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Occurrence and genetic diversity of *Cryptosporidium* and *Giardia* in urban wastewater treatment plants in north-eastern Spain

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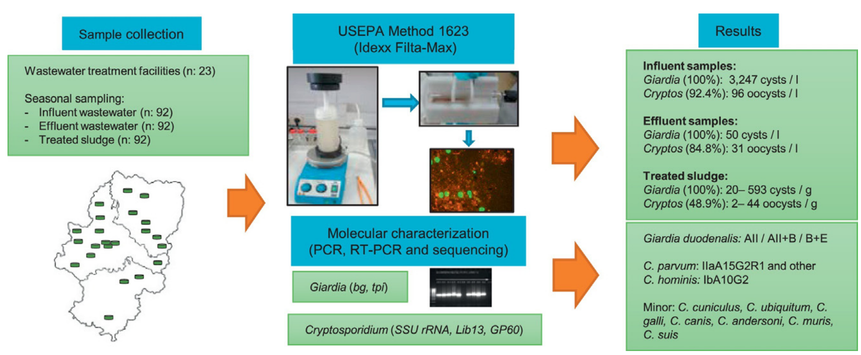
HIGHLIGHTS

- *Cryptosporidium* and *Giardia* are ubiquitous in urban sewage in north-eastern Spain.
- *Giardia* was removed more efficiently than *Cryptosporidium* by wastewater treatments.
- Potentially viable (oo)cysts were found in treated effluents and sludge.
- Nine *Cryptosporidium* species and three *G. duodenalis* variants (AII, B, E) were found.
- Both pathogens should be included in regulations for wastewater reclamation.

GRAPHICAL ABSTRACT



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ABSTRACT

This study was designed to investigate the presence and removal efficiency of *Cryptosporidium* and *Giardia* in wastewater treatment plants at the 20 most populated towns in Aragón (north-eastern Spain). Samples of influent and effluent wastewater and dewatered sewage sludge were collected seasonally from 23 plants and processed according to USEPA Method 1623. All samples from raw and treated wastewater tested positive for *Giardia*, at an average concentration of 3247 ± 2039 cysts/l and 50 ± 28 cysts/l, respectively. *Cryptosporidium* was identified in most samples from both raw (85/92) and treated (78/92) wastewaters in a concentration significantly lower than *Giardia*, at both influent (96 ± 105 oocysts/l) and effluent samples (31 ± 70 oocysts/l) ($P < 0.001$). The (oo)cyst counts peaked in summer in most plants. The removal efficiency was higher for *Giardia* (1.06-log to 2.34-log) than *Cryptosporidium* (0.35-log to 1.8-log). Overall, high removal efficiency values were found for *Giardia* after secondary treatment based on activated sludge, while tertiary treatment (microfiltration, chlorination and/or ultraviolet irradiation) was needed to achieve the greatest removal or inactivation of *Cryptosporidium*. Most samples of treated sludge were positive for *Giardia* (92/92) and *Cryptosporidium* (45/92), at an average concentration of 20–593 cysts/g and 2–44 oocyst/g, respectively. The molecular characterization of *Cryptosporidium* oocysts and *Giardia* cysts were attempted at the *SSU rRNA*/*GP60* and *bg*/*tpi* loci, respectively. *G. duodenalis* sub-assemblage AII was identified in all plants, with a large proportion of samples (15/47) harboring mixed assemblages (AII + B). Nine *Cryptosporidium* species and six subtypes were identified, with *C. parvum* IIaA15G2R1 being the most prevalent. The presence of significant numbers of (oo)cysts in samples of final effluents and treated sludge reveals the limited efficacy of conventional treatments in removing (oo)cysts and highlights the potential environmental impact and public health risks associated with disposal and reclamation of wastewater.

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1. Introduction

The environmental and public health risks associated with sewage disposal have focused attention on the importance of an efficient treatment of wastewater. Furthermore, reuse of reclaimed wastewater has emerged as a prominent option in the search for alternative sources of water (Mekala and Davidson, 2016). In addition to chemical contaminants, a wide range of bacteria, viruses, and parasites, pathogenic for humans and animals, end up in municipal sewage and should be reduced to acceptable levels (Montazeri et al. 2015). In addition to physical removal, potential pathogens can also be inactivated during wastewater treatment procedures. Wastewater management is, however, a challenging issue in the European Union, where legislation is fragmentary and in need of update, with some countries having more stringent regulations than those implemented by European Directives (Kelessidis and Stasinakis, 2012). The Urban Wastewater Treatment Directive 91/271/EC and the Sewage Sludge Directive 86/278/EC provide legal limits for physical and chemical parameters for the treatment of sewage effluents and sludge disposal in soil, respectively, but no pathogen standards are specified (CEC, 1986, 1991). The lack of pathogen standard protocols is greatly due to the inherent limitations of currently available methods to monitor these pathogens in water samples and to provide accurate, reliable and consistent concentration measures.

The major waterborne pathogens *Giardia duodenalis (*syn. *G. lamblia*, *G. intestinalis*) and *Cryptosporidium* spp. are among the most common parasites found in wastewater (Efstratiou et al., 2017). These intestinal protozoa are transmitted through environmentally-resistant cysts and oocysts, respectively, which are excreted in high numbers in the feces of infected hosts. The global emission of *Cryptosporidium* oocysts to surface waters has been estimated at 3×10^{17} oocysts per year, with comparable contributions from human wastewater and manure from livestock (Hofstra et al., 2013). Both *Giardia* and *Cryptosporidium*, particularly the latter, are resistant to chlorine-based disinfectants at the concentrations and exposure times commonly used in the water industry (Carmena, 2010). Additionally, many species and genotypes are infective to different livestock and companion animals, which may be a source for human infections and environmental contamination. At present, 31 *Cryptosporidium* species have been reported, although only two are responsible for the majority of human infections, including the anthroponotic species *C. hominis* and the zoonotic species *C. parvum* (Ryan et al., 2016). Subtyping at the highly polymorphic 60-kDa glycoprotein (*GP60*) gene, has enabled the identification of subtype families within *C. hominis* and *C. parvum*, as well as several subtypes within each family. Some of the *C. parvum* subtype families, such as IIa and IId, are responsible for zoonotic cryptosporidiosis, while other families, especially IIc, have so far only been found in humans (Xiao, 2010). Eight assemblages (A–H) and several sub-assemblages of *G. duodenalis* have been identified, but only two potentially zoonotic assemblages (A, B) are commonly found in humans (Ryan and Cacciò, 2013).

Studies conducted in some developed countries have reported the occurrence of *Giardia* and *Cryptosporidium* in raw wastewater, often at concentrations over 1000 cysts/l and 10 oocysts/l, respectively (Cacciò et al., 2003; McCuin and Clancy, 2006; Robertson et al., 2006; Cheng et al., 2009; Lobo et al. 2009; Kitajima et al., 2014; Taran-Benshoshan et al., 2015). However, quantitative data have shown that conventional treatment processes are not designed to completely remove both protozoa from wastewater. Efficiencies of (oo)cyst removal varying from 75.3 to 100% for *Giardia* and 40 to 100% for *Cryptosporidium* have been reported (Nasser et al., 2012; Nasser, 2016). Moreover, several studies have demonstrated that commonly used bacterial indicators of the hygienic quality of water do not necessarily correlate with the concentration of these protozoa (Bonadonna et al., 2002; Keeley and Faulkner, 2008).

Spain accounts for the largest proportion of reused treated wastewater in Europe (500 Mm^3/yr out of 1100 Mm^3/yr) and is among the greatest sewage sludge producers, with an annual production of 1,121,000 tons (Kelessidis and Stasinakis, 2012; BIO by Deloitte, 2015). In spite of this, the occurrence of *Giardia* and *Cryptosporidium* in Spanish wastewater treatment plants is not well documented and studies on the molecular characterization of isolates are limited. The scarcity of published data have shown that both protozoa are found in relatively high concentrations in wastewater, reclaimed water, sewage sludge, and even in fresh salad products, revealing the need to include them in regulations on urban wastewater reuse (Montemayor et al., 2005; Guzmán et al., 2007; Castro-Hermida et al., 2008, 2010; Galván et al., 2014; Amorós et al., 2010, 2016). However, no requirements are mentioned in current Spanish legislation, which only establishes certain limits for *Escherichia coli* and intestinal nematodes (Royal Decree 1620/2007). In this study, samples of raw wastewater, treated effluent and treated sewage sludge were seasonally investigated for the presence of *Giardia* and *Cryptosporidium* in municipal wastewater treatment plants in north-eastern Spain, in order to assess the occurrence, concentration and genetic diversity of both protozoa, and the reduction of pathogen load through different wastewater treatments.

2. Material and methods

2.1. Sample collection and processing

Over the period 2013–2015, samples were collected from 23 urban wastewater treatment plants located in the 20 most populated towns in Aragón (north-eastern Spain) (Fig. 1). This geographical area (42°56′ to 39°51′ N, 2°10′ O to 0°46′ E) is primarily agricultural, with an important ovine farming activity and an increasing industrial activity. These plants serve local settlements ranging from 5000 to over 660,000 inhabitants and treat wastewater from nearly 1 million people, which represents over 75% of the total population in Aragón. Only three plants served a population over 20,000. In addition to human wastewater, most facilities received industrial waste and seven plants also treated waste from slaughterhouses and/or farms. Most plants discharged the final effluents into rivers, although reclaimed water from three plants was also used to irrigate public parks, residential lawns, for street sweeping, or agricultural irrigation (Table 1). All facilities had biological reactor systems based on activated sludge and extended aeration with oxygen to improve the digestion of organic material by aerobic bacteria. Three plants used an Orbal® oxidation ditch process based on aerobic and anaerobic water depuration. Eleven plants had a Carrousel type reactor with canal configuration and vertical and superficial diffusers, typically used in low and medium load plants. Nine plants used biological reactors based on big and deep tanks with static diffusers commonly used in plants with high load. Only six plants used primary sedimentation and ten plants applied tertiary treatment, mostly based on chlorination, with two facilities using a combination of microfiltration and ultraviolet irradiation.

Samples of untreated influent and final effluent were collected from each wastewater treatment facility at four different times, each sampling time matching a different season (spring, summer, autumn, and winter). The holding times of each step in the treatment were taken into account during sampling, in order to examine the same wastewater at both points in the process. A sample of dewatered sewage sludge was also collected at each sampling time and kept for further analysis. Turbidity of influent and effluent samples was measured with a portable turbidimeter model HI93703 (Hanna Instruments, Spain) and the results were expressed in nephelometric turbidity units (NTU). The turbidity removal efficiency achieved by each plant was calculated using the following equation:

$$\text{Turbidity removal efficiency } (\%) = [(\text{turbidity influent} - \text{turbidity effluent})/(\text{turbidity influent})] \times 100$$