

Waterborne transmission of *Giardia* and *Cryptosporidium* at river beaches in Southern Europe (Portugal)

Cláudia Júlio, Cátia Sá, Idalina Ferreira, Susana Martins, Mónica Oleastro, Helena Ângelo, José Guerreiro and Rogério Tenreiro

ABSTRACT

Giardia and *Cryptosporidium* are the most frequent enteric protozoa causing gastroenteritis in humans worldwide. Intense recreational activity at Portuguese river beaches triggered the opportunity for a 2-year seasonal survey of 19 large river basin beaches. A total of 74 samples were collected and processed according to USEPA Method 1623 to detect *Cryptosporidium* and *Giardia* (oo)cysts. Faecal indicators (thermotolerant/total coliforms, *Escherichia coli*, and enterococci) and physicochemical parameters were also analysed according to the EU Bath Water Directive (BWD). Results pointed to a widespread presence of these protozoa at Portuguese river beaches. The percentage of samples testing positive for *Giardia* and *Cryptosporidium* were 85 and 82% respectively, with no significant differences between wet and dry seasons ($p > 0.05$). Although Portuguese river beaches present a very low exposure risk for infection with *Giardia* and *Cryptosporidium* (under 10^{-3}), a few particular cases revealed values over 0.2%, and were related to stormy wet events. The correlation between levels of *Giardia* and thermotolerant coliforms, *E. coli* and enterococci, was high ($r \geq 0.87$, $p < 0.001$), suggesting the need to carry out specific procedures for the detection of *Giardia* and *Cryptosporidium* whenever the values of those faecal indicators approach the maximum allowed level of the EU BWD.

Key words | *Cryptosporidium* spp., *Giardia* spp., Portugal, risk assessment, river beaches, waterborne transmission

Cláudia Júlio (corresponding author)

Idalina Ferreira

Susana Martins

Mónica Oleastro

Helena Ângelo

National Institute of Health Dr. Ricardo Jorge,
Department of Infectious Diseases,
National Reference Laboratory for Gastrointestinal
Infections,

Av. Padre Cruz 1649-016 Lisbon,
Portugal

E-mail: claudia.julio@insa.min-saude.pt

Cátia Sá

Rogério Tenreiro

Universidade de Lisboa,
Faculdade de Ciências,
Centro de Biodiversidade,
Genómica Integrativa e Funcional (BioFIG),
Edifício ICAT, Campus da FCUL,
Lisbon,
Portugal

José Guerreiro

Universidade de Lisboa,
Faculdade de Ciências,
Centro de Oceanografia,
Lisbon,
Portugal

INTRODUCTION

Giardia and *Cryptosporidium* are the most frequent worldwide enteric protozoa causing gastroenteritis in humans and are among one of the ten major human parasites (Thompson *et al.* 2000; Smith *et al.* 2007; Sunderland *et al.* 2007). *G. duodenalis* is the most frequent protozoan agent of intestinal disease worldwide causing an estimated 2.8×10^8 cases per year (Lane & Lloyd 2002). The annual incidence for *Cryptosporidium* in 2000 was estimated at 1.17 per 100,000 people (Groseclose *et al.* 2002).

The severity of the infection caused by these protozoa has particular consequences in some groups, such as young children (Yoder & Beach 2010) and immunocompromised people, including a high probability of mortality (Rose 1997; Smith & Ahmad 1997).

The (oo)cysts are transmitted by the faecal-oral route through contaminated food or water and are thus also considered to be a 'waterborne disease' (Tien & Earn 2010). These waterborne diseases occur worldwide and the outbreaks caused by water contamination can have both health and economic consequences for consumers. In particular, *Cryptosporidium* is considered to be responsible for half of the outbreaks caused by protozoa (Karanis *et al.* 2007; Baldursson & Karanis 2011). Outbreaks caused by contaminated drinking water are a major concern of national public health and environment agencies, as well as to the World Health Organization (WHO).

More recently, the use of recreational waters has been revealed to be a growing route of transmission, leading

WHO to launch Guidelines for Safe Recreational Water Environments (WHO 2003). Accordingly, surveys on *Giardia* and *Cryptosporidium* occurrence in swimming pools and coastal beaches have received increasing attention worldwide and a large set of international literature is now available. In contrast, river beaches have received comparatively little attention even though, according to Till et al. (2008), the presence of *Giardia* and *Cryptosporidium* is recorded when these environments are assessed and the recreational use of fresh water is considered to be a risk factor among users (Schets et al. 2008).

The reported outbreaks for freshwater recreational areas may have its origins in urban and non-urban runoff, industrial pollution, storm waters and human/animal-based wastewater discharges (Karanis et al. 2007). The origin of the contamination depends on the contribution of different features of the surrounding area such as livestock, discharge sewage points, and land use, among others.

Since the mid-1990s, government authorities in Portugal have developed a national policy promoting recreational bathing areas in most Portuguese river basins, which now encompasses more than 90 beaches. These beaches are in high demand, particularly in countryside regions, with some of the beaches being used by 100,000 people per year. Considering that several of these areas are located in close proximity to agricultural fields, there is the potential of contamination from runoff. Also, wastewater discharges located upriver have been shown to influence the contamination potential.

Water quality control of Portuguese river beaches initially followed the EU Bathing Water Directive 76/160/EEC, which set the standards for both physicochemical and microbiological parameters and the guidance and/or imperative values for some of them (coliforms, thermotolerant coliforms, streptococci and dissolved oxygen). Currently, a new EU Bathing Water Directive (BWD 2006/7/EC) is in force, which sets only enterococci and *E. coli* as mandatory parameters. Although the current BWD does not directly address the occurrence of both *Giardia* and *Cryptosporidium* (oo)cysts, it establishes a set of microbiological indicators that might be related to their occurrence in inland waters, particularly for intestinal enterococci and *E. coli*.

Little is known on the prevalence of these parasites in Portugal. Some studies have revealed the prevalence of

Giardia in asymptomatic school children to be 4% (Almeida et al. 2006) and Júlio et al. (2012) found a prevalence of 6.8% in asymptomatic children. The only recorded outbreak of *Giardia* in Portugal involved 1,400 American tourists on Madeira Island when 859 people were recorded experiencing diarrhea, with 39% of them testing positive (Lopez et al. 1978). For *Cryptosporidium*, Matos et al. (1998) found a prevalence of 8% in HIV patients with diarrhea (95% CI: 6–10). The only outbreak in Portugal was reported by Melo Cristino et al. (1998) when 27% (28/103) of children were infected with *Cryptosporidium* at a day-care hospital center. Analysis of raw water samples in five Portuguese north river basins revealed that 73% of the water samples tested positive for *Cryptosporidium* or *Giardia* (Almeida et al. 2010a). Nevertheless, no comprehensive study was ever carried out to assess the occurrence of *Giardia* and *Cryptosporidium* at these Portuguese river beaches. Thus, the objectives of this study were: i) to assess the occurrence and potential risk of *Giardia* and *Cryptosporidium* in river beaches and ii) to relate the occurrence of *Giardia* and *Cryptosporidium* with the mandatory profile information of the BWD.

MATERIALS AND METHODS

Site selection and sample collection

Sampling was designed based on a river basin approach. Nineteen beaches were selected (Figure 1) representing the major Portuguese river basins from north to south: Minho (2), Lima (1), Cávado (1), Douro (2), Mondego (3), Tejo (7), and Guadiana (3). Beaches were chosen proportionally by region, taking into consideration the representative environmental conditions of the surrounding area. The characteristics used for site selection included sewage discharge locations, land use, recreational use, and water quality under the BWD.

At each site samples were collected seasonally through both a wet and a dry season over a two-year period (2009–2010). This methodology was chosen following the suggestion of several authors who pointed out that one of the factors influencing major changes in the occurrence of these parasites would be storm runoff, particularly during

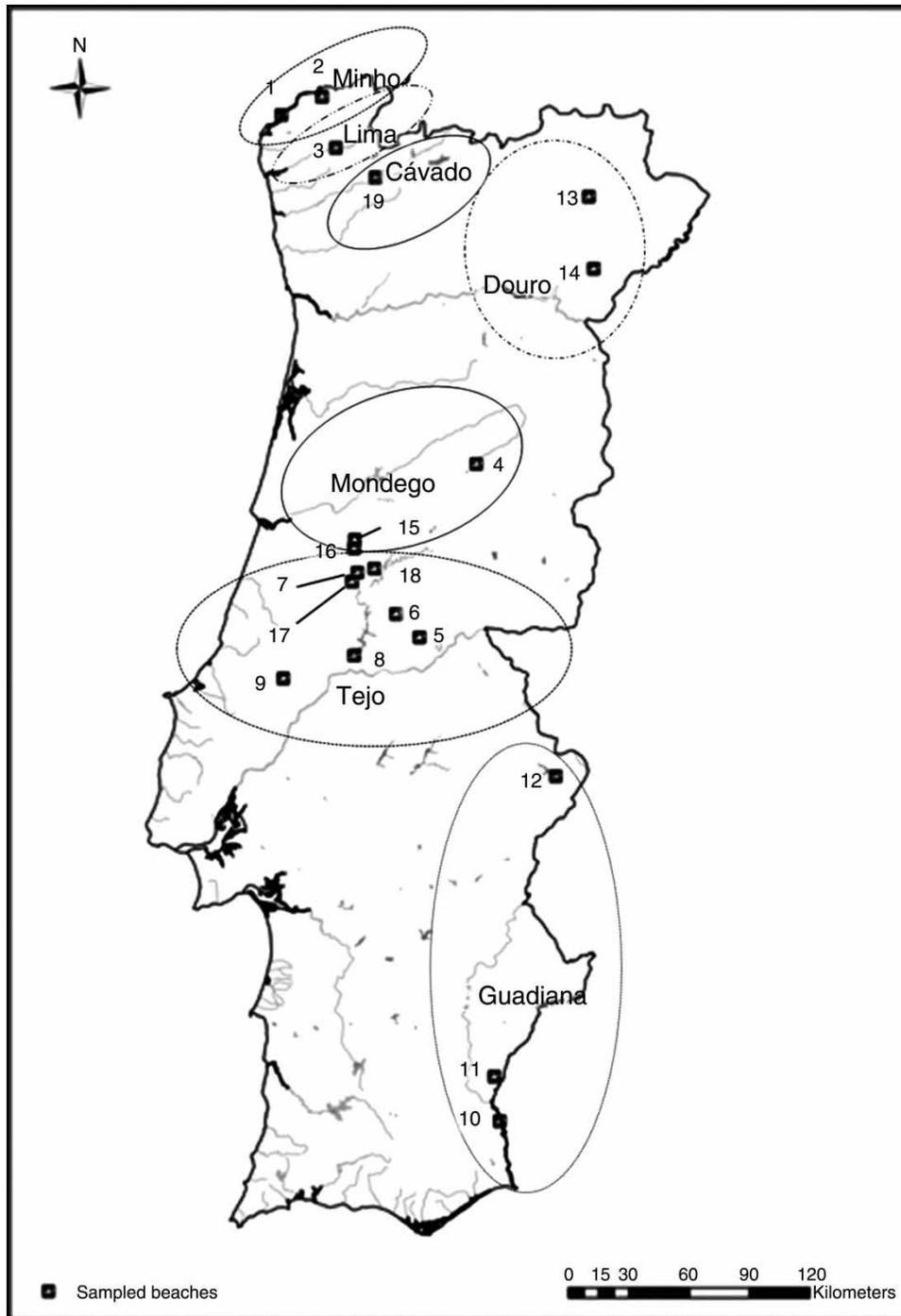


Figure 1 | Fluvial beaches sampled per different hydrographical basin.

the rainy season (Noble *et al.* 2003; Kim *et al.* 2005; Krometis *et al.* 2010). A total of 74 samples out of 76 collected at the identified sites were used as one of the beaches was revealed to be dry during summer.

The water samples were filtered with the IDEXX filters (IDEXX Laboratories, Inc., Westbrook, ME, USA; with 3 μ size pore) using a pump and an energy supply generator. The pump was located on the inlet side, assuring that the

flow rate and pressure (no higher than 4 bar) indicated by the manufacturer was followed. Water volume filtered ranged from 10 to 110 L as water turbidity led to filter clogging and inhibited the filtration at some sites. The filters were removed from the housing, preserved in refrigerated containers, away from the sunlight, and taken to the laboratory within 24 hours, under 4 °C conditions.

The water samples for bacteriological and physicochemical analysis were collected in appropriate containers and kept in a refrigerated environment until the laboratory analysis, which was within 24 hours of sample collection.

Bacteriological and physicochemical analysis

The microbiological parameters were analysed according to the following International Organization for Standardization (ISO) standards: coliforms, thermotolerant coliforms, and *E. coli* (CFU/100 mL) with ISO 9308-1:2000; enterococci (CFU/100 mL) with ISO 7899-2:2000; total suspended solids (TSS) (mg/L) with EN 872:2005-Whatman Filter 934-AH; conductivity ($\mu\text{S}/\text{cm}$) and biochemical oxygen demand (mg/L) with SMEWW 5210 B; chemical oxygen demand (mg/L) with SMEWW 5220 B; ammonia (mg/L) with molecular absorption spectrophotometry-LAE; and dissolved oxygen saturation (% saturation), nitrate, Kjeldhal nitrogen and phosphates (mg/L) with SMEWW 4500.

Cryptosporidium and *Giardia* analysis

Sample processing

Samples were processed with the filter-Max Manual system (IDEXX Laboratories, Inc., Westbrook, ME, USA). The filter elution and final concentration of the sample were performed following the manufacturer's instructions. The processing involves washing the filter twice with 600 mL of phosphate buffered saline (PBS) containing 0.01% Tween 20 (PBST). The washing solutions were filtered through a membrane under vacuum. The membranes were kneaded manually with 10 mL of PBST in a small sealable plastic bag. The washing solution was recovered into a 50 mL centrifuge tube and centrifuged at 1,500 g for

45 minutes. The supernatant was resuspended in 10 mL of PBST and transferred into a L10 tube.

Immunomagnetic separation

The immunomagnetic separation (IMS) was performed according to USEPA method 1623 (USEPA 2005). One millilitre each of 10 \times SL buffer A and buffer B (Dyna-beads[®] GC-Combo, Invitrogen, Dynal, Norway) and 100 μL of *Giardia* and *Cryptosporidium* IMS beads were added to each L10 tube. This was followed by incubation at room temperature for 1 hour with constant rotation. The L10 tubes were then placed in a magnetic particle concentrator, and after several washes the beads were recovered in 100 μL of 0.1 N HCl. The acid suspension was divided into two 50 μL parts, and one part was transferred to a slide containing 10 μL of 0.1 N NaOH. The remaining volume was maintained at -20 °C (for future DNA extraction).

Immunofluorescence

The slides were dried at room temperature and fixed with 50 μL of absolute methanol. After drying the sample, 50 μL of 4',6-diamidino-2-phenylindole (DAPI) staining (1 \times) were added to each sample for 1 minute at room temperature. After washing the slide with the wash buffer, the fluorescein isothiocyanate (FICT)-labelled monoclonal antibody (Aqua-Glo[™]G/C Direct Comprehensive Kit, Waterborne, USA) was applied; this was followed by incubation in a humid chamber for 30 minutes at 37 °C. The slide was washed once more, and 50 μL of Evans blue dye was applied to enhance contrast and non-specific background fluorescence reduction. Finally, the slides were washed, mounted with one drop of medium, and a cover glass applied. Slides were observed under epifluorescence optics (Zeiss Axioskop, Germany) at a magnification of 400 \times . *Cryptosporidium* and *Giardia* (oo)cysts were identified on the basis of their size and shape (*Giardia* cysts are oval to ellipsoid, measuring 8–19 μm (average 10–14 μm) and *Cryptosporidium* oocysts are rounded, measuring 4–6 μm), and the pattern and intensity of immunofluorescence assay staining (intense green fluorescent wall with blue nuclei).

The total number of parasites on each slide was used to extrapolate the concentration of parasites in 10 L of water. The Filta-Max system using the 1623 and 1622 EPA Method assures recovery rates $50.2\% \pm 13.8\%$ for *Cryptosporidium* oocysts and $41.2\% \pm 9.9\%$ for *Giardia* cysts, using the blinded matrix spike samples (McCuin & Clancy 2003).

Data analysis

Simple data analysis was carried out for average, standard deviation, minimum, and maximum values using EXCEL. The non-parametric Kolmogorov–Smirnov test was carried out to test the sample distribution and compare sampling periods. Pearson's correlation test was used to assess the correlation between parameters. Statistical significance of results was assumed using a confidence level of 95% ($\alpha = 0.05$).

Using NTSYSpc (Numerical Taxonomy System) software (version 2.20d; Exeter Software), principal component analysis (PCA) and cluster analysis were carried out. Since the dataset include the individual values obtained in the 19 beaches for the 15 variables (see list in Table 1), with PCA and cluster analysis all variables were simultaneously analysed (when comparing beaches) and all beaches were simultaneously compared (when analysing correlations amongst variables), allowing relationships among parameters and/or different beaches to be evaluated. PCA was performed after applying the Qs test to the data and the Log10 transformation to the microbiological data.

The infection risk was estimated by using the exponential dose-response model for *Cryptosporidium* and *Giardia* (Teunis et al. 1997; Ottoson & Stenström 2003; Veldhuis et al. 2010) through the equation:

$$P_{\text{inf.Single}} = 1 - e^{-r\mu}$$

where $P_{\text{inf.Single}}$ is the single exposure risk of infection by each protozoan, r is the organism-specific constant (0.0199 for *Giardia* and 0.0040 for *Cryptosporidium*) and μ is the pathogen dose. Computation considered a total ingested volume of 34 mL for adults and 51 mL for children (Schets et al. 2011).

RESULTS

Profile of the Portuguese fluvial beaches: occurrence of *Giardia* and *Cryptosporidium* and water quality

Cluster analysis (Figure 2) based on these results showed no distinctive clusters and no pattern could be distinguished among the 74 samples, thus revealing no relevant differences among beaches/river basins. Accordingly, samples were computed as a whole and the average values for microbiological and physicochemical parameters for each sampling season are shown in Table 1. According to the new BWD (2006/7/EC), which sets 660 CFU/100 mL for enterococci and 1,800 CFU/100 mL for *E. coli* as acceptable limits, only two out of the 74 samples (2.7%) were not considered suitable for recreational use, indicating a good bathing water quality. However, if we consider the former BWD (76/160/EEC), taking into account that mandatory levels are defined for coliforms (500 and 10,000 MPN/100 mL, as guidance and imperative values respectively) and thermotolerant coliforms (100 and 2,000 MPN/100 mL, as guidance and imperative values respectively), up to 36% (27 out of 74) of the samples would not be considered as 'suitable for recreational practice'. Particularly relevant are the higher values found at beaches 7–9 for *E. coli*, enterococci, thermotolerant coliforms, and coliforms (>2,000 CFU/100 mL), as well as beach 17 (≈ 500 CFU/100 mL). Some of the physicochemical parameters, such as TSS, phosphates, chemical oxygen demand, biochemical oxygen demand, ammonium, and Kjeldhal nitrogen, present values close to the detection limit indicating a very good water quality, thus fulfilling the requirements for good bathing water. When comparing the values during the sampling period, the results of the Kolmogorov–Smirnov test pointed out that no significant differences ($p < 0.05$) exist between seasons or years for all the parameters, considering both the overall picture as well as each individual beach. However, different concentration values were found from the first to the second year for *Cryptosporidium* and between the dry and wet season for *Giardia*.

PCA showed that the three first principal components (X, Y and Z axis) explained 68.1% of the variance present in the original dataset (Figure 3). The levels of bacteriological parameters (*E. coli*, thermotolerant coliforms, and

Table 1 | Average water quality parameters in river beaches during the sampling period. Values refer to average \pm standard deviation of the parameter in the set of 19 sampled beaches (minimum and maximum values in brackets)

Parameters	2009		2010	
	Wet season (N = 19)	Dry season (N = 18)	Wet season (N = 19)	Dry season (N = 18)
<i>Giardia</i> spp. (number/10 L)	4.9 \pm 10.76 (0.0, 46.7)	4.3 \pm 7.79 (0.0, 32.1)	188.8 \pm 747.17 (0.7, 3,272.0)	2.8 \pm 5.86 (0.0, 22.0)
<i>Cryptosporidium</i> spp. (number/10 L)	4.4 \pm 12.16 (0.0, 53.3)	1.7 \pm 2.16 (0.0, 6.8)	4.3 \pm 7.40 (0.0, 34.0)	1.0 \pm 1.20 (0.0, 4.0)
Coliforms (Log ₁₀ CFU/100 mL)	2.0 \pm 0.79 (1.0, 4.0)	2.6 \pm 0.82 (1.0, 4.3)	2.3 \pm 1.01 (1.0, 4.4)	2.0 \pm 0.66 (1.0, 3.6)
Thermotolerant coliforms (Log ₁₀ CFU/100 mL)	1.3 \pm 0.43 (1.0, 2.3)	1.5 \pm 0.51 (1.0, 2.4)	1.8 \pm 0.86 (1.0, 3.4)	1.4 \pm 0.46 (1.0, 2.4)
<i>E. coli</i> (Log ₁₀ CFU/100 mL)	1.3 \pm 0.43 (1.0, 2.3)	1.3 \pm 0.47 (1.0, 2.3)	1.8 \pm 0.86 (1.0, 3.4)	1.3 \pm 0.37 (1.0, 2.3)
Enterococci (Log ₁₀ CFU/100 mL)	1.4 \pm 0.53 (1.0, 2.30)	1.6 \pm 0.50 (1.0, 2.3)	1.8 \pm 0.80 (1.0, 3.5)	1.4 \pm 0.50 (1.0, 2.3)
Total suspended solids (mg/L)	3.3 \pm 1.75 (2.0, 7.2)	6.5 \pm 8.58 (2.0, 27.0)	12.1 \pm 22.53 (2.0, 92.0)	5.4 \pm 8.32 (2.0, 38.0)
Conductivity (μ S/cm)	114.1 \pm 104.32 (15.0, 430.0)	135.6 \pm 134.06 (12.0, 522.0)	96.1 \pm 86.76 (30.0, 371.0)	119.9 \pm 102.73 (30.0, 455.0)
Chemical oxygen demand (mg/L)	9.8 \pm 4.21 (8.0, 22.0)	10.6 \pm 5.12 (8.0, 24.0)	9.7 \pm 4.00 (8.0, 20.0)	10.3 \pm 6.27 (8.0, 33.0)
Dissolved oxygen (% saturation)	77.5 \pm 7.24 (67.0, 96.0)	67.2 \pm 8.26 (49.0, 80.0)	70.8 \pm 7.05 (56.0, 86.0)	62.2 \pm 15.00 (43.0, 93.0)
Biochemical oxygen demand (mg/L)	2.9 \pm 2.10 (2.0, 8.0)	2.3 \pm 0.95 (2.0, 5.4)	2.0 \pm 0.00 (2.0, 2.0)	2.22 \pm 0.73 (2.0, 5.0)
Ammonia (mg/L)	0.2 \pm 0.00 (0.2)	0.2 \pm 0.00 (0.2)	0.2 \pm 0.00 (0.2)	0.2 \pm 0.00 (0.2)
Kjedhal nitrogen (mg/L)	2.0 \pm 0.05 (2.0, 2.2)	2.0 \pm 0.00 (2.0)	2.2 \pm 0.64 (2.0, 4.8)	2.0 \pm 0.00 (2.0, 2.0)
Nitrate (mg/L)	4.4 \pm 2.09 (1.4, 9.2)	10.7 \pm 20.09 (0.8, 88.0)	10.1 \pm 5.79 (0.8, 18.0)	8.3 \pm 5.18 (1.2, 17.0)
Phosphates (mg/L)	0.2 \pm 0.06 (0.1, 0.4)	1.5 \pm 4.00 (0.1, 13.0)	0.2 \pm 0.02 (0.1, 0.2)	0.2 \pm 0.05 (0.1, 0.3)

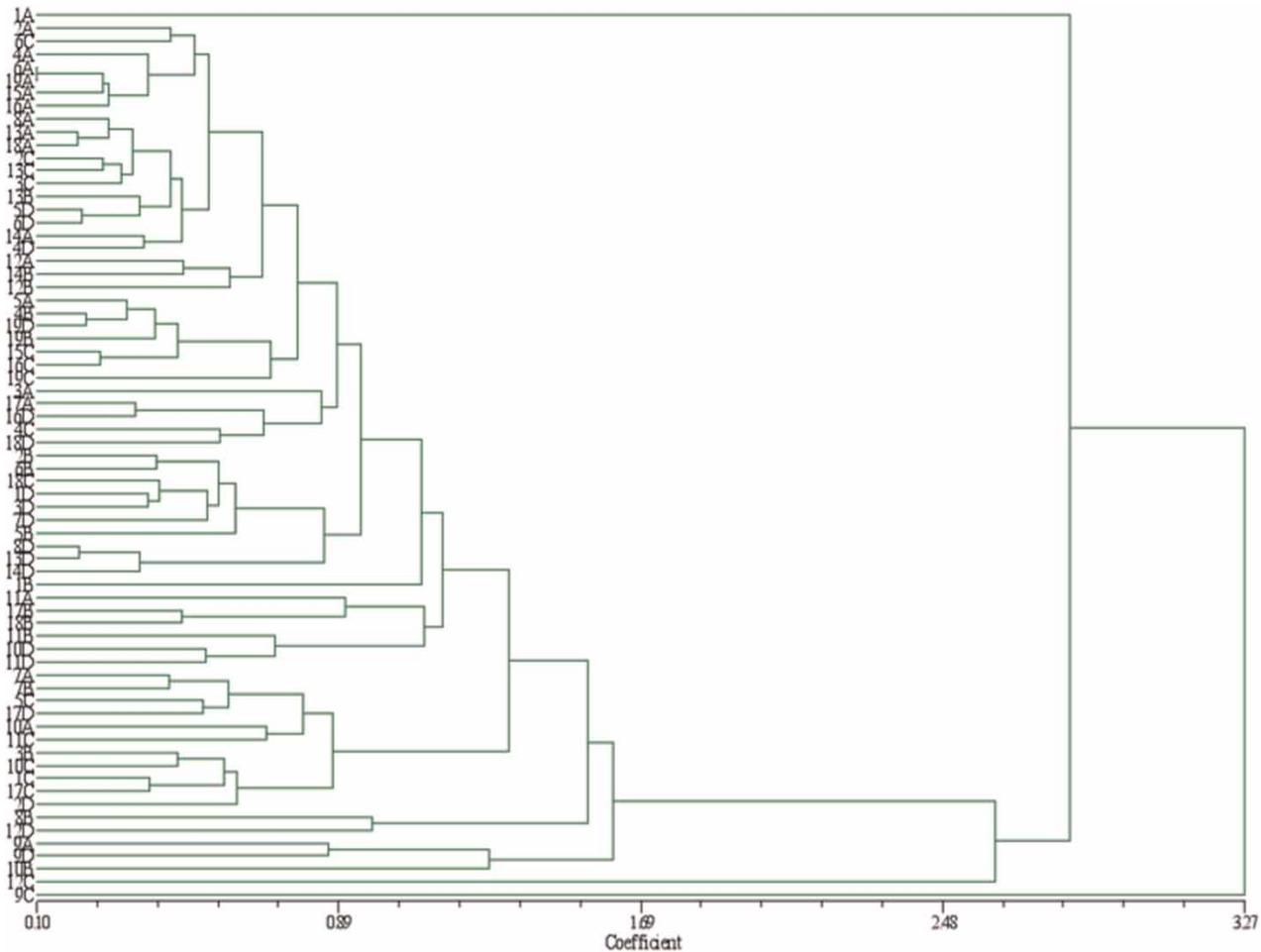


Figure 2 | Cluster analysis on the analysed parameters for the sampled beaches (1–19) in 2009 (A – wet season; B – dry season) and 2010 (C – wet season; D – dry season).

enterococci) and *Giardia* are strongly related to the principal component in the X-axis (positive end), while the physicochemical parameters (dissolved oxygen, TSS, conductivity) mostly explain the variance along the Y-axis (Figure 3(a)). A gradient is also perceived concerning *Cryptosporidium* and nitrate levels since these parameters show high correlations with the Z-axis and thus seem to explain the distribution of samples along it.

As shown in Figure 3(b), most of the samples are positioned near the centre of the co-ordinates. This highlights the weak relation between samples and high microbiological values (that project at the positive end of the axis) and points to a common good water quality.

The samples that are positioned more distantly (v.g. 9C, 12C) were collected during the wet season in the second

year when the precipitation values for Portugal peaked, which could influence runoff and increase contamination. The 1A sample (wet season first year) had a strong relationship with high values on nitrate that points to agriculture as a major source of contamination. During the wet season on the second year, beaches 8 and 9 revealed strong bacteriological and *Giardia* loadings, which suggest that the main source of contamination was wastewater discharge points in the vicinity of the beaches. In fact, the beach 9 area was used as a source of surface water supply but it was abandoned by the National Water Authority for this purpose during the course of the present study. This area is regularly compromised as the wastewater effluent discharges that feeds this beach system are at a high risk for faecal contamination.

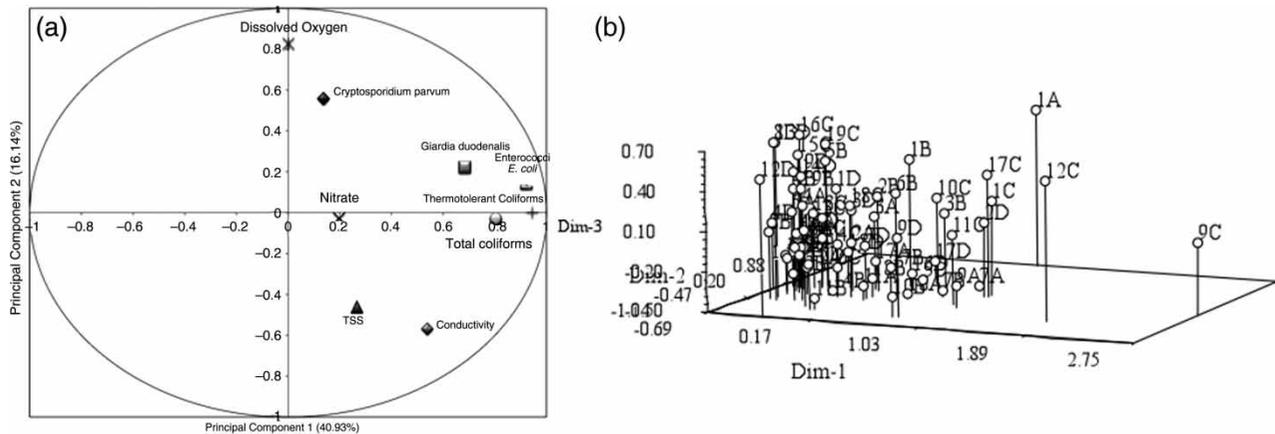


Figure 3 | Principal component analysis on the physicochemical and microbiological parameters (a) and distribution of samples on the PCA new dimensions (b). The numbers refers to the sampled beaches (1–19) and letters the sampled period (A – wet season 2009; B – dry season 2009; C – wet season 2010; D – dry season 2010).

Results for the occurrence of the protozoa pointed to their widespread presence in Portuguese river beaches. Using the IMS procedure, the percentage of samples testing positive for *Giardia* and *Cryptosporidium* is 85 and 82%, respectively. During the dry season, 83% of water samples tested positive for *Giardia* cysts while 87% tested positive through the wet season. During the dry season, 25% of the samples tested negative for *Cryptosporidium* but 89% tested positive during the wet season.

Potential risks to public health

This study highlights the correlations between TSS and coliform bacteria ($r = 0.54$, $p < 0.001$) and between thermotolerant coliforms and conductivity ($r = 0.40$, $p < 0.001$). The correlation between *Giardia* and some microbiological parameters, such as thermotolerant coliforms, *E. coli*, and enterococci were also high ($r \geq 0.87$, $p < 0.001$). Furthermore, a significant correlation between *Giardia* and *Cryptosporidium* was found ($r = 0.305$, $p < 0.05$), similar to the results of other studies (Brookes et al. 2005; Helmi et al. 2011). Results set out in Table 2 clearly indicate a higher risk for *Giardia* contamination than for *Cryptosporidium*. In particular, beaches 1, 8, 9 and 19 revealed values higher than 0.2% for adults and/or children. During the 2009 wet season, beach 8 presented extreme values, reaching 28.26% for children and 19.86% for adults. Further, during this period, absolute values for *Giardia* cysts were shown to be 327 cysts/L at beach 8; 12 cysts/L at beach 9;

3 cysts/L at beach 12, and 3 cysts/L at beach 19. Clearly, there was a higher risk associated with the 2009 wet season than the dry season when the probability for infection from these protozoa was generally less than 10^{-5} (0.1%). These values suggest that the probability of infection is very low risk for bathers during the peak period of recreational use in summer.

DISCUSSION

The levels of *Giardia* and *Cryptosporidium* found in this study along all major Portuguese river basins match the results found for five hydrographical river basins in the north of Portugal (Almeida et al. 2010b). Nevertheless the concentrations found in this study for both *Giardia* and *Cryptosporidium* are higher than those described elsewhere in the literature. This could be explained by the different focuses of these two studies: Almeida et al. (2010a) aimed to pinpoint the sources of surface water contamination, while the present study assumes a more intense human utilization at river beaches. Furthermore the high resilience to environmental stress conditions (Veldhuis et al. 2010), and the small diameter (Medema et al. 1998) and the low specific gravity are important factors enhancing extensive dissemination in water of these protozoa. However, other studies in similar environments revealed higher levels of contamination in recreational lake and river sites (Coupe et al. 2006; Schets et al. 2008) and in the Seine River, Paris, by Moulin et al. (2010).

Table 2 | Global risk of infection for *Giardia* and *Cryptosporidium* in adults and children in river beaches. Values refer to average \pm standard deviation of the single exposure risk of infection in the set of four seasonal samples

Beaches	<i>Giardia</i> risk (%)		<i>Cryptosporidium</i> risk (%)	
	Adults	Children	Adults	Children
1	0.148 \pm 0.142	0.228 \pm 0.212	0.020 \pm 0.034	0.033 \pm 0.052
2	0.043 \pm 0.072	0.070 \pm 0.100	0.005 \pm 0.006	0.005 \pm 0.006
3	0.050 \pm 0.044	0.073 \pm 0.061	0.008 \pm 0.010	0.008 \pm 0.010
4	0.025 \pm 0.044	0.040 \pm 0.061	0.005 \pm 0.006	0.005 \pm 0.006
5	0.013 \pm 0.019	0.035 \pm 0.035	0.010	0.010
6	0.055 \pm 0.064	0.023 \pm 0.033	0.000	0.010
7	0.003 \pm 0.006	0.085 \pm 0.093	0.000	0.000
8	0.233 \pm 0.412	0.007 \pm 0.012	0.000	0.000
9	0.018 \pm 0.015	0.368 \pm 0.602	0.000	0.000
10	0.033 \pm 0.059	0.023 \pm 0.021	0.000	0.005 \pm 0.006
11	0.058 \pm 0.115	0.048 \pm 0.088	0.000	0.000
12	0.003 \pm 0.005	0.088 \pm 0.168	0.010	0.010
13	0.030 \pm 0.054	0.005 \pm 0.006	0.005 \pm 0.006	0.005 \pm 0.006
14	0.010	0.045 \pm 0.077	0.050	0.070
15	0.010	0.010	0.000	0.010
16	0.040 \pm 0.039	0.068 \pm 0.049	0.010	0.010
17	0.033 \pm 0.040	0.048 \pm 0.057	0.000	0.010
18	0.020	0.010 \pm 0.014	0.000	0.010
19	0.240 \pm 0.120	0.095 \pm 0.183	0.000	0.010

In contrast, a large-scale survey of freshwater recreational and water supply sites in New Zealand (Till *et al.* 2008) had very low detection rates of *Giardia* and *Cryptosporidium* (8 and 5% respectively), thus revealing the changeability in concentration values in fresh water depending on the particular characteristics of each environment.

The fact that no significant seasonal variance was found in this study could be partly a consequence of an atypical 2009 meteorological pattern for Portugal. Three heat waves were recorded and the first wet sample period was one of the least rainy during the past 30 years; precipitation values during the 2009 dry season were much higher than those expected for that period (I.M. 2009). The differences found for these protozoa in the second year can be attributed to a more regular year, with a heavy and stormy rainy season (I.M. 2010a) and a more characteristic dry season, with low precipitation and high temperature values (I.M. 2010b). Both situations are described and may occur

elsewhere. While Dorner *et al.* (2007) did not record any seasonal variation in the Grand River (Ontario, Canada), Krometis *et al.* (2010) in the United States and Moulin *et al.* (2010) in the Seine River related stormy/rainy events to a higher occurrence of these protozoa. In addition, some studies have shown that the survival rate after several months (1–3) (deRegnier *et al.* 1989) and low temperatures may lead to higher survival rates for *Giardia* and *Cryptosporidium* (Brookes *et al.* 2004).

The correlation of these protozoa with *E. coli* and enterococci is similar to those found by Dorner *et al.* (2007) and is also related to turbidity and large precipitation events. High TSS values may result from erosion and transportation of erosion particulate matter and bacteria from the surrounding area due to intense rainfall events (Hansen & Ongerth 1991; Nagels *et al.* 2002). During these extreme events, coliform may be drained from non-point sources of contamination to the watershed, thus leading to high

correlation values. Beaches 8, 9, 17 and 19 (particularly in the second sampling period) seem to follow this latter pattern, which is also supported by the agricultural land use pattern and livestock farming in the vicinity. Furthermore, these river basins present several points of discharge of treated wastewater (#8: 235 discharge points; #9: 1 uncontrolled discharge point; #17: 12 discharge points; #19: 39 discharge points). The relationship between the higher risk of contamination due to agricultural use, livestock, and treated wastewater discharges is also reported by other authors (Keeley & Faulkner 2008; Till et al. 2008; Moulin et al. 2010). In fact, the Portuguese National Water Authority (INAG) removed these beaches from the national list of approved river beaches.

Several studies have shown similar results by finding a high correlation between some microbiological parameters and protozoa (Vernile et al. 2009; Graczyk et al. 2010) and between high microbiological parameter values and high (oo)cysts concentrations (Helmi et al. 2011). Thus the correlation between coliform contamination, TSS, and consequent turbidity could be indicative of a high organic content in water (Nnane et al. 2011), which could be useful for early warning of possible contamination by these protozoa as stated by Brookes et al. (2005). However, some studies advocate that the nonexistence of bacterial contamination does not necessarily point to the absence of protozoa while other studies consider that *E. coli* and enterococci are good indicators for faecal pollution but are inappropriate for protozoan pathogens risk assessment (Ashbolt et al. 2001; Helmi et al. 2011). The significant correlation between *Giardia* and *Cryptosporidium* matches similar results of other studies (Brookes et al. 2005; Helmi et al. 2011); despite the atypical year of 2009, this relationship is shown to be associated to wet season and rainfall events.

Results of the present study show that the application of the new EU BWD indicators suggest that *E. coli* and enterococci contamination rates could be high enough to require water quality monitoring to support recreational use, as does the correlation between the rate of swimming-related illnesses and those indicators (Mansilha et al. 2009). The high correlation found between *Giardia* and both new BWD indicators (*E. coli* and enterococci), suggests that high values of these indicators would require immediate controls on *Giardia* and *Cryptosporidium* concentrations.

The public health risk was shown to be low during summer months, with values generally under 10^{-5} during the summer period. However, these values are over 0.2% higher at some beaches, which is clearly related to stormy wet events, agricultural use, and wastewater discharges, as also pointed out by other authors (Till et al. 2008; Moulin et al. 2010). *Giardia* has been detected in at least 15 countries around the world, and for the last 100 years, more than 100 waterborne outbreaks have been reported worldwide. During outbreaks, the values are above 10 cysts/L and in recreational waters the numbers may be higher, reaching 722 cysts/L (Plutzer et al. 2010). The results of this study reveal that at-risk beaches 8 and 9 had values of *Giardia* that reached 327 and 12 cysts/L, respectively. In developed countries, the expansion of intensive agriculture production and the development of roads and settlements have contributed to the increase of non-point sources of protozoan contamination. Further, higher runoff volumes during extreme rain events are occurring and more frequent flooding events are expected as a result of climate change (Nnane et al. 2011). All of these events will possibly contribute to a higher level of waterborne transmission of both protozoa (Schets et al. 2008).

Even with the low probability revealed here, the risk may be higher due to some features that were not addressed by this study. Bathers themselves could be non-point contamination sources (Graczyk et al. 2010) and this risk could be higher than the estimate in this study (Sunderland et al. 2007). Moreover, in open waters a large proportion of the (oo)cysts can be attached to particles affecting the sedimentation velocity and that may result in accumulation of (oo)cysts in sediments (Medema et al. 1998). This is particularly relevant as the presence of bathers themselves may lead to re-suspension of bottom sediments (Graczyk et al. 2010) and to higher water protozoa concentrations.

Nevertheless possible contamination depends on water quantity ingested, and theoretically the ingestion of a single infectious cyst by a susceptible host should be sufficient to cause infection. Rendtorff's classic infection study with human volunteers showed a dose response of 10 cysts resulting in 100% of infected volunteers (Robertson et al. 2011). For *Cryptosporidium*, the dose response is nine viable oocysts (Smith et al. 2009); the water ingested during swimming recreational use is estimated at 18 mL

for the average adult and 34 mL is the highest average for children (Schets *et al.* 2004), thus reinforcing the low probability of contamination in Portuguese river beaches, particularly during the high summer season.

CONCLUSION

The intense recreational activity at the 90 Portuguese river beaches, most of them in rural areas, offered the opportunity for a 2-year large-scale survey of 19 river beaches in the larger Portuguese river basins. *Giardia* and *Cryptosporidium* were shown to be widespread in all the river basins, with a percentage of positive samples of 85% for *Giardia* and 82% for *Cryptosporidium*, with no significant differences among the river basins considered. Higher rates of these protozoa were recorded during the wet season, particularly during 2009, one of the rainiest years recorded, thus associating wet weather events with greater pathogenic loads.

Microbiological analysis revealed that, according to the EU BWD, only two beaches were considered unsuitable for bathing during winter as a result of runoff. A clear relationship emerged between the higher levels of *E. coli* and enterococci with *Giardia* peaks, particularly during the wet season and at beaches in closer vicinity to agricultural land uses, livestock farming, and treated wastewater discharges. Likewise, higher percentages of *Giardia* were matched with higher numbers for *Cryptosporidium* oocysts.

Portuguese river beaches present a very low risk of possible contamination with *Giardia* and *Cryptosporidium*, generally under 10^{-3} , particularly during the summer season. Higher risk levels, with the concentration of *Giardia* cysts considered to be over outbreak levels, were only recorded at two beaches and were associated with a very rainy wet season. Nevertheless, this study showed that compliance with legal standards for bathing water quality parameters does not guarantee the absence of protozoan parasites. The high levels of *E. coli* and enterococci suggest that public authorities should carry out specific procedures, for the detection of *Giardia* and *Cryptosporidium*, whenever the values of those faecal indicators approaches the maximum level allowed under the EU BWD.

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