal of Molecular Evolution. 73: 266-272.

Katz, L.A., 2012. Origin and Diversification of Eukaryotes. Annual Review Microbiology. 66: 411–427.

Kayış, S., Balta, F., Serezli, R. and Er, A. (2013). Parasites on different ornamental fish species in Turkey. Journal of FisheriesSciences.com. 7: 114-120.

Kazubsksi, L., and Migala, K. (1974). Studies on the distinctness of *Chilodonella cyprini* (Moroff) and *C. hexasticha* (Kiernik) (Chlamydodontidae, Gymnostomatida), ciliate parasites of fishes. Acta Protozoology. 13: 9-39.

Klausen, C., Jørgensen, N.O.G., Burford, M.A., O'Donohue, M. (2004). Actinomycetes may also produce taste and odour. Water. 31: 45–49.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Mentjies, P., Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 28(12): 1647–1649.

Keeling, P.J., Burki, F., Wilcox, H.M., Allam, B., Allen, E.E., Amaral-Zettler, L.A., Armbrust, E.V., et al. (2014). The Marine Microbial Eukaryote Transcriptome Sequencing Project

(MMETSP): Illuminating the Functional Diversity of Eukaryotic Life in the Oceans through Transcriptome Sequencing. PLoS Biology. 12: e1001889.

Kepner, R.L.Jr., Wharton, R.A.Jr. and Coats, D.W. (1999). Ciliated protozoa of two antarctic lakes: analysis by quantitative protargol staining and examination of artificial substrates. Polar Biology. 21: 285-294.

Kersters, K. De Vos, P., Gillis, M., Swings, J., Vanadamme, P. and Stackebrandt, E. (2006). Introduction to the Proteobacteria. The Prokaryotes: Volume 5: Proteobacteria: Alpha and Beta Subclasses. 3:37.

Kotob, M.H., Menanteau-Ledouble, S., Kumar, G., Abdelzaher, M., El-Matbouli, M. (2016). The impact of co-infections on fish: a review. Veterinary Research. 47: 98.

Kunttu, H.M.T., Sundberg, L.R., Pulkkinen, K., Valtonen, E.T. (2012). Environment may be the source of *Flavobacterium columnare* outbreaks at fish farms. Environmental Microbiolology Reports. 4:398–402.

Lahr, D.J.G., Laughinghouse, H.D., Oliverio, A.M., Gao, F. and Katz, L.A. (2014). How discordant morphological and molecular evolution among microorganisms can revise our notions of biodiversity on Earth. BioEssays. 36: 950-959.

Langdon, J.S., Gudkovs, N., Humphrey, J.D., Saxon, E.C. (1985). Deaths in Australian fresh-water fishes associated with *Chilodonella hexasticha* infection. Australian Veterinary Journal. 62: 409-413.

Laybourn-Parry, J. (1984). A functional biology of free-living protozoa. Functional Biology series, ed. P. Calow. London/Sydney: Croom Helm. 218 pp.

Leibovitz, L. (1980). Chilodonelliasis. Journal of the American Veterinary Medicine Association. 177: 222–223.

Li, C., Miller, T. L., Small, H. J., and Shields, J. D. (2011). *In vitro* culture and developmental cycle of the parasitic dinoflagellate *Hematodinium* sp. from the blue crab *Callinectes sapidus*. Parasitology. 138: 1924-1934.

Loch, T.P., Faisal, M. (2015). Emerging flavobacterial infections in fish: A review. Journal of Advanced Research. 6: 283-300.

Loh, R. and Landos, M. (2011). Fish Vetting Essentials. Richmond Publishing, Perth. 197 pp.

Lom, J. and Dyková, I. (1992). Protozoan parasites of fishes. Elsevier Science Publishers, Amsterdam, 315 pp.

Lom, J. and Nigrelli, R.F. (1970). *Brooklynella hostilis* n. g., n. sp., a Pathogenic Cyrtophorine Ciliate in Marine Fishes. The Journal of Protozoology. 17: 224-232.

Lynn, D.H. (2008). The ciliated protozoa: Characterization, classification, and guide to the literature, 3nd ed. Springer, New York, USA. 605 pp.

Macnab, V. and Barber, I. (2012). Some (worms) like it hot: fish parasites grow faster in warmer water, and alter host thermal preferences. Global Change Biology. 18:1540-1548.

Matz, C., Bergfeld, T., Rice, S.A. and Kjelleberg, S. (2004). Microcolonies, quorum sensing and cytotoxicity determine the survival of Pseudomonas aeruginosa biofilms exposed to protozoan grazing. Environmental Microbiology. 6: 218–226.

Martens, H. and Martens, M. (2000). Modified jack-knife estimation of parameter uncertainty in bilinear modelling by partial least squares regression. Food Quality and Preference 11: 5–16.

McKeever, D.J. and Rege, J.E.O. (1999). Vaccines and diagnostic tools for animal health: the influence of biotechnology. Livestock Production Science. 59:257-264.

Medlin, L., Elwood, H.J., Stickel, S., Sogin, M.L. (1988). The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene. 71: 491–499.

Miller, D.N., Bryant, J.E., Madsen, E.L., Ghiorse, W.C. (1999). Evaluation and Optimization of DNA Extraction and Purification Procedures for Soil and Sediment Samples. Applied Environmental Microbiology. 65: 4715-4724.

Miller, T. L., Adlard, R. D., Bray, R. A., Justine, J.-L., and Cribb, T. H. (2010a). Cryptic species of Euryakaina n. g. (Digenea: Cryptogonimidae) from sympatric lutjanids in the Indo-West Pacific. Systematic Parasitology. 77: 185-204.

Miller, T.L., and Cribb, T.H. (2007). Coevolution of Retrovarium n. gen. (Digenea: Cryptogonimidae) in Lutjanidae and Haemulidae (Perciformes) in the Indo-West Pacific. International Journal for Parasitology. 37: 1023-1045.

Miller, T.L. and Cribb, T.H. (2008). Eight new species of Siphoderina Manter, 1934 (Digenea, Cryptogonimidae) infecting Lutjanidae and Haemulidae (Perciformes) off Australia. Acta Parasitol. 53(4), 344–364.

Miller, T.L., Bray, R. A., Justine, J.-L., and Cribb, T. H. (2010b). Varialvus gen. nov. (Digenea: Cryptogonimidae), from species of Lutjanidae (Perciformes) off the Great Barrier Reef, New Caledonia and the Maldives. Acta Parasitologica. 55: 327-339.

Mitra, A.K. and Haldar, D.P. (2004). First record of *Chilodonella hexasticha* (Kiernik, 1909) Kahl, 1931 (Ciliophora: Chilodonellidae) infesting a freshwater fish *Nandus nandus* (Hamilton) from Gangetic West Bengal, India. Animal Biology. 54:111-118.

Mitra, A. K., Bandyopadhyay, P. K., and Gong, Y. (2013). Studies on trichodinid and chilodonellid ciliophorans (Protozoa: Ciliophora) in the Indian freshwater and estuarine fishes with description of *Trichodinella sunderbanensis* sp. nov. and *Trichodina nandusi* sp. nov. Parasitology Research. 112: 1077-1085.

Morley, N.J. and Lewis, J.W. (2010). Consequences of an outbreak of columnaris disease (*Flavobacterium columnare*) to the helminth fauna of perch (*Perca fluviatilis*) in the Queen Mary reservoir, south-east England. Journal of Helminthology. 84:186–192.

Mostafa, S.M. and Kumar, B.T. (2012). Aqua chemicals in shrimp farm: A study from south-west coast of Bangladesh. The Egyptian Journal of Aquatic Research. 38: 275-285.

Nikolic, V., Simonovic, P.D., Maric, S.P. (2006). Occurrence of *Chilodonella hexasticha* (Ciliophora, Protista) on farmed rainbow trout (*O*ncorhynchus mykiss) throughout the season. Acta Veterinaria-Beograd. 56: 55-61.

Nikolic, V.P. and Simonovic, P.D. (1996). Occurrence of parasitic ciliates (Protozoa) on perch (*Perca fluviatilis*) in Lake Vlasinsko. Annales Zoologici Fennici. 33: 707-710.

Noga, E.J. (2010). Fish Disease: Diagnosis and Treatment, 2nd ed. Wiley-Blackwell, Iowa, USA. 536pp.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. and Hase, T. (2000). Loop-mediated isothermal amplification of DNA. Nucleic Acids Research. 28: e63.

Nowak, B.F. (2007). Parasitic diseases in marine cage culture - an example of experimental evolution of parasites? International Journal for Parasitology. 37:581-588.

Ogram, A., Sayler, G.S., Barkay, T. (1987). The extraction and purification of microbial DNA from sediments. Journal of Microbiological Methods. 7: 57-66.

Oidtmann, B., Peeler, E., Lyngstad, T., Brun, E., Bang Jensen, B. and Stärk, K.D.C. (2013). Risk-based methods for fish and terrestrial animal disease surveillance. Preventive Veterinary Medicine. 112:13-26.

Oidtmann B.C., Thrush M.A., Denham K.L., Peeler E.J. (2011a). International and national biosecurity strategies in aquatic animal health. Aquaculture. 320: 22-33.

Oidtmann, B.C., Crane, C.N., Thrush, M.A., Hill, B.J. and Peeler, E.J. (2011b). Ranking freshwater fish farms for the risk of pathogen introduction and spread. Preventive Veterinary Medicine. 102:329-340.

Padua, S.B., Martins, M.L., Carrijo-Mauad, J.R., Ishikawa, M.M., Jeronimo, G.T., Dias-Neto, J. and Filarski, F. (2013). First record of *Chilodonella hexasticha* (Ciliophora: Chilodonellidae) in Brazilian cultured fish: A morphological and pathological assessment. Veterinary Parasitology. 191:154-160.

Paperna, I. and Van As, J.G. (1983). The pathology of *Chilodonella hexasticha* (Kiernik). Infections in cichlid fishes. Journal of Fish Biology. 23: 441-450.

Peeler, E.J. and Otte, M.J. (2016). Epidemiology and Economics Support Decisions about Freedom from Aquatic Animal Disease. Transboundary and Emerging Diseases. 63: 266-277.

Peeler, E.J. and Taylor N.G.H. (2011). The application of epidemiology in aquatic animal health -opportunities and challenges. Veterinary Research. 42(1): 94-94.

Piazza, R.S., Martins, M.L., Guiraldelli, L. and Yamashita, M.M. (2006). Parasitic diseases of freshwater ornamental fishes commercialized in Florianópolis, Santa Catarina, Brazil. Boletim do Instituto Pesca. 32(1): 51-57.

Pinheiro, M.O. and Bols, N. (2013). Use of cell cultures to study the interactions of ciliates with fish. Springer Science Reviews. 1:95-113.

Posada, D. (2008). jModelTest: phylogenetic model averaging. Molecular Biology and Evolution. 25(7): 1253–1256.

Prescott, D.M. (1994). The DNA of ciliated protozoa. Microbiological Reviews. 58:233-267.

Pulkkinen, K., Suomalainen, L.R., Read, A.F., Ebert, D., Rintamäki, P., Valtonen, E.T.(2010). Intensive fish farming and the evolution of pathogen virulence: the case of columnaris disease in Finland. Proceedings of the Royal Society B: Biological Sciences. 277: 593-600.

Qin, Y., Hou, J., Deng, M., Liu, Q., Wu, C., Ji, Y., He, X. (2016). Bacterial abundance and diversity in pond water supplied with different feeds. Scientific Reports. 6: 35232.

Read, P. (2007). Diagnosis, treatment and prevention of the diseases of the Australian freshwater fish silver perch (*Bidyanus bidyanus*). NSW Department of Primary Industries Grafton, Australia, N.S.W.

Řehulka, J. and Minařík, B. (2007). Blood parameters in brook trout *Salvelinus fontinalis* (Mitchill, 1815), affected by columnaris disease. Aquaculture Research. 38:1182–1197.

Riley, J.L. and Katz, L.A. (2001). Widespread distribution of extensive chromosomal fragmentation in ciliates. Molecular Biology and Evolution 18(7):1372-1377.

Rimmer, M.A. (2006). Cultured Aquatic Species Information Programme - *Lates calcarifer* (FAO Water Resources and Aquaculture Service - FIRI).

Rintamäki-Kinnunen, P. and Valtonen, E.T. (1997). Epizootiology of protozoans in farmed salmonids at northern latitudes. International Journal for Parasitology. 27:89-99.

Rintamäki, P., Torpström, H., Bloigu, A. (1994). *Chilodonella* spp. at Four Fish Farms in Northern Finland. Journal of Eukaryotic Microbiology. 41: 602-607.

Robson, H.L.A., Noble, T.H., Saunders, R.J., Robson, S.K.A., Burrows, D.W., Jerry, D.R. (2016). Fine-tuning for the tropics: application of eDNA technology for invasive fish detection in tropical freshwater ecosystems. Molecular Ecology Resources. 16: 922-932.

Roeselers, G., Mittge, E.K., Stephens, W.Z., Parichy, D.M., Cavanaugh, C.M., Guillemin, K., Rawls, J.F. (2011). Evidence for a core gut microbiota in the zebrafish. International Society for Microbial Ecology. 5: 1595-1608.

Rud, I., Kolarevic, J., Holan, A.B., Berget, I., Calabrese, S., Terjesen, B.F. (2016). Deep-sequencing of the bacterial microbiota in commercial-scale recirculating and semi-closed aquaculture systems for Atlantic salmon post-smolt production. Aquacultural Engineering. http://dx.doi.org/10.1016/j.aquaeng.2016.10.003

Rutvisuttinunt, W., Chinnawirotpisan, .P, Simasathien, S., Shrestha, S.K., Yoon, I.K., Klungthong, C., Fernandez, S. (2013). Simultaneous and complete genome sequencing of influenza A and B with high coverage by Illumina MiSeq Platform. Journal of Virology Methods. 193(2):394-404.

Salipante, S.J., Sengupta, D.J., Rosenthal, C., Costa, G., Spangler, J., Sims, E.H., Jacobs, M.A., Miller, S.I., Hoogestraat, D.R., Cookson, B.T., McCoy, C., Matsen, F.A., Shendure, J., Lee, C.C., Harkins, T.T., Hoffman, N.G. (2013). Rapid 16S rRNA Next-Generation Sequencing of Polymicrobial Clinical Samples for Diagnosis of Complex Bacterial Infections. PLoS ONE. 8: e65226.

Schisler, G.J., Walker. P.G., Chitum, L.A. and Bergersen, E.P. (1999). Gill ectoparasites of juvenile rainbow trout and brown trout in the upper Colorado river. Journal of Aquatic Animal Health. 11: 170–174.

Schlenk, D., Colley, W.C., El-Alfy, A., Kirby, R. and Griffin, B.R. (2000). Effects of the Oxidant Potassium Permanganate on the Expression of Gill Metallothionein mRNA and Its Relationship to Sublethal Whole Animal Endpoints in Channel Catfish. Toxicological Sciences. 54:177-182.

Scholz, T. (1999). Parasites in cultured and feral fish. Veterinary Parasitology. 84(3-4), 317-35.

Segner, H., Sundh, H., Buchmann, K., Douxfils, J., Sundell, K. S., Mathieu, C., Ruane, N., Jutfelt, F., Toften, H. and Vaughan, L. (2012). Health of farmed fish: its relation to fish welfare and its utility as welfare indicator. Fish Physiology and Biochemistry, 38, 85–105.

Shannon, C.E. (1948). A mathematical theory of communication. The Bell System Technical Journal. 27:379-423.

Shariff, M. (1984). Occurrence of *Chilodonella hexasticha* (Kiernik 1909) (Protozoa, Ciliata) on big head carp *Aristichthys nobilis* (Richardson) in Malaysia. Tropical Biomedicine. 1: 69–75.

Shinn, A.P., Pratoomyot, J., Bron, J.E., Paladini, G., Brooker, E.E. and Brooker, A.J. (2015). Economic costs of protistan and metazoan parasites to global mariculture. Parasitology. 142: 196-270.

Shukla, U., Gupta, P.K. (2001). Assemblage of Ciliated Protozoan Community in a Polluted and Non-polluted Environment in a Tropical Lake of Central Himalaya: Lake Naini Tal, India. Journal of Plankton Research. 23: 571-584.

Smith, K.F., Schmidt, V., Rosen, G.E., Amaral-Zettler, L. (2012). Microbial Diversity and Potential Pathogens in Ornamental Fish Aquarium Water. PLoS ONE. 7: e39971.

Sockett, R.E. (2009). Predatory lifestyle of *Bdellovibrio bacteriovorus*. Annual Review of Microbiology. 63:523–539.

Soto, E., Mauel, M.J., Karsi, A., Lawrence, M.L. (2008). Genetic and virulence characterization of *Flavobacterium columnare* from channel catfish (*Ictalurus punctatus*). Journal of Applied Microbiology. 104:1302–1310.

Spring, K.J., Pham, S. and Zufall, R.A. (2013). Chromosome copy number variation and control in the ciliate *Chilodonella uncinata*. Plos ONE. 8: e56413.

Stamatakis, A., Hoover, P. and Rougemont, J. (2008). A Rapid Bootstrap Algorithm for the RAxML Web Servers. Systematic Biology. 57(5): 758–771.

Stead, S.M. and Laird, L.M. (2002). Handbook of salmon farming. Springer-Praxis; Aberdeen, UK. ISBN 978-1-85233-119-1.

Stentiford, G.D., Sritunyalucksana, K., Flegel, T.W., Williams, B.A.P., Withyachumnarnkul, B., Itsathitphaisarn, O., Bass, D. (2017). New Paradigms to Help Solve the Global Aquaculture Disease Crisis. PLOS Pathogens. 13: e1006160.

Subasinghe, R.P. and Bondad-Reantaso, M.G. (2006). Biosecurity in Aquaculture: International Agreements and Instruments, their compliance, Prospects, and Challenges for Developing Countries. In: Aquaculture biosecurity: prevention, control and eradication of aquatic animal disease. In: Scarfe, A.D., Cheng-Sheng L. and O'Bryen, P.J. (ed.). Blackwell Publishing, Iowa, USA. pp. 9-16.

Subasinghe, R.P., Bondad-Reantaso, M.G. and McGladdery, S.E. (2001). Aquaculture development, health and wealth. In R.P. Subasinghe, P. Bueno, M.J. Phillips, C. Hough, S.E. McGladdery and J.R. Arthur, eds. Aquaculture in the Third Millennium. Technical Proceedings of the Conference on Aquaculture in the Third Millennium, Bangkok, Thailand, 20-25 February 2000. NACA, Bangkok and FAO, Rome. pp. 167-191.

Sugiura, M., Kawahara, S., Iio, H. and Harumoto, T. (2005). Developmentally and environmentally regulated expression of gamone 1: The trigger molecule for sexual reproduction in *Blepharisma japonicum*. Journal of Cell Science. 118: 2735–2741.

Summerfelt, S.T., Hochheimer, J.N. (1997). Review of Ozone Processes and Applications as an Oxidizing Agent in Aquaculture. The Progressive Fish-Culturist. 59: 94-105.

Sun, H.Y., Noe, J., Barber, J., Coyne, R.S., Cassidy-Hanley, D., Clark, T.G., Findly, R.C., Dickerson, H.W. (2009). Endosymbiotic bacteria in the parasitic ciliate *Ichthyophthirius multifiliis*. Applied Environmental Microbiology. 75:7445–7452.

Suomalainen, L.R., Bandilla, M., Valtonen, E.T. (2009). Immunostimulants in prevention of columnaris disease of rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Diseases. 32:723–726.

Taberlet, P., Cossaic, E., Hajibabaei, M., Rieseberg, L.H. (2012). Environmental DNA. Molecular Ecology. 21(8): 1789-1793.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution. 28(10): 2731–2739.

Tang, Y., Tao, P., Tan, J., Mu, H., Peng, L., Yang, D., Tong, S., Chen, L. (2014). Identification of Bacterial Community Composition in Freshwater Aquaculture System Farming of *Litopenaeus vannamei* Reveals Distinct Temperature-Driven Patterns. International Journal of Molecular Sciences. 15: 13663-13680.

Tsuchiya, C., Sakata, T., Sugita, H. (2008). Novel ecological niche of *Cetobacterium somerae*, an anaerobic bacterium in the intestinal tracts of freshwater fish. Letters in Applied Microbiology. 46: 43-48.

Tucker, C.S. (1989). Method for estimating potassium permanganate disease treatment rates for channel catfish. The Progressive Fish-Culturist. 51: 24-26.

Tucker, C.S. and Boyd, C.E. (1977). Relationship between potassium permanganate treatment and water quality. Transactions of the American Fisheries Society. 106: 481-488.

Urawa, S. (1996). The pathobiology of ectoparasitic protozoans on hatchery-reared Pacific salmon. Scientific Reports of the Hokkaido Salmon Hatchery. 0:1-99.

Urawa, S. and Yamao, S. (1992). Scanning Electron Microscopy and Pathogenicity of *Chilodonella piscicola* (Ciliophora) on Juvenile Salmonids. Journal of Aquatic Animal Health. 4: 188-197.

Valtonen, E.T. and Koskivaara, M. (1994). Relationships between the parasites of some wild and cultured fishes in two lakes and a fish farm in central Finland. International Journal of Parasitology. 24: 109-118.

Walker, P. and Subasinghe, R. (2000). DNA-based molecular diagnostic techniques: research needs for standardization and validation of the detection of aquatic animal pathogens and diseases. Report and proceedings of the Joint FAO/NACA/CSIRO/ACIAR/DFID Expert Workshop. Bangkok, Thailand, 7-9 February 1999. FAO Fisheries Technical Paper. N^{o} 395. Rome, FAO. pp. 93.

Walsh, J.B. (1987). Persistence of tandem arrays: implications for satellite and simples sequence DNAs. Genetics. 115:555–567.

Warren, A. (2013). *Chilodonella* Strand, 1928. World Ciliophora Database http://www.marinespecies.org/aphia.php?p=taxdetailsandid=415192 on 2016-04-11.

Watson, C. and Yanong, R.P.E. (1989). Use of copper in freshwater aquaculture and farm ponds. Institute of Food and Agricultural Sciences, University of Florida, Gainesville, USA.

Watts, M., Munday, B.L. and Burke, C.M. (2001). Immune responses of teleost fish. Australian Veterinary Journal. 79: 570–574.

Wei, Y., Wang, J., Liu, T., Kong, W., Chen, N., He, X.-Q., Jin, Y. (2015). Bacterial communities of Beijing surface waters as revealed by 454 pyrosequencing of the 16S rRNA gene. Environmental Science Pollution Research. 22: 12605-12614.

West-Eberhard, M. (1989). Phenotypic plasticity and the origins of diversity. Annual Rev Ecology Systematic. 20: 249–78.

Wiles, M., Cone, D.K. and Odense, P.H. (1985). Studies on *Chilodonella cyprini* and *C. hexasticha* (Protozoa, Ciliata) by scanning electron microscopy. Canadian Journal of Zoology. 63: 2483-2487.

Wooster, G.A., Martinez, C.M., Bowser, P.R. and O'Hara, D.S. (2005). Human Health Risks Associated with Formalin Treatments Used in Aquaculture: Initial Study. North American Journal of Aquaculture. 67: 111-113.

Wu, S., Wang, G., Angert, E.R., Wang, W., Li, W., Zou, H. (2012). Composition, Diversity, and Origin of the Bacterial Community in Grass Carp Intestine. PLoS ONE. 7: e30440.

Xu, D., Shoemaker, C.A., LaFrentz, B.R. (2014). Enhanced susceptibility of hybrid tilapia to Flavobacterium columnare after parasitism by *Ichthyophthirius multifiliis*. Aquaculture. 430: 44-49.

Xu, D., Shoemaker, C.A., Zhang, D. (2015). Treatment of *Trichodina* sp. reduced load of *Flavobacterium columnare* and improved survival of hybrid tilapia. Aquaculture Reports. 2: 126-131.

Yanong, R.P.E. (2008). Use of hydrogen peroxide in finfish aquaculture. Institute of Food and Agricultural Sciences, University of Florida, Gainesville, USA.

Yi, Z., Song, W., Clamp, J.C., Chen, Z., Gao, S., Zhang, Q., (2009). Reconsideration of systematic relationships within the order Euplotida (Protista, Ciliophora) using new sequences of the gene coding for small-subunit rRNA and testing the use of combined data sets to construct phylogenies of the Diophrys-complex. Molecular Phylogenetics and Evolution. 50(3): 599–607.

Young, N.D., Crosbie, P.B.B., Adams, M.B., Nowak, B.F., Morrison, R.N., (2007). *Neoparamoeba perurans* n. sp., an agent of amoebic gill disease of Atlantic salmon (*Salmo salar* L.). International Journal for Parasitology. 37: 1469–1481.

Zakrzewski, M., Proietti, C., Ellis, J.J., Hasan, S., Brion, M.-J., Berger, B., Krause, L. (2017). Calypso: a user-friendly web-server for mining and visualizing microbiome–environment interactions. Bioinformatics. 33: 782-783.

Zhang, J., Laakso, J., Mappes, J., Laanto, E., Ketola, T., Bamford, J.K.H., Kunttu, H., Sundberg, L.R. (2014). Association of colony morphotypes with virulence, growth and resistance against protozoan predation in the fish pathogen *Flavobacterium columnare*. FEMS Microbiology Ecology. 89: 553-562.

Zufall, R.A., Sturm, M. and Mahon, B.C. (2012). Evolution of Germline-Limited Sequences in Two Populations of the Ciliate *Chilodonella uncinata*. Journal of Molecular Evolution. 74:140-146.

2014 I. 16S Metagenomic App.

http://support.illumina.com/content/dam/illuminasupport/documents/documentation/software_documentation/basespace/16s-metagenomics-user-guide-15055860-a.pdf 2014.