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Cryptosporidium and *Giardia* safety margin increase in leafy green vegetables irrigated with treated wastewater

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ABSTRACT

The presence of *Cryptosporidium* and *Giardia* in waste water is a main concern because water reuse for irrigation can jeopardize human health. Spanish Legislation for water reuse does not oblige to analyze the presence of both pathogens *Cryptosporidium* and *Giardia* in reused water for irrigation. Therefore, the objective of this paper is to determine the influence of wastewater treatment in the increase of the consumer safety margin in relation to the presence of *Cryptosporidium* and *Giardia* in leafy green vegetables. With this aim in mind, a total of 108 samples from raw (influent) and treated wastewater (effluent) from three wastewater treatment plants in Spain were analysed according to USEPA Method 1623. Effluent results show that *Cryptosporidium* oocysts average counts ranged from 1.38 to 2.6/L oocysts and *Giardia* cysts ranged from 0.6 to 1.7/L cysts, which means a removal values of 2.7 log, 2.5 log and 1.8 log for Cryptosporidium oocysts and 1 log, 2 log and 2.2 log for *Giardia* cysts in the three wastewater treatment plants analysed. In relation to safety margin the highest probability that exposure exceed the dose response was observed for *Giardia*. In addition, the sensitivity analysis showed that (oo) cysts concentration present in the leafy green vegetables and the human dose-response were the most influential inputs in the safety margin obtained.

Significance and impact of the study

In this study has been applied a new tool in the field of risk assessment, the consumer safety margin, to measure the distance between the exposure and the dose-response. In this case the safety margin related to the presence of pathogen protozoa as *Cryptosporidium* and *Giardia* in leafy green vegetables when irrigated with contaminated waters has been evaluated. In relation to safety margin the highest probability that exposure exceed the dose response was observed for *Giardia*. In addition, the sensitivity analysis showed that (oo)cysts concentration present in the leafy green vegetables and the human dose-response were the most influential inputs in the safety margin obtained.

1. Introduction

In recent years, the non-potable water reuse for irrigation recreational areas and crops has become an interesting option to mitigate the consequences of an increasing water scarcity (Hachich et al., 2013). However, the existence of residual waterborne protozoan parasites is a potential problem in wastewater reuse, which can suppose a serious threat to human health (Ma et al., 2016; Plutzer and Karanis, 2016).

Giardia and *Cryptosporidium* are some of the most common parasites in wastewater. They have the potential to be transmitted from non-human to human hosts (zoonosis) and *vice versa*, enhancing the reservoir of cysts and oocysts markedly (Smith et al., 2007). CDC (2013) concluded that in developed countries, nearly 2% of adults and 6–8% of children are infected with *Giardia*, while one third of the people in the developing world have had giardiasis. Protozoan cysts including *Giardia* can survive for months in surface water and in soil. Even very small concentrations of virulent and infectious cysts may contribute a detectable outbreak (Plutzer et al., 2010). Furthermore, *Cryptosporidium* is the second most common waterborne pathogen worldwide, with an estimated 30,000 cases of cryptosporidiosis occurring annually in the USA (Yoder et al., 2012), and is identified in 2% of all diarrhoea cases in developed countries compared to a 7% rate in children and 14% in AIDS patients (Chen et al., 2002; Kotloff et al., 2013). All this can be influenced by the higher human *Giardia* prevalence estimated to be 10% in relation to the 3–5% of *Cryptosporidium* (Huang and White, 2006).

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Wastewater plants (WWTPs) usually have different treatment, although in general, all of them have a primary treatment to remove solid material and a secondary treatment to digest organic material, as well as nutrients such as nitrogen and phosphorus. However, more differences exist on the tertiary treatment, where in general consists on a UV treatment combined or not with a sand filtration. This stage in the wastewater plant has the aim of disinfection and to remove pathogenic microorganisms. Therefore, the effectiveness of the plant and the quality of final effluent depends directly on the treatments carried out (Robertson et al., 2000; Rodriguez-Manzano et al., 2012; Taran-Benshohan et al., 2015; Nasser, 2016; Ramo et al., 2017). Consequently, despite the wastewater treatments, effluents can contain pathogenic protozoa (Cacciò et al., 2003; Guy et al., 2003; Sulaiman et al., 2004; Spanakos et al., 2015) and as result, they can be found in food. In this field, European Directives (CEC, 1991), and Spanish Legislation for water reuse (RD, 2007), do not oblige to analyze the presence of both pathogens *Cryptosporidium* and *Giardia* in reused water for irrigation. However, there are a lot of studies, which demonstrate its presence in reused water and food. In Spain, it has been reported that lettuces and cabbages irrigated with contaminated waters presented *Cryptosporidium* oocysts and *Giardia* cysts (Amorós et al., 2010). Studies in Costa Rica and Peru (Monge and Arias, 1996; Ortega et al., 1997) have shown contamination of numerous raw vegetables, including basil, cabbage, celery, cilantro, green onions, leeks, lettuce, and parsley. In Vietnam, *Cryptosporidium* oocysts and *Giardia* cysts were found in water spinach, lettuce and coriander irrigated with sewage contaminated water (Nguyen et al., 2016). In Norway, Robertson and Gjerde (2001) detected *Cryptosporidium* oocysts and *Giardia* cysts in water samples concerned with field irrigation of bean sprouts.

In order to preserve consumer safety, risk assessment has been gradually introduced as a tool to support decision-making processes in food management policies (CAC, 2007). Following the recommendations made by the Codex alimentarius, risk assessment must include the appropriate uncertainty characterization and treatment (CAC, 2013).

In the context of quantitative microbiological risk assessment, food safety margin (FSM) was introduced by Doménech and Martorell (2016) as a new risk characterization metric, which at the same time, is able to address the effect of uncertainties. Therefore, the objective of this paper is to determine the influence of wastewater treatment plants in the increase of the consumer safety margin in relation to the presence of *Cryptosporidium* and *Giardia* in leafy green vegetables. With this aim in mind, the whole pathway from the wastewater until the fresh produce was studied. Thus, the leafy green vegetables contaminated by both parasites, as a consequence of its irrigation with treated and non-treated water, constituted the consumer exposure, which moreover was compared with the dose-response.

2. Material and methods

2.1. Sampling sites

Three urban wastewater treatment plants ($WWTP_1$, $WWTP_2$ and $WWTP_3$) located in Eastern Spain with different features and treatments (Table 1) have been monitored. A total of 108 samples from raw (influent) and treated wastewater (effluent) samples were collected from the three WWTPs: $WWTP_1$, $WWTP_2$, and $WWTP_3$ during 18 months. One liter, of influent samples, was collected in all the WWTPs studied. Effluent samples (10 L) were collected after UV disinfection in $WWTP_1$, $WWTP_2$ and $WWTP_3$, although in $WWTP_1$ was a previous sand filtration.

Pathway from wastewater treatment plants until human consumption has been represented in Fig. 1. In all the plants, ($WWTP_1$, $WWTP_2$ and $WWTP_3$), the first step of water treatment is the screening to remove large objects followed by the secondary treatment (Table 1). However, only $WWTP_1$ includes a tertiary treatment with sand filtration. All the studied plants have a final disinfection using UV lamps. The average UV dose in the WWTPs studied was 101.69 mj/cm^2

2.2. Detection of Giardia and Cryptosporidium

Secondary and tertiary wastewater effluent samples (10 L) were filtered through an Envirocheck HV filter (Pall Gelman Laboratory, Ann Arbor, MI, USA). Following the procedures described in Method 1623 of the U.S. Environmental Protection Agency (U.S. EPA, 2005). Trapped material on the filter was eluted in 250 mL eluate solution, and the eluate was centrifuged at 1500 × *g* for 15 min to concentrate oocysts and cysts (oo)cysts, and the pellet was resuspended in 10 mL of distilled water in a Leighton tube.

One liter of influent raw wastewater was concentrated by centrifugation at 2400 × *g* for 15 min and the pellet was resuspended in Leighton tubes. Immunomagnetic separation (IMS) was conducted using the commercially available Dynabeads GC-Combo kit (Life Technologies AS, Oslo, Norway) according to the manufacturer's instructions. The final concentrate from the IMS was dried overnight at room temperature and labeled with fluorescent monoclonal antibody for *Giardia* and *Cryptosporidium* immunofluorescence assay (IFA) according to the manufacturer's protocol (REAL®, Durviz Valencia, Spain). The internal structures of (oo)cysts were confirmed as follows. Fifty microliters of (4,6-diamidino-2-phenylindole dihydrochloride) DAPI (Sigma Aldrich, St. Louis, MO) solution (0.4 mg/ml in PBS) was placed in each well and allowed to stand at room temperature for 15 min; excess DAPI solution was removed by washing the slides twice in PBS. Slides were placed in the dark, mounted with mounting medium, and examined at 600× magnification using epifluorescence microscopy (Olympus BX 50, Tokyo, Japan). A blue filter (excitation, 480 nm; emission, 520 nm) was used to detect fluorescein isothiocyanate–conjugated MAb −labeled (oo) cysts.

The removal efficiency of (oo)cysts within each plant was calculated as follows:

Log removal = Log influent concentration − Log effluent concentration

2.3. Safety margin

2.3.1. Exposure assessment

Exposure assessment (E) for a microbiological hazard (H) is defined as the qualitative and/or quantitative evaluation of the likely intake of biological agents via food as well as exposures from other sources if relevant (FAO/WHO, 2006). In order to take into account the random uncertainty of input data, a quantitative evaluation was chosen (Doménech et al., 2007; WHO, 2004). Then, a standard Monte Carlo method was used to propagate the uncertainty due to variability from input parameters (parasite concentration and serving size consumed) to

Table 1
Main features of the studied wastewater treatment plants (WWTP).

Wastewater plant	Population served	Primary treatment	Secondary treatment	Tertiary treatment	Disinfection
$WWTP_1$	247297 inhabitants	Rake, screening, grit removal, sedimentation	Activated sludge, sedimentation	Sand filtration	UV
$WWTP_2$	164171 inhabitants	Rake, screening, grit removal, sedimentation	Activated sludge, sedimentation	None	UV
$WWTP_3$	40333 inhabitants	Rake, screening, grit removal, sedimentation	Activated sludge, sedimentation	None	UV

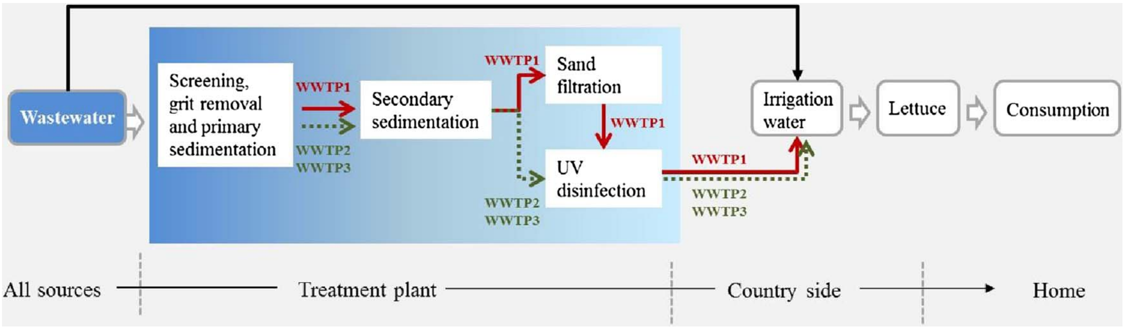

Fig. 1. Wastewater pathway from treatment plant until food consumption.

the output E(H) using Eq. (1).

$$E(H) = C_H * S \tag{1}$$

Where C_H is the probability distribution function (*pdf*) concentration for each hazard (H), herein oocyst/L and cyst/L for *Cryptosporidium* (C_C) and *Giardia* (C_G), respectively present in green leaves watered with reclaimed water. S is the serving size of leafy green vegetables consumed in grams.

In order to assess the exposure, two possible irrigation water were considered: a) before wastewater treatment plant (influent) and b) after (effluent). With this aim, the obtained values from our study have been fitted to a *pdf* using @Risk 5.7 software (Palisade, Middlesex UK). In addition, to obtain *pdf* more realistic, the highest concentration of protozoan parasites were truncated by up to two times their concentrations. Moreover, we adopted the same considerations made by Mota et al. (2009), where the volume of irrigation water retained on lettuce (i.e. rough produce) was 0.108 mL/g and all the (oo)cyst detected in the irrigation water were transferred to the product for a worst-case approach, Table 2. Furthermore, we took the serving size reported by Carrasco et al., 2010.

2.3.2. Dose-response

Dose-response quantifies the relationship between the amount of exposure to a microorganism and the effect that it can produce (FAO/WHO, 2009). To obtain the dose-response model, for both pathogens, the inverse transform sampling was used (Devroye 1986a,b). This methodology permits to generate sample numbers at random from any probability distribution given its cumulative distribution function. In this paper, an exponential dose response model was used for both pathogens, Eq 2, as it is one of the most referenced (Mota et al., 2009; Pujol et al., 2009; An et al., 2012; Xiao et al., 2013; Sato et al., 2013; Razzolini et al., 2016).

$$R(H) = (-1/r_H * LN(1 - Uniform(0;1))) \tag{2}$$

where R(H) is the dose-response for a microorganism (H) and r_H is the infectivity parameter for that organism-specified; herein, *Cryptosporidium* (r_C) and *Giardia* (r_G). Table 2 provides the infectivity parameters for both parasites considered, in this study, to assess the dose response. These data are based on WHO recommendation (Medema et al., 2009) considering that they are more appropriate for developing countries (Razzolini et al., 2016).

2.3.3. Formulation

The formulation of these metrics is inspired by the FSM developed for microbiological hazards (Doménech and Martorell, 2016), where the margin is defined as the probability of a load, E(H) exceeding the dose-response, R(H). Then, the FSM can be formulated in its classical form, as the Euclidean distance between E(H) and R(H), Eq. (3). Where the distance is divided by R(H) in order to obtain a value of c_FSM(H), which always ranges between zero and one. Thus, a value of this metric close to one would indicate a wide margin, which means that to have consequences for health is very unlikely. On the other hand, a margin close to zero would imply a narrow margin with a high possibility of adverse effects.

$$c\_FSM(Hi) = \begin{cases} 1 - \frac{E(H)}{R(H)} & if E(H) \leq R(H) \\ 0 & if E(H) \leq R(H) \end{cases} \tag{3}$$

Assessment of c_FSM(H) by Eq. (3) is carried out by a standard Monte Carlo method to propagate the variability from the inputs E(H) and D_i to the output, which yields a *pdf* of the margin for each parasite. Therefore, single statistics of the c_FSM(H) can be obtained, for example the mean value, percentile 5% or percentile 95%.

Alternatively, the FSM can be formulated in its probabilistic form (p_FSM), Eq (4).

$$pFSM = 1 - \Pr\{E(H) - R(H) > 0\} = 1 - \int_0^{\infty} [\int_H^{\infty} [f_E(y)dy] f_R(H)dH] \\ = 1 - EP(H) \tag{4}$$

where $f_E(y)$ and f_R (H) are the probability density function of the exposure and dose-response, respectively. EP (Exceedance Probability) represents the probability that a dose of a microorganism presents in a food, herein *Cryptosporidium* and *Giardia* in green leaves, exceed the infective dose. With this aim, again Monte Carlo was performed providing 100000 iterations and 100 simulations. The value obtained with

Table 2
Distribution data of leafy green vegetables concentration of each parasite, its infectivity, and the green leaves serving size.

Parameter	Description	Value	Source
C_C influent	*Cryptosporidium* concentration (oocyst/g)	Invgauss(257.6;16.103;RiskTruncate(0;7200)	This study
C_C effluent	*Cryptosporidium* concentration (oocyst/g)	Expon(3.7707; RiskTruncate(0;80)	This study
C_G influent	*Giardia* concentration (cyst/g)	RiskExpon(670.14;RiskTruncate(0;4200)	This study
C_G effluent	*Giardia* concentration (cyst/g)	Expon(2.4927;RiskTruncate(0;27)	This study
Wr	Irrigation water retained (mL/g)	0.108	Mota et al. (2009)
Tr	Transfer rate (%)	100	Mota et al. (2009)
S	Serving size (g)	Cumulative(25; 200; {28;55;123}; {0.5;0.75;0.95})	Carrasco et al. (2010)
r_C	Infectivity parameter for *Cryptosporidium*	Triang(0,0022;0,00419;0,00852)	Razzolini et al. (2016)
r_G	Infectivity parameter for *Giardia*	Triang(0,0098;0,0198;0,036)	Razzolini et al. (2016)

this metric also lies between zero and one.

2.3.4. Sensitivity and uncertainty analyses

The sensitivity and uncertainty analyses (SA and UA) aim to elucidate the dependency of the output, e.g. c_FSM(H) on the set of input parameters in Eqs. (1)–(3). In particular, a tornado graph was made in order to assess how the uncertainty of output depends on the uncertainty of inputs, i.e. to determine the relationship between the variability of the model inputs and outputs, (Martorell et al., 2014).

2.4. Statistical analyses

Descriptive analyses of the data were undertaken using Statgraphics Centurion XVI.II (Statpoint Technologies, Inc. Warrenton, Virginia). Relative proportions were compared using an analysis of variance (ANOVA). A probability value of less than 5% was deemed significant. The same program was used to carry out the box and whisker descriptive plot. This graph is a standardized way of displaying the distribution of data and it shows minimum, first quartile, median, third quartile, maximum value and possible outliers.

3. Results and discussion

3.1. (Oo)cysts counts in raw sewage

Average counts of *Cryptosporidium* oocysts found in WWTP influents ranged from 24.25 to 332/L oocysts and a 33.3% of all samples (n = 54) were negative (no oocysts detected by IMS-IFA). The highest counts of *Cryptosporidium* oocysts in influents, 580/L oocysts, were obtained in WWTP$_3$ (Fig. 2) despite this is the treatment plant serving the less populated town, but receives sewage from farms and agriculture, while sewage entering to WWTP$_1$ and WWTP$_2$ are from urban and industrial origin. Some outlier counts of 1820 oocysts and 3660/L oocysts were also observed in WWTP$_3$ and WWTP$_1$ respectively. The differences within the oocysts counts obtained in the three influents were statically significant (p-value 0.0000).

Giardia cysts average counts ranged from 100 to 806/L in the raw sewage of studied WWTPs, and the highest count, 2110/L cysts, was obtained in WWTP$_1$, although this data was considered an outlier count (Fig. 2). Some negative samplings occurred in WWTP$_1$ (22.2%) and WWTP$_3$ (11.1%). Significant differences (p-value 0.0293) were observed in cysts counts between the WWTP influents. The ingestion of as few as 10 cysts or 30 oocysts can cause giardiasis and cryptosporidiosis, respectively in humans (Adam, 1991; DuPont et al., 1995). Similar results of (oo)cysts counts in WWTPs have been reported by Ramo et al. (2017) in a study in north-eastern Spain where high counts of *Cryptosporidiun* were found in plants which receives water from agriculture and slaughter houses and the high counts of *Giardia* were obtained in the treatment plants serving the most populated towns. Robertson et al. (2000) detected significantly more *Giardia* cysts than *Cryptosporidium* oocysts in sewage influents of seven WWTP in Scotland. Ben-Ayed et al. (2010) also found more *Giardia* cysts than *Cryptosporidium* oocysts in sewage influents of WWTP in Tunisia. The concentration of *Giardia* cysts and *Cryptsoporidium* oocysts detected in sewage influent will be affected by the numbers of contributors (i.e. number of infected humans and animals in the community served by the WWTP), intensity of infection and dilution by other waste discharging to the WWTPs (Robertson et al., 2000).

3.2. (Oo)cysts counts in effluents

In the studied effluents, *Cryptosporidium* oocysts average counts ranged from 1.38 to 2.6/L oocysts and the highest count (11/L oocysts) was obtained in WWTP$_1$ (Fig. 3). Negative samplings (no oocysts detected by IMS-IFA) occurred in WWTP$_1$ (27.8%), WWTP$_2$ (38.9%) and WWTP$_3$ (22.2%). No significant differences between the oocysts values between the three WWTPs were found.

Cysts average in effluents ranged from 0.6 to 1.7/L cysts with a maximum of 6/L cysts and a minimum of 0 cysts. The highest levels of *Giardia* cysts, were obtained in WWTP$_1$ and WWTP2$_2$ (Fig. 3) which

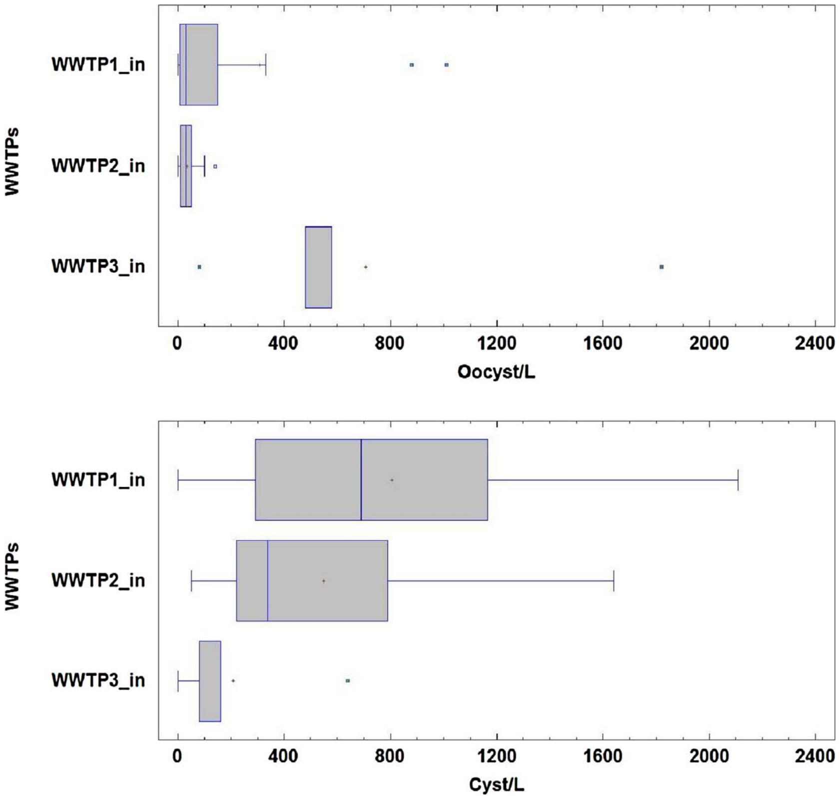

Fig. 2. *Cryptosporidium* oocyst and *Giardia* cyst concentrations in wastewater influents.

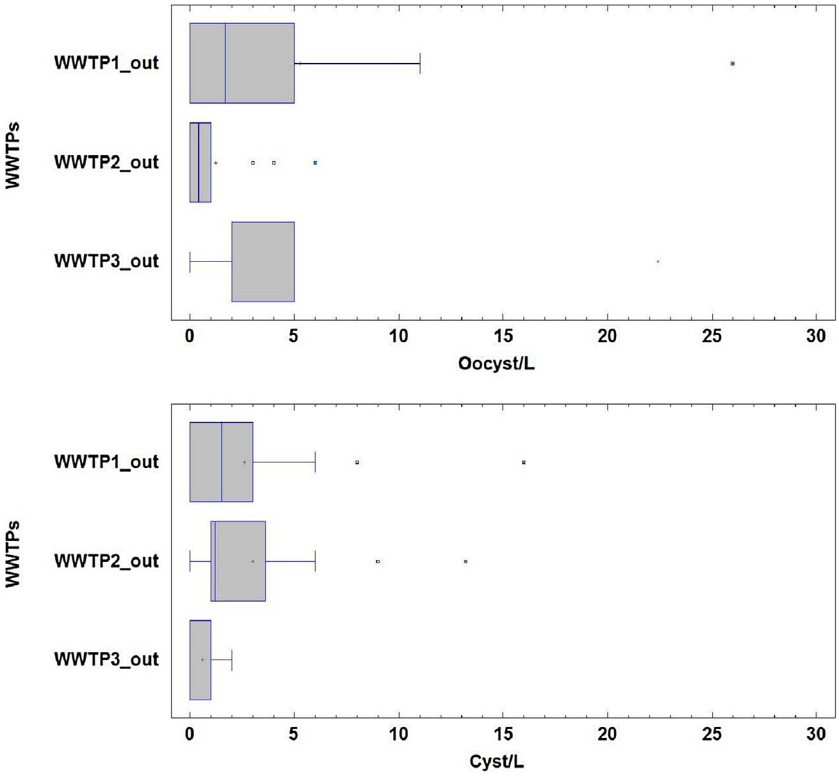

Fig. 3. *Cryptosporidium* oocyst and *Giardia* cyst concentrations in wastewater effluents.

present the highest population served. Outliers counts were observed in all the WWTPs (Fig. 3). Negative samplings (no cysts detected by IMS-IFA) occurred in WWTP$_1$ (27.8%,), WWTP$_2$ (22.2%) and WWTP$_3$ (61.1%). These results agree with other authors (Castro-Hermida et al., 2015; Rodriguez-Alvarez et al., 2015) who concluded that a water considered safe from the treatment process still contains a level of gastrointestinal pathogens enough to threat human health.

In the monitored WWTPs a reduction in the levels of pathogens in treated effluents has been achieved, through the different treatment processes. Highest reduction was observed in *Giardia* cysts (removal average 2.3 log) showing removal values of 2.7 log, 2.5 log and 1.8 log for WWTP$_1$, WWTP$_2$ and WWTP$_3$, respectively and less reduction (removal average 1.5 log) was achieved in *Cryptosporidium* oocysts with removal values of 1 log, 2 log and 2.2 log in WWTP$_1$, WWTP$_2$ and WWTP$_3$, respectively. Similar results were obtained in other studies (Rodriguez-Manzano et al., 2012) in two WWTPs in Spain, showing removal values of 2.33 log10 and 2.27 log10 in WWTP1 and 2.98 log10 and 1.75 log10 in WWTP2, for *Giardia* and *Cryptosporidium*, respectively. According to other authors (Taran-Benshohan et al., 2015) the highest removal of *Giardia* cysts by activated sludge can probably due to its greater sedimentation velocity compared to *Cryptosporidium* oocysts, moreover, the attachment of *Giardia* cysts to sewage particles may also enhance its removal efficiency in the activated sludge process.

Ramo et al. (2017) also found that *Giardia* cysts were removed more efficiently than *Cryptosporidium* oocysts and high removal values were found for *Giardia* by secondary treatment, while tertiary treatment was needed to achieve the greatest removal of *Cryptosporidium.* In our study, in contrast, the lowest *Cryptosporidium* removal was observed in WWTP1 the only plant that includes a tertiary treatment. Fu et al. (2010) found a 0.92 log removal (for *Cryptosporidium* oocysts) and 0.89 log removal (for *Giardia* cysts) after sand filtration tertiary treatment although other authors (Taran-Benshohan et al., 2015) found that the tertiary phase of treatment which consisted of sand filtration has been found inefficient for the removal of *Cryptosporidium* and *Giardia.* Data from Robertson et al. (2000) indicate that primary settlement has a mean removal efficiency of 41 ± 30% for *Giardia* cysts, and a mean removal efficiency of 40 ± 52% for *Cryptosporidium* oocysts. Ma et al. (2016) detected *Cryptosporidium oocysts* and *Giardia* cysts in a WWTP after activated sludge and UV effluent by IMS-IFA. *Giardia* counts ranged between 0.87–26.27 cysts/L while *Cryptosporidium* counts were lower, from 0 to 0.47 oocysts/L (Ma et al., 2016). Removal efficiency of *Giardia* cysts in differently operated WWTPs after activated sludge, secondary clarification and UV disinfection showed a 1.96–2.3 Log reduction (Neto et al., 2006; Khouja et al., 2010); 2.24–3.02 log reduction after activated sludge and sand filtration (Kistemann et al., 2008; Fu et al., 2010).

All the effluents from the studied WWTPs are reused for agriculture irrigation purposes. The wastewater treatment applied must be planned depending on the further use of the effluents and the public health risk involved (Taran-Benshohan et al., 2015). According to other authors (Plutzer et al., 2010; Taran-Benshohan et al., 2015; Spanakos et al., 2015), we also suggest a multiple-barrier approach (sedimentation, biological treatment, filtration and disinfection) to be applied to wastewater when reclaimed wastewater effluents are used for unrestricted irrigation of food crops.

3.3. Season influence in (oo)cysts counts

Average of highest levels of *Cryptosporidium* oocysts were found during spring in the raw influents (978 ± 1433/L) and in the treated effluents (13 ± 16.6/L) (Fig. 4). Differences between seasons were statistically significant in both influents (p-value 0.0002) and treated effluents (p-value 0.0446) showing a potential risk associated to the presence of *Cryptosporidium* oocysts in irrigation water depending on the season of the year.

Giardia cysts highest counts average in raw sewage were obtained in summer (980 ± 1160/L) and in treated effluents in winter (3.9 ± 4.5/L) but the differences between seasons were not significant neither in the influents (p-value 0.3566) nor the effluents (p-value 0.220). The potential risk derived of the presence of *Giardia* cysts can occur during all the seasons.

The seasonal distribution of *Cryptosporidium* in wastewater has been

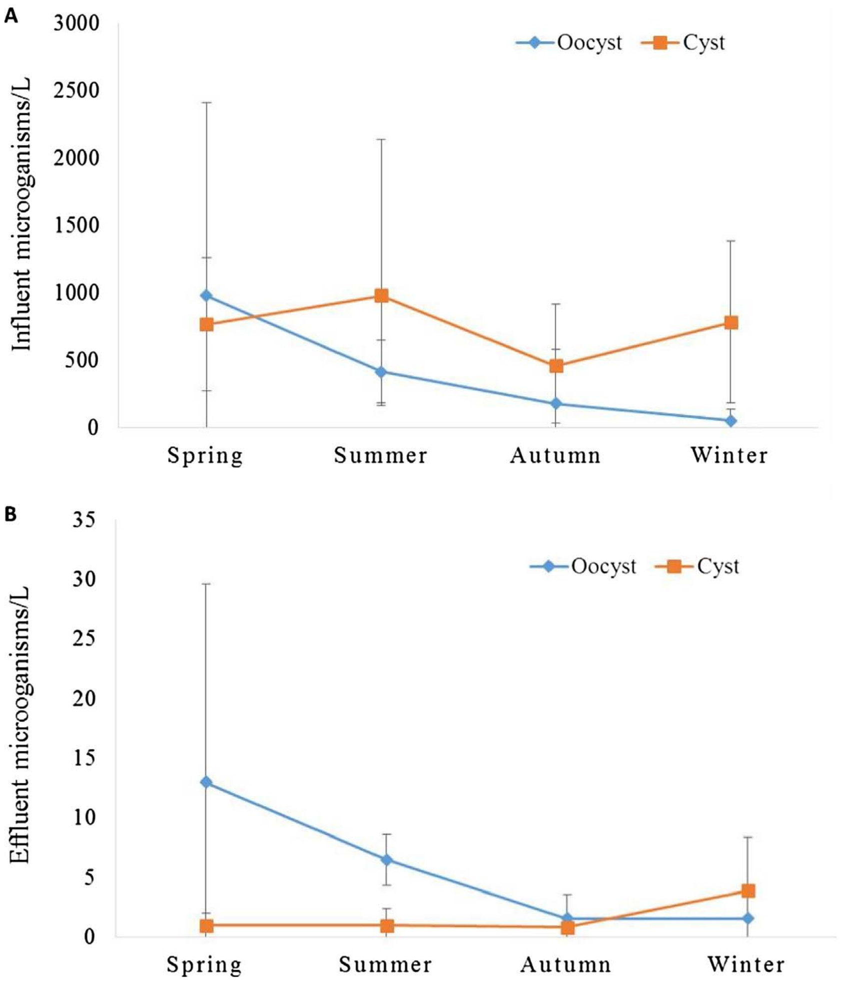

Fig. 4. Season prevalence of *Cryptosporidium* oocyst (O) and *Giardia* cyst (C) expressed as (oo)cysts/L. Where 4a influent and 4b effluent.

examined in various parts of the world. Several studies have investigated the seasonality of *Cryptosporidium* with diverse results, so it has not yet been defined if seasonality is a general feature of the contamination by *Cryptosporidium* spp. (Galván et al., 2014). In a meta-analysis on the seasonality of cryptosporidiosis, which was based on 61 published studies, increases in temperature and precipitation were associated with an increase in the incidence of cryptosporidiosis (Jagai et al., 2009). In temperate climates, the incidence of cryptosporidiosis peaked with an increase in temperature (Cacciò and Putignani, 2014). Ajonina et al. (2012) showed that oocysts of *Cryptosporidium* are predominant during autumn and winter in Germany. A study conducted in Italy has shown that the oocysts of *Cryptosporidium* were detected during the spring in raw wastewater (Caccio et al., 2005). Robertson et al. (2006) reported no pattern of seasonality in the occurrence of *Cryptosporidium* in Norway. However King et al. (2017) in a study in five WWTPs in Australia observed a direct relationship between the greatest *Cryptosporidium* oocysts densities and highest notification rates and the removals across the plants investigated were highly seasonal since the greatest oocysts challenges in these regions are more likely to occur in the summer/autumn months when demand for reuse is highest.

Different seasonal behavioural has also been described in Spain, with high frequencies detected during spring and autumn in raw and treated sewage and river water in north-eastern Spain (Montemayor et al., 2005); spring and summer in influent and effluent wastewater samples from Galicia (Castro-Hermida et al., 2008) and winter and summer in wastewater from the central area of Spain (Galván et al., 2014). Ramo et al. (2017) in north-eastern Spain reported that *Cryptosporidium* oocysts in influents peaked in summer while in effluents *Cryptosporidium* counts were significantly reduced ($P < 0.005$) in all the seasons.

In the present study, the highest counts of *Giardia* in the raw influent have been obtained in summer and in the final effluents in winter. Other authors have reported the highest prevalence of *Giardia* cysts in raw wastewater in the summer months while the lowest concentrations of *Giardia* cysts were observed in winter months (Taran-Benshohan et al., 2015). In the study in North Eastern Spain above described (Ramo et al., 2017) *Giardia* cysts presented a significant reduction ($p < 0.001$) after water treatment during all the seasons.

As observed in several studies the seasonality of *Cryptosporidium* and *Giardia* in wastewaters presents different patterns depending on the special country characteristics as weather, water temperatures linked to the epidemiological situation.

3.4. Consumer safety margin

The mean, 5th and 95th percentile for the classical FSM of exposure to *Cryptosporidium* oocyst and *Giardia* cyst in leafy green vegetables, corresponding to the *pdf* propagated by Monte Carlo method, for both irrigation water (influent, effluent) is showed in Table 3. In general, for both types of irrigation water, a higher mean margin can be observed for the first parasite, being this close to one. Focusing on influent water as irrigation water, the distance between the 5th and 95th percentiles also indicates a low uncertainty in the safety margin for oocyst however, no margin can be observed for *Giardia* at 5th. In relation to exceedance of probability (EP), findings show a low probability (0.005) to intake a leafy green vegetable contaminated with *Cryptosporidium* in a concentration high enough to be able to produce infection and

Table 3
Classic safety margin (c_FSM) and exceedance probability (EP) for *Cryptosporidium* and *Giardia* in leafy green vegetables. Mean, 5th and 95th percentiles.

Safety margin	5th	Mean	95th
Influent wastewater plant			
c_FSM *Cryptosporidium*	0.922	0.979	1
c_FSM *Giardia*	0	0.811	0.997
EP *Cryptosporidium*		0.005 ± 0.0006	
EP *Giardia*		0.069 ± 0.0017	
Effluent wastewater plant			
c_FSM *Cryptosporidium*	0.998	0.999	1
c_FSM *Giardia*	0.995	0.998	1
EP *Cryptosporidium*		0.0001 ± 0.00008	
EP *Giardia*		0.0003 ± 0.0001	

consequently to get sick. On the other hand, the probability for *Giardia* is around a 0.07. Moreover, when the effluent of the wastewater treatment plant is used as irrigation water, the safety margin for both microorganisms is close to one and very low uncertainty can be observed. In relation to exceedance of probability in both cases is also very low (*Cryptosporidium* 0.0001 and *Giardia* 0.0003).

Despite, there are not any other research papers with the estimation of safety margin in wastewater reuse, results can be compared with outbreaks data and risk studies. Sato *et al.* (2013), assessed the risk for drinking contaminated water. After to collect 206 samples throughout the 24 months from 28 locations concluded that the risk for *Giardia* was higher than for *Cryptosporidium* (0.29–2.47 and 0.15–0.29) in adults respectively, and in the same way for children (0.08–0.70 and 0.04–0.08). Also in water, the annual probability of infection in adults for *Giardia* was 0.07 and for *Cryptosporidium* 0.004 (Razzolini et al., 2016). Similar results were found for Mota et al. (2009) who found that the yearly risk *Cryptosporidium* infection associated with lettuce was 0.000755 and with regard to *Giardia* was 0.0109. Nevertheless, the assessed safety margin may also be overestimated since all (oo)cysts were considered viable and one hundred per cent of contamination in the retained water from the irrigation was considered transferred to the product. Therefore, to reduce the uncertainty further studies must be carried out.

A sensitivity analysis was performed to study the effects of the variation of input parameters on the final output, c_FSM for each parasite. In both cases, the parameters with the highest relative effects were considered the most sensitive input parameters. Results showed that, the most sensitive inputs were the (oo)cyst concentration present in the leafy green vegetables and the human dose-response. The rest of the parameters such as serving size and the specific infectivity parameter showed less influence. Similar results were found by Razzolini et al. (2016), who concluded that the concentration had the highest influence on the risk variability.

4. Conclusion

The traditional way to assess risk considering only mean values has de advantage of simplicity but does not allow the quantification of the margin in terms of the probability. The formulation introduced in this paper in order to consider the full probability distribution function of exposure and dose-response has permitted to quantify safety margin taking into account the uncertainties and estimate the probability that consumer exposure exceed the infective dose and causes health damage.

Wastewater treatments allows an important increase in the consumer safety margin since in treated effluents a high reduction in the (oo)cysts counts, compared with (oo)cysts counts in raw sewage, has been observed. However, deficiencies in the process performance, can reduce the treatment plant efficiency and occasionally high counts of (oo)cysts (outlier counts) can be detected in reused water. These results emphasise the importance of including both pathogens, *Cryptosporidium* and *Giardia*, in standard regulations for wastewater reclamation.

Conflict of interest

No conflict of interest declared.

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