Occurrence of *Cryptosporidium* and *Giardia* in raw and finished drinking water in north-eastern Spain

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Abstract

This paper collects the first large-sample-size study on the presence of *Cryptosporidium* oocysts and *Giardia* cysts in drinking water plants at the 20 most populated towns in Aragón (north-eastern Spain). Samples of influent raw water and effluent finished water were collected from each plant during different seasons and processed according to USEPA Method 1623. *Cryptosporidium* oocysts and *Giardia* cysts were detected in samples collected from 55% and 70% plants, respectively, with nine plants being positive for both protozoa and only four plants being negative over the study period. Both parasites were identified in the raw water throughout the year, with a lower frequency in autumn and a peak in winter, at a mean concentration of 67 ± 38 oocysts per 100 litres and 125 ± 241 cysts per 100 litres. The turbidity of raw water was not related to the presence or concentration of (oo)cysts, and the (oo)cyst removal efficiency was not related to the type of water treatment. One or both pathogens were identified in the finished water in 7 out of 11 plants with a conventional treatment process (coagulation, flocculation, sedimentation, filtration, and disinfection processes) compared to 4 out of 9 plants that did not apply one of the pre-chlorination treatment steps. Protozoa were detected in the finished water of positive plants at a mean concentration of 88 ± 55 oocysts per 100 litres and 37 ± 41 cysts per 100 litres, and most of them excluded propidium iodide so were considered potentially viable. The ubiquity of these parasites in the drinking water sources and the inefficiency of conventional water treatment in reducing/inactivating them may present a serious public health issue in this geographical area.

1. Introduction

Cryptosporidium and *Giardia* are the most common parasites involved in the etiology of waterborne diarrheic outbreaks in human populations in developed countries. Outbreaks have been associated with contaminated drinking water or recreational water and were most often due to the presence of *Giardia* in the United States, while *Cryptosporidium* predominates in Europe and Australia (Craun et al., 2010; Baldurson and Karanis, 2011). Outbreaks of these parasites can be particularly large in size, for example the massive cryptosporidiosis outbreak in 2003 affecting 403,000 people in Milwaukee (Wisconsin, USA), and the giardiasis outbreak in 2004 resulting in an estimated 2,500 cases in Bergen (Norway) (Mac Kenzie et al. 1994; Nygårdk et al. 2006). In Europe, most waterborne cryptosporidiosis and giardiasis infections have been reported in the United Kingdom and Ireland, although they have also been detected in the Nordic countries, France, Germany, Italy, Greece, and Spain (Semenza and Nichols, 2007; Baldursson and Karanis, 2011; Chalmers, 2012; Guzman-Herrador et al., 2015a).

Several factors have favored the emergence of these protozoa as waterborne pathogens, including their ubiquitous distribution, the environmental resistance of *Cryptosporidium* oocysts and *Giardia* cysts, the high (oo)cyst excretion rate from infected hosts and low infectious doses, and the presence of multiple hosts for some of the human pathogenic species and genotypes (Carmena, 2010; Escobedo et al. 2010). Furthermore, both parasites and particularly *Cryptosporidium* are not effectively removed by conventional water treatment processes because of their small size and resistance to disinfection. Outbreaks have been reported through drinking water that met the legal quality standards, including the WHO Guidelines for Drinking Water Quality (WHO, 2011)

and the European Drinking Water Directive (Directive 98/83/EC) (Chalmers, 2012). *Cryptosporidium* is considered a reference pathogen for drinking water (WHO, 2006), and is included in Directive 2003/99/EC of the European Parliament and the Council of the European Union on the monitoring of zoonoses and zoonotic agents. Human cryptosporidiosis cases should therefore be collected in Member States and communicated to the European Commission, but *Cryptosporidium* diagnosis is statutorily notifiable in only a few countries (Semenza and Nichols, 2007; Chalmers, 2012).

In Spain, *Cryptosporidium* and *Giardia* are significant causative agents of diarrhoeal disease in humans and young ruminants, which are major reservoirs of human-infective *Cryptosporidium* species and may harbor some *Giardia* genotypes/assemblages known to be infectious to humans (Navarro-i-Martínez et al., 2011; Carmena et al., 2012). Previous studies in regions of Spain have revealed the common occurrence of *Cryptosporidium* and *Giardia* in water (surface water, drinking water, wastewater, reclaimed, recreational) (Montemayor et al., 2005; Carmena et al., 2007; Castro-Hermida, 2008, 2009, 2015; Galván et al., 2014), and human cryptosporidiosis outbreaks linked to contaminated drinking water supplies or recreational water have occasionally been detected (Rodríguez-Salinas et al. 2000; Galmes et al., 2003, Soler, 2004). In spite of this, the potential of both protozoa for waterborne transmission has apparently been considered of minor significance, and specific legislative bodies established in some parts of the world (e.g. UK, USA) to regulate the monitoring of water for these parasites have not been introduced in Spain (Chalmers, 2012).

The region of Aragón (north-eastern Spain) has the highest number of notifications of human cases of cryptosporidiosis according to the Spanish Microbiological Information System (MIS, 2015). Two waterborne outbreaks involving an estimated 750 and 100 human cases, respectively, were reported in this region in 2000, which was also characterized by high rates of *Cryptosporidium* and *Giardia* infections in livestock farms (Quílez et al., 1996; 2008; Causapé et al., 2002; Anonymous, 2003). This study was designed to provide information on the occurrence and concentration of *Cryptosporidium* and *Giardia* in drinking water treatment plants in this geographical area. The efficiency of treatment plants in removing the protozoans was also investigated.

2. Materials and Methods

2.1. Site location and sampling

Over the period 2013-2015, water samples were collected at 20 municipal drinking water treatment plants located in the most populated towns in Aragón (north-eastern Spain) (Figure 1). These plants serve local settlements ranging from 7,000 to over 660,000 inhabitants and provide potable water to nearly 1 million people in total, which represents over 75% of the total population in Aragón (Table 1). From each water treatment facility, samples of untreated raw water (influent) and treated finished water (effluent) were collected at four different times, each sampling time matching a different season (spring, summer, autumn, and winter). Turbidity of each sample was measured with a portable turbidimeter model HI93703 (Hanna Instruments, Spain) and the results were expressed in nephelometric turbidity units (NTU).

Plants had different sources for their raw water, including surface water (river, reservoir) and groundwater. Eleven facilities applied conventional water treatment including coagulation-flocculation, clarification through sedimentation, filtration and chlorination (Table 1). Two types of filtration were used in the latter facilities, including a system combining sand and activated carbon filtering (six plants), or a rapid sand filtration system where water flows through several layers of coarse-grained sand and gravel to remove particles that have been trapped during the previous flocculation (five plants). Four additional facilities included slow filtration, which combines both physical and biological properties of the sand bed previous to the chlorination. Finally, five plants were small facilities that only applied sedimentation and chlorination. None of the plants had ultraviolet systems for water disinfection. The turbidity removal efficiency achieved by each plant was calculated using the following equation:

Turbidity removal efficiency (%) = [(turbidity influent –turbidity effluent) / (turbidity influent)] × 100

2.2. Detection and molecular characterization of Cryptosporidium and Giardia

A total of 160 water samples were analysed for the presence of *Cryptosporidium* oocysts and *Giardia* cysts according to the U.S. Environmental Protection Agency Method 1623 (USEPA, 2005). Briefly, samples of untreated (up to 50 litres of the raw influent) and treated (up to 100 litres of the finished effluent) water were filtered through Filta-Max filters (IDEXX Laboratories, Inc., Westbrook, ME, USA) using a motorized pump at recommended flow rates. The holding times of each step in the treatment were taking into account during sampling, in order to examine the same water

at both points in the process. The filters were transported to the laboratory in labelled and sealed plastic bags, and stored at 4°C. Elution procedures were carried out within 24 hours after collection with the Filta-Max Manual System (IDEXX Laboratories, Inc.) according to the manufacturer's instructions. After centrifugation at 1,500 × g for 15 min, the supernatant was aspirated to 20 ml and 10 ml of the resuspended pellet was subjected to immunomagnetic separation (IMS).

(Oo)cysts in concentrated samples of water were further purified from other particulates using the Dynal IMS procedure (Dynabeads GC-Combo, Invitrogen Dynal, A.S., Oslo, Norway) according to manufacturer's instructions. IMS-purified (oo)cysts were stained on well slides by fluorescein isothiocyanate (FITC)-conjugated anti-*Cryptosporidium* and anti-*Giardia* monoclonal antibodies (Crypto/Giardia Cel, Cellabs Pty Ltd, Australia). The internal structure of (oo)cysts was confirmed by staining with the nuclear fluorochrome 4′,6-diamidino-2-phenylindole (DAPI) (Sigma). Slides were examined using an epifluorescence microscope and (oo)cysts showing typical, confirmatory features (size, internal contents, fluorescence) were enumerated and numbers extrapolated to concentrations of parasite per 100 litres of water. Positive and negative staining controls were routinely included. All calculations were adjusted taking into account the recovery efficiency of *Cryptosporidium* and *Giardia* reported in our laboratory and the volume of water filtered.

The potential viability of (oo)cysts was estimated by staining with the vital dye propidium iodide (PI) (Sigma-Aldrich, USA). Stock solution was prepared by dissolving PI in distilled water (1mg/ml). A volume of 10 µl of PI working solution were added to each well and incubated at room temperature in the dark for 1 minute,

and excess PI solution was removed by washing the slides in distilled water. The (oo)cysts were counted according to whether they were PI-positive (PI+: permeable and presumably dead) or PI-negative (PI−: impermeable and presumably viable) (Jenkins et al., 1997).

The recovery efficiency of the method was determined by seeding 10 litres of distilled water with different turbidity values (0, 20, and 50 NTU) with a known number of (oo)cysts according to the instructions of the USEPA 1623 method. Manually enumerated (oo)cysts stained with FITC-labelled antibodies were used due to cost restrictions. This procedure was repeated three times for each turbidity value. The mean recovery efficiency was 19.8 ± 4.3% for *Cryptosporidium* and 49.2 ± 16.5% for *Giardia*, which meets the acceptance criteria described in this method (McCuin and Clancy, 2003; USEPA, 2005).

The removal efficiency of (oo)cysts by each plant and sampling time was calculated as follows:

Log removal = Log influent concentration – Log effluent concentration

Molecular characterization was attempted in positive samples with a concentration higher than 10 (oo)cysts per 100 litres. *Cryptosporidium* and *Giardia* DNA was extracted from IMS-purified (oo)cysts using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, incorporating an initial step of three freeze-thaw cycles (freezing in liquid nitrogen for 5 min and heating at 100ºC for 1 min) in the protocol. Previously described PCR protocols were

199 used to amplify the SSU rRNA and β-giardin genes of *Cryptosporidium* and *Giardia*,
200 respectively (Xiao et al. 2001; Lalle et al. 2005).
201
202 *2.3. Statistical analysis*
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204 Chi-squared and two-tailed Fisher's exact tests were used to evaluate possible
205 significant differences in the seasonal pattern of *Cryptosporidium* and *Giardia* positive
206 samples. The nonparametric Kruskall-Wallis test was used to evaluate significant
207 differences in the (oo)cyst counts between influent and effluent water and between
208 seasons. Analyses were performed using the SPSS statistical package for Windows
209 version 18 (SPSS Inc.). Values of $P<0.05$ were considered statistically significant.
210
211 **3. Results**
212
213 *Cryptosporidium* and *Giardia* were identified in water samples of 11 (55%) and 14
214 (70%) drinking water treatment plants, respectively, with nine plants being positive for
215 both protozoa and only four plants being negative over the study period (Tables 2 and
216 3). The analysis of results by season in the influent water samples revealed that winter
217 showed a higher rate of positive plants than other seasons did, although differences
218 were only statistically significant when compared to autumn, for both *Cryptosporidium*
219 (30% *versus* 0%; $P<0.01$) and *Giardia* (45% *vs.* 15%; $P<0.05$) (Figure 2). (Oo)cyst
220 counts in the influent samples revealed a mean concentration of 67 ± 38
221 *Cryptosporidium* oocysts and 125 ± 241 *Giardia* cysts per 100 l, with maximum values
222 being recorded in spring in the same plant (160 oocysts and 1759 cysts per 100 l).
223

All water treatment facilities positive for *Cryptosporidium* in the raw water (n = 8) achieved high removal efficiencies, since no oocysts were detected in the finished water. In contrast, *Cryptosporidium* was identified in the finished water of three additional plants where samples of raw water were negative for this protozoan. The water treatment also reduced the *Giardia* cyst counts in most plants, which exhibited a removal efficiency ranging from 0.01-log to >2.96-log, although some samples from four plants were only positive in the effluent water. Nevertheless, differences in the parasite concentration between the influent and effluent samples were not significant, which was probably due to the small number of (oo)cysts identified. Protozoa were detected in the finished water of positive plants at a mean concentration of 88 ± 55 oocysts per 100 l and 37 ± 41 cysts per 100 l. Based on the dye permeability staining, *Cryptosporidium* oocysts identified in all positive samples excluded propidium iodide and were thus considered potentially viable. Similarly, *Giardia* cysts identified in all but four samples from either raw or finished water were also impermeable to the fluorochrome. Sources of water in the positive facilities included both surface and groundwater, and the four plants negative for both protozoa collected water from rivers or reservoirs.

The (oo)cyst removal efficiency was not related to the type of water treatment. One or both protozoa were identified in the finished water of 7 out of 11 facilities with a conventional treatment process (coagulation, flocculation, sedimentation, filtration, and disinfection processes) compared to 4 out of 9 plants that did not apply one of the pre-chlorination steps. No obvious differences were seen in the removal efficiency in relation to the type of filtration, since (oo)cysts were seen in the effluent water of plants using rapid filtration (4/5), sand plus activated carbon filtering (3/6) or slow filtration

(2/4). The mean turbidity of the raw water samples ranged from 0 to 32 NTU and was not related to the presence or concentration of (oo)cysts, as demonstrated by the low turbidity values (< 1 NTU) in some positive samples and the high value (> 5 NTU) recorded in some negative plants (Table 1). Most water treatment facilities achieved 100% turbidity removal efficiency, although a high value (186 NTU) was detected in the finished water of a plant during spring, which was related to an accidental electricity failure causing contamination with sand in the effluent water. Repeated attempts to characterize *Cryptosporidium* and *Giardia* positive samples at the species level by PCR were unsuccessful.

4. Discussion

Results from this long-term study show that both *Cryptosporidium* and *Giardia* are common contaminants in the raw water of most water treatment facilities in Aragón (north-eastern Spain). The mean recovery percentage of the analytical procedure was close to 20% for *Cryptosporidium* and 50% for *Giardia*, which is considered acceptable for the USEPA method 1623 and was similar to data reported in other studies (USEPA, 2005; Ongerth, 2013). *Giardia* was identified in more than half of the facilities, being a much more prevalent protozoan than *Cryptosporidium* in all seasons, which is in agreement with observations reported for drinking water sources in Spain and other countries (Hörman et al., 2004; Carmena et al., 2007; Mons et al., 2009; Burnet et al., 2014; Castro-Hermida et al., 2015). Similarly, (oo)cyst counts revealed an overall higher concentration of *Giardia* cysts than *Cryptosporidium* oocysts, although differences were not statistically significant and contamination in the raw water rarely exceeded 100 cysts per 100 l.

These results are consistent with the ubiquity of *Cryptosporidium* and *Giardia*, which have been widely reported in the human population and are common pathogens in young livestock in Spain (Navarro-i-Martínez, 2011; Carmena et al. 2012). Some epidemiological surveys in diarrhoeic suckling ruminants have revealed infection rates by *Cryptosporidium* close to 60% and 80% in calves and lambs, respectively, and prevalence values close to 100% at farm level have been reported for *Giardia* infections in cattle and sheep farms in some Spanish regions (Causapé et al., 2002; Castro-Hermida et al., 2006; Quílez et al., 2008; Gómez-Muñoz et al., 2009). In humans, cryptosporidiosis and giardiasis have been nationally notifiable diseases since 2009, but routine testing is not carried out in many laboratories, and therefore many cases may remain unnoticed, especially for cryptosporidiosis (Martín-Ampudia et al., 2012). In fact, the number of cases of giardiasis and cryptosporidiosis reported to the Spanish Microbiological Information System in 2014 were 785 and 264 respectively (MIS, 2015), but studies focusing on the detection of these protozoa in symptomatic patients, mainly pediatric patients, have revealed prevalence rates up to 13-25% for giardiasis (Carmena et al., 2012). Likewise, incidence rates of human *Cryptosporidium* infections in some Spanish areas are larger than those typical of other European countries (Abal-Fabeiro et al., 2015).

Several factors could account for seasonal variations in the number of (oo)cysts present in the environment, including peaks of incidence in humans and animals, rainfall, agricultural practices, humidity and temperature (Putignani and Menichella, 2010; Guzman Herrador et al., 2015b). In livestock, the occurrence of *Cryptosporidium* infections have been reported to peak corresponding with the lambing/calving season,

and consistent seasonal patterns across national boundaries have also been found for human cryptosporidiosis or giardiasis albeit with variations between different areas and countries (Xiao and Ryan, 2008; Lal et al., 2012). In this study, both protozoa were identified in the influent of drinking water facilities throughout the year, with a lower frequency in autumn and a peak in winter. This finding is in agreement with the significantly higher prevalence of human cryptosporidiosis in winter, previously observed in children aged 1-4 years in this geographical area (Clavel et al., 1996), although other studies have recorded that human cases in Spain peaked in summer (Semenza and Nichols, 2007). Diverse results in the occurrence of *Cryptosporidium* and *Giardia* in samples from river water or drinking water supplies have also been reported in other Spanish areas, with the highest frequencies in spring and autumn in the north-east (Montemayor et al., 2005), in summer and autumn in the north (Carmena et al., 2007), in spring and summer in the north-west (Castro-Hermida et al., 2008, 2009), and in winter and spring in the central area (Galván et al., 2014).

The turbidity of water has been correlated significantly with the presence of *Cryptosporidium* and *Giardia* and is considered an indicator of filtration efficiency for removal of these pathogens (Hsu and Yeh 2003; Burnet et al. 2014). Moreover, recovery rates of *Cryptosporidium* and *Giardia* by filtration are positively correlated with the turbidity of samples, with a high turbidity being considered the major contributing factor to the poor recovery of (oo)cysts from the sample concentrate (Hu et al., 2004; Kothavade, 2012). In the current study, spiking experiments revealed that recovery efficiency of our detection method was certainly related to the turbidity of water, and the treatment process in most plants was consistently efficient in reducing turbidity to values below 0.4 NTU in the finished water. However, the turbidity of the

raw water was not related to the presence and/or concentration of (oo)cysts. Most plants with a turbidity value under 5 NTU in the influent water (11/12) were positive for either *Cryptosporidium* and/or *Giardia*, while a value over 5 NTU was recorded in the raw water of three of the four plants testing negative for both protozoa. These results contrast with Spanish legislation on the quality of drinking water intended for human consumption, which does not consider the routine detection of *Cryptosporidium* or other parasites but suggests its investigation only in the presence of enteric bacteria and turbidity values over 5 NTU (RD 140/2003).

Disinfection is the final step in the conventional water treatment process, with chlorine being the most common disinfectant. Most viruses and bacteria are effectively inactivated by chlorination, but some protozoa such as *Giardia* may require prolonged contact times at high chlorine residuals to achieve 3-log inactivation, and chlorine-based disinfectants are generally not effective at inactivation of *Cryptosporidium* (Betancourt and Rose, 2004). For this reason, emphasis is placed on removing protozoa in the treatment process preceding chlorination. Nevertheless, waterborne outbreaks caused by these protozoa have occurred in systems employing conventional water treatments consisting of coagulation, flocculation, sedimentation, and filtration, revealing the inability of this technology to completely remove (oo)cysts from water (Clancy and Hargy, 2008). Several studies at pilot- and full-scale conventional water treatment plants have showed removal efficiencies ranging from 1.4 to 4.0-log for *Cryptosporidium* and 1.5-log to 4.0-log for *Giardia* (Betancourt and Rose, 2004).

In this study, the removal efficiency was similar in plants with a complete water treatment and those lacking one treatment step preceding chlorination. Drinking water

facilities were effective in retaining most (oo)cysts as demonstrated by the reduction in the number of positive plants and (oo)cyst counts in the treated water. However, some plants achieved less than 1-log removal efficiency and small numbers of *Cryptosporidium* oocysts (40-166 per 100 l) and *Giardia* cysts (12-148 per 100 l) were still identified in the finished water of three and nine facilities respectively. Additionally, (oo)cysts in most samples from both raw and finished water were found to be impermeable to propidium iodide and thus considered potentially viable, suggesting that viability is not reduced by the water treatment. Nevertheless, these findings should be taken with caution due to the small number of (oo)cysts and limitations of vital dye assays resulting in overestimation of viability (Jenkins et al. 1997). It is significant to note that none of the facilities had ultraviolet radiation, which has been reported as an alternative disinfection procedure to inactivate (oo)cysts in water at appropriate levels (Clancy and Hargy, 2008). It is also worth mentioning the apparent lack of (oo)cysts in the raw water of four plants which tested positive for the finished water, a finding previously reported in drinking water facilities, that could be related to the small volume of raw water filtered because of filter clogging, revealing the limitations of analytical methods for recovering these organisms from water samples (Castro-Hermida et al., 2008; Ongerth, 2013).

The presence of potentially viable *Cryptosporidium* oocysts and *Giardia* cysts in the finished water of some municipalities in this study may present a serious public health issue. The (oo)cyst concentrations may not be enough to induce human infections since the infectious dose (ID50) has been estimated to be 10-83 oocysts for *Cryptosporidium* (Chappell et al., 2006) and 19-50 cysts for *Giardia* (Adam, 2001), but serve as a warning with regard to the potential risk of waterborne outbreaks and the significance of

protecting water sources from contamination. Unfortunately, the presence of zoonotic *Cryptosporidium* spp / *Giardia* spp could not be confirmed, since the PCR amplification was unsuccessful for all positive specimens selected for molecular characterization, a finding which might be related to the low parasite load or the presence of PCR inhibitors in the water. In northern and central Spain, conventional water treatment plants were highly efficient and no protozoa were detected in the finished water, but a high proportion of samples of effluent water from small facilities, including rapid filtration and/or disinfection processes only, were positive at average concentrations of 2.3-7.8 oocysts / 100 l and 1.3-2 cysts / 100 l (Carmena et al., 2007; Galván et al., 2014). In contrast, conventional water treatment facilities were inefficient in reducing/inactivating these pathogens in drinking water in the north-west of the country, where up to 190 oocysts per 100 l and 330 cysts per 100 l were estimated to be present in the treated drinking water of some facilities, and 90-95% of (oo)cysts were potentially viable according to a vital-dye assay (Castro-Hermida et al., 2015).

5. Conclusions

The current study highlights the occurrence of *Cryptosporidium* and *Giardia* in the drinking water facilities of the most populated towns in Aragón region of Spain, and the limited efficiency of conventional water treatment processes in removing (oo)cysts. Intensive efforts should be made to prevent the contamination of water sources with these pathogens and new regulations should be implemented to evaluate the need of testing for *Cryptosporidium* and *Giardia* in water, including monitoring tools as an alternative to turbidity readings. Our results also reveal the need for improving

analytical methods to identify these protozoa and test their viability/infectivity in contaminated water.

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References

Anonymous, 2003. Epidemiological monitoring of cryptosporidiosis in Spain (Vigilancia epidemiológica de la criptosporidiosis en España). Weekly Epidemiological Record, 11(24), 277–284. Available online: http://gesdoc.isciii.es/gesdoccontroller?action=download&id=20/03/2013-153858f564. Accessed 20 July 2016.

Abal-Fabeiro, J.L., Maside, X., Llovo, J., Bartolomé, C., 2015. Aetiology and epidemiology of human cryptosporidiosis cases in Galicia (NW Spain), 2000–2008. Epidemiol. Infect. 143, 3022-3035. http://dx.doi.org/10.1017/S0950268815000163

Adam, R.D., 2001. Biology of *Giardia lamblia*. Clin Microbiol Rev. 14, 447–75.

Baldursson, S., Karanis, P., 2011. Waterborne transmission of protozoan parasites: Review of worldwide outbreaks - An update 2004-2010. Water Res. 45, 6603–6614. http://dx.doi.org/10.1016/j.watres.2011.10.013

Betancourt, W.Q., Rose, J.B., 2004. Drinking water treatment processes for removal of *Cryptosporidium* and *Giardia*. Vet Parasitol. 126, 219–234. http://dx.doi.org/10.1016/j.vetpar.2004.09.002

Burnet, J.B., Penny, C., Ogorzaly, L., Cauchie, H.M., 2014. Spatial and temporal distribution of *Cryptosporidium* and *Giardia* in a drinking water resource: implications for monitoring and risk assessment. Sci Total Environ. 472, 1023–35. http://dx.doi.org/10.1016/j.scitotenv.2013.10.083

Carmena, D., Aquinagalde, X., Zigorraga, C., Fernández-Crespo, J.C., Ocio, J.A., 2007. Presence of Giardia cysts and Cryptosporidium oocysts in drinking water supplies in northern Spain. J Appl Microbiol. 102, 619–629. http://dx.doi.org/10.1111/j.1365-2672.2006.03193.x

Carmena, D., 2010. Waterborne transmission of *Cryptosporidium* and *Giardia*: detection, surveillance and implications for publich health. In: Mendez-Vilas A, (Ed.). Current Research, Technology and Education Topicas in Applied Microbiology and Microbial Biotechnology. Formatex microbiology series, vol 2. Formatex Research Center, Badajoz, Spain. pp. 3–14.

448 Carmena, D., Cardona, G.A., Sánchez-Serrano, L.P., 2012. Current situation of *Giardia*
449 infection in Spain: implications for public health. World J Clin Infect Dis. 2, 1–12.
450 http://dx.doi.org/10.5495/wjcid.v2.i1.1
451
452 Castro-Hermida, J.A., Carro-Corral, C., González-Warleta, M., Mezo., 2006.
453 Prevalence and intensity of infection of *Cryptosporidium* spp. and *Giardia duodenalis*
454 in dairy cattle in Galicia. J Vet Med B Infect Dis Vet Publich Health. 53, 244–246.
455 http://dx.doi.org/10.1111/j.1439-0450.2006.00946.x
456
457 Castro-Hermida, J.A., Garcia-Presedo, I., Almeida, A., Gonzalez-Warleta, M., Correia
458 Da Costa, J.M., Mezo, M., 2008. Presence of *Cryptosporidium* spp. and *Giardia*
459 *duodenalis* through drinking water. Sci Total Environ. 405, 45–53.
460 http://dx.doi.org/10.1016/j.scitotenv.2008.06.040
461
462 Castro-Hermida, J.A., García-Presedo, I., Almeida, A., González-Warleta, M., Da
463 Costa, J.M., Mezo, M., 2009. Detection of *Cryptosporidium* spp. and *Giardia*
464 *duodenalis* in surface water: a health risk for humans and animals. Water Res. 43,
465 4133–4142. http://dx.doi.org/10.1016/j.watres.2009.06.020
466
467 Castro-Hermida, J.A., González-Warleta, M., Mezo, M. 2015. *Cryptosporidium* spp.
468 and *Giardia duodenalis* as pathogenic contaminants of water in Galicia, Spain: The
469 need for safe drinking water. Int J Hyg Environ Health. 218, 132–138.
470 http://dx.doi.org/10.1016/j.ijheh.2014.09.001.
471
472 Causapé, A.C., Quílez, J., Sánchez-Acedo, C., del Cacho, E., López-Bernad, F., 2002.

Prevalence and analysis of potential risk factors for *Cryptosporidium parvum* infections in lambs in Zaragoza (northeastern Spain). Vet Parasitol. 104, 287-298. http://dx.doi.org/10.1016/S0304-4017(01)00639-2

Chalmers, R.M., 2012. Waterborne outbreaks of cryptosporidiosis. Ann Ist Super Sanita. 48, 429-446. http://dx.doi.org/10.4415/ANN_12_04_10.

Chappell, C.L., Okhuysen, P.C., Langer-Curry, R., Widmerg, G., Akiyoshi, D.E., Tanriverdi, S., Tzipori, S., 2006. *Cryptosporidium hominis*: experimental challenge of healthy adults. Am J Trop Med Hyg. 75, 851–857.

Clavel, A., Olivares, J.L., Fleta, J., Castillo, J., Varea, M., Ramos, F.J, Arnal, A.C., Quílez, J., 1996. Seasonality of cryptosporidiosis in children. Eur J Clin Microbiol Infect Dis. 15, 77–79. http://dx.doi.org/10.1007/BF01586190

Clancy, J.L., Hargy, T.M., 2008. Waterborne: Drinking water. In: Fayer, R., Xiao, L. (eds.). *Cryptosporidium* and cryptosporidiosis, 2nd edn. IWA Publishing/CRC Press, Boca Ratón, pp. 305–333

Craun, G.F., Brunkard, J.M., Yoder, J.S., Roberts, V.A., Carpenter, J., Wade, T., Calderon, R.L., Roberts, J.M., Beach, M.J., Roy, S.L., 2010. Causes of outbreaks associated with drinking water in the United States from 1971 to 2006. Clin Microbiol Rev. 23(3), 507–528. http://dx.doi.org/10.1128/CMR.00077–09

497 Diaz, P., Quilez, J., Chalmers, R.M., Panadero, R., Lopez, C., Sanchez-Acedo, C.,
498 Morrondo, P., Díez-Baños, P., 2010a. Genotype and subtype analysis of
499 *Cryptosporidium* isolates from calves and lambs in Galicia (NW Spain). Parasitology.
500 137, 1187–93. http://dx.doi.org/10.1017/S0031182010000181
501
502 Escobedo, A.A., Almirall P., Robertson L.J., Franco, R.M.B., Hanevik, K., Mørch, K.,
503 Cimerman, S., 2010. Giardiasis: The Ever-Present Threat of a Neglected Disease. Infect
504 Disord Drug Targets. 10, 329–348. http://dx.doi.org/10.2174/187152610793180821
505
506 Galmes, A., Nicolau, A., Arbona, G. Gomis., E., Guma, M., Smith-Palmer, A., Soler,
507 P., 2003. Cryptosporidiosis outbreak in British tourists who stayed at a hotel in Majorca,
508 Spain. Euro Surveill. 7, pii: 2275
509
510 Galván, A.L., Magnet, A., Izquierdo, F., Fernández-Vadillo, C., Peralta, R.H., Angulo,
511 S., Fenoy, S., del Aguila, C., 2014. A year-long study of *Cryptosporidium* species and
512 subtypes in recreational, drinking and wastewater from the central area of Spain. Sci
513 Total Environ. 468–469, 368-375. http://dx.doi.org/10.1016/j.scitotenv.2013.08.053
514
515 Gómez-Muñoz, M.T., Navarro, C., Garijo-Toledo, M.M., Dea-Ayuela, M.A,
516 Fernández-Barredo, S., Pérez-Gracia, M.T., Domínguez-Márquez, M.V., Borrás, R.,
517 2009. Ocurrence and genotypes of *Giardia* isolated from lambs in Spain. Parasitol Int.
518 58, 297–299. http://dx.doi.org/10.1016/j.parint.2009.06.005
519
520 Guzman-Herrador, B.R., Carlander, A., Ethelberg, S., Freiesleben de Blasio, B., Kuusi,
521 M., Lund, V., Löfdahl, M., MacDonald, E., Nichols, G., Schönning, C., Sudre, B.,

522 Trönnberg, L., Vold, L., Semenza, J.C., Nygård, K., 2015a. Waterborne outbreaks in the
523 Nordic countries 1998 to 2012. Euro Surveill. 20, pii: 21160
524
525 Guzman-Herrador, B.R., de Blasio, B.F., MacDonald, E., Nichols, G., Sudre, B., Vold,
526 L., Semenza, J.S., Nygård, K., 2015b. Analytical studies assessing the association
527 between extreme precipitation or temperature and drinking water-related waterborne
528 infections: a review. Environ Health. 14, 29. http://dx.doi.org/10.1186/s12940-015-
529 0014-y
530
531 Hörman, A., Rimhanen-Finne, R., Maunula, L., von Bonsdorff, C.H., Torvela, N.,
532 Heikinheimo, A., Hänninen, M.L., 2004. *Campylobacter* spp., *Giardia* spp.,
533 *Cryptosporidium* spp., noroviruses, and indicator organisms in surface water in
534 southwestern Finland, 2000-2001. Appl Environ Microbiol. 70, 87–95.
535 http://dx.doi.org/10.1128/AEM.70.1.87-95.2004
536
537 Hsu, B.M., Yeh, H.H., 2003. Removal of *Giardia* and *Cryptosporidium* in drinking
538 water treatment: a pilot-scale study. Water Res. 37, 1111–1117.
539 http://dx.doi.org/10.1016/S0043-1354(02)00466-9
540
541 Hu, J., Feng, Y., Ong, Sl.L., Ng, W.J., Song, L., Tan, X., Chu, X., 2004. Improvement
542 of recoveries for the determination of protozoa *Cryptosporidium* and *Giardia* in water
543 using Method 1623. J. Microbiol. Methods. 58, 321–325.
544 http://dx.doi.org/10.1016/j.mimet.2004.04.013
545

546 Jenkins, M.B., Anguish, L.J., Bowman, D,D., Walker, M.J., Ghiorse, W.C., 1997.
547 Assessment of a dye permeability assay for determination of inactivation rates of
548 *Cryptosporidium parvum* oocysts. Appl Environ Microbiol. 63, 3844–50
549
550 Kothavade, R.J., 2012. Potential molecular tools for assessing the public health risk
551 associated with waterborne *Cryptosporidium* oocysts. J. Med. Microbiol. 61, 1039–
552 1051. http://dx.doi.org/10.1099/jmm.0.043158-0
553
554 Lal, A., Hales, S., French, N., Baker, M.G., 2012. Seasonality in human zoonotic enteric
555 diseases: a systematic review. PLoS One, 7,e31883.
556 http://dx.doi.org/10.1371/journal.pone.0031883
557
558 Lalle, M., Pozio, E., Capelli, G., Bruschi, F., Crotti, D., Caccio, S. M., 2005. Genetic
559 heterogeneity at the beta-giardin locus among human and animal isolates of *Giardia*
560 *duodenalis* and identification of potentially zoonotic subgenotypes. Int. J. Parasitol. 35,
561 207–213. http://dx.doi.org/10.1016/j.ijpara.2004.10.022
562
563 McCuin, R.M., Clancy, J.L., 2003. Modifications to United States Environmental
564 Protection Agency Methods 1622 and 1623 for detection of *Cryptosporidium* oocysts
565 and *Giardia* cysts in water. Appl Environ Microbiol. 69, 267–274.
566
567 MacKenzie, W.R., Hoxie, N.J., Proctor, M.E., Gradus, M.S., Blair, K.A., Peterson,
568 D.E., Kazmierczak, J.J., Addiss, D.G., Fox, K.R., Rose, J.B., Davis, J.P.,1994. A
569 massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the

570 public water supply. N Engl J Med. 331, 161–167.
571 http://dx.doi.org/10.1056/NEJM199407213310304
572
573 Martín-Ampudia, M., Mariscal, A., Lopez-Gigosos, R.M., Mora, L., Fernandez-
574 Crehuet,J., 2012. Under-notification of cryptosporidiosis by routine clinical and
575 laboratory practices among non-hospitalised children with acute diarrhoea in Southern
576 Spain. Infection 40, 113–119. http://dx.doi.org/10.1007/s15010-011-0188-3
577
578 Montemayor, M., Valero, F., Jofre, J., Lucena, F.. 2005. Occurrence of
579 *Cryptosporidium* spp. oocysts in raw and treated sewage and river water in north-
580 eastern Spain. J Appl Microbiol. 99, 1455–1462. http://dx.doi.org/10.1111/j.1365-
581 2672.2005.02737.x
582
583 Mons, C., Dumètre, A., Gosselin, S., Galliot, C., Moulin, L., 2009. Monitoring of
584 *Cryptosporidium* and *Giardia* river contamination in Paris area. Water Res. 43, 211–
585 217. http://dx.doi.org/10.1016/j.watres.2008.10.024
586
587 Navarro-i-Martinez, L., del Águila, C., Bornay-Llinares, F.J., 2011. *Cryptosporidium*: a
588 genus in revisión. The Situation in Spain (*Cryptosporidium*: un género en revisión.
589 Situación en España). Enferm Infecc Microbiol Clin. 29, 135–143.
590 http://dx.doi.org/10.1016/j.eimc.2010.12.002
591
592 Nygård, K., Schimmer, B., Søbstad, Ø., Walde, A., Tveit, I., Langeland, N., Hausken,
593 T., Aavitsland, P., 2006. A large community outbreak of waterborne giardiasis-delayed
594 detection in a non-endemic urban area. BMC Public Health, 6, 141.

595 http://doi.org/10.1186/1471-2458-6- 141

596

597 Ongerth, J.E., 2013. The concentration of *Cryptosporidium* and *Giardia* in water--the
598 role and importance of recovery efficiency. Water Res. 47, 2479–2488.
599 http://dx.doi.org/10.1016/j.watres.2013.02.015

600

601 Putignani, L., Menichella, D., 2010. Global distribution, public health and clinical
602 impact of the protozoan pathogen *Cryptosporidium* Interdiscip Perspect Infect Dis pii:
603 753512. http://dx.doi.org/10.1155/2010/753512

604

605 Quílez, J., Sánchez-Acedo, C., del Cacho, E., Clavel, A., Causapé, A.C., 1996.
606 Prevalence of *Cryptosporidium* and *Giardia* infections in cattle in Aragón (northeastern
607 Spain). Vet Parasitol. 66, 139–146. http://dx.doi.org/10.1016/S0304-4017(96)01015-1

608

609 Quílez, J., Torres, E., Chalmers, R.M., Robinson, G., Del Cacho, E., Sánchez-Acedo,
610 C., 2008. *Cryptosporidium* species and subtype analysis from dairy calves in Spain.
611 Parasitology. 135, 1613–1620. http://dx.doi.org/10.1017/S0031182008005088

612

613 RD 140/2003. Royal Decree 140/2003, February 7th, by which health criteria for
614 drinking water quality are stablished. (Real Decreto 140/2003, de 7 de febrero, por el
615 que se establecen los criterios sanitarios de la calidad del agua de consumo humano.
616 BOE no. 45). http://www.boe.es/boe/dias/2003/02/21/pdfs/A07228-07245.pdf.
617 Accessed 20 July 2016

618

619 Rodríguez-Salinas, P.E., Aragon-Pena, A.J., Allue, T.M., Lopaz-Perez, M.A., Jimenez,
620 M.M., Dominguez-Rodriguez, M.J., 2000. Outbreak of cryptosporidiosis in Guadarrama
621 (Autonomous Community of Madrid) [In Spanish]. Rev Esp Salud Publica. 74, 527–
622 536.
623
624 Semenza, J.C., Nichols, G., 2007. Cryptosporidiosis surveillance and water-borne
625 outbreaks in Europe. Euro Surveill. 12, pii: 711
626
627 Soler, P. 2004. Surveillance of cryptosporidiosis in Spain from 1995 to 2003. Euro
628 Surveill. 8, pii: 2402.
629
630 MIS (Microbiological Information System), 2015. Annual Report from System of
631 Microbiological Information 2014. National Centre of Epidemiology. Spain.
632 http://www.isciii.es/ISCIII/es/contenidos/fd-servicios-cientifico-tecnicos/fd-vigilancias-
633 alertas/fd-sistema-informacion-
634 microbiologica/pdf_2015/Informe_anual_2014_20150918.pdf Accessed 20 July 2016
635
636 USEPA (U.S. Enviromental Protection Agency), 2005. Method 1623: *Cryptosporidium*
637 and *Giardia* in Water by Filtration/IMS/FA. Office of Water, U.S. Environmental
638 Protection Agency, Washington, D.C. https://www.epa.gov/sites/production/files/2015-
639 07/documents/epa-1623.pdf. Accessed 20 July 2016.
640
641 WHO (World Health Organization), 2006. Guidelines for Drinking Water Quality
642 *Cryptosporidium*.http://www.who.int/water_sanitation_health/gdwqrevision/cryptodraft
643 2.pdf. Accessed 20 July 2016.

WHO (World Health Organization), 2011. Guidelines for drinking-water quality. Fourth Edition. http://www.who.int/water_sanitation_health/publications/2011/dwq_guidelines/en/. Accessed 20 July 2016.

Xiao, L., Singh, A., Limor, J., Graczyk, T.K., Gradus, S., Lal, A., 2001. Molecular characterization of *Cryptosporidium* oocysts in samples of raw surfacewater and wastewater. Appl. Environ. Microbiol. 67, 1097–1101. http://dx.doi.org/10.1128/AEM.67.3.1097-1101.2001

Xiao, L., Ryan, U.M., 2008. Molecular epidemiology. In: Fayer, R., Xiao, L. (Eds.). *Cryptosporidium* and cryptosporidiosis, 2nd edn. IWA Publishing/CRC Press, Boca Ratón, pp. 119–171.

667 **Figure 1.** Geographic location of drinking water facilities sampled in Aragón (north-
668 eastern Spain). The served population, source of water and type of water treatment for
669 each plant are indicated in Table 1.
670
671
672 **Figure 2.** Number of positive plants for *Cryptosporidium* and *Giardia* in samples from
673 20 drinking water treatment facilities in north-eastern Spain. Samples from the influent
674 and effluent water were collected at each plant in spring, summer, autumn and winter.
675

Table 1
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Table 1. Main features of drinking water treatment plants at the most populated towns in Aragón (north-eastern Spain). Average values and ranges (maximum and minimum) in the turbidity and turbidity removal efficiency are indicated. Values of turbidity are expressed in nephelometric turbidity units (NTU)

Plant[a]	Served population	Source of water	Water treatment	Turbidity (NTU)		Turbidity removal efficiency (%)
				Influent	Effluent	
1	664,953	River	P1A	32 (6–42)	0	100
2	12,141	River, reservoir	P1B	6 (0–12)	0	100
3	7,820	Reservoir	P1B	3 (0–9)	0	100
4	7,014	River, reservoir	P2	5 (0–18)	0	100
5	6,941	Reservoir	P3	6 (0–22)	0.1 (0–0.4)	98.3
6	16,754	Reservoir	P1B	7 (4–9)	0	100
7	19,724	Reservoir	P1A	5 (0–19)	0	100
8	7,680	River	P3	0	0	0
9	10,759	River	P1B	6 (0–11)	0	100
10	9,867	River	P3	0.2 (0–1)	0.4 (0–1)	NR
11	52,239	River	P3	3 (0–6)	0	100
12	13,088	River	P1A	0.1 (0–0.3)	46 (0–186)	-----
13	9,598	Underground river	P3	0	0	0
14	17,020	River	P1B	3 (2–3)	2 (0–7)	33.3
15	17,260	Reservoir	P1B	24 (4–49)	0	100
16	14,921	Reservoir	P2	3 (0–12)	0	100
17	9,439	Reservoir	P2	2 (0–6)	0	100
18	35,590	Groundwater	P1A	0	0	0
19	8,065	Groundwater	P2	0	0	0
20	16,230	River	P1A	4 (0–9)	0	100

P1: conventional water treatment facilities that include coagulation, flocculation, sedimentation, filtration and chlorination processes. Two types of filtration systems were used, including rapid sand filtration (A) or a combination of sand and activated carbon filtering (B)

P2: water treatment facilities that include slow sand filtration and chlorination only

P3: water treatment facilities that include sedimentation and/or chlorination only

Table 2
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Table 2. *Cryptosporidium* oocyst counts [arithmetic mean and ranges (maximum and minimum)] and removal efficiency (removal log) of oocysts in drinking water treatment plants in north-eastern Spain. Samples from influent and effluent water were collected four times from each plant, each sampling time matching with a different season.

Plant	Influent		Effluent		Removal log
	Positive season[a]	Oocysts / 100 litres	Positive season[a]	Oocysts / 100 litres	
1	W, SM	65 (30–101)	–	0	> 1.8
2	W	40	–	0	> 1.6
3	W	40	–	0	> 1.6
4	–	0	–	0	–
5	SM	40	–	0	> 1.6
6	–	0	–	0	–
7	SP	160	–	0	> 2.2
8	–	0	–	0	–
9	W, SP	70 (40–101)	–	0	> 1.8
10	W	81	–	0	> 1.9
11	W	40	–	0	> 1.6
12	–	0	A	166	NR[b]
13	–	0	A	40	NR[b]
14	–	0	–	0	–
15	–	0	–	0	–
16	–	0	–	0	–
17	–	0	SP	60	NR[b]
18	–	0	–	0	–
19	–	0	–	0	–

20	−	0	−	0	−

[a] SP: Spring; SM: summer; A: autumn; W: Winter

[b] NR: No removal: oocyst were only detected in the finished water.

Table 3
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Table 3. *Giardia* cyst counts [arithmetic mean and ranges (maximum and minimum)] and removal efficiency (removal log) of cysts in drinking water treatment plants in north-eastern Spain. Samples from influent and effluent water were collected four times from each plant, each sampling time matching with a different season.

Plant[a]	Influent		Effluent		Removal log
	Positive season[a]	Cysts / 100 litres	Positive season[a]	Cysts / 100 litres	
1	W, SM, SP	94 (41−142)	W	12	0.89
2	−	0	SP	24	NR[b]
3	SM	33	SM	16	0.31
4	−	0	−	0	−
5	W, SP	49 (33−65)	−	0	> 1.69
6	−	0	−	0	−
7	W, SP	915 (53−1769)	−	0	> 2.96
8	−	0	−	0	−
9	W, SM, A, SP	152 (33−407)	SP	148	0.01
10	A, SP	49 (33−65)	A	16	0.48
11	−	0	−	0	−
12	W, SM, A	33	−	0	> 1.51
13	−	0	−	0	−
14	W, SM	33	−	0	> 1.51
15	−	0	−	0	−
16	SM	33	A	16	0.31
17	−	0	SP	49	NR[b]
18	W	24	W, SM, A	33 (16−49)	− 0.13
19	W, SP	49 (33−65)	−	0	> 1.69

20	W, SM	33	SP	16	0.31

[a] SP: Spring; SM: summer; A: autumn; W: Winter

[b] NR: No removal: cysts were only detected in the finished water.

Figure 1

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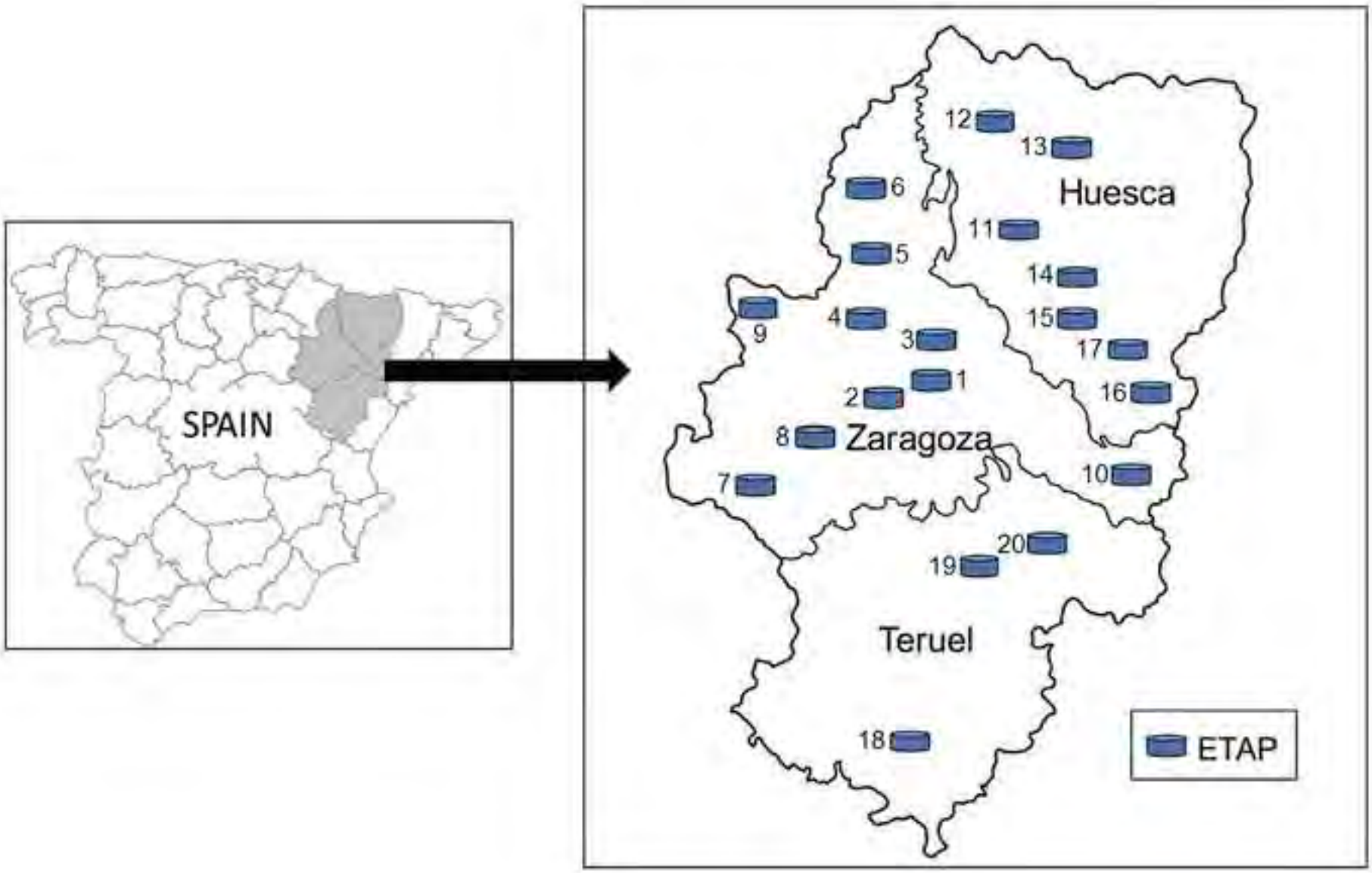

Figure 2

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