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

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ABSTRACT

Globally, disease accounts for around 40% of lost aquaculture production. Commonly, disease occurs due to an inability of farm managers to accurately quantify disease risk due to the abundance of the pathogenic agent in their production systems, along with a poor understanding of how outbreaks are linked to changes in water quality parameters. Environmental DNA (eDNA) sampling methodology associated with molecular techniques is suited to rapidly assess the background presence of pathogens in fish farms, thereby providing managers with critical information that can be used to mitigate disease threats. Adopting the ciliate protozoan *Chilodonella hexasticha* as a model, this study examined the relationship between environmental DNA of *C. hexasticha*, critical water parameters and the occurrence of disease outbreaks on a commercial barramundi *Lates calcarifer* farm, where water was sampled monthly over a 1 year timeframe. A qPCR assay based on the small subunit ribosomal DNA gene was used to monitor the abundance of *C. hexasticha* in pond water (SSU-rDNA copies/μL). Increased *C. hexasticha* eDNA levels were found to be highly correlated with occurrence of later fish mortality events ($r = 0.402$; $P < 0.001$) and also with size of fish ($r = -0.189$; $P < 0.05$); smaller fish were more prone to being impacted by an epizootic of the parasite. However, no correlations were found between any of the water quality parameters measured (rainfall, water temperature and dissolved oxygen) and abundance of this parasite, although there were significantly more fish mortalities observed during the warmer, wetter monsoonal season compared to the cooler, dry season (1280 vs. 135 mortalities, respectively; $P < 0.05$). This study highlights the potential of an eDNA approach as a management tool to quickly assess parasite loads in water and minimise the risk of disease outbreaks in aquaculture systems.

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

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(Goldberg et al., 2015; 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; 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; Bastos Gomes et al., 2016), with *Chilodonella* spp. (Phyllopharyngea:Chilodonellidae) being particularly harmful to farmed freshwater fishes (Padua et al., 2013; 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 (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.

2. Material and methods

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.

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 (200 ×) 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 (Bastos Gomes et al., 2017). 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.

2.3. DNA extractions

A direct eDNA precipitation and extraction method was adopted, whereby above mentioned sample tubes containing pond water and preservative were centrifuged at 3200*g* for 60 min at 6 °C 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 Edwards et al., 1991) and resuspended in 60 μL of 1 × 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.*