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Occurrence and removal efficiency of parasitic protozoa in Swedish wastewater treatment plants

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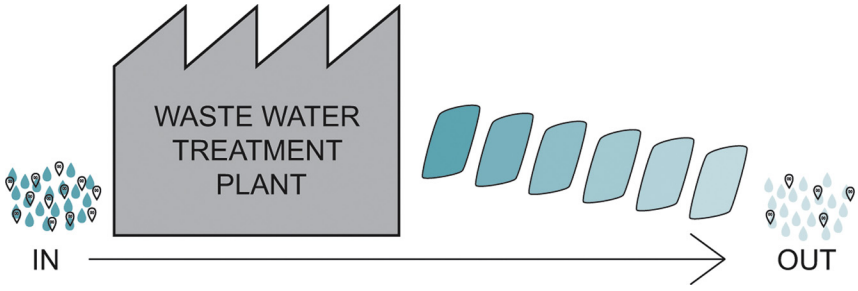
HIGHLIGHTS

- High prevalence of parasitic protozoa in influent wastewater in Sweden
- *Giardia intestinalis*, *Entamoeba dispar* and *Dientamoeba fragilis* detected
- Wastewater treatment removal efficiency low for parasitic protozoa

GRAPHICAL ABSTRACT



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ABSTRACT

Giardia intestinalis, *Cryptosporidium* spp., *Entamoeba histolytica* and *Dientamoeba fragilis* are parasitic protozoa and causative agents of gastroenteritis in humans. *G. intestinalis* and *Cryptosporidium* spp. in particular are the most common protozoa associated with waterborne outbreaks in high-income countries. Surveillance of protozoan prevalence in wastewater and evaluation of wastewater treatment removal efficiencies of protozoan pathogens is therefore imperative for assessment of human health risk. In this study, influent and effluent wastewater samples from three wastewater treatment plants in Sweden were collected over nearly one year and assessed for prevalence of parasitic protozoa. Quantitative real-time PCR using primers specific for the selected protozoa *Cryptosporidium* spp., *G. intestinalis*, *E. histolytica*, *Entamoeba dispar* and *D. fragilis* was used for protozoan DNA detection and assessment of wastewater treatment removal efficiencies. Occurrence of *G. intestinalis, E. dispar* and *D. fragilis* DNA was assessed in both influent (44, 30 and 39 out of 51 samples respectively) and effluent wastewater (14, 9 and 33 out of 51 samples respectively) in all three wastewater treatment plants. Mean removal efficiencies of *G. intestinalis*, *E. dispar* and *D. fragilis* DNA quantities, based on all three wastewater treatment plants studied varied between 67 and 87%, 37–75% and 20–34% respectively. Neither *E. histolytica* nor *Cryptosporidium* spp. were detected in any samples. Overall, higher quantities of protozoan DNA were observed from February to June 2012. The high prevalence of protozoa in influent wastewater indicates the need for continued monitoring of these pathogens in wastewater-associated aquatic environments to minimise the potential risk for human infection.

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1. Introduction

Gastroenteritis is a common disease which can be caused by a number of different waterborne pathogens. One of the main groups of aetiological agents is parasitic protozoa, among which *Cryptosporidium* spp. and *Giardia intestinalis* are the two most commonly associated with waterborne outbreaks (Baldursson and Karanis, 2011). *Cryptosporidium* spp. and *G. intestinalis* are eliminated via faeces of infected humans and animals in environmentally resilient forms, i.e. oocysts and cysts, and are therefore suitable for dissemination through the wastewater systems (Fletcher et al., 2012). Studies on *Cryptosporidium* spp. occurrence in wastewater have been performed in countries including USA (Kitajima et al., 2014), Germany (Gallas-Lindemann et al., 2013), Poland (Sroka et al., 2013), Tunisia (Khouja et al., 2010) and Sweden (Ottoson et al., 2006). *G. intestinalis* is also prevalent in wastewater. In a meta-review from 2012 (Nasser et al., 2012), it was concluded that 23 out of 30 studies (77%) reported *G. intestinalis* in all wastewater samples collected.

Entamoeba histolytica is a cyst-forming parasitic protozoan capable of causing amoebiasis, a type of gastroenteritis. Microscopy-based methods of detection and quantification, which are widely employed in environmental prevalence studies of protozoa, cannot distinguish between *E. histolytica* and *Entamoeba dispar* (Fletcher et al., 2012). As such, while the prevalence of *Entamoeba* spp. has been reported in several studies, including in wastewater from Iran (Hatam-Nahavandi et al., 2015) and Tunisia (Ben Ayed et al., 2009; Khouja et al., 2010), the specific prevalence of *E. histolytica* in wastewater remains unclear. While amoebiasis caused by *E. histolytica* is one of the parasitic diseases responsible for most deaths worldwide, *E. dispar* has traditionally been considered to be non-pathogenic. However, this view has recently been challenged (Oliveira et al., 2015) and further studies of *E. dispar* are warranted to elucidate its potential pathogenicity.

Dientamoeba fragilis is a protozoa suspected of causing gastroenteritis in humans (Ögren et al., 2015). The mode of transmission of *D. fragilis* is a contested issue. *D. fragilis* trophozoites die quickly outside of a host and no cyst form has been unequivocally demonstrated. A few studies have however reported pseudocysts and cyst-like states (Munasinghe et al., 2013; Stark et al., 2014), which could indicate the potential for *D. fragilis* to disseminate via wastewater. For now, the general scientific consensus is that no cyst forms exist and the currently leading hypothesis is that *D. fragilis* is transmitted via pinworm ova (Clark et al., 2014; Ögren et al., 2013). One study has detected *D. fragilis* in wastewater (Stark et al., 2012), although the authors were unable to establish if the detected specimen was viable or infectious. Depending on its environmental viability and infectiousness, *D. fragilis* presence in wastewater could potentially constitute a health risk.

In order to assess the health risk associated with infectious protozoa transmitted via wastewater, it is imperative to survey the protozoan prevalence and evaluate the capacities of wastewater treatment plants (WWTPs) to remove protozoa during wastewater treatment. WWTP removal efficiencies of various parasitic protozoa are usually reported in the range of 81%–99% (Ottoson et al., 2006; Ben Ayed et al., 2009; Kitajima et al., 2014), although one study has reported as low as 35% removal efficiency for *Cryptosporidium* oocysts (Castro-Hermida et al., 2010). On the other hand, prevalence and removal rates in wastewater treatment systems of *D. fragilis* and *E. dispar* in particular are largely unknown due to a scarcity of studies. The aim of this study was to bridge this knowledge gap as well as to elucidate the prevalence and removal efficiencies in WWTPs of other parasitic protozoa in Sweden. Real-time PCR was used to investigate the prevalence of *Cryptosporidium* spp., *G. intestinalis*, *E. histolytica*, *E. dispar* and *D. fragilis* in influent and effluent wastewater collected from three different WWTPs located along the river Göta älv, Sweden. WWTP removal rates for the investigated protozoa were calculated to assess the potential health risk associated with the occurrence of these protozoa in wastewater treatment systems. Furthermore, WWTP removal and seasonal variation of these gastroenteritis-causing parasitic protozoa were assessed.

2. Materials and methods

2.1. Sampling sites and sampling

Influent and effluent wastewater samples (1000 mL each) were collected from three WWTPs located along the river Göta älv in Sweden: Holmängen (WWTP A), Arvidstorp (WWTP B) and Ellbo (WWTP C) which treat wastewater from Vänersborg, Trollhättan and Lilla Edet respectively. WWTPs A, B and C treat wastewater from approximately 27,000, 47,000 and 6200 people, respectively. The flows handled by the WWTPs were approximately $6200 \cdot 10^3$ m^3/year for WWTP A, $11{,}000 \cdot 10^3$ m^3/year for WWTP B and $840 \cdot 10^3$ m^3/year for WWTP C. The treatment at these WWTPs included mechanical, chemical and biological steps. At WWTP A, the treatment included: mechanical treatment using screens, grit chambers and primary sedimentation; biological treatment for removal of organic matter and nitrogen using a trickling filter and a separate denitrification step; chemical treatment in a flocculation tank and a sedimentation tank. At WWTP B, the treatment included: mechanical treatment using screens, grit chambers and primary sedimentation; chemical treatment, which is integrated with the mechanical steps; biological treatment for removal of organic matter and nitrogen using activated sludge process. At WWTP C, the treatment included: mechanical treatment using screens and grit chambers; biological treatment for removal of organic matter using activated sludge process; chemical treatment, which is integrated with the activated sludge step.

The samples (1000 mL) were collected continuously over 24 h every two weeks from 31th August 2011 to 5th June 2012 for WWTP B and C (21 samples from each WWTP), and once every month from 26th November 2011 to 5th June 2012 for WWTP A (9 samples). Samples were collected using automatic flow rate controlled samplers and were stored in sterile glass containers at 4 °C before pre-treatment.

2.2. Pre-treatment of water samples and DNA extraction

The pre-treatment of the wastewater samples was performed as follows: from the 1000 mL wastewater samples, pellets were accumulated by centrifugation of 400 mL from each sample at 4000*g* at 4 °C for 30 min. The pellets were resuspended in PBS (pH 7.4) and subsequently centrifuged for 15 min at 4300*g* at 4 °C, suspended in 280 μL Buffer AL (Qiagen, Hilden, Germany) and 20 μL Proteinase K (Qiagen), incubated at 56 °C for 60 min and lastly incubated at −80 °C for 30 min. After the pre-treatment, total DNA was extracted using the MagNa Pure LC Total Nucleic Acid Isolation Kit with the MagNa Pure LC 2.0 Instrument (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Extracted DNA was stored at −20 °C before subsequent analyses.

2.3. Detection and quantification of protozoan DNA with real-time PCR

Two multiplex real-time PCR assays were employed to detect and quantify DNA fragments specific to protozoan parasites (Table 1). The reaction mixture for the first assay consisted of primers and probes specific for *E. histolytica*, *E. dispar* and *D. fragilis* (Verweij et al., 2004; Visser et al., 2006; Verweij et al., 2007). For the second assay, the reaction mixture consisted of primers and probes specific for *Cryptosporidium parvum/hominis* and *G. intestinalis* (Fontaine and Guillot, 2002; Verweij et al., 2004). Phocine herpesvirus 1 (PhHV-1) and primers and probes complementary to PhHV-1 were added to the reaction mixture of the second assay to serve as inhibition control (Niesters, 2004). 5 μL of template was added to each reaction mixture to a total volume of 25 μL. Primers, probes and concentrations used are presented in Table 1. The cycling protocol used was as follows: an initial heating step at 95 °C for 5 min followed by 55 cycles of 95 °C for 5 s, 60 °C for 15 s and 72 °C for 15 s. Gene quantities were calculated using a standard curve consisting of serial dilutions of plasmids (Genexpress, Berlin,