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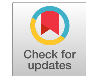
Profiling the diversity of *Cryptosporidium* species and genotypes in wastewater treatment plants in Australia using next generation sequencing



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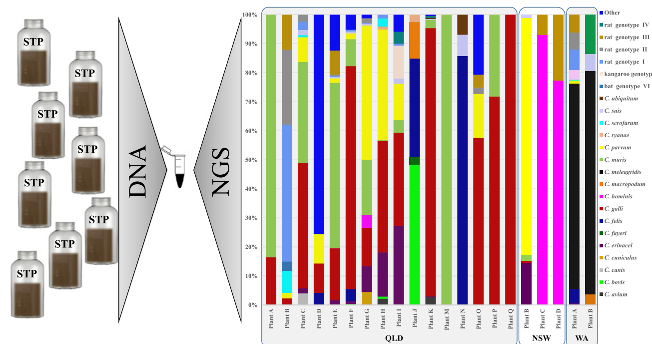
HIGHLIGHTS

- NGS analysis of *Cryptosporidium* spp. in raw wastewater samples ($n = 730$)
- Detection of large diversity of *Cryptosporidium* spp. and genotypes in wastewater
- Identification of *C. hominis*, *C. parvum* and *C. meleagridis* in untreated wastewater
- Potential contribution of livestock, wildlife and birds to wastewater contamination

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater recycling is an increasingly popular option in worldwide to reduce pressure on water supplies due to population growth and climate change. *Cryptosporidium* spp. are among the most common parasites found in wastewater and understanding the prevalence of human-infectious species is essential for accurate quantitative microbial risk assessment (QMRA) and cost-effective management of wastewater. The present study conducted next generation sequencing (NGS) to determine the prevalence and diversity of *Cryptosporidium* species in 730 raw influent samples from 25 Australian wastewater treatment plants (WWTPs) across three states: New South Wales (NSW), Queensland (QLD) and Western Australia (WA), between 2014 and 2015. All samples were screened for the presence of *Cryptosporidium* at the 18S rRNA (18S) locus using quantitative PCR (qPCR), oocyst numbers were determined directly from the qPCR data using DNA standards calibrated by droplet digital PCR, and positives were characterized using NGS of 18S amplicons. Positives were also screened using *C. parvum* and *C. hominis* specific qPCRs. The overall *Cryptosporidium* prevalence was 11.4% (83/730): 14.3% (3/21) in NSW; 10.8% (51/470) in QLD; and 12.1% (29/239) in WA. A total of 17 *Cryptosporidium* species and six genotypes were detected by NGS. In NSW, *C. hominis* and *Cryptosporidium* rat genotype III were the most prevalent species (9.5% each). In QLD, *C. galli*, *C. muris and C. parvum* were the three most prevalent species (7.7%, 5.7%, and 4.5%,

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respectively), while in WA, *C. meleagridis* was the most prevalent species (6.3%). The oocyst load/Litre ranged from 70 to 18,055 oocysts/L (overall mean of 3426 oocysts/L: 4746 oocysts/L in NSW; 3578 oocysts/L in QLD; and 3292 oocysts/L in WA). NGS-based profiling demonstrated that *Cryptosporidium* is prevalent in the raw influent across Australia and revealed a large diversity of *Cryptosporidium* species and genotypes, which indicates the potential contribution of livestock, wildlife and birds to wastewater contamination.

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1. Introduction

Australia is the driest of the world's inhabited continents, with the lowest percentage of rainfall as run-off and the lowest amount of water in rivers (Anonymous, 2004). Drinking water resources are under considerable strain as a result of major shifts in long-term climate change, and climate predictions for all Australian States and Territories suggest increasing temperatures, a decline in average rainfall, but increasing severity and frequency of storm events (Garnaut Review, 2008). Consequently, there is increasing pressure for more efficient use of water resources, both in urban and rural environments (Toze, 2006a). Recycling wastewater will help address these challenges and is a prominent option among the various alternative sources of water in both developing and developed countries (Miller, 2006; Mekala and Davidson, 2016). However, infection with pathogenic microorganisms is a major risk factor (Rodriguez-Manzano et al., 2012) and therefore water destined for reuse must be fit for purpose (Toze, 2006b).

The waterborne parasite *Cryptosporidium* represents an important public health concern for water utilities, as it is a major cause of diarrhoea and there is neither a vaccine nor an effective treatment (Ryan et al., 2016; Zahedi et al., 2016a). *Cryptosporidium* is particularly suited to waterborne transmission as the oocyst stage is highly resistant to chlorine disinfection and can penetrate and survive routine water and wastewater treatment systems (King and Monis, 2007; King et al., 2017; Ryan et al., 2017a). The parasite has been responsible for numerous large-scale waterborne outbreaks worldwide (Efstratiou et al., 2017) and is highly prevalent in wastewater (Amorós et al., 2016; Ma et al., 2016).

Cryptosporidium species are currently monitored in wastewater using standard detection methodologies (i.e. fluorescence microscopy using EPA method 1623 - USEPA, 2012), however, this method cannot discriminate between different *Cryptosporidium* species. Of the 37 recognised *Cryptosporidium* species, *C. hominis* and *C. parvum* are the dominant species that infect humans (Ryan et al., 2016; Zahedi et al., 2017a; Čondlová et al., 2018; Kváč et al., 2018). As not all species of *Cryptosporidium* are infectious to humans (Ryan et al., 2016), understanding the diversity of *Cryptosporidium* in wastewater is crucial for more accurate quantitative microbial risk assessment (QMRA), for proper management of wastewater and its recycling. Due to the complex composition, abundance, and distribution patterns of *Cryptosporidium* species present in wastewater samples, molecular techniques such as conventional PCR and Sanger sequencing-based genotyping methods are unable to resolve complex DNA mixtures due to mixed sequencing chromatograms and are also unable to detect low abundance species or variants of *Cryptosporidium* (which typically appear as a “bumpy baseline” in Sanger chromatograms) (Murray et al., 2015; Paparini et al., 2015; Grinberg and Widmer, 2016).

Next-generation sequencing (NGS) technologies have allowed the comprehensive characterization and deep coverage of microbial community structure and diversity in environmental samples such as soil, water, the atmosphere and other environments (Cruaud et al., 2014). NGS is also more sensitive for the detection of less abundant species within microbial communities (Salipante et al., 2013). Recently, NGS approaches have been described that examine the composition and diversity of microbial communities (Shanks et al., 2013; Ma et al., 2015; Newton et al., 2015; Xu et al., 2017), adenovirus (Ogorzaly et al., 2015), norovirus (Prevost et al., 2015), astrovirus (Brinkman et al., 2013) and protists (Maritz et al., 2017) in sewage. However, to the best of the authors' knowledge, to date no large scale longitudinal studies have been undertaken to investigate the composition and diversity of *Cryptosporidium* species in wastewater using high-throughput amplicon NGS. As the costs of NGS continue to decrease and the bioinformatics analysis of data continues to improve, NGS screening of wastewater samples has become more feasible (Muir et al., 2016).

Therefore, the aim of the present study was to use NGS, for the first time on a large scale, to more accurately determine the prevalence and composition of *Cryptosporidium* species in Australian WWTPs across three states: New South Wales (NSW), Queensland (QLD) and Western Australia (WA).

2. Materials and methods

2.1. Study sites and sample collection

In NSW, WWTP samples (250 mL raw influent) were collected on a monthly interval over five months (April 2015 to August 2015). A total of 21 WWTP samples were collected from four wastewater plants within the WaterNSW area of operations (greater Sydney) (Table 1). In QLD, a total of 470 WWTP samples (250 mL raw influent) were collected on fortnightly intervals from WWTP sites ($n = 19$) across south east Queensland (Table 1) over a year (January 2014 to January 2015). In WA, a total of 239 WWTP samples (250 mL raw influent) were collected from two treatment plants on weekly intervals from December 2014 to December 2015 (Table 1). All raw influent WWTP samples were collected into individual 250 mL collection pots and stored at 4 °C until required and samples collected in NSW and QLD were shipped to Murdoch University for analysis.

2.2. Sample processing and DNA isolation

All 250 mL WWTP samples were transferred to 50 mL centrifuge tubes, and evenly weighed tubes ($n = 5$) were prepared from the same samples. These samples were centrifuged at 10,000 ×g for 20 min and pellets from the same samples were mixed together again. DNA was extracted from aseptically separated 250 mg aliquots of each sample (pellet), using a Power Soil DNA Kit (MO BIO, Carlsbad, California, USA) (Walden et al., 2017). An extraction blank (no WWTP sample) and a positive extraction control (a *Cryptosporidium* positive faecal sample from a kangaroo), was included in each extraction batch, as a process control for extraction efficiency. Purified DNA was stored at −20 °C prior to molecular analyses.

2.3. qPCR and oocyst enumeration

All WWTP sample extracts were screened for the presence of *Cryptosporidium* at the 18S rRNA (18S) locus using a quantitative PCR (qPCR) as previously described (King et al., 2005; Yang et al., 2014). A spike analysis of the 18S qPCR assay (addition of 0.5 μL of positive control DNA into test samples) was conducted on randomly selected negative samples from each group of DNA extractions, to determine if negative results were due to PCR inhibition by comparing the cycle threshold (Ct) values of the spike and the positive control (both with same

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