Direct and indirect QMRA of infectious *Cryptosporidium* oocysts in reclaimed water

M. Agulló-Barceló, R. Casas-Mangas and F. Lucena

ABSTRACT

Water scarcity leads to an increased use of reclaimed water, which in turn calls for an improvement in water reclamation procedures to ensure adequate quality of the final effluent. The presence of infectious *Cryptosporidium* oocysts (IOO) in reclaimed water is a health hazard for users of this resource. Here, we gathered information on *Cryptosporidium* (concentrations, infectivity and genotype) in order to perform quantitative microbial risk assessment (QMRA). Moreover, data concerning the spores of sulphite-reducing clostridia (SRC) were used to undertake QMRA at a screening level. Our results show that the probability of infection (PI) by *Cryptosporidium* depends on the tertiary treatment type. The mean PI using the exponential dose-response model was 3.69×10^{-6} in tertiary effluents (TE) treated with UV light, whereas it was 3 $\log_{10}$ units higher, 1.89×10^{-3}, in TE not treated with this disinfection method. With the β-Poisson model, the mean PI was 1.56×10^{-4} in UV-treated TE and 2 $\log_{10}$ units higher, 4.37×10^{-2}, in TE not treated with UV. The use of SRC to perform QMRA of *Cryptosporidium* showed higher PI than when using directly IOO data. This observation suggests the former technique is a conservative method of QMRA.

Key words | *Cryptosporidium*, infectivity, QMRA, reclaimed water, sulphite-reducing clostridia spores, UV irradiation

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INTRODUCTION

Water reclamation is a new and sustainable process that provides non-potable water for a variety of uses. Fluctuations in drought periods in the Mediterranean area are common, and Catalonia (northeast Spain) has suffered several periods of water shortage over recent years. The most recent shortage took place between 2007 and 2008 and resulted in the government imposing severe water restrictions. Climate change is expected to increase the frequency and intensity of extreme events, such as drought and floods, as well as to reduce water availability (IPCC 2007; Falloon & Betts 2010). Accordingly, improvements in water management and in water reclamation and reuse will be essential in the near future.

Microbiological criteria for reclaimed water quality in Spanish legislation (RD 1620/2007) are based mainly on the detection of *E. coli* and nematode eggs. In addition, depending on the use made of the water, analyses of *Legionella* spp., *Salmonella*, *Taenia saginata* and *T. solium* are required. Nevertheless, a huge range of pathogens, such as enteric viruses and other pathogenic bacteria or protozoan parasites, can be found in reclaimed water, thus posing a health risk to final users. Alternative indicators, such as bacteriophages and spores of sulphite-reducing clostridia (SRC), have been proposed as suitable candidates for modelling the presence or behaviour of certain pathogens in water (Payment & Franco 1993; Harwood *et al.* 2005; Mandilara *et al.* 2006; Costán-Longares *et al.* 2008).

Cryptosporidium is a ubiquitous protozoan parasite that causes a type of gastroenteritis known as cryptosporidiosis and has produced several waterborne outbreaks of this illness. The most extensive waterborne outbreak caused by *Cryptosporidium* on record was in Milwaukee in 1993 (MacKenzie *et al.* 1994). Since this incident, *Cryptosporidium* has become an important target of water research

([WHO 2009](#)). Given several of its characteristics, such as high infectivity, resistance to chemical disinfection and long survival in the environment, this parasite is now widely considered a reference pathogen. Therefore, efforts have been focused on water treatments designed to decrease *Cryptosporidium* infectivity. As a result, one of the most common strategies for obtaining reclaimed water has been the use of multi-barrier systems (combinations of several physicochemical processes) to achieve adequate quality of the final effluent. Moreover, as *Cryptosporidium* is highly resistant to chlorine, even at very high doses ([Betancourt & Rose 2004](#)), disinfection processes using ozone and UV irradiation have been widely applied in combination with other techniques. UV treatment is highly effective at inactivating protozoa ([Hijnen et al. 2006](#)). The combination of UV treatment with a secondary disinfectant (such as chlorine) has shown synergistic disinfection effects ([Ballester & Malley 2004](#); [Shang et al. 2007](#); [Montemayor et al. 2008](#); [Wang et al. 2011](#)), thereby indicating its utility as a disinfection treatment in reclamation processes.

Quantitative microbial risk assessment (QMRA) allows quantification of the potential risks associated with the presence of pathogens in water. The approach was initially developed to assess drinking water safety; however, it has been widely applied to reclaimed water. Reports on the use of QMRA have increased considerably in the last five years and it has been repeatedly proven that QMRA is a useful tool for identifying potential human health risks associated with the presence of certain pathogens in reclaimed water ([Rose et al. 1991](#); [Asano et al. 1992](#); [Hamilton et al. 2006](#)). Furthermore, indirect QMRA can be performed using the relationship between a given faecal indicator and a pathogen, in contrast to direct QMRA, which uses pathogen concentrations ([Craig et al. 2003](#); [Van Lieverloo et al. 2007](#)). Thus, indirect QMRA may be an advantage when working with water samples with low concentrations of pathogens, such as tertiary effluents (TE).

Here we performed direct QMRA related to the presence of *Cryptosporidium* in TE samples treated with UV light and samples not treated thus. For this purpose we examined the following: (i) *Cryptosporidium* oocyst occurrence; (ii) *Cryptosporidium* inactivation with the different tertiary treatments studied; and (iii) genotype identification

data. Finally, we performed indirect QMRA at a screening level using the relationship between SRC and *Cryptosporidium* in reclaimed water.

MATERIALS AND METHODS

Collection of samples and sampling sites

Samples of raw wastewater (RW), secondary effluent (SE) and TE from seven wastewater treatment plants (WWTPs) were taken in order to: (i) monitor the concentrations of total and infectious oocysts (TOO and IOO) throughout the treatment process; (ii) assess the efficiency of tertiary treatments in inactivating infectious oocysts; (iii) study the relationship between SRC and *Cryptosporidium* oocysts; (iv) identify the genotype of the oocysts; and (v) perform QMRA analyses directly with *Cryptosporidium* data or with SRC concentrations, ratios or reductions.

The WWTPs were located in three provinces of Catalonia (Spain). The population served by these plants varied from 6000 to 1,093,000 inhabitants. All of them used biological processes plus sedimentation as secondary treatment and five of them produced reclaimed water. Tertiary treatments had multiple barrier systems for water reclamation purposes, including: coagulation-flocculation, sand filtration or microfiltration; and combined disinfection steps with UV treatment (dose ranged from 18 to 80 mJ/cm^2) and/or chlorination (1–5 ppm). Reclaimed water from these plants was used for a number of purposes, such as golf course irrigation, environmental restoration (aquifer recharge, maintenance of water environment to prevent sea water intrusion), public garden irrigation and other non-potable urban uses.

Detection of *Cryptosporidium* oocysts and SRC

Total *Cryptosporidium* oocyst concentration and elution

A range of sample volumes were taken depending on the turbidity of the sample. Volumes ranged from 0.05 to 1 L for RW, 0.5 to 3 L for SE and 10 to 100 L for TE. Samples were processed as described by [Montemayor et al. (2005)](#), with minor modifications and following [USEPA guidelines](#)

(2005). Briefly, water samples were filtered through a 160-μm and a 38-μm sieve to remove large particles before concentration. The elution step of RW and SE samples was performed twice to improve the recovery efficiency of the filtration.

Oocysts purification

Oocysts were purified using immunomagnetic separation with a Dynabeads Anti-*Cryptosporidium* kit (Dynal, A.S., Oslo, Norway) and following a modified version of the manufacturer's instructions. Modifications consisted of the following: (i) a double step of bead capture, to improve recovery; (ii) two dissociation steps (Reynolds *et al.* 1999); (iii) two sample washing – centrifugation steps with PBS to ensure pH neutralisation; and (iv) homogenisation of the final volume, which was then divided equally into tubes and stored at 4 °C for further analyses (detection, infectivity assay or genotype identification).

Detection of *Cryptosporidium* oocysts by laser scanning cytometry

Cryptosporidium oocysts were stained with anti-*Cryptosporidium* EasyStain™ antibody following the manufacturer's instructions and examined by laser scanning cytometry, as described in Montemayor *et al.* (2005).

Infectivity assay

Infectivity assays were performed by inoculating an aliquot of the purified oocysts in a HCT8 cell monolayer to detect infectious oocysts, using the focus detection method as described by Slifko *et al.* (1997) but with the following modifications: (i) after bleach pre-treatment (1/10 bleach solution of 4.5% sodium hypochlorite, for 8 min at 4 °C), samples were washed three times with PBS to ensure bleach removal; (ii) well chamber slides (Lab-Tek®II Chamber Slide™ System; Nalge Nunc International, Naperville, IL) were plated with 5×10^4 to 5×10^5 cells per well; (iii) one sample per well was added; and (iv) after incubation, the slide was washed, fixed, rehydrated and labelled with the A600FLR-20X Sporo-Glo antibody and the C101 counterstain reagent (Waterborne™, Inc., New Orleans, LA),

following the manufacturer's instructions. Each slide was mounted and foci were immediately counted under an epifluorescence microscope. The infection foci fluoresced an apple-green colour against a relatively red background (caused by the C101 reagent) of uninfected cells.

Genotype identification

A subsample of purified oocysts was used for DNA extraction with the QIAamp DNA blood mini kit (QIAGEN GmbH, Hilden, Germany), following the manufacturer's instructions. Next, the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) procedure described by Xiao *et al.* (2001) was performed to identify the genotype. When the RFLP profile indicated *C. hominis* genotype, we amplified and sequenced the gp60 gene (Peng *et al.* 2001; Sulaiman *et al.* 2001; Chalmers *et al.* 2005). However, when the profile indicated a different genotype, the small-subunit rRNA gene was sequenced to confirm the RFLP result.

SRC detection

SRC were cultured in SPS agar medium following the procedure described by Bufton (1959) and were then incubated for 20 ± 4 h at 44 °C.

Quantitative microbial risk assessment

QMRA was performed to assess the risk associated with *Cryptosporidium* as a result of accidental ingestion of reclaimed water produced in each reclamation plant. Moreover, a possible failure of UV disinfection was considered. Such a failure would lead to reclaimed water being disinfected only with chlorine rather than with the combination of chlorine and UV irradiation. In addition, a single (one ingestion of reclaimed water) and a cumulative (more than one ingestion) PI were determined. Risk of infection by *Cryptosporidium* was calculated using the exponential model described by Haas *et al.* (1996) and the β-Poisson dose-response model based on data published by Chappell *et al.* (2006) and optimised by Enger (2011). @RISK software version 5.5 (Palisade Corporation) was used to perform QMRA.

Statistical analyses

Statgraphics Plus software version 5.1 (Statistical Graphics Corporation, Herndon, VA, USA) was used for statistical analyses. A *p*-value <0.05 was considered statistically significant. Results under the detection limit were taken as the value of the detection limit except when using the effective volume strategy (Parkhurst & Stern 1998).

RESULTS AND DISCUSSION

Cryptosporidium and SRC concentrations and ratios along the treatment train

Cryptosporidium and SRC concentrations showed a significant reduction from influent wastewater to SE and from SE to TE (see Figure 1).

Cryptosporidium and SRC in RW

Forty RW samples were analysed. Oocysts were found in most of the samples (97.5% positive samples) with a minimum of 1.33 and a maximum of 7,460 oocysts/L. Previously published data on *Cryptosporidium* concentrations in northeast Spain showed higher percentages of positive samples as well as a much narrower range of oocysts/L (Montemayor *et al.* 2005). This variability is probably due to the diversity of the treatment plants studied (size of population served,

Figure 1 | Mean concentrations and 95% confidence intervals of total *Cryptosporidium* oocysts (TOO), infectious oocysts (IOO) and spores of sulphite-reducing clostridia (SRC) expressed in log$_{10}$ units of oocysts/L and CFU/100 mL respectively. RW: raw wastewater; SE: secondary effluent; TE: tertiary effluent.

different areas or sampling seasons). The mean concentration of *Cryptosporidium* TOO per litre was 1.76 log$_{10}$ units, while IOO per litre registered 1.09 log$_{10}$ and SRC 5.37 log$_{10}$ units CFU/100 mL. The mean ratios between the concentrations of SRC and TOO and between SRC and IOO were 3.83 ± 0.53 (SD) and 4.65 ± 0.47 log$_{10}$ units, respectively.

Cryptosporidium and SRC in SE

Seventy-four SE samples were analysed. Oocysts were found in 91.9% of the samples, with values ranging from 0.5 to 496 oocysts/L. Mean concentrations of *Cryptosporidium* in SE were 1.02 log$_{10}$ units of TOO/L and 0.54 log$_{10}$ units of IOO/L. SRC concentrations were 3.81 log$_{10}$ units of CFU/100 mL. With regard to the concentrations of *Cryptosporidium* as well as the percentage of positive samples, our results are similar to those described previously (Harwood *et al.* 2005; Montemayor *et al.* 2005; Costán-Longares *et al.* 2008). The mean ratios between the concentrations of SRC and TOO or SRC and IOO were 2.85 ± 0.61 and 3.44 ± 0.51 log$_{10}$ units respectively.

Cryptosporidium and SRC in TE

Sixty-seven samples of reclaimed water were analysed. The mean concentrations of oocysts as well as the percentage of positive samples were significantly lower in TE than in SE. The percentage of positive samples for TOO was 83.6%, which is higher than other reports in TE samples (Harwood *et al.* 2005; Montemayor *et al.* 2005; Costán-Longares *et al.* 2008). However, the mean concentrations were −0.04 log$_{10}$ units of TOO/L, −1.09 log$_{10}$ units of IOO/L and 2.64 log$_{10}$ units of CFU/100 mL of SRC. The mean ratios between SRC and TOO were the lowest (2.42 ± 0.87 log$_{10}$ units), whereas they were similar but higher between SRC and IOO (3.66 ± 0.75 log$_{10}$ units). This increment in the ratio with IOO suggests that tertiary treatments (probably disinfection processes) are more effective at decreasing oocyst infectivity than SRC viability.
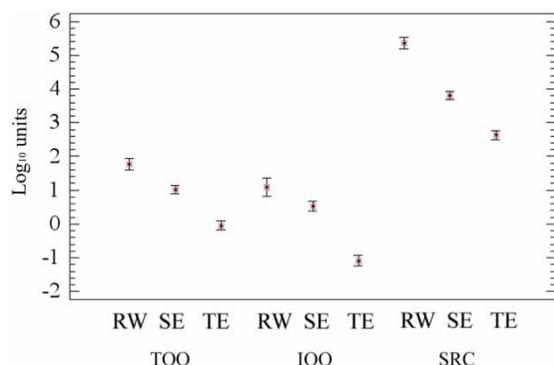
UV treatment: effect on the inactivation of IOO and SRC

Samples of TE were grouped depending on the tertiary treatment, namely with or without UV irradiation.

Concentrations of IOO were significantly higher when tertiary treatment did not include UV light as part of the disinfection process. The mean concentrations of IOO in tertiary treatments with and without UV irradiation were $-1.41 \log_{10}$ units and $-0.38 \log_{10}$ units respectively. The effective volume-averaged concentration of IOO/L was $< -4.32 \log_{10}$ units. In contrast, an effective volume-averaged concentration of $-0.45 \log_{10}$ units of IOO/L was found when tertiary treatment excluded the application of UV light. These values were calculated from:

$$C = \frac{\sum_{i=1}^{n} OO_i}{\sum_{i=1}^{n} V_i} \tag{1}$$

where C is the concentration of *Cryptosporidium* (oocysts/L), n is the number of samples, OO_i is the number of total or infectious oocysts detected in a given sample and V_i is the volume (L) analysed in the given sample.

No IOO were detected out of 7,481 TOO in a total sample volume (V) of 896.8 L from UV-treated samples. On the other hand, in tertiary treatments without UV irradiation, 124 IOO were detected out of 1,088 TOO counted in a total sample volume of 345.6 L (V). Furthermore, the percentage of positive samples was calculated in accordance to:

$$INF = \frac{\sum_{i=1}^{n} IOO_i}{\sum_{i=1}^{n} TOO_i} \times 100 \tag{2}$$

where INF is the infectivity percentage, n is the number of samples, IOO_i is the number of infectious oocysts detected in a given sample and TOO is the number of total oocysts detected in the given sample.

Using Equation (2), TE samples subjected to UV irradiation showed 0.013% of infectivity while those samples not treated with this disinfection method registered 11.4% infectivity. When UV treatment was applied, there was a 5.41-$\log_{10}$ reduction in the IOO from RW to TE. The concentrations of IOO as well as infectivity percentages were $3 \log_{10}$ units higher when UV irradiation was not used in the tertiary treatment. The efficiency of UV irradiation to inactivate *Cryptosporidium* has previously been described (Hijnen *et al.* 2006; Montemayor *et al.* 2008).

Data from two representative treatment plants that apply UV irradiation and from two other representative WWTPs without this disinfection process were selected to compare the reductions of SRC and IOO in the two treatment options (see Figure 2).

Mean $\log_{10}$ reductions of SRC were 1.49 ± 0.71 (SD) $\log_{10}$ units when UV light was included in the tertiary treatment and $0.42 \pm 0.69 \log_{10}$ units without this disinfection regime. IOO mean $\log_{10}$ reductions were $1.73 \pm 0.56 \log_{10}$ units with UV irradiation and $0.57 \pm 0.59 \log_{10}$ units without. There were no significant differences between mean $\log_{10}$ reductions of IOO and SRC in either case (with or without UV irradiation). In contrast, significantly higher reductions in IOO and SRC were detected when UV treatment was used compared to mean reductions without this disinfection procedure. These results indicate that SRC behave in a similar manner to IOO in the tertiary treatments studied here.

Genotype identification of the circulating *Cryptosporidium* oocysts

We analysed the genotypes of the purified oocysts in order to gather epidemiological data on *Cryptosporidium* in northeast Spain. Of the samples, 76% (31/41) were positive by the nested PCR-RFLP procedure and 87% of these showed RFLP profiles that belonged to *C. hominis* (two of them were mixed profiles of *C. hominis* and *C. muris*). Only two samples showed RFLP profiles belonging exclusively to *Cryptosporidium* of animal origin: one sample had a
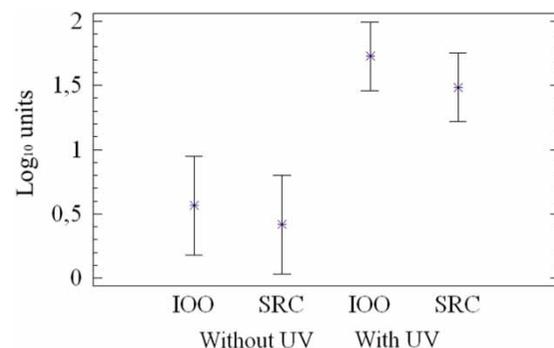

Figure 2 │ Mean reduction and 95% confidence intervals of infectious oocysts (IOO) and spores of sulphite-reducing clostridia (SRC) from secondary to tertiary effluent expressed in $\log_{10}$ units of IOO/L and $\log_{10}$ units of CFU/100 mL respectively.

C. muris profile (infects rodents and some mammals) and the other had a *C. andersoni* profile (infects cattle). The *C. andersoni* profile came from a WWTP receiving wastewater from cattle abattoirs and it was confirmed that the slaughter-house was in operation on the sampling day. The gp60 gene was amplified and sequenced when the resulting RFLP pro-files belonged to *C. hominis*. Family IbA10G2R2 was found in 100% of the gp60-positive samples. However, 19% (5/27) of the samples were repeatedly negative in the PCR for the gp60 gene. Genotype identification revealed that most of the samples were of human origin, thereby indicating that oocysts are continuously shed throughout the study area (ill people plus asymptomatic carriers). This notion is in agreement with the human carriage rates of *Cryptospori-dium* reported in Spain, which can range from 0.4 to 12.8% depending on the population, age range and region (Compañ-Barco *et al.* 1991). Another recent study in the north of Spain has described prevalence of from 1 to 3.1% in children (Cardona *et al.* 2011). That study also reported that *C. hominis* was the only genotype infecting these chil-dren. However, the annual mean number of reported cases of cryptosporidiosis from 2000 to 2010 was 102 (data from the BES, weekly Spanish epidemiological bulletins). This number is consistent with the low prevalence described but contrasts with the high concentrations of *Cryptospori-dium* oocysts detected in RW in Spain. This observation could be attributed to failure to diagnose the illness and is supported by the fact that cryptosporidiosis is not a notifiable disease in Spain. Furthermore, population immu-nity may increase as a result of high rates of endemic *Cryptosporidium* infections (Bonadonna *et al.* 2002). As epi-demiological studies on *Cryptosporidium* in Catalonia have not been published to date, our data should be taken into account for risk assessment purposes; however, more studies are required to further our understanding of water-borne transmission of *Cryptosporidium* in this area.

QMRA analyses

The *Cryptosporidium* data gathered in this study were used to perform QMRA linked to the presence of *Cryptospori-dium* in reclaimed water. The worst-case scenario was selected for the calculation, namely the accidental ingestion of reclaimed water. This scenario was chosen because one of

the treatment plants studied has a distribution network of reclaimed water, which can be used for private purposes, and therefore there is a real risk of accidental ingestion. In addition, direct ingestion of reclaimed water is one of the simplest scenarios for risk assessment since there are fewer assumptions to consider (such as decay rates or time from release to consumption), thus, final results carry fewer uncertainties. Hazard was identified as the presence of IOO, and PI was calculated using *Cryptosporidium* con-centrations in TE and also SRC data in SE and TE.

Use of direct *Cryptosporidium* concentrations

Data were grouped into samples: (i) treated with UV light and (ii) not treated with UV light, with infectivity percen-tages of 0.013 and 11.4% respectively. Concentrations of IOO/L were calculated as follows: the infectivity percentage was applied to TOO/L and new estimated values of IOO/L were obtained. This was selected as the best approach to work with the data because of the great number of values under the detection limit for IOO. We then assigned a prob-ability distribution function (PDF) of IOO/L to each treatment plant. Two other parameters were taken into account to calculate the dose: the recovery efficiency (%) and the volume per ingestion of reclaimed water. For the recovery rate, a normal distribution with parameters mean = 33.6% and SD = 20.2% was selected (Montemayor 2007). For the volume per ingestion of reclaimed water, a triangular distribution with the following parameters was used: most likely = 0.125 L; minimum = 0.010 L, and maximum = 0.2 L. The equation used to calculate the dose was:

$$\text{Dose} = C \times (100 \div R) \times V \qquad (3)$$

where *C* is the PDF of concentration of IOO (IOO/L), *R* is the PDF of recovery percentage and *V* is the PDF of volume (L) per ingestion.

Single and multiple exposures were studied. It was con-sidered that accidental ingestion of reclaimed water in a private-use scenario would be more likely to occur in the summer than in colder seasons. Therefore, the multiple exposure scenario was calculated on the basis of 12 exposures (one intake per week during 12 summer weeks). *C. hominis* was chosen as the only genotype present in

reclaimed water on the basis of our epidemiological results. Furthermore, in the worst-case context, it was assumed that 100% of the population is susceptible to the disease (WHO 2006) and that the ratio of illness to infection is 1. Mean PIs by *Cryptosporidium* and 95% percentiles were calculated for each treatment plant (see Table 1).

The mean PI was 1 to 2 $\log_{10}$ units higher with the β-Poisson dose-response model than with the exponential model regardless of the degree of exposure. When all treatment plants using UV irradiation were pooled, the mean PI was 3.69×10^{-6} (single exposure, exponential dose-response model) while it was approximately 3 $\log_{10}$ units higher (1.89×10^{-3}) without the inclusion of UV light in the tertiary treatment. These results are consistent with the IOO concentrations found in TE, which were 3 $\log_{10}$ units lower in UV-treated effluents. When the β-Poisson model was used, the same difference was observed: mean PI of 4.40×10^{-5} in treatment plants using UV irradiation and 1.78×10^{-2} in those not applying this disinfection procedure. Ryu *et al.* (2007) found similar results with regard to the risk of infection in reclaimed water treated with UV light, or not. In that study, the mean risk of infection was 3 to 4 $\log_{10}$ units higher in treatment plants using only chlorine for disinfection.

Use of SRC data for QMRA analyses

Due to the low densities of TOO and IOO in final effluents, QMRA performed directly with *Cryptosporidium* data in TE samples may carry uncertainties derived from the imprecision inherent to the methodology, namely the use of several steps such as oocyst concentrations, elution, purification and cell line infection. Therefore, QMRA analyses were also performed at a screening level using the following SRC data: concentrations of SRC, ratios between SRC and *Cryptosporidium*, and reductions from SE to TE. Risk assessment performed with SRC was calculated in a single exposure scenario and using the data from the treatment plant with the highest number of TE samples (plant 1, with UV treatment). Using the concentrations of SRC, we applied two approaches:

(A) Using the concentrations of SRC in SE samples; the ratio between SRC and IOO in SE, and the reduction of IOO from SE to TE. The formula used to calculate the estimated IOO concentrations was:

$$\mathrm{Log}_{10}\mathrm{eIOO} = (C_{\mathrm{SRCSE}} - \mathrm{Ra}_{\mathrm{SRC:IOO\ SE}}) - \mathrm{Red}_{\mathrm{SRC\ SE-TE}} \quad (4)$$

Table 1 | Probability of infection by *Cryptosporidium* and 95 percentiles (Perc. 95%) calculated for each treatment plant with two dose-response models

Dose-response model	Exposure	Treatment plants with UV			Treatment plants without UV		
		1	2	3	4	5	1[d]
Exponential[a]	Single						
	Mean PI	1.10×10^{-5}	5.24×10^{-8}	3.54×10^{-8}	9.58×10^{-4}	2.74×10^{-3}	1.97×10^{-3}
	Perc. 95%	2.35×10^{-5}	1.20×10^{-7}	7.13×10^{-8}	2.38×10^{-3}	6.93×10^{-3}	4.30×10^{-3}
	Multiple[c]						
	Mean PI	1.31×10^{-4}	6.29×10^{-7}	4.24×10^{-7}	8.05×10^{-3}	2.63×10^{-2}	1.90×10^{-2}
	Perc. 95%	2.81×10^{-4}	1.44×10^{-6}	8.56×10^{-7}	2.82×10^{-2}	8.01×10^{-2}	5.04×10^{-2}
β-Poisson[b]	Single						
	Mean PI	4.64×10^{-4}	2.42×10^{-6}	1.63×10^{-6}	1.69×10^{-2}	6.40×10^{-2}	5.03×10^{-2}
	Perc. 95%	1.08×10^{-3}	5.53×10^{-6}	3.29×10^{-6}	8.81×10^{-2}	1.90×10^{-1}	1.38×10^{-1}
	Multiple						
	Mean PI	4.51×10^{-3}	2.89×10^{-5}	1.94×10^{-5}	7.69×10^{-2}	2.72×10^{-1}	2.51×10^{-1}
	Perc. 95%	1.26×10^{-2}	6.64×10^{-5}	3.95×10^{-5}	3.80×10^{-1}	5.21×10^{-1}	4.60×10^{-1}

[a]Exponential dose-response model (Haas *et al.* 1996).
[b]β-Poisson dose-response model (Chappell *et al.* 2006; Enger 2011).
[c]12 hazardous events were taken to calculate multiple exposures.
[d]UV irradiation was stopped manually in treatment plant 1.

where eIOO is the estimated IOO concentration, $C_{SRC\ SE}$ is the PDF of concentration of SRC ($\log_{10}$ units of SRC/100 mL) in SE samples, $Ra_{SRC:\ IOO\ SE}$ is the PDF of the ratio between SRC and IOO in SE (in $\log_{10}$ units), and $Red_{SRC\ SE-TE}$ is the PDF of the reduction from SE to TE of SRC (in $\log_{10}$ units).

There were no significant differences between true IOO concentrations in SE and estimated IOO concentrations with the SRC:IOO ratio in SE. However, we detected significant differences when true IOO concentrations in TE were compared to estimated IOO concentrations in TE (using SRC reductions from SE to TE). True IOO concentrations in this effluent were 0.6 $\log_{10}$ units lower than the estimated concentrations.

(B) Using the concentrations of SRC in TE samples and the ratio between SRC and IOO in TE, the formula used to calculate the estimated IOO concentrations was:

$$Log_{10}eIOO = C_{SRCTE} - Ra_{SRC:IOO\ TE} \qquad (5)$$

where C_{SRCTE} is the PDF of concentration of SRC (in $\log_{10}$ units of SRC/100 mL) in TE samples and $Ra_{SRC:\ IOO\ TE}$ is the PDF of the ratio between SRC and IOO in the TE (in $\log_{10}$ units).

There were no significant differences between true IOO concentrations in TE and estimated IOO concentrations with the SRC:IOO ratio in this effluent.

Using SRC data, mean PIs were 1 to 2 $\log_{10}$ units higher than when *Cryptosporidium* was used directly depending on the model and on the method (see Table 2). The PI in treatment plant 1 calculated with approach A would meet the annual acceptable risk of infection of 1×10^{-4} (USEPA 1989; WHO 2006). In contrast, using approach B, the PI would not meet this value. However, in treatment plant 1, no infectious oocysts were detected when UV irradiation was used for disinfection, despite the considerably higher concentrations of TOO (1.3 $\log_{10}$ units of TOO/L). Therefore, these results suggest that the use of indicators to indirectly calculate the risk of infection by pathogens could be a conservative method for QMRA.

Nevertheless, there are a few facts that could limit the applicability of these approaches such as the variability in

Table 2 | Comparison of mean probabilities of infection (PI) and 95 percentiles (Perc. 95%) in treatment plant 1 (with UV irradiation) using SRC (sulphite-reducing clostridia) data for the calculation

Dose-response model	Treatment plant 1 (with UV)		
	Direct[c]	A[d]	B[e]
Exponential[a]			
Mean PI	1.10×10^{-5}	5.50×10^{-4}	5.83×10^{-5}
Perc. 95%	2.35×10^{-5}	2.53×10^{-3}	2.24×10^{-4}
β-Poisson[b]			
Mean PI	4.64×10^{-4}	1.65×10^{-2}	2.53×10^{-3}
Perc. 95%	1.08×10^{-3}	8.70×10^{-2}	1.01×10^{-2}

[a]Exponential dose-response model (Haas *et al.* 1996).
[b]β-Poisson dose-response model (Chappell *et al.* 2006; Enger 2011).
[c]Using *Cryptosporidium* oocysts concentrations.
[d]Using PDFs (probability distribution functions) of SRC concentrations in secondary effluents (SE), PDF of ratios between SRC and infectious oocysts (IOO) in SE, and PDF of reductions of IOO from SE to tertiary effluent (TE).
[e]Using PDF of SRC concentrations in TE samples and PDF of ratios between SRC and IOO in TE.

the reductions of SRC from SE to TE; that their use does not provide any epidemiological data for *Cryptosporidium*, which is an important issue in terms of health risks and the fact that the presence of SRC does not imply the presence of *Cryptosporidium*. Thus, these results justify further research to achieve more reliable risk assessment of *Cryptosporidium* using SRC data and to determine the proper circumstances to apply it.

To sum up, when treatment plants were studied separately, great differences (3 $\log_{10}$ units) in the PI were observed, even when the plants used the same disinfection treatment. This finding is not surprising because they are located in different geographic areas, and *Cryptosporidium* concentrations in influent water as well as in reclaimed water varied significantly from one plant to another (data not shown). Moreover, a theoretical outage of the UV lamps in a treatment plant could have serious consequences regarding the risk of *Cryptosporidium* infection. In fact, risk of infection in plants that do not apply UV treatment would not meet the limit of less than one infection per 10,000 people per year (1×10^{-4}). However, this limit is only a reference value for drinking water, and some authors have considered it overly restrictive (Haas *et al.* 1996). Moreover, all the assumptions for the QMRA made in the present study were highly conservative.

CONCLUSIONS

C. hominis family IbA10G2R2 is the most common circulating genotype in the study area. The WWTPs studied achieved a significant reduction of *Cryptosporidium* oocysts through treatment. In fact, the highest reductions of IOO were obtained when UV light was included in tertiary treatments. Accordingly, the mean PIs associated with *Cryptosporidium* were 3 $\log_{10}$ units lower when UV light was applied for disinfection purposes, regardless of the dose-response model used. Thus, multiple barrier tertiary treatments using UV disinfection, in combination with other processes, can better guarantee reclaimed water quality. Moreover, QMRA analyses performed with SRC data (concentrations, ratios and reductions) resulted in higher mean PIs in a treatment where no infectious oocysts were ever detected. Therefore, the use of indicators in QMRA could be a conservative method for risk calculation.

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