Laboratory of Parasitology

Department of Virology, Parasitology and Immunology

Faculty of Veterinary Medicine

Ghent University

Waterborne and foodborne transmission of *Cryptosporidium* and *Giardia* in Northern Greece

Panagiota Ligda

Promoters:

Prof. Dr. E. Claerebout

Dr. S. Sotiraki

Dissertation submitted in fulfillment of the requirements for the degree of Doctor in Philosophy (PhD) in Veterinary Sciences

2020

to my parents

Table of contents

List of abbreviations

BLAST	Basic Local Alignment Search Tool
bg	β-giardin
COWP	*Cryptosporidium* oocyst wall protein
CP47	47 kDa protein
CP56	56 kDa trans-membrane protein
DALYs	Disability-Adjusted Life Years
DAPI	4’6 diamindino-2-phenyl indole
ddPCR	droplet digital polymerase chain reaction
DIC	differential interference contrast
DNA	deoxyribonucleic acid
DW	purified drinking water
DZ-HRGP	hydroxyproline-rich glycoprotein
EC	electrical conductivity
ef1-a	elongation factor 1 alpha
FAO	Food and Agriculture Organization
FISH	fluorescent *in situ* hybridization
FITC	fluorescein isothiocyanate
FNU	Formazin Nephelometric units
FW	filtered water
GBD	Global Burden of Disease
gdh	glutamate dehydrogenase
GLORF-C4	*Giardia lamblia* open reading frame-C4
gp60	60 kDa glycoprotein
HIV	human immunodeficiency virus

hsp70	70 kDa heat shock protein
IBS	irritable bowel syndrome
IFAT	immunofluorescent antibody testing
IMS	immunomagnetic separation
ISO	International Standard Method
ITS	internal transcribed spacer
IW	irrigational water
LAMP	loop mediated isothermal amplification
LDFA	Linear Discriminant Function Analysis
mAb	monoclonal antibody
MSC6-7	serine repeat antigen
Mucin1	mucin-like protein
NaHMP	sodium hexametaphosphate
PCC	proportional chance criterion
PI	propidium iodine
QMRA	Quantitative Microbial Risk Assessment
qPCR	quantitative PCR
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
RIW	raw intake water
RNA	ribonucleic acid
RPGR	hypothetical retinis pigmentosa GTPase regulator
RW	raw water
SSU rRNA	small subunit ribosomal RNA
TDS	total dissolved solids
tpi	triose phosphate

TWW	treated wastewater
US EPA	U.S. Environmental Protection Agency
VNTR	variable number tandem repeat
WHO	World Health Organization
WWTP	wastewater treatment plant

Chapter 1

Literature review

Waterborne parasites: *Cryptosporidium* and *Giardia*

The protozoan parasites *Cryptosporidium* and *Giardia* are of great importance, as they infect both humans and animals worldwide, causing gastrointestinal disease. Both parasites are of increasing concern during the last decades, not only for their impact on human and animal health but also for their socio-economic consequences (Kirk et al., 2015). As *Cryptosporidium* and *Giardia* can be transmitted via multiple routes (directly through the faecal-oral route or indirectly via contaminated water and food) and contamination can originate from both human and animal sources, there is great interest in their transmission dynamics and their zoonotic potential.

The aim of this literature review is to first introduce *Cryptosporidium* and *Giardia* by addressing their taxonomy, life cycle, their presence in humans and animals, and their zoonotic potential. Subsequently, the transmission routes of these parasites are reviewed, discussing also the factors that allow them to transmit through multiple routes. Waterborne and foodborne transmission of these parasitic protozoa are discussed in depth and an overview of worldwide documented waterborne and foodborne outbreaks of cryptosporidiosis and giardiasis is presented. Finally, a review is given of widely used methods for the detection of *Cryptosporidium* and *Giardia* in water and on/in food matrices.

1.1 *Cryptosporidium* spp.

1.1.1 Introduction

The genus *Cryptosporidium* was first discovered by Ernest Edward Tyzzer in 1907, who described the species *C. muris* found in gastric glands of laboratory mice (Tyzzer, 1907). However, it was only characterized as a medically and economical important (due to production losses) parasite more than 50 years later (Abeywardena et al., 2015).

Cryptosporidium (phylum Apicomplexa) is a genus of intestinal protozoan parasites which affects a wide range of vertebrates, including humans (Chalmers and Katzer, 2013; Fayer, 2004; Thompson et al., 2005). It infects the microvillus region of the epithelial cells in the digestive tract, causing gastrointestinal symptoms, e.g., diarrhoea, nausea, vomiting. It can also infect the respiratory tract, however this is rare (Sponseller et al., 2014).

Cryptosporidiosis has been included in the Neglected Diseases Initiative of the World Health Organization (WHO) since 2004, because of the severity of the symptoms and the related economical losses (Savioli et al., 2006; WHO, 2004).

1.1.2 Taxonomy

Organisms of the genus *Cryptosporidium* are classified in the phylum Apicomplexa, class Gregarinomorphea and sub-classs Cryptogregaria (Ryan et al., 2016a). *Cryptosporidium* species appear to be mostly host-adapted. To date, based on morphological, biological and molecular analyses 31 *Cryptosporidium* species and more than 40 genotypes have been recognized in various hosts (Certad et al., 2017; Fayer, 2010) (Table 1). Among them, *C. hominis* and *C. parvum* are responsible for over 90% of human cryptosporidiosis cases in most industrialized countries, whereas also *C. meleagridis*, *C. canis* and *C. felis* have been identified as the causative agents of human disease (Xiao, 2010; Xiao and Feng, 2008). Although humans are the major host species for *C. hominis,* it has been also isolated from domestic and wildlife animals (e.g., sheep, goats, cattle, kangaroos, non-human primates) and fish (Ryan et al., 2016b). *C. parvum* is considered the major zoonotic species and is closely linked to clinical disease in neonatal calves that can lead to mortality due to dehydration, as a result of watery diarrhoea (Ryan et al., 2014; Santín, 2013).

Table 1. *Cryptosporidium* species classification (genotypes have not been included since they are updated to species) (Feng et al., 2018; Holubová et al., 2016; Khan et al., 2018; Kváč et al., 2016; Ryan et al., 2016a, 2014; Zahedi et al., 2016b).

Species	***Major hosts***
C. andersoni	cattle
C. apodemi	mice
C. avium	birds
C. baileyi	poultry
C. bovis	cattle
C. canis	dogs
C. cuniculus	rabbits
C. ditrichi	mice
C. erinacei	hedgehogs, horses
C. fayeri	marsupials
C. felis	cats
C. fragile	toads
C. galli	birds
C. hominis (9 subtypes: Ia, Ib, Id, Ie …. Ij)	humans
C. huwi	fish
C. macropodum	marsupials
C. meleagridis	birds, mammals, humans
C. molnari	fish
C. muris	rodents
C. occultus	rodents
C. ornithophilus	birds
C. parvum (14 subtypes: IIa-IIo)*	mammals (including humans)
C. proliferans	rodents
C. rubeyi	squirrels
C. ryanae	cattle
C. scrofarum	pigs
C. scophthalmi	turbot
C. serpentis	reptiles
C. suis	pigs
C. tyzzeri	rodents
C. ubiquitum	ruminants, rodents, primates
C. varanii	lizards
C. viatorum	humans

C. wrairi	guinea pigs
C. xiaoi	sheep, goats

** C. parvum subtype IIc has only been found in humans and has been suggested to be a separate sub-species (C. parvum antroponosum); Nader et al., 2019*

1.1.3 Life cycle

Cryptosporidium has a relatively complicated life cycle, consisting of both sexual and asexual developmental stages (Fig. 1). The infection begins with the ingestion by the host of the sporulated oocysts, either directly via the faecal-oral route, or indirectly through contaminated water or food. Inhalation of the oocysts may also occur. The oocyst contains 4 sporozoites, which after excystation, in the intestinal lumen or the respiratory tract, emerge and invade the epithelial cells and develop into trophozoites. The trophozoites undergo asexual division (merogony) and form Type I meronts, consisting of 8 merozoites. Some of these merozoites form Type II meronts, which contain 4 merozoites and initiate the sexual phase of the life cycle. Macrogametocytes and microgametocytes are formed, fertilize and produce the zygote. Most of the zygotes develop into oocysts, the thick ones with a two-layered wall that are released into the environment and the thin-walled that facilitate autoinfection.

The pre-patent period varies among species, for example the pre-patent period of *C. parvum* ranges from 7 to 21 days (Current and Reese, 1986; Thompson et al., 2005; Tyzzer, 1910; Vetterling et al., 1971).

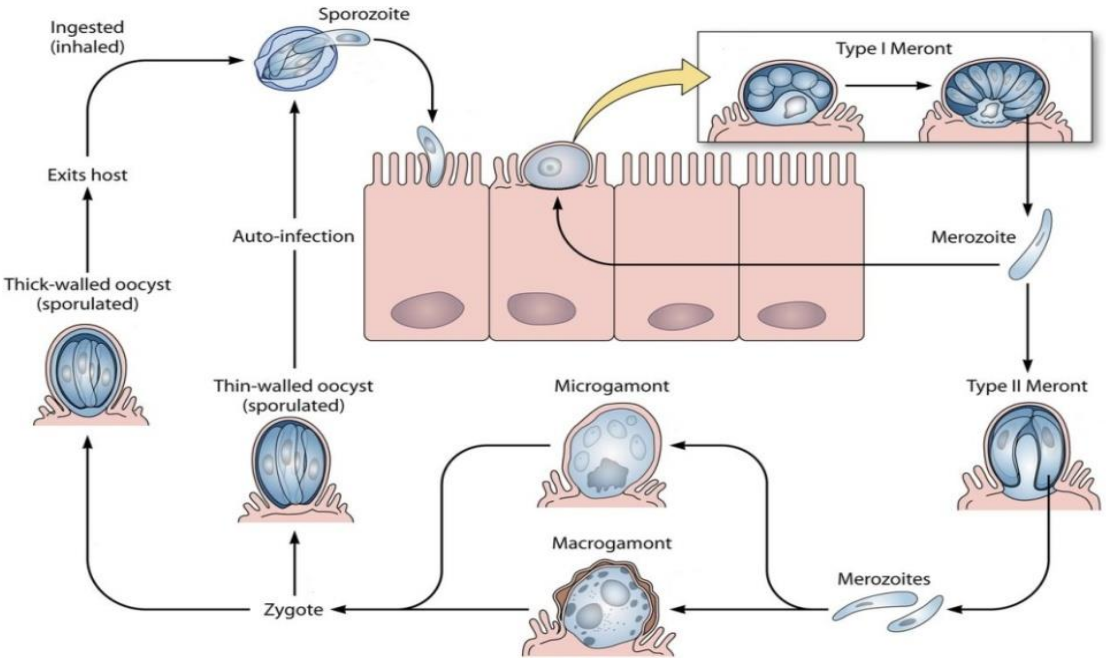


Figure 1. *Cryptosporidium* spp. life cycle *(as shown by Bouzid et al., 2013)*

1.1.4 *Cryptosporidium* in humans and animals

Cryptosporidium is globally recognized as an important **human** foodborne and waterborne parasite, ranked 5th according to the FAO/WHO Multicriteria-based ranking of foodborne parasites (when transmitted via food) for risk management report, due to its significant impact on human health (FAO/WHO, 2014). According to WHO estimates, only in 2010 cryptosporidiosis resulted in 2,159,331 Disability-Adjusted Life Years (DALYs) due to foodborne infections (Kirk et al., 2015). It is the most common diarrhoea-causing protozoan parasite worldwide, especially in developing countries, where hygiene, sanitation and water quality are inadequate (Squire and Ryan, 2017). In industrialized countries, *Cryptosporidium* is less common and accounts for approximately 9% of diarrhoeal episodes in children (Fletcher et al., 2012; Ryan et al., 2016b). Cryptosporidiosis is estimated to be the 5th leading diarrhoeal aetiology among children under the age of 5 years old, causing more than 48,000 deaths and resulting in more than 4,2 million DALYs in this age class, according to the Global Burden of Disease study (GBD) for 2016 (Khalil et al., 2018). It is also an important cause of diarrhoea in immunocompromised patients, with a prevalence ranging between 3% and 16% among HIV-infected individuals with diarrhoea in developed countries (Putignani and Menichella, 2010). Young children and immunocompromised individuals, which are more

susceptible to cryptosporidiosis (Bouzid et al., 2013; Cacciò and Chalmers, 2016; Thompson et al., 2005), may excrete high numbers of oocysts, ranging between 5.0 x 10^3 to 9.2 x 10^5 oocysts/mL, and may experience severe symptoms (Goodgame et al., 1993). Long-term symptoms, such as irritable bowel syndrome (IBS), other IBS-like syndromes, diarrhea, fatigue, abdominal pain or joint paint are among the most commonly reported post-infection symptoms of cryptosporidiosis (Carter et al., 2019; Hunter et al., 2004; Iglói et al., 2018; Insulander et al., 2013; Rehn et al., 2015; Stiff et al., 2017). In contrast, the disease in healthy individuals is mainly self-limiting and sometimes even asymptomatic (Bouzid et al., 2013; Cacciò and Chalmers, 2016; Thompson et al., 2005).

Although different *Cryptosporidium* species (e.g., *C. hominis*, *C. parvum*, *C. viatorum, C. felis*, *C. meleagridis*, *C. ubiquitum* and *C. canis*) have been reported in humans, *C. parvum* and *C. hominis* are the most common species reported worldwide (Feng et al., 2018). The distribution of the species differs among geographic areas and socioeconomic conditions. For example, in Europe and New Zealand, both species are commonly detected in humans, whereas *C. parvum* is the dominant species in humans in the Middle East and *C. hominis* the dominant species in developing countries and other industrialized nations (Feng et al., 2018; Ryan et al., 2016b, 2014; Xiao, 2010). In the U.S, England and Wales *C. hominis* is more common than *C. parvum*. Infections in rural areas are commonly due to *C. parvum*, while *C. hominis* is more common to urban areas (Xiao and Fayer, 2008). Nevertheless, *C. hominis* is responsible for more outbreaks than *C. parvum* worldwide (Xiao, 2010). *C. hominis* subtype Id, *C. parvum*, *C. canis* and *C. felis* have been associated with a more severe disease in HIV/AIDS patients. Among *C. hominis* subtypes, subtype Id was associated with a greater risk of diarrhoea compared to subtypes Ib and Ia, whereas individuals infected with *C. meleagridis* showed no clinical signs and excreted low numbers of oocysts (Certad et al., 2017).

Cryptosporidium is also an important parasite for many animal species, as it may cause severe disease that can lead to death.

Ruminants are on top of that list, as *Cryptosporidium* is one of the most frequently diagnosed enteropathogen in these animals. **Cattle** are commonly infected with *C. parvum*, *C. andersoni*, *C. bovis* and *C. ryanae* (Xiao, 2010), with *C. parvum* being the most common species in calves causing diarrhoea during the first weeks of their life (Thompson et al., 2008). *C. bovis* and *C. ryanae* predominate in post-weaned calves, and *C. andersoni* in older calves and adult cattle

(Fayer et al., 2007, 2006; Santín, 2020; Santín et al., 2008; Thompson et al., 2008), although these species have not been associated with illness (Santín, 2013). In endemic herds, morbidity rates are usually 100%, but mortality is infrequently observed; calves can die from dehydration and cardiovascular collapse (Thompson et al., 2008).

Sheep and **goats** are also infected with *Cryptosporidium* species, mainly during the first 3 weeks of their life. The predominant species are *C. ubiquitum*, *C. xiaoi* and *C. parvum*, while other species such as *C. hominis*, *C. andersoni* and *C. suis* have been reported sporadically (Santín, 2020, 2013). Small ruminants can shed large numbers of oocysts (10^8 - 10^{10}) for a long period of time, while animals can be also asymptomatic (Santín, 2013; Thompson et al., 2005).

In **dogs** and **cats**, prevalence rates range between 0.5% to 44.1% and 0% and 29.4%, respectively (Lucio-Forster et al., 2010; Taghipour et al., 2020). Most infections are caused by the host-specific *C. canis* and the zoonotic *C. parvum* in dogs and *C. felis* in cats (Bowman and Lucio-Forster, 2010; Scorza and Tangtrongsup, 2010; Taghipour et al., 2020). Asymptomatic infection usually occurs, while watery diarrhoea is the most common manifestation of clinical disease (Scorza and Tangtrongsup, 2010). Infected dogs and cats can shed oocysts for several months (Hamnes et al., 2007; Santín, 2013; Thompson et al., 2005).

Cryptosporidium has been also documented in **horses**, with most infections being asymptomatic, but severe diarrhoea can occur in foals and immunocompromised animals (Santín, 2013; Thompson et al., 2005; Veronesi et al., 2010). *Cryptosporidium* horse genotype and *C. parvum* have been mostly detected (Chalmers et al., 2005; Grinberg et al., 2008a; Kostopoulou et al., 2015; Ryan et al., 2003; Veronesi et al., 2010).

Pigs are mostly infected with *C. scrofarum* and *C. suis* (Kváč et al., 2013; Ryan et al., 2004), although *C. parvum*, *C. muris*, *C. andersoni* and *C. tyzzeri* have been also isolated from pigs worldwide (Farzan et al., 2011; Lin et al., 2015; Petersen et al., 2015; Schubnell et al., 2016; Yui et al., 2014). *C. scrofarum* is more frequently found in older animals (>5 weeks), while *C. suis* preferentially infects suckling piglets (Johnson et al., 2008; Kváč et al., 2013; Langkjær et al., 2007; Yui et al., 2014). The disease is usually subclinical (Xiao, 2010), while clinical signs seem to depend on the species that cause the infection and co-infection with other enteropathogens (Santín, 2013), although the pathogenicity of the species remains equivocal (Enemark et al., 2003; Yui et al., 2014).

Cryptosporidium has been also found in other animal species, including **wild mammals**, **birds**, **fish**, **shellfish**, **reptiles** and **marsupials** (Appelbee et al., 2005; Feng, 2010; Zahedi et al., 2016b; Ryan et al., 2014). Species commonly detected include: *C. cuniculus* (rabbits and marsupials), *C. ubiquitum* (deer, raccoons, foxes), *C. andersoni* (mink, camels, rodents, panda), *C. muris* (wild rodents, such as species of rats, squirrels, hamsters), *C. canis* (foxes, coyotes) and *C. fayeri* (bandicoots, kangaroos, koalas) (Ryan et al., 2014; Zahedi et al., 2016b). However, also the human specific *C. hominis* (dugong, kangaroos, marsupials, deer, foxes) and zoonotic *C. parvum* (racoons, mustangs, bamboo rats, deer, canids, camelids, fish, dolphins, shellfish) have been isolated (Barrera et al., 2020; Certad et al., 2019; Dowle et al., 2013; Fayer et al., 1998; Giangaspero et al., 2014, 2005; Gomez-Bautista et al., 2000; Gómez-Couso et al., 2006, 2004; Graczyk et al., 2001, 1998; Hattori et al., 2018; Marquis et al., 2015; Ng et al., 2011; Parsons et al., 2015; Reboredo-Fernández et al., 2014; Traversa et al., 2004; Wagnerová et al., 2016; Wei et al., 2019; Xie et al., 2019; Zahedi et al., 2016b), thus potentially contributing to the contamination of water systems (Zahedi et al., 2016b). Finally, wild fish are infected mostly with host-specific species (*C. molnari, C. scophthalmi*), however also the human infectious species, *C. hominis* and *C. parvum* have been detected in fish (Robertson et al., 2019a). Even if the presence of *Cryptosporidium* oocysts has been reported in all the above-mentioned animal species, there are no/limited data on the prevalence rates.

1.1.5 Molecular evidence of zoonotic potential

At least 20 distinct *Cryptosporidium* species have been identified as the causative agents of human cryptosporidiosis (Elwin et al., 2012a; Ng et al., 2012; Ryan et al., 2014; Xiao, 2010). Among them, infections are most commonly due to *C. hominis* and *C. parvum*. The occurrence of *C. hominis* in humans is most likely due to anthroponotic transmission, as it infects almost exclusively humans and non-human primates. On the other hand, the presence of *C. parvum* in a population can be a result of both anthroponotic and zoonotic transmission, as it infects humans, livestock, pigs and horses, and thus it is considered a potential zoonotic pathogen (Xiao, 2010; Xiao and Feng, 2008).

Molecular diagnostic tools have helped us to better evaluate the zoonotic or anthroponotic nature of *Cryptosporidium* species and characterize its transmission dynamics. The small subunit (SSU) rRNA is the most commonly used marker for *Cryptosporidium* species

identification in different types of samples (i.e., animal and human faeces, water, food), because of its multicopy nature and the presence of both conserved and highly polymorphic regions, that allows the design of specific primers (Xiao et al., 1999). Other loci, such as the internal transcribed spacers (ITS) ribosomal DNA (rDNA) (Morgan et al., 1999), the 70 kDa heat shock protein (hsp70) (Sulaiman et al., 2000), the actin (Sulaiman et al., 2002) and the *Cryptosporidium* oocyst wall protein (COWP) (Xiao et al., 2000), are also used for the *Cryptosporidium* species identification.

Subtyping tools, particularly for *C. hominis* and *C. parvum* have been developed and used in population genetic studies (Alves et al., 2003; Gatei et al., 2007, 2006; Kato et al., 2003; Sulaiman et al., 2002, 2001; Xiao et al., 1999). One of the most widely used markers is the 60 kDa glycoprotein (gp60, also known as gp40/15) gene that has several tandem repeats of a serine-coding tri-nucleotide TCA, TCG or TCT and multiple sequence divergent non-repeat regions (Gatei et al., 2006; Leoni et al., 2007b, 2007a; Wielinga et al., 2008). With the use of this genetic marker, several subtypes have been revealed. For *C. hominis* 9 subtype groups, from Ia, Ib, Id to Ij, have been described, whereas for *C. parvum* 14 subtypes, from IIa to IIo, have been detected (Ryan et al., 2014). The name of gp60 subtypes starts with the subtype family description (e.g., Ia, Ib, etc. for *C. hominis* and IIa, IIb, etc. for *C. parvum*) followed by the number of tri-nucleotide repeats represented by a letter (i.e. A for TCA, G for TCG or T for TCT). Thus, for instance the name IbA10G2 indicates that the parasite belongs to *C. hominis* subtype family Ib and has 10 copies of the TCA repeat and 2 copies of the TCG repeat in the tri-nucleotide repeat region of the gp60 gene (Sulaiman et al., 2005). In addition to these variations, there are sequence differences in non-repeat regions that are represented with the letter “R” and the number of repetitions. Thus, in *C. hominis* subtype family Ia, subtypes are further identified by the number of a 15 bp repetitive sequence AA(A/G)ACGGTGGTAAGG (the last repeat is always the 13 bp: AAAACGGTGAAGG). In *C. parvum* subtype family IIa, some subtypes after the tri-nucleotide repeats also have repetitions of the sequence ACATCA (Xiao, 2010).

In order to increase the resolution of the gp60 typing, several minisatellite and microsatellite targets have been also used, although from only few studies, including the 47 kDa protein (CP47) gene, the 56 kDa trans-membrane protein (CP56), the mucin-like protein (Mucin1) gene, the serine repeat antigen (MSC6-7) gene, the hypothetical retinis pigmentosa GTPase

regulator (RPGR), and the hydroxyproline-rich glycoprotein (DZ-HRGP) gene (Gatei et al., 2007, 2006). These targets seemed to offer a superior tool for *C. hominis* subtyping, as new subtypes were revealed, compared to the ones from the gp60 analyses, and clustered according to their geographical origin (Gatei et al., 2006). The CP47 marker showed higher resolution compared to gp60 for both length polymorphisms and multilocus sequence typing, and thus could be used to distinguish *C. hominis* and *C. parvum* by size with no overlap in the number of tri-nucleotide repeats. Also, as MSC6-7 has many polymorphic sites and strong linkage disequilibrium to gp60 and CP47, it could be a useful target for the subtyping of both *C. hominis* and *C. parvum* (Gatei et al., 2007). Furthermore, other variable number tandem repeat (VNTR) loci have been identified, especially for *C. parvum*, as promising tools for differentiation among species and identification of zoonotic species, however their application still needs standardization (Chalmers et al., 2017; Pérez-Cordón et al., 2016).

C. hominis subtype families Ia, Ib and Ie are responsible for most of the human cases worldwide. In developing nations, higher heterogeneity of these subtypes has been observed, compared to developed countries and this is possibly an indicator of intensive and chronic transmission of the parasite in these areas (Xiao, 2010). The subtype Ib is the main causative agent of diarrhoea among immunocompetent individuals in the USA and Europe, and is also responsible for most disease outbreaks worldwide (Efstratiou et al., 2017b; Khan et al., 2018). Even if *C. hominis* is considered a human parasite (Ryan et al., 2014; Xiao, 2010), it has been also isolated from many wildlife hosts. The subtype IbA10G2, that has been implicated in many waterborne outbreaks (Chalmers et al., 2019; Mayne et al., 2011; Ng-Hublin et al., 2015; Widerström et al., 2014), has been also found in kangaroos and cattle in Australia (Zahedi et al., 2016a, 2016b).

The use of gp60-based subtyping, multilocus subtyping and multilocus sequence typing tools has increased our appreciation of anthroponotic transmission of *C. parvum* (Xiao, 2010; Xiao and Feng, 2008). Studies where PCR product length polymorphism analysis of minisatellite and microsatellite markers was used, revealed the presence of different distinct groups of *C. parvum* in humans and cattle. Genotypes clustered in human-specific groups were not found in any of the animals genotypes, and humans were infected with a greater spectrum of *C. parvum* multilocus types than cattle, suggesting that humans were not infected from cattle

(Grinberg et al., 2008b; Leoni et al., 2007b; Mallon et al., 2003b, 2003a; Morrison et al., 2008; Wielinga et al., 2008).

Subtypes IIa and IIc are the 2 most common families, with the IIa subtype commonly found both in humans and animals (e.g., calves), and thus considered zoonotic. On the contrary, subtype IIc has only been found in humans and not in animals, which demonstrates its anthroponotic nature of transmission (Feng et al., 2018; Widmer, 2009; Xiao, 2010; Xiao and Feng, 2008). It has been also suggested to classify IIc as a separate sub-species under the name *C. parvum antroponosum* (Nader et al., 2019). *C. parvum* subtype family IId that is commonly found in sheep and goats, is the dominant subtype also in humans in Middle East countries (Feng et al., 2018; Nazemalhosseini-Mojarad et al., 2012). In Portugal, the IId subtype has been also detected in HIV patients, lambs and calves of the same areas (Alves et al., 2006). Unlike industrialized nations, in developing countries, such as India, Malawi, Uganda, and Kenya, *C. parvum* subtypes IIb and IIe have been identified in humans (and not in sympatric animals of these areas), although less frequently, due to the dominance of *C. hominis* in these regions (Akiyoshi et al., 2006; Cama et al., 2007; Essid et al., 2018; Feng et al., 2018; Muthusamy et al., 2006; Peng et al., 2003).

Other species, such as *C. meleagridis*, *C. felis*, *C. canis*, *C. ubiquitum*, *C. cuniculus* and *C. viatorum* are less common in humans. The remaining *Cryptosporidium* species (Table 1) have been only found in few human cases (Elwin et al., 2012b, 2012a; Kváč et al., 2014; Nichols et al., 2006; Rašková et al., 2013). The distribution of these species in humans usually depends on socioeconomic conditions, and as a result differs among geographic areas. *C. felis* and *C. canis* have been mostly detected in humans in developing countries, but at a low rate, indicating a minimal risk for public health (Lucio-Forster et al., 2010; Thompson et al., 2008) Such host-specific species may infect humans when the immune status of the host, socio-economic and environmental conditions favour the transmission (Xiao and Feng, 2008). Instead, *C. ubiquitum* has been mostly detected in industrialized countries, including the United Kingdom, Canada and the United States and *C. cuniculus* mainly in the United Kingdom (Feng et al., 2018; Xiao, 2010).

1.2 *Giardia duodenalis*

1.2.1 Introduction

The first detailed description of *Giardia* was published in 1859, however Antonie van Leeuwenhoek has first observed it back in 1681 (Abeywardena et al., 2015).

Giardia (phylum Metamonada) is a single-celled parasitic protist, which comprises many species. It infects the intestinal tract of many vertebrate hosts, including humans, domestic animals, rodents and wildlife animals. Among the species, *G. duodenalis* (also known as *G. intestinalis* and *G. lamblia*) is known to cause disease to many mammals, including humans, suggesting a zoonotic transmission (Di Genova and Tonelli, 2016).

Since *Giardia* has been isolated from many hosts through the years, the WHO has recognized its zoonotic significance in 1979 (WHO, 1979). In 2004 giardiasis was added to the Neglected Diseases, due to the impact on human health and mainly among children in developing countries (Savioli et al., 2006; WHO, 2004).

1.2.2 Taxonomy

Organisms of the genus *Giardia* are classified in the phylum Metamonada, class Trepomonadea and sub-class Diplozoa (Plutzer et al., 2010). To date, according to the morphology and ultrastructure of trophozoites and/or cysts, six species of *Giardia* are accepted by most researchers (Table 2). These species are found in amphibians (*G. agilis*), a water monitor (*G. varani),* birds (*G. psittaci* and *G. ardeae*), rodents (*G. muris* and *G. microti*) and mammals (*G. duodenalis*) (Adam, 2001; Cacciò et al., 2005; McRoberts et al., 1996; Plutzer et al., 2010; Upton and Zien, 1997). *G. duodenalis* has been isolated from many mammals, including livestock and companion animals, but it is the only species found in humans (Feng and Xiao, 2011; Ryan and Cacciò, 2013).

According to multigenetic sequence analyses, *G. duodenalis* is now considered a multispecies complex, whose members have little variation to their morphology and are divided into 8 distinct genetic assemblages (A to H). Assemblages A and B are found in a wide range of mammals and are responsible for human infection. Due to further genetic variation, these two assemblages are also divided into sub-assemblages: AI, AII, AIII and AIV for assemblage A and BI, BII, BIII and BIV for assemblage B (Monis et al., 2003). Sub-assemblages AI, AIII and AIV

can be found in many animal species (e.g., livestock, companion animals), whereas sub-assemblage AII is mainly found in humans. Although host distribution of assemblage B is predominantly in humans, sub-assemblages BI and BII have been isolated from animals (e.g. dogs, monkeys), whereas sub-assemblages BIII and BIV have been described in humans (Ryan and Cacciò, 2013; Sprong et al., 2009).

The remaining assemblages (C to H) have a more restricted host specificity, as assemblages C and D are found in dogs, E in livestock and other hoofed animals, F in cats, G in rodents and assemblage H in marine mammals (Heyworth, 2016).

Table 2. *Giardia* species classification (Heyworth, 2016; Thompson et al., 2008; Thompson and Monis, 2004).

Species	***Major hosts***
G. duodenalis Assemblage A	humans and other primates, dogs, cats, livestock, rodents and other wild mammals
G. duodenalis Assemblage B *(G. enterica)*	humans, ruminants and other hoofed livestock, dogs, rabbits, marsupials, marine mammals, rodents, ferrets, rock hyrax, non-human primates, chicken, ostrich, gull
G. duodenalis Assemblage C/ D *(G. canis)*	dogs
G. duodenalis Assemblage E *(G. bovis)*	cattle and other hoofed livestock
G. duodenalis Assemblage F *(G. cati)*	cats
G. duodenalis Assemblage G *(G. simondi)*	rats, mice
G. duodenalis Assemblage H	marine mammals
G. agilis	amphibians
G. ardeae	birds
G. microti	voles and muskrats
G. muris	rodents
G. psittaci	birds
G. varani	lizards

1.2.3 Life cycle

Giardia spp. has a simple and direct life cycle consisting of two morphogenetic stages: the infectious cyst (Fig. 2b), which is resistant to many environmental conditions and the trophozoite (Fig. 2a), which colonizes the small intestine and causes the disease. Infection

initiates after oral ingestion of viable cysts through either direct contact (via the faecal-oral route) or indirect via contaminated water or food. After passing through the stomach, cysts begin the excystation process and release two trophozoites in the upper part of the small intestine. The trophozoites attach to the epithelial cells of the small intestine with their adhesive ventral disk, where they multiply asexually by binary fission (Fig. 3). Once the environmental conditions are suitable (*i.e.*, increased bile salt concentration and cholesterol deprivation), the trophozoites transform into cysts (encystation process) which are then passed with the faeces and excreted to the environment (Di Genova and Tonelli, 2016). After the ingestion of *Giardia* cysts, the pre-patent period is usually 1-2 weeks, but can range from 1 to 45 days (Jokipii and Jokipii, 1977; Ortega and Adam, 1997; Rosa et al., 2007).

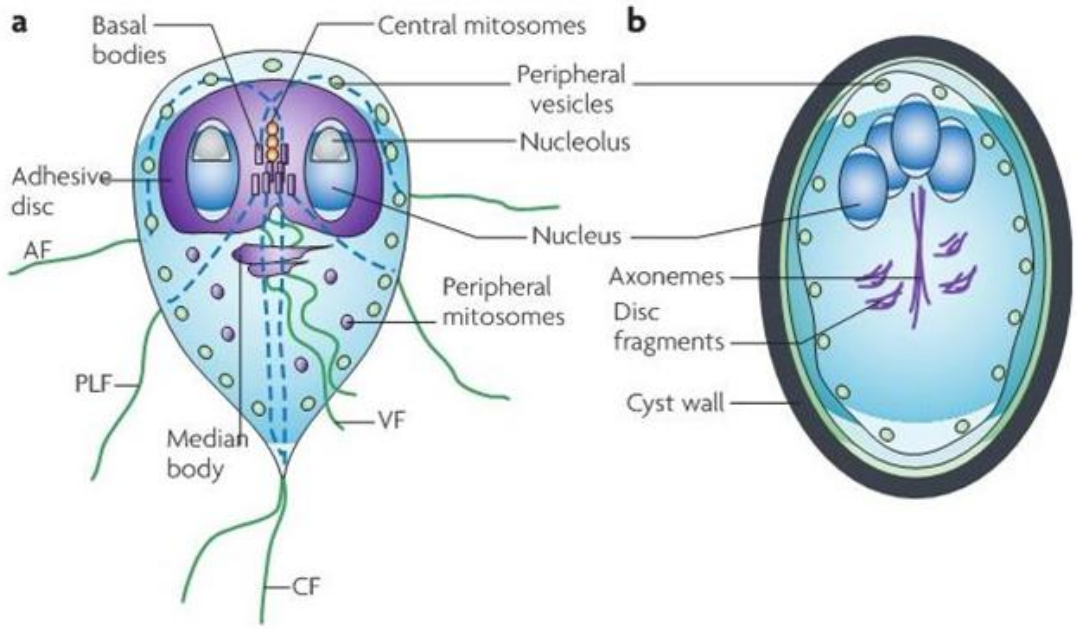


Figure 2. **(a)** *Giardia* spp. trophozoite; **(b)** *Giardia* spp. cyst *(as shown by Ankarklev et al., 2010)*

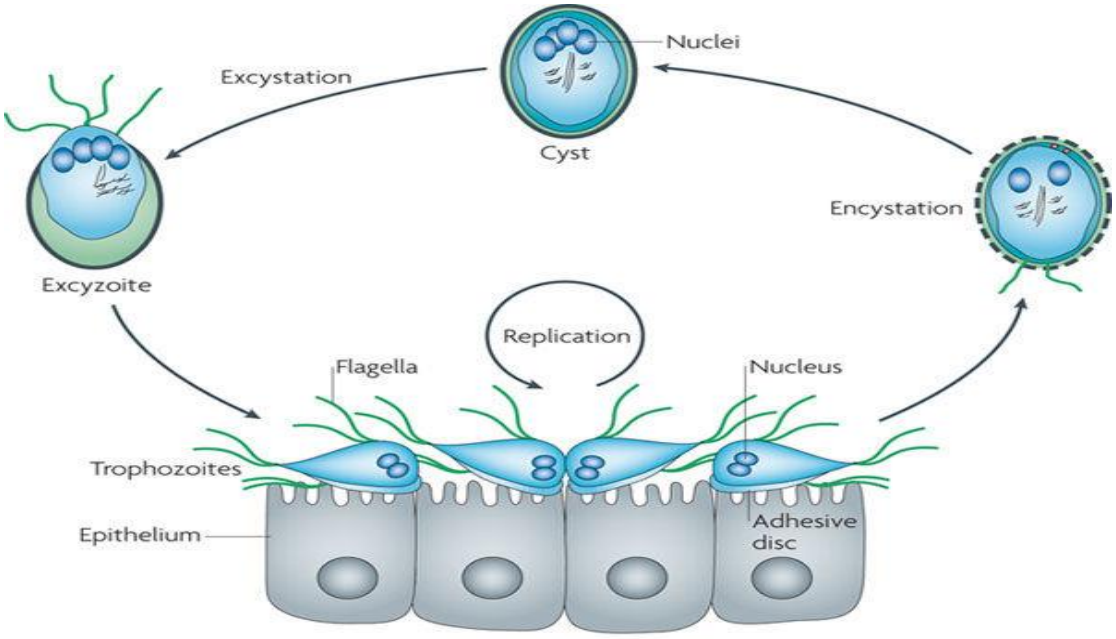


Figure 3. *Giardia* spp. life cycle *(as shown by Ankarklev et al., 2010)*

1.2.4 *Giardia* in humans and animals

G. duodenalis is among the important **human** parasites globally, considered as the most common cause of acute diarrhoea among travellers, especially to those returning from developing countries (Broglia et al., 2013; Gautret et al., 2012; Muhsen and Levine, 2012). According to estimates, more than 200 million people worldwide have symptoms of giardiasis and about 280 million new cases occur annually (Lane and Lloyd, 2002; WHO, 1996). Only in 2010 giardiasis resulted in 171,100 DALYs (Kirk et al., 2015) and as a result, for all the above reasons, some years later *Giardia* ranked 11th to the FAO/WHO Multicriteria-based ranking of foodborne parasites (when transmitted via food) for risk management report (FAO/WHO, 2014). Although infections are reported globally, higher infection rates are documented in developing countries (up to 30%) compared to developed ones (up to 7.5%) (Feng and Xiao, 2011). It is estimated that only in the United States, more than 3,000 hospitalizations and 1.2 million cases of the disease occur annually (Scallan et al., 2011). There have been several giardiasis outbreaks, mostly as point source infections, reported in different European countries, such as Belgium, Greece, UK, the Netherlands and Nordic countries, during the past years (Braeye et al., 2015; Guzman-Herrador et al., 2015; Hadjichristodoulou et al., 1998; Hardie et al., 1999; Smith et al., 2006).

According to genotyping analyses of *G. duodenalis*, assemblages A and B are primarily associated with human infections (Heyworth, 2016; Lebbad et al., 2011; Muhsen and Levine, 2012). Infections are more commonly due to assemblage B worldwide (about 58% of the cases), compared to assemblage A (about 37% of the cases), without differentiation in distribution among developed and developing countries (Einarsson et al., 2016). However, in developing countries mixed infections with both assemblages A and B are commonly observed (about 5%) compared to industrialized ones (about 3%) (Einarsson et al., 2016; Ryan and Cacciò, 2013). While some studies support that assemblage A was more often associated with the presence of symptoms compared to assemblage B (Aydin et al., 2004; Breathnach et al., 2010; Read et al., 2004; Sahagún et al., 2008; Samie et al., 2020), the opposite finding was observed in other studies (Gelanew et al., 2007; Homan and Mank, 2001; Pelayo et al., 2008; Wang et al., 2019). However, as many factors (e.g. the infection dose, the dominance of a specific assemblage in the region, the immune status and the age of the individual infected, etc.), can contribute to the virulence of the assemblages, such findings need further investigation (Feng and Xiao, 2011; Robertson et al., 2010). There is also diversity among the sub-assemblages of the same assemblage. Sub-assemblage AII is most prevalent and distributed globally compared to sub-assemblage AI, except for Asia and Australia. On the other hand, sub-assemblages BIII and BIV are equally distributed in Europe and Australia, whereas in Africa and Asia sub-assemblage BIII is predominant, but BIV is predominant in North America (Feng and Xiao, 2011; Sprong et al., 2009).

The infection is typically characterized by diarrhoea, but can be either asymptomatic or responsible for a broad spectrum of clinical manifestations (e.g., nausea, vomiting, bloating), with symptoms ranging from acute to chronic. Post-infectious fatigue syndrome, IBS, IBS-like symptoms, functional dyspepsia, reactive and post-infectious arthritis and intestinal allergies are among the most common long-term consequences of *Giardia* infection (Allain and Buret, 2020; Farthing, 1996; Halliez and Buret, 2013; Litleskare et al., 2019, 2018; Minetti et al., 2016; Mørch et al., 2009b, 2009a; Nash et al., 1987; Robertson et al., 2010). Young children and immunocompromised patients are more vulnerable to clinical manifestations (Halliez and Buret, 2013).

Giardia is commonly found in **cattle**. Higher prevalence rates are recorded in young calves up to 6 months old, compared to older animals (Becher et al., 2004; Nydam et al., 2001; Ralston

et al., 2003; Xiao, 1994). However, prevalence rates vary significantly, reaching sometimes 100% depending on the age and the number of the animals included in the studies, the management conditions, as well as the diagnostic tools used (Feng and Xiao, 2011; Geurden et al., 2010; Robertson, 2009). *G. duodenalis* assemblage E is predominant, whereas also isolates of assemblage A are common, as well as mixed infections of assemblages A and E (Geurden et al., 2008; Sprong et al., 2009). Infections are mostly asymptomatic but can be also associated with mild diarrhoea and ill thrift, leading to production losses (Abeywardena et al., 2015).

The prevalence of *Giardia* in **sheep** and **goats** also depends on the age and the number of the animals tested, the management practices of the farm, the geographical area and the diagnostic tools used, thus the infection rates can vary significantly (Feng and Xiao, 2011; Geurden et al., 2010; Robertson, 2009; Santín, 2020). Assemblages A and E are most frequently detected, with E being the predominant genotype in most areas, however also infections with assemblage B have been recorded (Robertson, 2009; Santín, 2020). Asymptomatic infection usually occurs, but when diarrhoea persists, this can lead to production losses (Feng and Xiao, 2011).

Giardia is one of the most common intestinal parasites of **dogs** and **cats** in developed countries (Ballweber et al., 2010; Barutzki and Schaper, 2011; Claerebout et al., 2009; Kostopoulou et al., 2017; Pallant et al., 2015; Palmer et al., 2008; Zanzani et al., 2014). The prevalence rates often range between 1% and 45% in healthy or clinically ill dogs or cats (Claerebout et al., 2009; Julien et al., 2019; Kim et al., 2019; Kostopoulou et al., 2017; Pan et al., 2018; Ryan and Zahedi, 2019; Tangtrongsup et al., 2020; Zanzani et al., 2014), although the prevalence rates can vary considerably depending on the study population, the study area, the diagnostic method used, as well as the health status of the animals (Tangtrongsup and Scorza, 2010). The most prevalent species in dogs are the host-specific assemblages C and D (Cacciò and Ryan, 2008; Ryan and Zahedi, 2019), but also other assemblages such as sub-assemblages AI, AII, BIII and BIV have been isolated (Claerebout et al., 2009; Palmer et al., 2008; Pan et al., 2018; Ryan and Zahedi, 2019; Sprong et al., 2009). Similarly, in cats the host-specific assemblage F is predominant, but also assemblages C, D, E, AI, AII, BIII and BIV-like have been detected (Kostopoulou et al., 2017; Palmer et al., 2008; Pan et al., 2018; Papini et al., 2007; Ryan and Zahedi, 2019; Souza et al., 2007; Sprong et al., 2009; Tangtrongsup et

al., 2020). The infection in companion animals is mainly sub-clinical, although chronic diarrhoea and other clinical manifestations (e.g., abdominal pain, weight loss) can also occur. The severity of the signs depends on several factors including the age and the immune status of the animal, as well as the presence of other pathogens (Adell-Aledón et al., 2018; Tangtrongsup and Scorza, 2010; Tysnes et al., 2014).

Infections of **horses** with *Giardia* have been reported from many areas worldwide, while the prevalence rates vary significantly (0.5%-35%) (Kostopoulou et al., 2015; Olson et al., 1997; Santín et al., 2013; Traversa et al., 2012; Veronesi et al., 2010; Xiao, 1994). Infection occurs mainly with assemblages A, AI, B, BIV and E (Kostopoulou et al., 2015; Traub et al., 2005; Traversa et al., 2012; Veronesi et al., 2010). Although the prevalence of infection with *G. duodenalis* in horses is not related with age (Olson et al., 1997; Qi et al., 2015; Santín et al., 2013), foals are more prone to disease (Johnson et al., 1997; Veronesi et al., 2010; Xiao and Herd, 1994).

Prevalence rates of *G. duodenalis* in **pigs** range between 1% and 51% (Armson et al., 2009; Budu-Amoako et al., 2012; Farzan et al., 2011). Assemblages A, B, and E have commonly been detected from piglets in different areas (Armson et al., 2009; Budu-Amoako et al., 2012; Farzan et al., 2011; Langkjær et al., 2007; Minetti et al., 2014). However, also the canine-specific assemblages C and D and the feline-specific assemblage F have been occasionally isolated from pigs (Armson et al., 2009; Langkjær et al., 2007; Minetti et al., 2014). Infections are usually asymptomatic, however diarrhoea has occasionally been associated with the presence of assemblage E (Armson et al., 2009; Ryan and Zahedi, 2019).

Giardia spp. have been also isolated from other animal species, including **wild mammals**, **amphibians**, **reptiles**, **birds** and **fish**, causing asymptomatic or mild to severe disease (Feng and Xiao, 2011; Ryan and Zahedi, 2019). Beavers, chinchillas, muskrats, wild rodents, monkeys, deer, coyotes foxes, seals and dolphins are among the animals in which *Giardia* species have been detected (Appelbee et al., 2005; Chagas et al., 2019; Delport et al., 2014; Feng and Xiao, 2011; Helmy et al., 2018; Reboredo-Fernández et al., 2014; Zhang et al., 2020). Host-adapted species, such as *G*. *muris*, *G. microti*, *G. agilis*, *G. psittaci* and the *G. duodenallis* specific assemblages G and H have been commonly detected in these animals (Cacciò et al., 2018; Feng and Xiao, 2011; Ryan and Zahedi, 2019), as well as the zoonotic assemblages A (I,II

and III) and B (BIII and BIV) (Feng and Xiao, 2011; Li et al., 2017; Robertson et al., 2019a; Ryan and Zahedi, 2019; Zhang et al., 2020).

Also many **shellfish** (*i.e.* clams, oysters, mussels) have been documented to be contaminated with different *G. duodenalis* assemblages (Adell et al., 2014; Coupe et al., 2018; Giangaspero et al., 2014, 2007; Gómez-Couso et al., 2004, 2005; Schets et al., 2007; Tei et al., 2016), although prevalence rates of this parasite in such species are not known.

1.2.5 Molecular evidence of zoonotic potential

Although *Giardia* was recognized as a zoonotic pathogen over forty years ago by the WHO (WHO, 1979), direct evidence for zoonotic transmission is lacking, and thus its zoonotic importance remains under debate, mainly due to recent advances in genotyping (Ryan and Cacciò, 2013; Thompson, 2004). Molecular typing tools have been extensively used to study the complex epidemiology of giardiasis, with focus on its zoonotic transmission.

G. duodenalis assemblages A and B are associated with human infections. Protein polymorphisms of 23 loci revealed the presence of distinct sub-assemblages between assemblages A (sub-assemblage AI, AII, AIII & AIV) and B (sub-assemblage BI, BII, BIII & BIV) (Monis et al., 2003). Widely used markers for genetic characterization of *G. duodenalis* isolates are some of the well-conserved eukaryotic genes, such as the small subunit ribosomal RNA (SSU-rRNA) and the elongation factor 1-alfa (ef1-a), the conserved (although with divergent regions) glutamate dehydrogenase (gdh) and the triose phosphate (tpi) gene (Monis et al., 1999), or genes uniquely associated with *Giardia,* such as the β-giardin (bg) gene (Lalle et al., 2005b, 2005a).

Sub-assemblage AI is preferentially found in companion animals and livestock, sub-assemblage AII is mainly found in humans, while sub-assemblages AIII and AIV are almost exclusively isolated from animals. Human isolates revealed the presence of both sub-assemblages AI and AII, with AII most commonly detected (Feng and Xiao, 2011). Although only sub-assemblage AI was considered to be zoonotic and sub-assemblage AII to be human specific (Cacciò and Lalle, 2015; Ryan and Cacciò, 2013), many studies identified sub-assemblage AII in animals (e.g. cattle and dogs) (Adell-Aledón et al., 2018; Fantinatti et al., 2018; Gultekin et al., 2017; Kostopoulou et al., 2017; Ryan and Zahedi, 2019) and in both

humans and animals (Lee et al., 2017). Zoonotic transmission of sub-assemblage AI appears to be minimal in developed countries, but the situation is different in endemic regions, for example in localised foci where humans and animals live in close contact (Cacciò and Lalle, 2015). The assemblage AIII is almost exclusively detected in wild animals, particularly wild ruminants and is considered to be a host-adapted genotype (Cacciò et al., 2008; Lalle et al., 2007; Sprong et al., 2009). Multilocus sequencing and phylogenetic analysis, based on the analysis of the bg, gdh and tpi genes (but not the SSU-rRNA gene because of its highly conserved nature and the little intra-assemblage variability in both assemblages A and B), supported the previous findings (Cacciò et al., 2008; Lalle et al., 2007; Sprong et al., 2009). However, these markers show little variability between the subtypes of assemblage A and thus have a low resolution (Lebbad et al., 2011, 2010; Ryan and Cacciò, 2013).

Human isolates appeared to form two clusters (BIII and BIV) within assemblage B, whereas animal isolates belonged mainly to sub-assemblages BI and BII, when allozyme electrophoretic analysis of multiple loci was used as a tool of differentiation. Therefore, the zoonotic potential appears to be minimum (Monis et al., 2003). However, multilocus sequence analysis does not support the clustering of human and animal isolates into distinct clusters (Cacciò et al., 2008; Lasek-Nesselquist et al., 2009). Multilocus sequence analysis of assemblage B isolates revealed mixed sequencing profiles with ambiguous nucleotides or overlapping signals, and high genetic diversity. As a result, no clear genotypes have been inferred for assemblage B and consequently no distinction between zoonotic and host-adapted genotypes has been made (Cacciò et al., 2008; Sprong et al., 2009; Xiao and Feng, 2017).

The remaining *G. duodenalis* assemblages C, D, E and F have been found rarely in human cases. Assemblage C that is mainly isolated from dogs, was first reported in an immunocompromised adult from Egypt (Soliman et al., 2011). A severe giardiasis case, where assemblage C was detected, was reported in Slovakia (Štrkolcová et al., 2015). The dog-specific assemblage D was isolated from German tourists, who travelled in southern Asia (Broglia et al., 2013). Assemblage E was detected in a survey of a poor community in Brazil with limited drinking water supply, absence of a sewage network and contact with stray animals (Fantinatti et al., 2016). Moreover, it has been isolated from individuals with diarrhoea from urban and rural areas in Australia (Zahedi et al., 2017) and from children in

agricultural areas of Egypt (Abdel-Moein and Saeed, 2016). Importantly, assemblages A and E are close phylogenetically; assemblage A is more similar to assemblage E at the genome level than assemblage B (Brynildsrud et al., 2018; Franzén et al., 2009; Jerlström-Hultqvist et al., 2010). Moreover, recombination between assemblages A and E has been identified (Ankarklev et al., 2018; Xu et al., 2012) and this could be explained by the fact that often these assemblages co-infect sheep and goats (Ryan and Cacciò, 2013), thus the zoonotic potential of assemblage E cannot be excluded. Finally, the cat-specific assemblage F was detected in humans from rural and urban areas in Ethiopia (Gelanew et al., 2007) and children who lived in poor socio-economic conditions in Slovakia (Pipiková et al., 2020). Even though these findings demonstrate that assemblages C to F can indeed infect humans, thus suggesting less host-specificity, the fact that these infections are rare suggests that these findings should be interpreted with caution, as classification among the assemblages is influenced by the choice of markers (Cacciò et al., 2018).

The systematic use of more sensitive subtyping tools and, more importantly, whole genome sequencing approaches in epidemiological studies in both endemic and epidemic areas, will help us to better understand the complexity of the host-specificity and transmission cycles of *Giardia* (Ankarklev et al., 2018; Brynildsrud et al., 2018; Durigan et al., 2018; Lecová et al., 2019; Ryan and Cacciò, 2013).

1.3 Transmission

1.3.1 Routes and factors favouring transmission

Cryptosporidium and *Giardia* share common transmission routes. (Oo)cysts are the stages of the parasites that are transmitted from an infected host to a susceptible one by the faecal-oral route. Routes of transmission can be (a) human-to-human, (b) animal-to-animal, (c) human-to-animal, (d) animal-to-human, (e) waterborne and (f) foodborne (Fayer et al., 2000; Ryan et al., 2016b; Thompson, 2004). Also mechanical transmission through vectors, such as arthropods or birds has been reported (Fayer et al., 2000; Graczyk et al., 1999; Thompson et al., 2005).

Human-to-human transmission can occur after direct contact with infected individuals, especially in areas where sanitation standards are inadequate (e.g., in developing countries)

or in places such as day care centers where hygiene levels may be compromised (Fayer et al., 2000; Thompson, 2004). People can be also infected by animals, after direct contact with their manure in combination with lack of hygiene practices (e.g., hand washing) (Cacciò and Chalmers, 2016; Feng and Xiao, 2011; Heyworth, 2016; Ryan et al., 2016b). However, it is a matter of debate whether transmission between animals and humans occurs via direct contact or if the presence of a common contaminated source initiates infection (Baneth et al., 2016).

Indirect transmission of *Cryptosporidium* and *Giardia* through water or food is most common. Especially in cases of massive outbreaks of cryptosporidiosis or giardiasis, individuals became infected after accidental ingestion of parasites' (oo)cysts mostly via contaminated drinking water, but sometimes also from recreational water, or contaminated foodstuff (Efstratiou et al., 2017b; Ryan et al., 2019, 2018).

Numerous factors in the biology of *Cryptosporidium* and *Giardia* make them suitable for waterborne and foodborne transmission.

First, the (oo)cysts that are excreted into the environment are robust, fully infective and no further maturation or intermediate hosts are required (Ryan et al., 2019, 2018). Both parasites are resistant to common disinfectants (*i.e*., chlorine) (King and Monis, 2007; Korich et al., 1990; Peeters et al., 1989; Winiecka-Krusnell and Linder, 1998) and can survive under various environmental conditions for prolonged periods of time and still remain viable and infectious, especially in cool and damp areas (DeRegnier et al., 1989; Feng and Xiao, 2011; Olson et al., 1999; Peng et al., 2008; Robertson et al., 1992; Robertson and Gjerde, 2006). Interestingly infectious (oo)cysts of both parasites have been isolated from vegetables and fruits maintained in household refrigerators for several days (Hohweyer et al., 2016; Macarisin et al., 2010; Utaaker et al., 2017b). In general, *Cryptosporidium* oocysts are more environmentally resistant compared to *Giardia* cysts, while both are more vulnerable when temperature rises. In water and cattle faeces *Cryptosporidium* oocysts can remain viable for more than 12 weeks at -4 °C, and in soil for up to 10 weeks, while *Giardia* cysts remain infectious in these matrices for less than a week at -4 °C (Olson et al., 1999). On the contrary, both parasites are more vulnerable to higher temperatures (DeRegnier et al., 1989; Olson et al., 1999; Peng et al., 2008). *Cryptosporidium* oocysts remain infectious up to 10 weeks in

water and up to 4 weeks in soil and faeces at 25 °C, while *Giardia* cysts are viable up to 2 weeks in water and for a week in soil and faeces at the same temperature (Olson et al., 1999).

The fact that infected hosts have the ability to shed large numbers of (oo)cysts (up to billions), is one of the important factors contributing to the continuous environmental pressure with these parasites. For example, neonatal calves can excrete up to 30 billion *Cryptosporidium* oocysts over a 1-2 week period (Kuczynska and Shelton, 1999) and ruminants, at the peak of infection, can excrete up to 10^6 *G. duodenalis* cysts per gram of faeces (O'Handley and Olson, 2006; Xiao, 1994). Interestingly, it has been reported that infected humans may excrete up to 9.2 x 10^5 *Cryptosporidium* oocysts/mL (Goodgame et al., 1993) and up to 10^7 *Giardia* cysts/g faeces (Danciger and Lopez, 1975).

Another factor that facilitates the transmission of *Cryptosporidium* and *Giardia* is the low infectious dose, with 10-100 (oo)cysts (even the ingestion of a single *Giardia* cyst proved having a 2% probability of causing disease; Teunis et al., 1996) to be able to cause disease (DuPont et al., 1995; Okhuysen et al., 1999; Rendtorff, 1954).

Furthermore, the small size of the (oo)cysts, 4-7 µm in length for *Cryptosporidium* (Thompson et al., 2005) and 7-10 µm in length for *Giardia* (Adam, 2001), allows them to penetrate and survive water filters, such as sand filters, which are commonly used by the water industry for the production of drinking water (Ryan et al., 2019). They can be also filtered by shellfish through their feeding process and thus be concentrated and remain viable it their tissues (Robertson, 2007).

1.3.2 Waterborne transmission

Cryptosporidium oocysts and *Giardia* cysts can be transported easily to surface waters through different ways, such as drainage from manure storage areas, direct contamination by livestock and wild animals, agricultural runoffs, wastewater overflows, and non-functional sewage systems (Bodley-Tickell et al., 2002; Cacciò et al., 2003; Kistemann et al., 2012; Robertson et al., 2006b; Sischo et al., 2000; Slifko et al., 2000). Thus, different water matrices including surface waters, rivers, canals, lakes that are used for the production of drinking water can become contaminated with human, zoonotic or host-specific parasite species and assemblages that, in combination with insufficient drinking water treatment processes, may

lead to disease outbreaks. Climate change may also affect the distribution of *Cryptosporidium* oocysts and *Giardia* cysts in the environment. An increase in the number and force of extreme rainfall events will likely increase surface runoffs that may contain zoonotic species and assemblages of the parasites (Semenza et al., 2012). As a matter of fact, rainfall events have been documented in several studies being a major factor contributing to faecal contamination of water matrices (Atherholt et al., 1998; Dias et al., 2018; Fayer et al., 2002; Mons et al., 2009; Sischo et al., 2000).

Protozoan parasites along with liquid sewage from improperly arranged toilets, septic tanks and livestock farms penetrate into the soil and aquifers. (Oo)cysts, due to their biological characteristics mentioned above, may have the ability to contaminate also the underground water supplies, that are considered to be the most reliable in terms of sanitary. Under extreme events that confining strata of the underground water supplies are disrupted, the (oo)cysts can penetrate and contaminate these water supplies (Omarova et al., 2018).

Waterborne transmission of these parasites can also occur through contaminated water of recreational facilities, such as swimming and wading pools, thermal and other natural springs, fresh and marine waters, interactive fountains, and other venues where water contact or activities take place. Contamination in such facilities is often associated with accidental faecal contamination but can be also caused by poorly constructed or maintained plumbing, poor filtration systems and insufficient use of disinfectants. Swimming is one of the most popular recreational activities worldwide, with over 350 million persons-events estimated to take place annually only in the US. Sources of contamination in such cases are considered to be bathers themselves. The likelihood of a faecal accident at recreational facilities increases when these facilities are used by diapered children, toddlers and incontinent persons (Fayer, 2004).

1.3.3 Waterborne outbreaks

Several outbreaks of waterborne cryptosporidiosis and giardiasis have been documented (Baldursson and Karanis, 2011; Efstratiou et al., 2017b; Fayer, 2004; Karanis et al., 2007; McClung et al., 2018; Semenza and Nichols, 2007), often associated with high numbers of infections. Interestingly, most cryptosporidiosis and giardiasis waterborne outbreaks have been reported from developed countries where detection and monitoring systems have been

legislated. As these protozoan parasites are more likely to be endemic in less developed countries, where those infrastructures that are necessary for a safe drinking water supply (*i.e.*, intact sewage disposal system, effective catchment control measures and efficient water treatments) may be suboptimal, it is likely that these countries are at even greater risk of disease outbreaks. However, lack of detection and surveillance systems in less developed countries, both at the water treatment and public health level, makes detection of these parasites and vehicles of transmission difficult (Robertson, 2014, 2013). As a result, waterborne protozoan outbreaks have not yet been reported in Africa and South America. On the contrary, during 2011 to 2016, almost half (49%) of the reported waterborne parasitic protozoan outbreaks occurred in Australia and New Zealand (48% only in New Zealand), 41% in North America and only 9% of them have been documented in Europe. In Asia only 3 (1%) outbreaks were reported (Efstratiou et al., 2017b).

Until 2016, about 524 cryptosporidiosis and 344 giardiasis waterborne outbreaks had been reported worldwide (Baldursson and Karanis, 2011; Efstratiou et al., 2017b; Karanis et al., 2007), with more than 577,242 and 86,938 cases, respectively (Efstratiou et al., 2017b). Better surveillance systems and the application of standardized techniques for the detection of these parasitic protozoa, led to an increase in the number of the reported outbreaks during the last years. For example, during a period of more than a hundred years (from the start of the last century until 2004), 297 waterborne outbreaks of both parasites were reported (Karanis et al., 2007), while in a period of only 13 years (2004-2016), 571 outbreak reports were documented (Baldursson and Karanis, 2011; Efstratiou et al., 2017b).

The first documented waterborne cryptosporidiosis outbreak was in 1983 (Galbraith et al., 1987), while the largest outbreak was in Milwaukee, USA, in 1993. This single outbreak resulted in an estimated 400,000 infected individuals by *Cryptosporidium*, due to failure in the filtration process during the production of drinking water (MacKenzie et al., 1994). About 100 deaths and an estimated illness-associated cost of $96.2 million were the outcome of this large outbreak (Corso et al., 2003). The second largest outbreak of cryptosporidiosis occurred in 2010 in Östersund, Sweden, with an estimated 27,000 individuals infected with *C. hominis*, due to an ineffective municipal drinking water treatment plant (Widerström et al., 2014). One year later (2011), Sweden faced again a huge waterborne outbreak. About 20,000 individuals were infected with *C. hominis*, because drinking water production from surface water lacked

sufficient barriers for parasites (Bjelkmar et al., 2017; Guzman-Herrador et al., 2015; Rehn et al., 2015). *C. parvum* and *C. hominis* are responsible for most outbreaks (Efstratiou et al., 2017b; Xiao, 2010), with *C. hominis* being predominant, mostly in large waterborne outbreaks (Guzman-Herrador et al., 2015; Waldron et al., 2011; Widerström et al., 2014). *C. hominis* subtype families Ia (Cantey et al., 2012; Feng et al., 2012), Ib (Gertler et al., 2015; Mayne et al., 2011; Ng-Hublin et al., 2015; Waldron et al., 2011; Widerström et al., 2014) and Id (Cope et al., 2015) were identified in outbreaks where subtyping analysis was performed. *C. parvum* was also responsible for quite large numbers of infected individuals. In the most recent outbreak in Oregon, USA, in 2013, about 2,780 cases of gastrointestinal disease were reported, due to absence of filtration at the drinking water treatment facility. *C. parvum* subtype IIaA15G2R1 was identified as the aetiological agent (DeSilva et al., 2015).

Giardia has been also implicated in numerous waterborne outbreaks. In the United States, 74.8% of the 242 *Giardia* outbreaks that have been reported from 1971 to 2011, affecting around 41,000 people, were associated with water. Of them 74.6% were due to contaminated drinking water, while 18.2% were linked with recreational water (Adam et al., 2016). The largest giardiasis outbreak occurred in 1955 in Portland, where about 50,000 individuals were infected, probably because chlorination was the only treatment of the drinking water (Meyer, 1973; Veazie, 1969). In the USA also another great outbreak occurred, in 1977 in Berlin, New Hampshire, with 7,000 estimated cases (Lippy, 1978). The largest giardiasis outbreak since 2000, occurred in 2004, in Bergen, Norway, where approximately 48,000 people were exposed to contaminated drinking water. Out of them, 2,500 received medical treatment, whereas 1,300 cases were confirmed by laboratory analysis, with *G. duodenalis* assemblage B isolated from faecal samples (Nygård et al., 2006; Robertson et al., 2006a, 2006c). Assemblage B was responsible for most outbreaks investigated with subtyping tools (Daly et al., 2010; Karon et al., 2011; Nygård et al., 2006; Robertson et al., 2006c), whereas up to date only in one waterborne outbreak, in 1994 in Ontario, Canada, assemblage A was identified in stool samples (van Keulen et al., 2002). The main cause identified in most giardiasis outbreaks (over 75%) were deficiencies either a) in the processes for the production of drinking water (over 75%), including insufficient barriers and inadequate or poorly operated treatment and disinfection systems, or b) in the distribution system (over 12%). When these deficiencies

happen along with the presence of sufficient viable human infectious cysts in the water, massive waterborne outbreaks may occur (Robertson and Lim, 2011).

With the implementation of more stringent treatment of drinking water, the number of outbreaks due to contaminated drinking water is in decline in developed countries, and most outbreaks in the USA and Australia are now associated with recreational water (Painter et al., 2015; Ryan et al., 2017). From the beginning of the last century until 2004, *Cryptosporidium* was responsible for 50.3% of the outbreaks due to contaminated recreational water, while *Giardia* was responsible for about 13.6% of them. Cryptosporidiosis is considered the most frequently reported gastrointestinal disease in outbreaks associated with treated recreational water in the USA (Hlavsa et al., 2015; Yoder and Beach, 2007), with 4,232 cases and 183 hospitalizations during 2009-2017 (Gharpure et al., 2019). Swimming pools were mostly (64.1%) implicated in these outbreaks, and kiddie/wading pools (7.1%) and water playgrounds (6.4%) were also responsible (Gharpure et al., 2019). The largest recreational water outbreak occurred in 1995 at a water park in Georgia, USA, causing an estimated 5,449 cases after a probable faecal accident in the children's pool. Stool specimens of infected individuals were positive for both *Cryptosporidium* and *Giardia* (Levy et al., 1998). A year later, in 1996, about 3,000 individuals acquired cryptosporidiosis after being exposed to untreated water at a swimming pool and water from a jet-ski spray in California (Levy et al., 1998).

1.3.4 Foodborne transmission

Cryptosporidium oocysts and *Giardia* cysts have been recovered from a wide variety of ingredients and food products, including fruits (e.g., apples, strawberries) and vegetables (e.g., lettuce, kale, chicory, rocket, spinach, cilantro, dill, tomatoes, cabbage, onions) (Amorós et al., 2010; Caradonna et al., 2017; Dixon et al., 2013; Duedu et al., 2014; Dziedzinska et al., 2018; Hernández-Arango et al., 2019; Li et al., 2019; Mohamed et al., 2016; Robertson et al., 2002; Robertson and Gjerde, 2001a; Sakkas et al., 2020; Utaaker et al., 2017a), dairy products (e.g., milk) (Robertson and Chalmers, 2013), meat (Ryan et al., 2018; Yoshida et al., 2007) and shellfish (Adell et al., 2014; Coupe et al., 2018; Giangaspero et al., 2014; Gomez-Bautista et al., 2000; Gómez-Couso et al., 2005, 2004; Robertson, 2007; Robertson and Gjerde, 2008; Schets et al., 2007; Tei et al., 2016).

Fresh products, such as vegetables and fruits that are usually consumed raw, can become contaminated with *Cryptosporidium* and/or *Giardia* during the irrigation process or washing of produce with faecally contaminated water (Budu-Amoako et al., 2011; EFSA, 2018; Iqbal et al., 2015; Rafael et al., 2017; Ryan et al., 2019, 2018). Contamination of the fresh products can also occur during harvest, packaging, transport, food packing or preparation from infected food handlers, either ill or asymptomatic. Industrialization of food processing increases the likelihood of contamination, as food matrices are handled by several people and large amounts of the final product are distributed to the market. Thus, contaminated products can easily be distributed even in several batches of a product within a short period of time (Ryan et al., 2019, 2018). Poor personal hygiene of infected food handlers is a major issue contributing to the transmission of cryptosporidiosis and/or giardiasis (EFSA, 2018; Greig et al., 2007), as (oo)cysts of the parasites have been frequently detected in faecal samples and under their nails (Abdel-Dayem et al., 2014; Baswaid and Al-Haddad, 2008; Colli et al., 2015; Figgatt et al., 2017; Kheirandish et al., 2014; Kostopoulou, 2018; Saeed and Hamid, 2010; Takizawa et al., 2009; Zaglool et al., 2011). Insufficient washing of produce is also of importance. Moreover, *Cryptosporidium* oocysts are sticky and can survive on fresh fruits and leafy vegetables and as a result even vigorous washing does not always removes them (Macarisin et al., 2010).

Not only fruits and vegetables are important food products for the distribution of these parasites, but also shellfish are considered as a risk for disease outbreaks, as both parasites have been isolated from shellfish tissues (Coupe et al., 2018; Giangaspero et al., 2014; Pagoso and Rivera, 2017; Santos et al., 2018; Tei et al., 2016; Tryland et al., 2014; Willis et al., 2013). Marine bivalves have the ability to filter large volumes of water, between 20 and 100 L of water a day depending on species, and they commonly thrive in sheltered marine environments, usually near-shore, shallow, estuarine waters (e.g., around river estuaries, wastewater discharges) where there is a high concentration of nutrients, but also a potentially high concentration of protozoan parasites. Thus, transmission of *Cryptosporidium* and/or *Giardia* from shellfish to humans can occur, as they are usually consumed lightly cooked or even raw (Robertson, 2007; Ryan et al., 2019).

Other factors contributing to foodborne transmission of *Cryptosporidium* and *Giardia* include the global trade of foodstuff that leads to the rapid international distribution of products

(Robertson et al., 2014b), new dietary habits and trends, with the consumption of raw food, eating outside of the home (Broglia and Kapel, 2011; EFSA, 2018) and higher proportions of the population that are immunologically compromised due to an increase in individuals with immunosuppressive diseases and/or treatments and an increasingly elderly population (Newell et al., 2010; Torgerson et al., 2015).

1.3.5 Foodborne outbreaks

Only few foodborne cryptosporidiosis and giardiasis outbreaks have been reported, compared to waterborne outbreaks (Robertson and Chalmers, 2013; Ryan et al., 2019, 2018; Smith et al., 2007).

Out of the food matrices mentioned above, dairy products, fresh salads, unpasteurized milk and apple cider are the main food items implicated in foodborne cryptosporidiosis outbreaks (FAO/WHO, 2014; Gharpure et al., 2019; Ryan et al., 2018; Ursini et al., 2020). In the USA, for the period 2009-2017, 22 out of the 444 cryptosporidiosis outbreaks were foodborne, with unpasteurized milk (40.9%) and unpasteurized apple cider (18.2%) the most commonly associated food types (Gharpure et al., 2019). In most outbreaks for which genotyping was conducted (13 out of 16), *C. parvum* IIa and IId subtype families, which are commonly found in livestock (Xiao, 2010) were isolated and the causative agents were fresh salads (Åberg et al., 2015; Gherasim et al., 2012; Insulander et al., 2013, 2008; Johansen et al., 2015; McKerr et al., 2015; Rimšelienė et al., 2011), apple cider (Blackburn et al., 2006; Robertson et al., 2019b), goat milk (Rosenthal et al., 2015) and meat (Collier et al., 2011; Yoshida et al., 2007). *C. hominis* has been implicated in 2 outbreaks caused by infected food handlers (Ethelberg et al., 2009; Quiroz et al., 2000). Also *C. ubiquitum* has been identified in ozonated apple cider that caused a disease outbreak (Blackburn et al., 2006). Although zoonotic transmission appears to predominate in foodborne outbreaks, it is important to note that relatively few genotyping studies have been conducted on foodborne outbreaks and identification of those species does not necessarily indicate an animal origin of contamination. Thus, further molecular subtyping studies are needed in order to better understand the sources of contamination and transmission dynamics of foodborne cryptosporidiosis outbreaks (Ryan et al., 2018).

Up to date only 38 foodborne outbreaks of giardiasis have been reported, all of them in the USA (Adam et al., 2016). Only few have been investigated thoroughly and genotyping analysis has been performed for only 2 outbreaks. Infected food handlers were considered to be the source of infection, however the implicated food matrix could not be determined. *G. duodenalis* assemblage B (sub-assemblage BIII for the outbreak reported by Figgatt et al., 2017) has been identified as the causative agent in both outbreaks (Feng and Xiao, 2011; Figgatt et al., 2017; Sulaiman et al., 2003). For most giardiasis foodborne outbreaks the vehicle of infection was not identified, but different vegetables (Mintz et al., 1993; Robertson, 2013; Rose and Slifko, 1999; Ryan et al., 2019), salads (e.g., lettuce-based salads, noodle salad) (Petersen et al., 1988; Robertson, 2013; Ryan et al., 2019), fruits (Porter et al., 1990), dairy products (Robertson, 2013; Ryan et al., 2019), shellfish (Ryan et al., 2019; Smith-DeWaal et al., 2001) and salmon (Osterholm et al., 1981) have been listed for others. Interestingly, the source in all documented giardiasis outbreaks was infected food handlers (asymptomatic or not).

Most foodborne outbreaks are never recognized, or if recognized they are poorly investigated, and often go unreported (Robertson, 2014). This is likely due to lack of appropriate tools for detection of *Cryptosporidium* and *Giardia* (oo)cysts in food matrices. Only recently the International Standard Method (ISO) 18744 (ISO, 2016) for the detection and enumeration of *Cryptosporidium* oocysts and *Giardia* cysts on/in leafy green vegetables and berry fruits, has been developed. However, this method does not include any molecular analysis that is essential in tracking transmission of foodborne cryptosporidiosis and/or giardiasis (Ryan et al., 2018, 2016b). Also, the low numbers of (oo)cysts that may be present in foodstuffs and the differences in food matrices require the development of food-specific detection methods (Cacciò and Lalle, 2015). Under-reporting and under-diagnosis is also due to the fact that illness due to foodborne parasites appears more sporadically and commonly involves a single household or only a few individuals, thus these cases are not recognized as being part of an outbreak (Briggs et al., 2014; Ryan et al., 2019; Smith et al., 2007). In addition, food matrices will also no longer be available for analysis when the foodborne outbreak occurs or, if so, it is usually difficult for the individuals to remember all types of food consumed during the incubation period of the infection (van de Venter et al., 2015), taking into account

the long incubation periods of both cryptosporidiosis (up to 12 days or more) and giardiasis (1-3 weeks) (Cacciò and Lalle, 2015; Ryan et al., 2018).

1.4 Detection methods for water and food matrices

Detection of contamination with *Cryptosporidium* and *Giardia* of water or food relies on isolation and identification of the (oo)cysts or DNA of the (oo)cysts in or on the contamination vehicle. Furthermore, the number of (oo)cysts that are usually concentrated from contaminated water or food matrix are low, and thus the detection is much more difficult than in faecal samples (Robertson, 2014, 2013).

1.4.1 Water analysis

Access to safe drinking water is a fundamental human right and as *Cryptosporidium* and *Giardia* are of great importance for public health, research activity has been focused for many years on the standardization of methods for detecting these parasitic protozoa in water samples. For this reason, standardized detection methods have been available for many years.

The most commonly used methods are the U.S Environmental Protection Agency Method 1623 (US EPA, 2005) and its updated edition 1623.1 (US EPA, 2012): *Cryptosporidium* and *Giardia* in water by Filtration/IMS/FA, and the ISO 15553 (ISO, 2006): Isolation and identification of *Cryptosporidium* oocysts and *Giardia* cysts from water. In both methods first a relatively large volume of water (10-1000 L) is concentrated by filtration (flocculation and sedimentation are also used, but less frequently) and the concentrated particles that are approximately the size of *Cryptosporidium* oocysts and *Giardia* cysts or larger, are eluted from the filter into a smaller volume and further concentrated with centrifugation. Then the (oo)cysts are concentrated using immunomagnetic separation (IMS), dissociated from the beads and dried on microscope slides (usually 50 µl of the IMS eluate on every slide) for IFAT detection. The (oo)cysts are labelled on the slides using a monoclonal antibody (mAb) fluorescent marker, mostly the fluorescein isothiocyanate (FITC). Sometimes a nucleic acid stain, usually the 4'6 diamindino-2-phenyl indole (DAPI) is further used, in order to increase the identification of the (oo)cysts. Finally, the sample is examined by fluorescence microscopy (immunofluorescent antibody testing; IFAT) for the presence of the labelled (oo)cysts and if

DAPI stain has been added, also differential interference contrast (DIC) microscopy is used to assist in identification. When viability assessment of the (oo)cysts is required, staining in suspension with vital dyes, such as DAPI and propidium iodine (PI) is preferred (Robertson et al., 2014a).

While IMS is most commonly used for isolation, it is an expensive method, and other cheaper separation techniques, such as density gradient flotation, are sometimes used (Robertson, 2014, 2013). An alternative method that combines both concentration and detection steps, is flow cytometry (fluorescent-activated cell sorting) (Keserue et al., 2011). However, the high cost of equipment that is required, the need of skilled laboratory personnel, along with the low specificity (because of the presence of the other particles of the same size at the matrix that may cross-react) and thus the potential of false positives, are among the disadvantages of this method that does not allow its wide acceptance and application. On the other hand, the combination of flow cytometry with IMS (increasing at the same time the cost of the method), may have a potential for this technique to become more sensitive and reliable for detection *Cryptosporidium* and *Giardia* (oo)cysts in water samples (Keserue et al., 2012).

Even though these methods result in high recovery efficiencies, although variable (<10% and >80%) according to the matrix tested (Efstratiou et al., 2017a), they do not provide information regarding species/assemblages and subtypes of the parasites, which is essential for understanding transmission dynamics and unravel outbreak cases (Ryan et al., 2019, 2016b). Thus molecular tools ,i.e. polymerase chain reaction (PCR)-based methods, are also applied for detecting contamination in water samples, providing important information on the genotypic profile of the parasites isolated and quantification of the contamination in the relevant transmission vehicle (Cacciò et al., 2005; Smith et al., 2006). DNA extraction of the (oo)cysts for molecular identification can be performed either before (oo)cysts are dissociated from IMS beads or after their enumeration on microscope slides by scraping the slides (Robertson et al., 2009; Smith and Nichols, 2010; Sunnotel et al., 2006).

For both parasites multi-copy genes are widely used for detection in water samples, due to the higher sensitivity to detect contamination even when low numbers of (oo)cysts are present (Smith and Nichols, 2010). For this reason, the multi-copy conserved SSU rRNA gene is most commonly used for both parasites (Xiao, 2010; Xiao and Feng, 2017). The hsp70 and the COWP genes are popular markers for the detection of *Cryptosporidium* (Sulaiman et al.,

2000; Xiao et al., 2000), while also the actin gene has been used (Sulaiman et al., 2002). Finally, the gp60 gene is commonly used for subtyping analysis of the isolates, because of its sequence heterogeneity and relevance to parasite biology (Xiao, 2010). Where possible, a multilocus approach to characterizing *Cryptosporidium* isolates should be used to increase the accuracy of identification (Cacciò et al., 2005; Smith et al., 2006), as many single copy loci may not have sufficient sensitivity to detect low numbers (<10) of oocysts that usually are present in environmental samples (Smith and Nichols, 2010). For *Giardia* identification, apart from the SSU rRNA gene, the bg, tpi and gdh genes are commonly used, also for sub-assemblage classification, whereas the ef1-a, the variable surface protein (vsp), the *G. lamblia* open reading frame-C4 (GLORF-C4) and the internal transcribed spacers (ITS) of ribosomal DNA are more rarely used (Koehler et al., 2014).

Nested PCRs targeting the previously mentioned genes followed by sequencing for species/genotypes identification of parasites in different water matrices have been widely used (Almeida et al., 2015; Castro-Hermida et al., 2015; Ćirković et al., 2020; Ehsan et al., 2015b, 2015a; Prystajecky et al., 2014; Stokdyk et al., 2019; Ulloa-Stanojlović et al., 2016) and are preferred than conventional PCRs, as they offer increased sensitivity (Adeyemo et al., 2018). Also analyses of PCR-restriction fragment length polymorphisms (RFLP) has been used as an alternative option for classification into (sub)species/(sub)assemblages of the parasites in environmental samples (Ćirković et al., 2020; El-Latif et al., 2020; Xiao et al., 2001). However as environmental samples are more likely to contain more mixtures of (sub)species/(sub)assemblages of both parasites than clinical samples, the usefulness of this approach is limited (Smith et al., 2006; Smith and Nichols, 2010). Other approaches, such as multiplex TaqMan-probe based qPCR assays (Guy et al., 2003; Li et al., 2010) and next generation sequencing (Xiao and Feng, 2017; Zahedi et al., 2019) may be promising for use in environmental samples, although with increased cost.

Another drawback of using only molecular analyses for the detection of contamination in environmental samples (instead of using a combination of IFAT with molecular analyses) is the presence of non-nucleated parasites in these samples that will remain undetected. Even though the presence of non-nucleated *Cryptosporidium* and *Giardia* (oo)cysts is of no public health importance, they indicate that the material being investigated was contaminated with these parasitic protozoa. Finally the presence of various PCR inhibitors in environmental

samples are a major issue, meaning that adjusted PCR conditions for every matrix tested are required (Robertson, 2014, 2013). Alternatively, other PCR-based methods that are not sensitive to inhibitors, such as the droplet digital PCR (ddPCR) or the loop mediated isothermal amplification (LAMP) could be appropriate (Gallas-Lindemann et al., 2016; Yang et al., 2014).

1.4.2 Food analysis

In principal, the approach to analyse food matrices for contamination with *Cryptosporidium* oocysts and *Giardia* cysts is the same as the one used for water. However, unlike water samples, filtration of large volumes is not possible (also filtration of colloidal liquids such as milk is impractical), meaning that a relatively smaller (in terms of portion size) amount of product can be analysed. Moreover, one other difficulty in analysing food matrices, is the physical and biochemical nature of the different food products, that can influence the removal of the parasites during elution. Consequently, variations in both physical and biochemical characteristics of different foodstuff, from fruits and vegetables to shellfish, dairy products and meat, do not allow the application of a unique method for all the different food matrices. However, immunofluorescence remains the preferred method for the detection of the (oo)cysts of the parasites on/in food matrices (Robertson, 2014, 2013). PCR methods, that are considered to be more sensitive for detection, have been also used for the detection of (oo)cysts in different food samples. However the presence of inhibitors in foodstuff and the fact that they may sometimes detect only the parasite's DNA and not intact (oo)cysts (thus overestimating the infection risk), are important limitations for their solely use (Ryan et al., 2019, 2018). Similar to water analysis, methods less sensitive to PCR inhibitors, such as ddPCR or LAMP could be promising tools for detection of *Cryptosporidium* and *Giardia* in food samples (Gallas-Lindemann et al., 2016; Yang et al., 2014). Another important aspect for food analyses, is that current detection methods do not provide information on (oo)cysts viability. Currently applied methods for assessing viability are not entirely adjusted to be used in food matrices, however the use of simple vital dye inclusion/exclusion assays (that sometimes overestimate the viability) along with molecular methods for species/genotypes identification could be reliably applied (Rousseau et al., 2018).

As mentioned above, most foodborne cryptosporidiosis and giardiasis outbreaks have been related with the consumption of **fruits** or **vegetables** (Ryan et al., 2019, 2018), and thus research interest has been focused on the standardization of an analytical method to detect *Cryptosporidium* and *Giardia* on/in these products. Recently, the ISO method 18744 (ISO, 2016) for detection and enumeration of these parasites in fresh leafy green vegetables and berry fruits, has been released. This method is based on the standard water protocol (ISO 15553, 2006) or variations of this method (Cook et al., 2007; Robertson and Gjerde, 2001b, 2000). First, elution of the produce into a specified medium is being performed, either through agitation of the produce by shaking or by stomaching with a stomacher. Then the eluate is concentrated by centrifugation, the parasites are isolated using IMS and finally they are detected with IFAT. Although this method has been developed for analysis of leafy vegetables and berry fruits, it has also been used for other varieties of fruits and vegetables including baby sweet corn, carrots, chillies, asparagus, peppers, parsley, dill, leeks, mushrooms, onions, tomatoes and sprouted seeds of different varieties (Robertson, 2013). However, as there are many differences in the biochemical composition and the structure of the different vegetables or berries, this method has to be further validated for other varieties (Chalmers et al., 2020). Furthermore, one important limitation of the ISO method, is that because it involves determination of the recovery efficiency by spiking (oo)cysts into the samples, genetic characterisation cannot be performed. This drawback of the method is crucial and needs to be refined along with the absence of viability assessment, as these are important information to determine public health risk (Ryan et al., 2019). PCR detection methods, targeting the genes widely used for other matrices (i.e. water and faeces), have been also used for the detection of these parasites in fresh vegetables and fruits, assessing also the parasites species/genotypes (Dixon et al., 2013; Dziedzinska et al., 2018; Hohweyer et al., 2016; Hong et al., 2014; Li et al., 2019; Ramirez-Martinez et al., 2015; Tiyo et al., 2016; Utaaker et al., 2017a). Recently, a multiplex PCR assay has been generated for the simultaneous detection of both parasites, as well as other protozoan parasites (i.e. *Cyclospora cayetanensis* and *Toxoplasma gondii*) on leafy greens, although without species/assemblages identification (Shapiro et al., 2019).

Although only one small outbreak of giardiasis (Smith-DeWaal et al., 2001) and no cryptosporidiosis outbreak has been associated with bivalve **shellfish** consumption, this food

matrix is recognized as a potential transmission vehicle. As a result, different research groups have tried to develop standardized methods for analysing this product (Robertson and Gjerde, 2008; Schets et al., 2013), based again on protocols used for water analysis. However a standardized method has yet to be described (Robertson, 2007). In principal, homogenized tissues (gills, glands, intestinal tracts), haemolymph or whole shellfish first undergo a procedure for lipid removal (i.e. sieving, pepsin-digestion), then the (oo)cysts are concentrated (frequently with centrifugation), isolated/purified (i.e. with IMS, flotation on caesium chloride or sucrose gradient or lipid extraction) and are finally detected with an IFAT. Although IFAT is the preferred detection method used by most researchers, some research groups have used other techniques or combined IFAT with other techniques such as fluorescent *in situ* hybridization (FISH) and PCR (Robertson, 2014, 2013) or multiplex PCR (Marangi et al., 2015). As already mentioned, PCR based methods still are not the methods of choice for detection of parasite (oo)cysts, due to different limitations, such as PCR inhibitors in the matrix and presence of non-nucleated (oo)cysts that will remain undetected.

There have been few reported cryptosporidiosis outbreaks due to **beverages**, specifically apple cider (ozonated or fresh) (Blackburn et al., 2006; Millard et al., 1994; Robertson et al., 2019b; Ryan et al., 2018) and milk (Gelletlie et al., 1997; Harper et al., 2002; Robertson and Chalmers, 2013; Rosenthal et al., 2015; Ryan et al., 2018; Soave et al., 1989; Ursini et al., 2020), and thus there has been some research directed towards the development of analytical methods for detection of contamination with *Cryptosporidium* oocysts in beverages. However, there are currently no widely used protocols or a standardized ISO method. Sucrose flotation proved more efficient in recovering *Cryptosporidium* oocysts from apple cider, compared to formalin-ethyl acetate sedimentation, followed by IMS concentration and detection with IFAT (Deng and Cliver, 2000). However, in an outbreak of cryptosporidiosis due to contaminated apple cider, direct centrifugation for the concentration of the oocysts was used in combination with molecular detection (Blackburn et al., 2006). In a more recent outbreak, the apple juice was first concentrated with centrifugation, then IMS was performed and the oocysts were detected with IFAT (Robertson et al., 2019b). Also centrifugation of fruit juices, followed by Sheather's sugar flotation and staining with modified Ziehl-Neelsen were used, but validation data of the method (i.e., recovery efficiency and detection limits) were not provided (Mossallam, 2010). Analysis of dairy products has

been performed on liquid milk, while yogurt and ice cream were only analysed in one study (Deng and Cliver, 1999). The analyses are based first on different approaches for defatting the sample, using either ether, trypsin or detergents, followed by IMS, flotation or microfiltration (Chalmers et al., 2020), with the addition of IMS resulting in higher recovery efficiencies (Deng et al., 2000; Deng and Cliver, 1999; Di Pinto and Tantillo, 2002) and detection by IFAT or PCR (Chalmers et al., 2020). As no giardiasis outbreaks associated with beverages (apart from water) have been reported, only limited research (Mossallam, 2010; Rai et al., 2008) has been focused on the standardization of methods for detection of *Giardia* cysts in these products. However, it is likely that methods similar to those used for *Cryptosporidium* could be adapted for analysis of samples for *Giardia* (Robertson, 2013).

References

Abdel-Dayem, M., Al Zou'bi, R., Hani, R.B., Arm, Z.S., 2014. Microbiological and parasitological investigation among food handlers in hotels in the Dead Sea area, Jordan. J. Microbiol. Immunol. Infect. 47, 377–380. https://doi.org/10.1016/j.jmii.2013.06.001

Abdel-Moein, K.A., Saeed, H., 2016. The zoonotic potential of *Giardia intestinalis* assemblage E in rural settings. Parasitol. Res. 115, 3197–3202. https://doi.org/10.1007/s00436-016-5081-7

Åberg, R., Sjöman, M., Hemminki, K., Pirnes, A., Räsänen, S., Kalanti, A., Pohjanvirta, T., Cacciò, S.M., Pihlajasaari, A., Toikkanen, S., Huusko, S., Rimhanen-Finne, R., 2015. *Cryptosporidium parvum* caused a large outbreak linked to frisée salad in Finland, 2012. Zoonoses Public Health 62, 618–624. https://doi.org/10.1111/zph.12190

Abeywardena, H., Jex, A.R., Gasser, R.B., 2015. A perspective on *Cryptosporidium* and *Giardia*, with an emphasis on bovines and recent epidemiological findings, in: Rollinson, D., Stothard, J.D. (Eds.), Adv Parasitol. Elsevier Ltd, pp. 243–301. https://doi.org/10.1016/bs.apar.2015.02.001

Adam, E.A., Yoder, J.S., Gould, L.H., Hlavsa, M.C., Gargano, J.., 2016. Giardiasis outbreaks in the United States, 1971-2011. Epidemiol. Infect. 144, 2790–2801. https://doi.org/10.1017/S0950268815003040.

Adam, R.D., 2001. Biology of *Giardia lamblia*. Clin. Microbiol. Rev. 14, 447–475. https://doi.org/10.1128/CMR.14.3.447-475.2001

Adell-Aledón, M., Köster, P.C., de Lucio, A., Puente, P., Hernández-de-Mingo, M., Sánchez-Thevenet, P., Dea-Ayuela, M.A., Carmena, D., 2018. Occurrence and molecular epidemiology of *Giardia duodenalis* infection in dog populations in eastern Spain. BMC Vet. Res. 14, 1–11. https://doi.org/10.1186/s12917-018-1353-z

Adell, A.D., Smith, W.A., Shapiro, K., Melli, A., Conrad, P.A., 2014. Molecular epidemiology of *Cryptosporidium* spp. and *Giardia* spp. in mussels (*Mytilus californianus*) and California sea lions (*Zalophus californianus*) from central California. Appl. Environ. Microbiol. 80, 7732–7740. https://doi.org/10.1128/AEM.02922-14

Adeyemo, F.E., Singh, G., Reddy, P., Stenström, T.A., 2018. Methods for the detection of *Cryptosporidium* and *Giardia*: from microscopy to nucleic acid based tools in clinical and environmental regimes. Acta Trop. 184, 15–28. https://doi.org/10.1016/j.actatropica.2018.01.011

Akiyoshi, D.E., Tumwine, J.K., Bakeera-Kitaka, S., Tzipori, S., 2006. Subtype analysis of *Cryptosporidium* isolates from children in Uganda. J. Parasitol. 92, 1097–1100. https://doi.org/10.1645/ge-843r.1

Allain, T., Buret, A.G., 2020. Pathogenesis and post-infectious complications in giardiasis. Adv. Parasitol. 107, 173–199. https://doi.org/10.1016/bs.apar.2019.12.001

Almeida, J.C., Martins, F.D.C., Ferreira Neto, J.M., Santos, M.M. dos, Garcia, J.L., Navarro, I.T., Kuroda, E.K., Freire, R.L., Almeida, J.C., Martins, F.D.C., Ferreira Neto, J.M., Santos, M.M. dos, Garcia, J.L., Navarro, I.T., Kuroda, E.K., Freire, R.L., 2015. Occurrence of *Cryptosporidium* spp. and *Giardia* spp. in a public water-treatment system, Paraná, Southern Brazil. Rev. Bras. Parasitol. Veterinária 24, 303–308. https://doi.org/10.1590/S1984-29612015051

Alves, M., Xiao, L., Antunes, F., Matos, O., 2006. Distribution of *Cryptosporidium* subtypes in humans and domestic and wild ruminants in Portugal. Parasitol. Res. 99, 287–292. https://doi.org/10.1007/s00436-006-0164-5

Alves, M., Xiao, L., Sulaiman, I., Lal, A.A., Matos, O., Antunes, F., 2003. Subgenotype analysis of *Cryptosporidium* isolates from humans, cattle, and zoo ruminants in Portugal. J. Clin. Microbiol. 41, 2744–2747. https://doi.org/10.1128/jcm.41.6.2744-2747.2003

Amorós, I., Alonso, J.L., Cuesta, G., 2010. *Cryptosporidium* oocysts and *Giardia* cysts on salad products irrigated with contaminated water. J. Food Prot. 73, 1138–1140.

Ankarklev, J., Jerlström-Hultqvist, J., Ringqvist, E., Troell, K., Svärd, S.G., 2010. Behind the smile: cell biology and disease mechanisms of *Giardia* species. Nat. Rev. Microbiol. 8, 413–422. https://doi.org/10.1038/nrmicro2317

Ankarklev, J., Lebbad, M., Einarsson, E., Franzén, O., Ahola, H., Troell, K., Svärd, S.G., 2018. A novel high-resolution multilocus sequence typing of *Giardia intestinalis* assemblage A isolates reveals zoonotic transmission, clonal outbreaks and recombination. Infect. Genet. Evol. 60, 7–16. https://doi.org/10.1016/j.meegid.2018.02.012

Appelbee, A.J., Thompson, R.C.A., Olson, M.E., 2005. *Giardia* and *Cryptosporidium* in mammalian wildlife - current status and future needs. Trends Parasitol. 21, 370–376. https://doi.org/10.1016/j.pt.2005.06.004

Armson, A., Yang, R., Thompson, J., Johnson, J., Reid, S., Ryan, U.M., 2009. *Giardia* genotypes in pigs in Western Australia: prevalence and association with diarrhea. Exp. Parasitol. 121, 381–383. https://doi.org/10.1016/j.exppara.2009.01.008

Atherholt, T.B., LeChevallier, M.W., Norton, W.D., Rosen, J.S., 1998. Effect of rainfall on *Giardia* and *Crypto*. J. / Am. Water Work. Assoc. 90, 66–80. https://doi.org/10.1002/j.1551-8833.1998.tb08499.x

Aydin, A.F., Besirbellioglu, B.A., Avci, I.Y., Tanyuksel, M., Araz, E., Pahsa, A., 2004. Classification of *Giardia duodenalis* parasites in Turkey into groups A and B using restriction fragment length polymorphism. Diagn. Microbiol. Infect. Dis. 50, 147–151. https://doi.org/10.1016/j.diagmicrobio.2004.06.001

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

Ballweber, L.R., Xiao, L., Bowman, D.D., Kahn, G., Cama, V.A., 2010. Giardiasis in dogs and cats: update on epidemiology and public health significance. Trends Parasitol. 26, 180–189. https://doi.org/10.1016/j.pt.2010.02.005

Baneth, G., Thamsborg, S.M., Otranto, D., Guillot, J., Blaga, R., Deplazes, P., Solano-Gallego, L., 2016. Major parasitic zoonoses associated with dogs and cats in Europe. J. Comp. Pathol. 155, S54–S74. https://doi.org/10.1016/j.jcpa.2015.10.179

Barrera, J.P., Carmena, D., Rodríguez, E., Checa, R., López, A.M., Fidalgo, L.E., Gálvez, R., Marino, V., Fuentes, I., Miró, G., Montoya, A., 2020. The red fox (*Vulpes vulpes*) as a potential natural reservoir of human cryptosporidiosis by *Cryptosporidium hominis* in Northwest Spain. Transbound. Emerg. Dis. https://doi.org/10.1111/tbed.13569

Barutzki, D., Schaper, R., 2011. Results of parasitological examinations of faecal samples from cats and dogs in Germany between 2003 and 2010. Parasitol. Res. 109, 45–60. https://doi.org/10.1007/s00436-011-2402-8

Baswaid, S.H., Al-Haddad, A., 2008. Parasitic infections among restaurant workers in Mukalla (Hadhramout/Yemen). Iran. J. Parasitol. 3, 37–41.

Becher, K.A., Robertson, I.D., Fraser, D.M., Palmer, D.G., Thompson, R.C.A., 2004. Molecular epidemiology of *Giardia* and *Cryptosporidium* infections in dairy calves originating from three sources in Western Australia. Vet. Parasitol. 123, 1–9. https://doi.org/10.1016/j.vetpar.2004.05.020

Bjelkmar, P., Hansen, A., Schönning, C., Bergström, J., Löfdahl, M., Lebbad, M., Wallensten, A., Allestam, G., Stenmark, S., Lindh, J., 2017. Early outbreak detection by linking health advice line calls to water distribution areas retrospectively demonstrated in a large waterborne outbreak of cryptosporidiosis in Sweden. BMC Public Health 17, 328. https://doi.org/10.1186/s12889-017-4233-8

Blackburn, B.G., Mazurek, J.M., Hlavsa, M., Park, J., Tillapaw, M., Parrish, M.K., Salehi, E., Franks, W., Koch, E., Smith, F., Xiao, L., Arrowood, M., Hill, V., Da Silva, A., Johnston, S., Jones, J.L., 2006. Cryptosporidiosis associated with ozonated apple cider. Emerg. Infect. Dis. 12, 684–686. https://doi.org/10.3201/eid1204.050796

Bodley-Tickell, A.T., Kitchen, S.E., Sturdee, A.P., 2002. Occurrence of *Cryptosporidium* in agricultural surface waters during an annual farming cycle in lowland UK. Water Res. 36, 1880–1886.

Bouzid, M., Hunter, P.R., Chalmers, R.M., Tyler, K.M., 2013. *Cryptosporidium* pathogenicity and virulence. Clin. Microbiol. Rev. 26, 115–134. https://doi.org/10.1128/CMR.00076-12

Bowman, D.D., Lucio-Forster, A., 2010. Cryptosporidiosis and giardiasis in dogs and cats: Veterinary and public health importance. Exp. Parasitol. 124, 121–127. https://doi.org/10.1016/j.exppara.2009.01.003

Braeye, T., De Schrijver, K., Wollants, E., Van Ranst, M., Verhaegen, J., 2015. A large community outbreak of gastroenteritis associated with consumption of drinking water contaminated by river water, Belgium, 2010. Epidemiol. Infect. 143, 711–719. https://doi.org/10.1017/S0950268814001629

Breathnach, A.S., McHugh, T.D., Butcher, P.D., 2010. Prevalence and clinical correlations of genetic subtypes of *Giardia lamblia* in an urban setting. Epidemiol. Infect. 138, 1459–1467. https://doi.org/10.1017/S0950268810000208

Briggs, A.D.M., Boxall, N.S., Van Santen, D., Chalmers, R.M., McCarthy, N.D., 2014. Approaches to the detection of very small, common, and easily missed outbreaks that together contribute substantially to human *Cryptosporidium* infection. Epidemiol. Infect. 142, 1869–1876. https://doi.org/10.1017/S0950268814000673

Broglia, A., Kapel, C., 2011. Changing dietary habits in a changing world: Emerging drivers for the transmission of foodborne parasitic zoonoses. Vet. Parasitol. 182, 2–13. https://doi.org/10.1016/j.vetpar.2011.07.011

Broglia, A., Weitzel, T., Harms, G., Cacciò, S.M., Nöckler, K., 2013. Molecular typing of *Giardia duodenalis* isolates from German travellers. Parasitol. Res. 112, 3449–3456. https://doi.org/10.1007/s00436-013-3524-y

Brynildsrud, O., Tysnes, K.R., Robertson, L.J., Debenham, J.J., 2018. *Giardia duodenalis* in primates: classification and host specificity based on phylogenetic analysis of sequence data. Zoonoses Public Health 65, 637–647. https://doi.org/10.1111/zph.12470

Budu-Amoako, E., Greenwood, S.J., Dixon, B.R., Barkema, H.W., Hurnik, D., Estey, C., McClure, J.T., 2012. Occurrence of *Giardia* and *Cryptosporidium* in pigs on Prince Edward Island, Canada. Vet. Parasitol. 184, 18–24. https://doi.org/10.1016/j.vetpar.2011.07.047

Budu-Amoako, E., Greenwood, S.J., Dixon, B.R., Barkema, H.W., McClure, J.T., 2011. Foodborne illness associated with *Cryptosporidium* and *Giardia* from livestock. J. Food Prot. 74, 1944–1955. https://doi.org/10.4315/0362-028X.JFP-11-107

Cacciò, S.M., Beck, R., Lalle, M., Marinculic, A., Pozio, E., 2008. Multilocus genotyping of *Giardia duodenalis* reveals striking differences between assemblages A and B. Int. J. Parasitol. 38, 1523–1531. https://doi.org/10.1016/j.ijpara.2008.04.008

Cacciò, S.M., Chalmers, R.M., 2016. Human cryptosporidiosis in Europe. Clin. Microbiol. Infect.

22, 471–480. https://doi.org/10.1016/j.cmi.2016.04.021
Cacciò, S.M., De Giacomo, M., Aulicino, F.A., Pozio, E., 2003. *Giardia* cysts in wastewater treatment plants in Italy. Appl. Environ. Microbiol. 69, 3393–3398. https://doi.org/10.1128/AEM.69.6.3393-3398.2003
Cacciò, S.M., Lalle, M., 2015. Giardia, in: Xiao, L., Ryan, U., Feng, Y. (Eds.), Biology of Foodborne Parasites. CRP Press, USA, pp. 175–193.
Cacciò, S.M., Lalle, M., Svärd, S.G., 2018. Host specificity in the *Giardia duodenalis* species complex. Infect. Genet. Evol. 66, 335–345. https://doi.org/10.1016/j.meegid.2017.12.001
Cacciò, S.M., Ryan, U., 2008. Molecular epidemiology of giardiasis. Mol. Biochem. Parasitol. 160, 75–80. https://doi.org/10.1016/j.molbiopara.2008.04.006
Cacciò, S.M., Thompson, R.C.A., McLauchlin, J., Smith, H. V., 2005. Unravelling *Cryptosporidium* and *Giardia* epidemiology. Trends Parasitol. 21, 430–437. https://doi.org/10.1016/j.pt.2005.06.013
Cama, V.A., Ross, J.M., Crawford, S., Kawai, V., Chavez-Valdez, R., Vargas, D., Vivar, A., Ticona, E., Ñavincopa, M., Williamson, J., Ortega, Y., Gilman, R.H., Bern, C., Xiao, L., 2007. Differences in clinical manifestations among *Cryptosporidium* species and subtypes in HIV-infected persons. J. Infect. Dis. 196, 684–691. https://doi.org/10.1086/519842
Cantey, P.T., Kurian, A.K., Jefferson, D., Moerbe, M.M., Marshall, K., Blankenship, W.R., Rothbarth, G.R., J., H., Hall, R., Yoder, J., Brunkard, J., Johnston, S., Xiao, L., Hill, V.R., Sarisky, J., Zarate-Bermudez, M.A., Otto, C., Hlavsa, M.C., 2012. Outbreak of cryptosporidiosis associated with a man-made chlorinated lake - Tarrant County, Texas, 2008. J. Environ. Health 75, 14–19.
Caradonna, T., Marangi, M., Del Chierico, F., Ferrari, N., Reddel, S., Bracaglia, G., Normanno, G., Putignani, L., Giangaspero, A., 2017. Detection and prevalence of protozoan parasites in ready-to-eat packaged salads on sale in Italy. Food Microbiol. 67, 67–75. https://doi.org/10.1016/j.fm.2017.06.006
Carter, B.L., Stiff, R.E., Elwin, K., Hutchings, H.A., Mason, B.W., Davies, A.P., Chalmers, R.M., 2019. Health sequelae of human cryptosporidiosis—a 12-month prospective follow-up study. Eur. J. Clin. Microbiol. Infect. Dis. 38, 1709–1717. https://doi.org/10.1007/s10096-019-03603-1
Castro-Hermida, J.A., González-Warleta, M., Mezo, M., 2015. *Cryptosporidium* spp. and *Giardia duodenalis* as pathogenic contaminants of water in Galicia, Spain: The need for safe drinking water. Int. J. Hyg. Environ. Health 218, 132–138. https://doi.org/10.1016/j.ijheh.2014.09.001
Certad, G., Follet, J., Gantois, N., Hammouma-Ghelboun, O., Guyot, K., Benamrouz-Vanneste, S., Fréalle, E., Seesao, Y., Delaire, B., Creusy, C., Even, G., Verrez-Bagnis, V., Ryan, U., Gay, M., Aliouat-Denis, C., Viscogliosi, E., 2019. Prevalence, molecular identification, and risk factors for *Cryptosporidium* infection in edible marine fish: a survey across sea areas surrounding France. Front. Microbiol. 10, 1–15. https://doi.org/10.3389/fmicb.2019.01037
Certad, G., Viscogliosi, E., Chabé, M., Cacciò, S.M., 2017. Pathogenic mechanisms of *Cryptosporidium* and *Giardia*. Trends Parasitol. 33, 561–576. https://doi.org/10.1016/j.pt.2017.02.006
Chagas, C.R.F., Gonzalez, I.H.L., Salgado, P.A.B., Rodrigues, B., Ramos, P.L., 2019. *Giardia* spp., ten years of parasitological data in the biggest zoo of Latin America. Ann. Parasitol. 65, 35–51. https://doi.org/10.17420/ap6501.181

Chalmers, R.M., Katzer, F., 2013. Looking for *Cryptosporidium*: The application of advances in detection and diagnosis. Trends Parasitol. 29, 237–251. https://doi.org/10.1016/j.pt.2013.03.001

Chalmers, R.M., Robertson, L.J., Dorny, P., Jordan, S., Kärssin, A., Katzer, F., La Carbona, S., Lalle, M., Lassen, B., Mladineo, I., Rozycki, M., Bilska-Zajac, E., Schares, G., Mayer-Scholl, A., Trevisan, C., Tysnes, K., Vasilev, S., Klotz, C., 2020. Parasite detection in food: current status and future needs for validation. Trends Food Sci. Technol. 99, 337–350. https://doi.org/10.1016/j.tifs.2020.03.011

Chalmers, R.M., Robinson, G., Elwin, K., Elson, R., 2019. Analysis of the *Cryptosporidium* spp. and gp60 subtypes linked to human outbreaks of cryptosporidiosis in England and Wales, 2009 to 2017. Parasit Vectors 12, 95. https://doi.org/10.1186/s13071-019-3354-6

Chalmers, R.M., Robinson, G., Hotchkiss, E., Alexander, C., May, S., Gilray, J., Connelly, L., Hadfield, S.J., 2017. Suitability of loci for multiple-locus variable-number of tandem-repeats analysis of *Cryptosporidium parvum* for inter-laboratory surveillance and outbreak investigations. Parasitology 144, 37–47. https://doi.org/10.1017/S0031182015001766

Chalmers, R.M., Thomas, A.L., Butler, B.A., Davies Morel, M.C.G., 2005. Identification of *Cryptosporidium parvum* genotype 2 in domestic horses. Vet. Rec. 156, 49–50. https://doi.org/10.1136/vr.156.2.49

Ćirković, V., Klun, I., Utaaker, K.S., Uzelac, A., Tysnes, K.R., Robertson, L.J., Djurković-Djaković, O., 2020. Surface waters as a potential source of *Giardia* and *Cryptosporidium* in Serbia. Exp. Parasitol. 209, 107824. https://doi.org/10.1016/j.exppara.2019.107824

Claerebout, E., Casaert, S., Dalemans, A.C., De Wilde, N., Levecke, B., Vercruysse, J., Geurden, T., 2009. *Giardia* and other intestinal parasites in different dog populations in Northern Belgium. Vet. Parasitol. 161, 41–46. https://doi.org/10.1016/j.vetpar.2008.11.024

Colli, C.M., Bezagio, R.C., Nishi, L., Ferreira, E.C., Falavigna-Guilherma, A.L., Gomes, M.L., 2015. Food handlers as a link in the chain of transmission of *Giardia duodenalis* and other protozoa in public schools in southern Brazil. Trans. R. Soc. Trop. Med. Hyg. 109, 601–603. https://doi.org/10.1093/trstmh/trv062

Collier, S.A., Smith, S., Lowe, An., Hawkins, P., McFarland, P., Salyers, M., Rocco, P., Bumby, G., Maillard, J.-M., Williams, C., Fleischauer, A., Radke, V., Roberts, J.M., Hightower, A.W., Bishop, H.S., Mathison, B.A., da Silva, A.J., Hayden, A.S., Hlavsa, M.C., Xiao, L., Roberts, V.A., Brunkard, J., Beach, M.J., Hill, V., Yoder, J., Dunbar, E.L., Dearen, T., Bopp, C., Humphrys, M.S., Phillips, G., Chang, L., Meites, E.M., 2011. Cryptosporidiosis outbreak at a summer camp - North Carolina, 2009, MMWR. Morbidity and mortality weekly report.

Cook, N., Nichols, R.A.B., Wilkinson, N., Paton, C.A., Barker, K., Smith, H. V., 2007. Development of a method for detection of *Giardia duodenalis* cysts on lettuce and for simultaneous analysis of salad products for the presence of *Giardia* cysts and *Cryptosporidium* oocysts. Appl. Environ. Microbiol. 73, 7388–7391. https://doi.org/10.1128/AEM.00552-07

Cope, J.R., Prosse, A., Nowicki, S., Roberts, M.W., Scheer, D., Anderson, C., Longsworth, A., Parsons, C., Goldschmidt, D., Johnston, S., Bishop, H., Xiao, L., Hill, V., Beach, M., Hlavsa, M.C., 2015. Preventing community-wide transmission of *Cryptosporidium*: a proactive public health response to a swimming pool-associated outbreak - Auglaize county, Ohio, USA. Epidemiol. Infect. 143, 3459–3467. https://doi.org/10.1017/S0950268815000813

Corso, P.S., Kramer, M.H., Blair, K.A., Addiss, D.G., Davis, J.P., Haddix, A.C., 2003. Cost of illness

in the 1993 waterborne *Cryptosporidium* outbreak, Milwaukee, Wisconsin. Emerg. Infect. Dis. 9, 426–431.

Coupe, A., Howe, L., Burrows, E., Sine, A., Pita, A., Velathanthiri, N., Vallée, E., Hayman, D., Shapiro, K., Roe, W.D., 2018. First report of *Toxoplasma gondii* sporulated oocysts and *Giardia duodenalis* in commercial green-lipped mussels (*Perna canaliculus*) in New Zealand. Parasitol. Res. 117, 1453–1463. https://doi.org/10.1007/s00436-018-5832-8

Current, W.L., Reese, N.C., 1986. A comparison of endogenous development of three isolates of *Cryptosporidium* in suckling mice. J. Protozool. 33, 98–108.

Daly, E.R., Roy, S.J., Blaney, D.D., Manning, J.S., Hill, V.R., Xiao, L., Stull, J.W., 2010. Outbreak of giardiasis associated with a community drinking-water source. Epidemiol. Infect. 138, 491–500. https://doi.org/10.1017/S0950268809990744

Danciger, M., Lopez, M., 1975. Numbers of *Giardia* in the feces of infected children. Am. J. Trop. Med. Hyg. 24, 237–242.

Delport, T.C., Asher, A.J., Beaumont, L.J., Webster, K.N., Harcourt, R.G., Power, M.L., 2014. *Giardia duodenalis* and *Cryptosporidium* occurrence in Australian sea lions (*Neophoca cinerea*) exposed to varied levels of human interaction. Int. J. Parasitol. Parasites Wildl. 3, 269–275. https://doi.org/10.1016/j.ijppaw.2014.09.001

Deng, M.Q., Cliver, D.O., 2000. Comparative detection of *Cryptosporidium parvum* oocysts from apple juice. Int. J. Food Microbiol. 54, 155–162. https://doi.org/10.1016/S0168-1605(99)00200-7

Deng, M.Q., Cliver, D.O., 1999. *Cryptosporidium parvum* studies with dairy products. Int. J. Food Microbiol. 46, 113–121. https://doi.org/10.1016/S0168-1605(98)00187-1

Deng, M.Q., Lam, K.M., Cliver, D.O., 2000. Immunomagnetic separation of *Cryptosporidium parvum* oocysts using MACS MicroBeads and high gradient separation columns. J. Microbiol. Methods 40, 11–17. https://doi.org/10.1016/S0167-7012(99)00127-X

DeRegnier, D.P., Cole, L., Schupp, D.G., Erlandsen, S.L., 1989. Viability of *Giardia* cysts suspended in lake, river, and tap water. Appl. Environ. Microbiol. 55, 1223–1229. https://doi.org/10.1128/aem.55.5.1223-1229.1989

DeSilva, M.B., Schafer, S., Scott, M.K., Robinson, B., Hills, A., Buser, G.L., Salis, K., Gargano, J., Yoder, J., Hill, V., Xiao, L., Roellig, D., Hedberg, K., 2015. Communitywide cryptosporidiosis outbreak associated with a surface water-supplied municipal water system – Baker City , Oregon, 2013. Epidemiol. Infect. 144, 274–284. https://doi.org/10.1017/S0950268815001831

Di Genova, B.M., Tonelli, R.R., 2016. Infection strategies of intestinal parasite pathogens and host cell responses. Front. Microbiol. 7, 1–16. https://doi.org/10.3389/fmicb.2016.00256

Di Pinto, A., Tantillo, M.G., 2002. Direct detection of *Cryptosporidium parvum* oocysts by immunomagnetic separation-polymerase chain reaction in raw milk. J. Food Prot. 65, 1345–1348. https://doi.org/10.4315/0362-028X-65.8.1345

Dias, A.P., Calegar, D., Carvalho-Costa, F.A., Alencar, M. de F.L., Ignacio, C.F., da Silva, M.E., Henrique, A., de Moraes Neto, A.H.A., 2018. Assessing the influence of water management and rainfall seasonality on water quality and intestinal parasitism in rural northeastern Brazil. J. Trop. Med. 2018. https://doi.org/10.1155/2018/8159354

Dixon, B., Parrington, L., Cook, A., Pollari, F., Farber, J., 2013. Detection of *Cyclospora*, *Cryptosporidium*, and *Giardia* in ready-to-eat packaged leafy greens in Ontario, Canada. J. o f Food Prot. 76, 307–313. https://doi.org/10.4315/0362-028X JFP-12-282

Dowle, M., Hill, N.J., Power, M.L., 2013. *Cryptosporidium* from a free-ranging marsupial host:

bandicoots in urban Australia. Vet. Parasitol. 198, 197–200. https://doi.org/10.1016/j.vetpar.2013.08.017

Duedu, K.O., Yarnie, E.A., Tetteh-Quarcoo, P.B., Attah, S.K., Donkor, E.S., Ayeh-Kumi, P.F., 2014. A comparative survey of the prevalence of human parasites found in fresh vegetables sold in supermarkets and open-aired markets in Accra, Ghana. BMC Res. Notes 7, 1–6. https://doi.org/10.1186/1756-0500-7-836

DuPont, H.L., Chappell, C.L., Sterling, C.R., Okhuysen, P.C., Rose, J.B., Jakubowski, W., 1995. The infectivity of *Cryptosporidium parvum* in healthy volunteers. N. Engl. J. Med. 332, 855–859.

Durigan, M., Cardoso-Silva, C.B., Ciampi-Guillardi, M., Toledo-Silva, G., Mori, G.M., Franco, R.M.B., Souza, A.P., 2018. Molecular genotyping, diversity studies and high-resolution molecular markers unveiled by microsatellites in *Giardia duodenalis*. PLoS Negl. Trop. Dis. 12, 1–18. https://doi.org/10.1371/journal.pntd.0006928

Dziedzinska, R., Vasickova, P., Hrdy, J., Slany, M., Babak, V., Moravkova, M., 2018. Foodborne bacterial, viral, and protozoan pathogens in field and market strawberries and environment of strawberry farms. J. Food Sci. 83, 3069–3075. https://doi.org/10.1111/1750-3841.14401

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazzards), Koutsoumanis, K., Allende, A., Alvarez-Ordóñez, A., Bolton, D., Bover-Cid, S., Chemaly, M., Davies, R., De Cesare, A., Herman, L., Hilbert, F., Lindqvist, R., Nauta, M., Peixe, L., Ru, G., Simmonis, M., Skandamis, P., Suffredini, E., Cacciò, S., Chalmers, R., Deplazes, P., Devleesschaiwer, B., Innes, E., Romig, T., van der Giessen, J., Hempen, M., Van der Stede, Y., Robertson, L., 2018. Scientific Opinion on the public health risks associated with food-borne parasites. EFSA J. 16, 13–29. https://doi.org/10.2903/j.efsa.2018.5495

Efstratiou, A., Ongerth, J., Karanis, P., 2017a. Evolution of monitoring for *Giardia* and *Cryptosporidium* in water. Water Res. 123, 96–112. https://doi.org/10.1016/j.watres.2017.06.042

Efstratiou, A., Ongerth, J.E., Karanis, P., 2017b. Waterborne transmission of protozoan parasites: Review of worldwide outbreaks - An update 2011–2016. Water Res. 114, 14–22. https://doi.org/10.1016/j.watres.2017.01.036

Ehsan, A., Casaert, S., Levecke, B., Van Rooy, L., Pelicaen, J., Smis, A., De Backer, J., Vervaeke, B., De Smedt, S., Schoonbaert, F., Lammens, S., Warmoes, T., Geurden, T., Claerebout, E., 2015a. *Cryptosporidium* and *Giardia* in recreational water in Belgium. J. Water Health 13, 870–878. https://doi.org/10.2166/wh.2015.268

Ehsan, A., Geurden, T., Casaert, S., Paulussen, J., De Coster, L., Schoemaker, T., Chalmers, R., Grit, G., Vercruysse, J., Claerebout, E., 2015b. Occurrence and potential health risk of *Cryptosporidium* and *Giardia* in different water catchments in Belgium. Environ. Monit. Assess. 187. https://doi.org/10.1007/s10661-014-4157-z

Einarsson, E., Ma'ayeh, S., Svärd, S.G., 2016. An up-date on *Giardia* and giardiasis. Curr. Opin. Microbiol. 34, 47–52. https://doi.org/10.1016/j.mib.2016.07.019

El-Latif, N.F.A., El-Taweel, H.A., Gaballah, A., Salem, A.I., El-Malek, A.H.M.A., 2020. Molecular characterization of *Giardia intestinalis* detected in humans and aater samples in Egypt. Acta Parasitol. https://doi.org/10.2478/s11686-020-00176-4

Elwin, K., Hadfiel, S.J., Robinson, G., Chalmers, R.M., 2012a. The epidemiology of sporadic human infections with unusual cryptosporidia detected during routine typing in England and Wales, 2000-2008. Epidemiol. Infect. 140, 673–683. https://doi.org/10.1017/S0950268811000860

Elwin, K., Hadfield, S.J., Robinson, G., Crouch, N.D., Chalmers, R.M., 2012b. *Cryptosporidium viatorum* n. sp. (Apicomplexa: Cryptosporidiidae) among travellers returning to Great Britain from the Indian subcontinent, 2007-2011. Int. J. Parasitol. 42, 675–682. https://doi.org/10.1016/j.ijpara.2012.04.016

Enemark, H.L., Ahrens, P., Bille-Hansen, V., Heegaard, P.M.H., Vigre, H., Thamsborg, S.M., Lind, P., 2003. *Cryptosporidium parvum*: infectivity and pathogenicity of the "porcine" genotype. Parasitology 126, 407–416. https://doi.org/10.1017/S0031182003003032

Essid, R., Menotti, J., Hanen, C., Aoun, K., Bouratbine, A., 2018. Genetic diversity of *Cryptosporidium* isolates from human populations in an urban area of Northern Tunisia. Infect. Genet. Evol. 58, 237–242. https://doi.org/10.1016/j.meegid.2018.01.004

Ethelberg, S., Lisby, M., Vestergaard, L.., Enemark, H.., Olsen, K.E.., Stensvold, C.., Nielsen, H.., Porsbo, L.., Plesner, A.., Mølbak, K., 2009. A foodborne outbreak of *Cryptosporidium hominis* infection. Epidemiol. Infect. 137, 348–356. https://doi.org/10.1017/S0950268808001817

Fantinatti, M., Bello, A.R., Fernandes, O., Da-Cruz, A.M., 2016. Identification of *Giardia lamblia* assemblage E in humans points to a new anthropozoonotic cycle. J. Infect. Dis. 214, 1256–1259. https://doi.org/10.1093/infdis/jiw361

Fantinatti, M., Caseca, A.C., Bello, A.R., Fernandes, O., Da-Cruz, A.M., 2018. The presence of *Giardia lamblia* assemblage A in dogs suggests an anthropozoonotic cycle of the parasite in Rio de Janeiro, Brazil. Infect. Genet. Evol. 65, 265–269. https://doi.org/10.1016/j.meegid.2018.07.025

FAO/WHO, 2014. Multicriteria-based ranking for risk management of food-borne parasites, Microbiological Risk Assessment Series.

Farthing, M.J.G., 1996. Giardiasis. Gastroenterol. Clin. North Am. 25, 493–515. https://doi.org/10.1016/S0889-8553(05)70260-0

Farzan, A., Parrington, L., Coklin, T., Cook, A., Pintar, K., Pollari, F., Friendship, R., Farber, J., Dixon, B., 2011. Detection and characterization of *Giardia duodenalis* and *Cryptosporidium* spp. on swine farms in Ontario, Canada. Foodborne Pathog. Dis. 8, 1207–1213. https://doi.org/10.1089/fpd.2011.0907

Fayer, R., 2010. Taxonomy and species delimitation in *Cryptosporidium*. Exp. Parasitol. 124, 90–97. https://doi.org/10.1016/j.exppara.2009.03.005

Fayer, R., 2004. *Cryptosporidium*: a water-borne zoonotic parasite. Vet. Parasitol. 126, 37–56. https://doi.org/10.1016/J.VETPAR.2004.09.004

Fayer, R., Graczyk, T.K., Lewis, E.J., Trout, J.M., Parley, C.A., 1998. Survival of infectious *Cryptosporidium parvum* oocysts in seawater and Eastern oysters (*Crassostrea virginica*) in the Chesapeake Bay. Appl. Environ. Microbiol. 64, 1070–1074.

Fayer, R., Morgan, U., Upton, S.J., 2000. Epidemiology of *Cryptosporidium*: transmission, detection and identification. Int. J. Parasitol. 30, 1305–1322. https://doi.org/10.1016/S0020-7519(00)00135-1

Fayer, R., Santin, M., Trout, J.M., 2007. Prevalence of *Cryptosporidium* species and genotypes in mature dairy cattle on farms in eastern United States compared with younger cattle from the same locations. Vet. Parasitol. 145, 260–266. https://doi.org/10.1016/j.vetpar.2006.12.009

Fayer, R., Santín, M., Trout, J.M., Greiner, E., 2006. Prevalence of species and genotypes of *Cryptosporidium* found in 1-2-year-old dairy cattle in the eastern United States. Vet. Parasitol. 135, 105–112. https://doi.org/10.1016/j.vetpar.2005.08.003

Fayer, R., Trout, J.., Lewis, E.., Xiao, L., Lal, A., Jenkins, M.., Graczyk, T.., 2002. Temporal

variability of *Cryptosporidium* in the Chesapeake Bay. Parasitol. Res. 88, 998–1003. https://doi.org/10.1007/s00436-002-0697-1

Feng, Y., 2010. *Cryptosporidium* in wild placental mammals. Exp. Parasitol. 124, 128–137. https://doi.org/10.1016/j.exppara.2008.11.005

Feng, Y., Ryan, U.M., Xiao, L., 2018. Genetic diversity and population structure of *Cryptosporidium*. Trends Parasitol. 34, 997–1011. https://doi.org/10.1016/j.pt.2018.07.009

Feng, Y., Wang, L., Duan, L., Gomez-Puerta, L.A., Zhang, L., Zhao, X., Hu, J., Zhang, N., Xiao, L., 2012. Extended outbreak of cryptosporidiosis in a pediatric hospital, China. Emerg. Infect. Dis. 18, 312–314. https://doi.org/10.3201/eid1802.110666

Feng, Y., Xiao, L., 2011. Zoonotic potential and molecular epidemiology of *Giardia* species and giardiasis. Clin. Microbiol. Rev. 24, 110–140. https://doi.org/10.1128/CMR.00033-10

Figgatt, M., Mergen, K., Kimelstein, D., Mahoney, D.M., Newman, A., Nicholas, D., Ricupero, K., Cafiero, T., Corry, D., Ade, J., Kurpiel, P., Madison-Antenucci, S., Anand, M., 2017. Giardiasis outbreak associated with asymptomatic food handlers in New York State, 2015. J. Food Prot. 80, 837–841. https://doi.org/10.4315/0362-028X.JFP-16-415

Fletcher, S.M., Stark, D., Harkness, J., Ellis, J., 2012. Enteric protozoa in the developed world: a public health perspective. Clin. Microbiol. Rev. 25, 420–449. https://doi.org/10.1128/CMR.05038-11

Franzén, O., Jerlström-Hultqvist, J., Castro, E., Sherwood, E., Ankarklev, J., Reiner, D.S., Palm, D., Andersson, J.O., Andersson, B., Svärd, S.G., 2009. Draft genome sequencing of *Giardia intestinalis* assemblage B isolate GS: is human giardiasis caused by two different species? PLoS Pathog. 5, e1000560. https://doi.org/10.1371/journal.ppat.1000560

Galbraith, N.S., Barrett, N.J., Stanwell-Smith, R., 1987. Water and disease after croydon: a review of water-borne and water-associated disease in the UK 1937-86. Water Environ. J. 1, 7–21. https://doi.org/10.1111/j.1747-6593.1987.tb01184.x

Gallas-Lindemann, C., Sotiriadou, I., Plutzer, J., Noack, M.J., Mahmoudi, M.R., Karanis, P., 2016. *Giardia* and *Cryptosporidium* spp. dissemination during wastewater treatment and comparative detection via immunofluorescence assay (IFA), nested polymerase chain reaction (nested PCR) and loop mediated isothermal amplification (LAMP). Acta Trop. 158, 43–51. https://doi.org/10.1016/j.actatropica.2016.02.005

Gatei, W., Das, P., Dutta, P., Sen, A., Cama, V., Lal, A.A., Xiao, L., 2007. Multilocus sequence typing and genetic structure of *Cryptosporidium hominis* from children in Kolkata, India. Infect. Genet. Evol. 7, 197–205. https://doi.org/10.1016/j.meegid.2006.08.006

Gatei, W., Hart, C.A., Gilman, R.H., Das, P., Cama, V., Xiao, L., 2006. Development of a multilocus sequence typing tool for *Cryptosporidium hominis*. J. Eukaryot. Microbiol. 53, 43–48. https://doi.org/10.1111/j.1550-7408.2006.00169.x

Gautret, P., Cramer, J.P., Field, V., Caumes, E., Jensenius, M., Gkrania-Klotsas, E., de Vries, P.J., Grobusch, M.P., Lopez-Velez, R., Castelli, F., Schlagenhauf, P., Hervius Askling, H., von Sonnenburg, F., Lalloo, D.G., Loutan, L., Rapp, C., Basto, F., Santos O'Connor, F., Weld, L., Parola, P., Network, E., 2012. Infectious diseases among travellers and migrants in Europe, EuroTravNet 2010. Eurosurveillance 17. https://doi.org/10.2807/ese.17.26.20205-en

Gelanew, T., Lalle, M., Hailu, A., Pozio, E., Cacciò, S.M., 2007. Molecular characterization of human isolates of *Giardia duodenalisi* from Ethiopia. Acta Trop. 102, 92–99. https://doi.org/10.1016/j.actatropica.2007.04.003

Gelletlie, R., Stuart, J., Soltanpoor, N., Armstrong, R., Nichols, G., 1997. Cryptosporidiosis

associated with school milk. Lancet 350, 1005–1006.
Gertler, M., Dürr, M., Renner, P., Poppert, S., Askar, M., Breidenbach, J., Frank, C., Preußel, K., Schielke, A., Werber, D., Chalmers, R., Robinson, G., Feuerpfeil, I., Tannich, E., Gröger, C., Stark, K., Wilking, H., 2015. Outbreak of *Cryptosporidium hominis* following river flooding in the city of Halle (Saale), Germany, August 2013. BMC Infect. Dis. 15, 1–10. https://doi.org/10.1186/s12879-015-0807-1
Geurden, T., Geldhof, P., Levecke, B., Martens, C., Berkvens, D., Casaert, S., Vercruysse, J., Claerebout, E., 2008. Mixed *Giardia duodenalis* assemblage A and E infections in calves. Int. J. Parasitol. 38, 259–264. https://doi.org/10.1016/j.ijpara.2007.07.016
Geurden, T., Vercruysse, J., Claerebout, E., 2010. Is *Giardia* a significant pathogen in production animals? Exp. Parasitol. 124, 98–106. https://doi.org/10.1016/j.exppara.2009.03.001
Gharpure, R., Perez, A., Miller, A.D., Wikswo, M.E., Silver, R., Hlavsa, M.C., 2019. Cryptosporidiosis outbreaks — United States, 2009 – 2017. Morb. Mortal. Wkly. Rep. 68, 568–572.
Gherasim, A., Lebbad, M., Insulander, M., Decraene, V., Kling, A., Hjertqvist, M., Wallensten, A., 2012. Two geographically separated food-borne outbreaks in Sweden linked by an unusual *Cryptosporidium parvum* subtype, October 2010. Eurosurveillance 17, 1–8. https://doi.org/10.2807/ese.17.46.20318-en
Giangaspero, A., Berrilli, F., Brandonisio, O., 2007. *Giardia* and *Cryptosporidium* and public health: the epidemiological scenario from the Italian perspective. Parasitol. Res. 101, 1169–1182. https://doi.org/10.1007/s00436-007-0598-4
Giangaspero, A., Molini, U., Iorio, R., Traversa, D., Paoletti, B., Giansante, C., 2005. *Cryptosporidium parvum* oocysts in seawater clams (*Chamelea gallina*) in Italy. Prev. Vet. Med. 69, 203–212. https://doi.org/10.1016/J.PREVETMED.2005.02.006
Giangaspero, A., Papini, R., Marangi, M., Koehler, A. V., Gasser, R.B., 2014. *Cryptosporidium parvum* genotype IIa and *Giardia duodenalis* assemblage A in *Mytilus galloprovincialis* on sale at local food markets. Int. J. Food Microbiol. 171, 62–67. https://doi.org/10.1016/j.ijfoodmicro.2013.11.022
Gomez-Bautista, M., Ortega-Mora, L.M., Tabares, E., Lopez-Rodas, V., Costas, E., 2000. Detection of infectious *Cryptosporidium parvum* oocysts in mussels (*Mytilus galloprovincialis*) and cockles (*Cerastoderma edule*). Appl. Environ. Microbiol. 66, 1866–1870. https://doi.org/10.1128/AEM.66.5.1866-1870.2000
Gómez-Couso, H., Freire-santos, F., Amar, C.F.L., Grant, K.A., Williamson, K., Ares-Mazás, M.E., McLauchlin, J., 2004. Detection of *Cryptosporidium* and *Giardia* in molluscan shellfish by multiplexed nested-PCR. Int. J. Food Microbiol. 91, 279–288. https://doi.org/10.1016/j.ijfoodmicro.2003.07.003
Gómez-Couso, H., Méndez-Hermida, F., Castro-Hermida, J.A., Ares-Mazás, E., 2006. *Cryptosporidium* contamination in harvesting areas of bilvave mollusks. J. Food Prot. 69, 185–190. https://doi.org/10.4315/0362-028X-69.1.185
Gómez-Couso, H., Méndez-Hermida, F., Castro-Hermida, J.A., Ares-Mazás, E., 2005. Occurrence of *Giardia* cysts in mussels (*Mytilus galloprovincialis*) destined for human consumption. J. Food Prot. 68, 1702–1705.
Goodgame, R.W., Genta, R.M., White, A.C., Chappell, C.L., 1993. Intensity of infection in AIDS-associated cryptosporidiosis. J. Infect. Dis. 167, 704–709. https://doi.org/10.1093/infdis/167.3.704
Graczyk, T.K., Cranfield, M.R., Fayer, R., Bixler, H., 1999. House flies (*Musca domestica*) as

transport hosts of *Cryptosporidium parvum*. Am. J. Trop. Med. Hyg. 61, 500–504. https://doi.org/10.4269/ajtmh.1999.61.500

Graczyk, T.K., Fayer, R., Cranfield, M.R., Conn, D.B., 1998. Recovery of waterborne *Cryptosporidium parvum* oocysts by freshwater benthic clams (*Corbicula fluminea*). Appl. Environ. Microbiol. 64, 427–430.

Graczyk, T.K., Marcogliese, D.J., De Lafontaine, Y., Da Silva, A.J., Mhangami-Ruwende, B., Pieniazek, N.J., 2001. Cryptosporidium parvum oocysts in zebra mussels (*Dreissena polymorpha*): evidence from the St. Lawrence River. Parasitol. Res. 87, 231–234. https://doi.org/10.1007/s004360000293

Greig, J.D., Todd, E.C.D., Bartleson, C.A., Michaels, B.S., 2007. Outbreaks where food workers have been implicated in the spread of foodborne disease. Part 1. Description of the problem, methods, and agents involved. J. Food Prot. 70, 1752–1761. https://doi.org/10.4315/0362-028X-70.7.1752

Grinberg, A., Learmonth, J., Kwan, E., Pomroy, W., Lopez Villalobos, N., Gibson, I., Widmer, G., 2008a. Genetic diversity and zoonotic potential of *Cryptosporidium parvum* causing foal diarrhea. J. Clin. Microbiol. 46, 2396–2398. https://doi.org/10.1128/JCM.00936-08

Grinberg, A., Lopez-Villalobos, N., Pomroy, W., Widmer, G., Smith, H., Tait, A., 2008b. Host-shaped segregation of the *Cryptosporidium parvum* multilocus genotype repertoire. Epidemiol. Infect. 136, 273–278. https://doi.org/10.1017/S0950268807008345

Gultekin, M., Ural, K., Aysul, N., Ayan, A., Balikci, C., Akyildiz, G., 2017. Prevalence and molecular characterization of *Giardia duodenalis* in dogs in Aydin, Turkey. Int. J. Environ. Health Res. 27, 161–168. https://doi.org/10.1080/09603123.2017.1310187

Guy, R.A., Payment, P., Krull, U.J., Horgen, P.A., 2003. Real-time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage. Appl. Environ. Microbiol. 69, 5178–5185. https://doi.org/10.1128/AEM.69.9.5178

Guzman-Herrador, B., Carlander, A., Ethelberg, S., Freiesleben de Blasio, B., Kuusi, M., Lund, V., Löfdahl, M., MacDonald, E., Nichols, G., Schönning, C., Sudre, B., Trönnberg, L., Vold, L., Semenza, J.C., Nygård, K., 2015. Waterborne outbreaks in the Nordic countries, 1998 to 2012. Eurosurveillance 20, 1–10.

Hadjichristodoulou, C., Gkikas, A., Pediatidis, Y., Tselentis, Y., 1998. Outbreak of giardiasis among English tourists in Crete. Lancet 351, 65–66. https://doi.org/10.1016/s0140-6736(05)78057-0

Halliez, M.C.M., Buret, A.G., 2013. Extra-intestinal and long term consequences of *Giardia duodenalis* infections. World J. Gastroenterol. 19, 8974–8985. https://doi.org/10.3748/wjg.v19.i47.8974

Hamnes, I.S., Gjerde, B.K., Robertson, L.J., 2007. A longitudinal study on the occurrence of *Cryptosporidium* and *Giardia* in dogs during their first year of life. Acta Vet. Scand. 49, 1–10. https://doi.org/10.1186/1751-0147-49-22

Hardie, R.M., Wall, P.G., Gott, P., Bardhan, M., Bartlett, C.L.R., 1999. Infectious diarrhea in tourists staying in a resort hotel. Emerg. Infect. Dis. 5, 168–171. https://doi.org/10.3201/eid0501.990123

Harper, C.M., Cowell, N.A., Adams, B.C., Langley, A.J., Wohlsen, T.D., 2002. Outbreak of *Cryptosporidium* linked to drinking unpasteurised milk. Commun. Dis. Intell. Q. Rep. 26.

Hattori, K., Donomoto, T., Manchanayake, T., Shibahara, T., Sasai, K., Matsubayashi, M., 2018. First surveillance and molecular identification of the *Cryptosporidium* skunk genotype and *Cryptosporidium parvum* in wild raccoons (*Procyon lotor*) in Osaka, Japan. Parasitol. Res. 117, 3669–3674. https://doi.org/10.1007/s00436-018-6089-y

Helmy, Y.A., Spierling, N.G., Schmidt, S., Rosenfeld, U.M., Reil, D., Imholt, C., Jacob, J., Ulrich, R.G., Aebischer, T., Klotz, C., 2018. Occurrence and distribution of *Giardia* species in wild rodents in Germany. Parasit Vectors 11, 1–13. https://doi.org/10.1186/s13071-018-2802-z

Hernández-Arango, N., Pinto, V., Muñoz-Sanchez, D., Lora-Suarez, F., Gómez-Marín, J.E., 2019. Detection of *Giardia* spp. with formalin/ether concentration in *Brassica oleracea* (cabbage) and *Lactuca sativa* (lettuce). Heliyon 5. https://doi.org/10.1016/j.heliyon.2019.e02377

Heyworth, M.F., 2016. *Giardia duodenalis* genetic assemblages and hosts. Parasite 23. https://doi.org/10.1051/parasite/2016013

Hlavsa, M.C., Roberts, V.A., Kahler, A.M., Hilborn, E.D., Mecher, T.R., Beach, M.J., Wade, T.J., Yoder, J.S., 2015. Outbreaks of illness associated with recreational water - United States, 2011-2012. Morb. Mortal. Wkly. Rep. 64, 668–672. https://doi.org/10.1111/ajt.13473

Hohweyer, J., Cazeaux, C., Travaille, E., Languet, E., Dumetre, A., Aubert, D., Terryn, C., Dubey, J.P., Azas, N., Houssin, M., Loïc, F., Villena, I., La Carbona, S., 2016. Simultaneous detection of the protozoan parasites *Toxoplasma*, *Cryptosporidium* and *Giardia* in food matrices and their persistence on basil leaves. Food Microbiol. 57, 36–44. https://doi.org/10.1016/j.fm.2016.01.002

Holubová, N., Sak, B., Horčičková, M., Hlásková, L., Květoňová, D., Menchaca, S., McEvoy, J., Kváč, M., 2016. *Cryptosporidium avium* n. sp. (Apicomplexa: Cryptosporidiidae) in birds. Parasitol. Res. 115, 2243–2251. https://doi.org/10.1007/s00436-016-4967-8

Homan, W.L., Mank, T.G., 2001. Human giardiasis: genotype linked differences in clinical symptomatology. Int. J. Parasitol. 31, 822–826. https://doi.org/10.1016/S0020-7519(01)00183-7

Hong, S., Kim, K., Yoon, S., Park, W.Y., Sim, S., Yu, J.R., 2014. Detection of *Cryptosporidium parvum* in environmental soil and vegetables. J. Korean Med. Sci. 29, 1367–1371. https://doi.org/10.3346/jkms.2014.29.10.1367

Hunter, P.R., Hughes, S., Woodhouse, S., Raj, N., Syed, Q., Chalmers, R.M., Verlander, N.Q., Goodacre, J., 2004. Health sequelae of human cryptosporidiosis in immunocompetent patients. Clin. Infect. Dis. 39, 504–510. https://doi.org/10.1086/422649

Iglói, Z., Mughini-Gras, L., Nic Lochlainn, L., Barrasa, A., Sane, J., Mooij, S., Schimmer, B., Roelfsema, J., van Pelt, W., Kortbeek, T., 2018. Long-term sequelae of sporadic cryptosporidiosis: a follow-up study. Eur. J. Clin. Microbiol. Infect. Dis. 37, 1377–1384. https://doi.org/10.1007/s10096-018-3268-9

Insulander, M., de Jong, B., Svenungsson, B., 2008. A food-borne outbreak of cryptosporidiosis among guests and staff at a hotel restaurant in Stockholm county, Sweden, September 2008. Eurosurveillance 13.

Insulander, M., Silverlås, C., Lebbad, M., Karlsson, L., Mattsson, J.G., Svenungsson, B., 2013. Molecular epidemiology and clinical manifestations of human cryptosporidiosis in Sweden. Epidemiol. Infect. 141, 1009–1020. https://doi.org/10.1017/S0950268812001665

Iqbal, A., Labib, M., Muharemagic, D., Sattar, S., Dixon, B.R., Berezovski, M. V., 2015. Detection of *Cryptosporidium parvum* oocysts on fresh produce using DNA aptamers. PLoS One 10, 1–13. https://doi.org/10.1371/journal.pone.0137455

ISO, 2016. ISO 18744: Microbiology of the food chain – Detection and enumeration of *Cryptosporidium* and *Giardia* in fresh leafy vegetables and berry fruits. International Organization for Standardization, Geneva, Switzerland.

ISO, 2006. ISO 15553: Water quality - Isolation and identification of *Cryptosporidium* oocysts and *Giardia* cysts from water. International Organization for Standardization, Geneva, Switzerland.

Jerlström-Hultqvist, J., Franzén, O., Ankarklev, J., Xu, F., Nohýnková, E., Andersson, J.O., Svärd, S.G., Andersson, B., 2010. Genome analysis and comparative genomics of a *Giardia intestinalis* assemblage E isolate. BMC Genomics 11. https://doi.org/10.1186/1471-2164-11-543

Johansen, H.Ø., Hanevik, K., Thrana, F., Carlson, A., Stachurska-Hagen, T., Skaare, D., Robertson, L.J., 2015. Symptomatic and asymptomatic secondary transmission of *Cryptosporidium parvum* following two related outbreaks in schoolchildren. Epidemiol. Infect. 143, 1702–1709. https://doi.org/10.1017/S095026881400243X

Johnson, E., Atwill, E.R., Filkins, M.E., Kalush, J., 1997. The prevalence of shedding of *Cryptosporidium* and *Giardia* spp. based on a single fecal sample collection from each of 91 horses used for backcountry recreation. J. Vet. Diagnostic Investig. 9, 56–60. https://doi.org/10.1177/104063879700900110

Johnson, J., Buddle, R., Reid, S., Armson, A., Ryan, U.M., 2008. Prevalence of *Cryptosporidium* genotypes in pre and post-weaned pigs in Australia. Exp. Parasitol. 119, 418–421. https://doi.org/10.1016/j.exppara.2008.04.009

Jokipii, A.M.M., Jokipii, L., 1977. Prepatency of giardiasis. Lancet 1, 1095–1097. https://doi.org/10.1016/S0140-6736(77)92345-5

Julien, D.A., Sargeant, J.M., Guy, R.A., Shapiro, K., Imai, R.K., Bunce, A., Sudlovenick, E., Chen, S., Li, J., Harper, S.L., 2019. Prevalence and genetic characterization of *Giardia* spp. and *Cryptosporidium* spp. in dogs in Iqaluit, Nunavut, Canada. Zoonoses Public Health 66, 813–825. https://doi.org/10.1111/zph.12628

Karanis, P., Kourenti, C., Smith, H. V., 2007. Waterborne transmission of protozoan parasites: A worldwide review of outbreaks and lessons learnt. J. Water Health 5, 1–38. https://doi.org/10.2166/wh.2006.002

Karon, A.E., Hanni, K.D., Mohle-Boetani, J.C., Beretti, R.A., Hill, V.R., Arrowood, M., Johnston, S.P., Xiao, L., Vugia, D.J., 2011. Giardiasis outbreak at a camp after installation of a slow-sand filtration water-treatment system. Epidemiol. Infect. 139, 713–717. https://doi.org/10.1017/S0950268810001573

Kato, S., Lindergard, G., Mohammed, H.O., 2003. Utility of the *Cryptosporidium* oocyst wall protein (COWP) gene in a nested PCR approach for detection infection in cattle. Vet. Parasitol. 111, 153–159. https://doi.org/10.1016/S0304-4017(02)00353-9

Keserue, H.A., Füchslin, H.P., Egli, T., 2011. Rapid detection and enumeration of *Giardia lamblia* cysts in water samples by immunomagnetic separation and flow cytometric analysis. Appl. Environ. Microbiol. 77, 5420–5427. https://doi.org/10.1128/AEM.00416-11

Keserue, H.A., Füchslin, H.P., Wittwer, M., Nguyen-Viet, H., Nguyen, T.T., Surinkul, N., Koottatep, T., Schürch, N., Egli, T., 2012. Comparison of rapid methods for detection of *Giardia* spp. and *Cryptosporidium* spp. (oo)cysts using transportable instrumentation in a field deployment. Environ. Sci. Technol. 46, 8952–8959. https://doi.org/10.1021/es301974m

Khalil, I.A., Troeger, C., Rao, P.C., Blacker, B.F., Brown, A., Brewer, T.G., Colombara, D. V., De Hostos, E.L., Engmann, C., Guerrant, R.L., Haque, R., Houpt, E.R., Kang, G., Korpe, P.S., Kotloff, K.L., Lima, A.A.M., Petri Jr, W.A., Platts-Mills, J.A., Shoultz, D.A., Forouzanfar, M.H., Hay, S.I., Reiner Jr, R.C., Mokdad, A.H., 2018. Morbidity, mortality, and long-term

consequences associated with diarrhoea from *Cryptosporidium* infection in children younger than 5 years: a meta-analyses study. Lancet Glob. Heal. 6, e758–e768. https://doi.org/10.1016/S2214-109X(18)30283-3

Khan, A., Shaik, J.S., Grigg, M.E., 2018. Genomics and molecular epidemiology of *Cryptosporidium* species. Acta Trop. 184, 1–14. https://doi.org/10.1016/j.actatropica.2017.10.023

Kheirandish, F., Tarahi, M., Ezatpour, B., 2014. Prevalence of intestinal parasites among food handlers in western Iran. Rev. do 56, 111–114. https://doi.org/10.1590/S0036-46652014000200004

Kim, H.Y., Lee, H., Lee, S.H., Seo, M.G., Yi, S., Kim, J.W., Kim, C.H., Lee, Y.R., So, B.J., Kwon, O.D., Kwak, D., 2019. Multilocus genotyping and risk factor analysis of *Giardia duodenalis* in dogs in Korea. Acta Trop. 199, 105113. https://doi.org/10.1016/j.actatropica.2019.105113

King, B.J., Monis, P.T., 2007. Critical processes affecting *Cryptosporidium* oocyst survival in the environment. Parasitology 134, 309–323. https://doi.org/10.1017/S0031182006001491

Kirk, M.D., Pires, S.M., Black, R.E., Caipo, M., Crump, J.A., Devleesschauwer, B., Döpfer, D., Fazil, A., Fischer-Walker, C.L., Hald, T., Hall, A.J., Keddy, K.H., Lake, R.J., Lanata, C.F., Torgerson, P.R., Havelaar, A.H., Angulo, F.J., 2015. World Health Organization Estimates of the Global and Regional Disease Burden of 22 Foodborne Bacterial, Protozoal, and Viral Diseases, 2010: A Data Synthesis. PLoS Med. 12, 1–21. https://doi.org/10.1371/journal.pmed.1001921

Kistemann, T., Rind, E., Koch, C., Claßen, T., Lengen, C., Exner, M., Rechenburg, A., 2012. Effect of sewage treatment plants and diffuse pollution on the occurrence of protozoal parasites in the course of a small river. Int. J. Hyg. Environ. Health 215, 577–583. https://doi.org/10.1016/j.ijheh.2011.12.008

Koehler, A. V., Jex, A.R., Haydon, S.R., Stevens, M.A., Gasser, R.B., 2014. *Giardia*/giardiasis - A perspective on diagnostic and analytical tools. Biotechnol. Adv. 32, 280–289. https://doi.org/10.1016/j.biotechadv.2013.10.009

Korich, D.G., Mead, J.R., Madore, M.S., Sinclair, N.A., Sterling, C.R., 1990. Effects of ozone, chlorine dioxide, chlorine, and monochloramine on *Cryptosporidium* parvum oocyst viability. Appl. Environ. Microbiol. 56, 1423–1428.

Kostopoulou, D., 2018. Prevalence and zoonotic potential of *Cryptosporidium* spp and *Giardia duodenalis* in different host species in Greece. Ghent University.

Kostopoulou, D., Casaert, S., Tzanidakis, N., van Doorn, D., Demeler, J., von Samson-Himmelstjerna, G., Saratsis, A., Voutzourakis, N., Ehsan, A., Doornaert, T., Looijen, M., De Wilde, N., Sotiraki, S., Claerebout, E., Geurden, T., 2015. The occurrence and genetic characterization of *Cryptosporidium* and *Giardia* species in foals in Belgium, The Netherlands, Germany and Greece. Vet. Parasitol. 211, 170–174. https://doi.org/10.1016/j.vetpar.2015.04.018

Kostopoulou, D., Claerebout, E., Arvanitis, D., Ligda, P., Voutzourakis, N., Casaert, S., Sotiraki, S., 2017. Abundance, zoonotic potential and risk factors of intestinal parasitism amongst dog and cat populations: The scenario of Crete, Greece. Parasit Vectors 10. https://doi.org/10.1186/s13071-017-1989-8

Kuczynska, E., Shelton, D.R., 1999. Method for detection and enumeration of *Cryptosporidium parvum* oocysts in feces, manures, and soils. Appl. Environ. Microbiol. 65, 2820–2826. https://doi.org/10.1128/aem.65.7.2820-2826.1999

Kváč, M., Havrdová, N., Hlásková, L., Daňková, T., Kanděra, J., Ježková, J., Vítovec, J., Sak, B.,

Ortega, Y., Xiao, L., Modrý, D., Chelladurai, J.R.J.J., Prantlová, V., McEvoy, J., 2016. *Cryptosporidium proliferans* n . sp . (Apicomplexa: Cryptosporidiidae): molecular and biological evidence of cryptic species within gastric *Cryptosporidium* of mammals. PLoS One 11, 1–24. https://doi.org/10.1371/journal.pone.0147090

Kváč, M., Kestřánová, M., Pinková, M., Květoňová, D., Kalinová, J., Wagnerová, P., Kotková, M., Vítovec, J., Ditrich, O., McEvoy, J., Stenger, B., Sak, B., 2013. *Cryptosporidium scrofarum* n. sp. (Apicomplexa: Cryptosporidiidae) in domestic pigs (*Sus scrofa*). Vet. Parasitol. 191, 218–227. https://doi.org/10.1016/j.vetpar.2012.09.005

Kváč, M., Saková, K., Květoňová, D., Kicia, M., Wesolowska, M., McEvoy, J., Sak, B., 2014. Gastroenteritis caused by the *Cryptosporidium* hedgehog genotype in an immunocompetent man. J. Clin. Microbiol. 52, 347–349. https://doi.org/10.1128/JCM.02456-13

Lalle, M., di Regalbono, A.F., Poppi, L., Nobili, G., Tonanzi, D., Pozio, E., Cacciò, S.M., 2007. A novel *Giardia duodenalis* assemblage A subtype in fallow deer. J. Parasitol. 93, 426–428. https://doi.org/10.1645/ge-983r.1

Lalle, M., Jimenez-Cardosa, E., Cacciò, S.M., Pozio, E., 2005a. Genotyping of *Giardia duodenalis* from humans and dogs from Mexico using a β-giardin nested polymerase chain reaction assay. J. Parasitol. 91, 203–205. https://doi.org/10.1645/ge-3405rn

Lalle, M., Pozio, E., Capelli, G., Bruschi, F., Crotti, D., Cacciò, S.M., 2005b. Genetic heterogeneity at the β-giardin locus among human and animal isolates of *Giardia duodenalis* and identification of potentially zoonotic subgenotypes. Int. J. Parasitol. 35, 207–213. https://doi.org/10.1016/j.ijpara.2004.10.022

Lane, S., Lloyd, D., 2002. Current trends in research into the waterborne parasite *Giardia*. Crit. Rev. Microbiol. 28, 123–147. https://doi.org/10.1080/1040-840291046713

Langkjær, R.B., Vigre, H., Enemark, H.L., Maddox-Hyttel, C., 2007. Molecular and phylogenetic characterization of *Cryptosporidium* and *Giardia* from pigs and cattle in Denmark. Parasitology 134, 339–350. https://doi.org/10.1017/S0031182006001533

Lasek-Nesselquist, E., Welch, D.M., Thompson, R.C.A., Steuart, R.F., Sogin, M.L., 2009. Genetic exchange within and between assemblages of *Giardia duodenalis*. J. Eukaryot. Microbiol. 56, 504–518. https://doi.org/10.1111/j.1550-7408.2009.00443.x

Lebbad, M., Mattsson, J.G., Christensson, B., Ljungström, B., Backhans, A., Andersson, J.O., Svärd, S.G., 2010. From mouse to moose: multilocus genotyping of Giardia isolates from various animal species. Vet. Parasitol. 168, 231–239. https://doi.org/10.1016/j.vetpar.2009.11.003

Lebbad, M., Petersson, I., Karlsson, L., Botero-Kleiven, S., Andersson, J.O., Svenungsson, B., Svärd, S.G., 2011. Multilocus genotyping of human *Giardia* isolates suggests limited zoonotic transmission and association between assemblage B and flatulence in children. PLoS Negl. Trop. Dis. 5, 1–10. https://doi.org/10.1371/journal.pntd.0001262

Lecová, L., Tůmová, P., Nohýnková, E., 2019. Clone-based haplotyping of *Giardia intestinalis* assemblage B human isolates. Parasitol. Res. 118, 355–361. https://doi.org/10.1007/s00436-018-6161-7 PROTOZOOLOGY

Lee, M.F., Cadogan, P., Eytle, S., Copeland, S., Walochnik, J., Lindo, J.F., 2017. Molecular epidemiology and multilocus sequence analysis of potentially zoonotic *Giardia* spp. from humans and dogs in Jamaica. Parasitol. Res. 116, 409–414. https://doi.org/10.1007/s00436-016-5304-y

Leoni, F., Gómez-Couso, H., Ares-Mazás, M.E., McLauchlin, J., 2007a. Multilocus genetic analysis of *Cryptosporidium* in naturally contaminated bivalve molluscs. J. Appl.

Microbiol. 103, 2430–2437. https://doi.org/10.1111/j.1365-2672.2007.03508.x
Leoni, F., Mallon, M.E., Smith, H. V., Tait, A., McLauchlin, J., 2007b. Multilocus analysis of *Cryptosporidium hominis* and *Cryptosporidium parvum* isolates from sporadic and outbreak-related human cases and *C. parvum* isolates from sporadic livestock cases in the United Kingdom. J. Clin. Microbiol. 45, 3286–3294. https://doi.org/10.1128/JCM.02536-06
Levy, D.A., Bens, M.S., Craun, G.F., Calderon, R.L., Herwaldt, B.L., 1998. Surveillance for waterborne disease outbreaks: United States, 1995-1996. Morb. Mortal. Wkly. Rep. 47, 1–34.
Li, J., Shi, K., Sun, F., Li, T., Wang, R., Zhang, S., Jian, F., Ning, C., Zhang, L., 2019. Identification of human pathogenic *Enterocytozoon bieneusi*, *Cyclospora cayetanensis*, and *Cryptosporidium parvum* on the surfaces of vegetables and fruits in Henan, China. Int. J. Food Microbiol. 307, 108292. https://doi.org/10.1016/j.ijfoodmicro.2019.108292
Li, W., Deng, L., Wu, K., Huang, X., Song, Y., Su, H., Hu, Y., Fu, H., Zhong, Z., Peng, G., 2017. Presence of zoonotic *Cryptosporidium scrofarum*, *Giardia duodenalis* assemblage A and *Enterocytozoon bieneusi* genotypes in captive Eurasian wild boars (*Sus scrofa*) in China: potential for zoonotic transmission. Parasit Vectors 10, 1–8. https://doi.org/10.1186/s13071-016-1942-2
Li, W., Zhang, N., Gong, P., Cao, L., Li, J., Su, L., Li, S., Diao, Y., Wu, K., Li, H., Zhang, X., 2010. A novel multiplex PCR coupled with Luminex assay for the simultaneous detection of *Cryptosporidium* spp., *Cryptosporidium parvum*and *Giardia duodenalis*. Vet. Parasitol. 173, 11–18. https://doi.org/10.1016/j.vetpar.2010.05.024
Lin, Q., Wang, X.Y., Chen, J.W., Ding, L., Zhao, G.H., 2015. *Cryptosporidium suis* infection in post-weaned and adult pigs in Shaanxi Province, Northwestern China. Korean J. Parasitol. 53, 113–117. https://doi.org/10.3347/kjp.2015.53.1.113
Lippy, E.C., 1978. Tracing a giardiasis outbreak at Berlin, New Hampshire. J. Am. Water Works Assoc. 70, 512–520. https://doi.org/10.1002/j.1551-8833.1978.tb04230.x
Litleskare, S., Rortveit, G., Eide, G.E., Emberland, K.E., Hanevik, K., Langeland, N., Wensaas, K.A., 2019. Quality of life and its association with irritable bowel syndrome and fatigue ten years after giardiasis. Neurogastroenterol. Motil. 31, 1–8. https://doi.org/10.1111/nmo.13559
Litleskare, S., Rortveit, G., Eide, G.E., Hanevik, K., Langeland, N., Wensaas, K.A., 2018. Prevalence of irritable bowel syndrome and chronic fatigue 10 years after *Giardia* infection. Clin. Gastroenterol. Hepatol. 16, 1064–1072. https://doi.org/10.1016/j.cgh.2018.01.022
Lucio-Forster, A., Griffiths, J.K., Cama, V.A., Xiao, L., Bowman, D.D., 2010. Minimal zoonotic risk of cryptosporidiosis from pet dogs and cats. Trends Parasitol. 26, 174–179. https://doi.org/10.1016/j.pt.2010.01.004
Macarisin, D., Santín, M., Bauchan, G., Fayer, R., 2010. Infectivity of *Cryptosporidium parvum* oocysts after storage of experimentally contaminated apples. J. Food Prot. 73, 1824–1829. https://doi.org/10.4315/0362-028X-73.10.1824
MacKenzie, W.R., Hoxie, N.J., Proctor, M.E., Gradus, M.S., A., B.K., Peterson, D.E., Kazmierczak, J.J., Addiss, D.G., Fox, K.R., Rose, J.B., Davis, J.P., 1994. A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. N. Engl. J. Med. 331, 161–167. https://doi.org/10.1056/NEJM199407213310304
Mallon, M.E., MacLeod, A., Wastling, J., Smith, H., Reilly, B., Tait, A., 2003a. Population structures and the role of genetic exchange in the zoonotic pathogen *Cryptosporidium*

parvum. J. Mol. Evol. 56, 407–417. https://doi.org/10.1007/s00239-002-2412-3
Mallon, M.E., MacLeod, A., Wastling, J.M., Smith, H., Tait, A., 2003b. Multilocus genotyping of *Cryptosporidium parvum* Type 2: population genetics and sub-structuring. Infect. Genet. Evol. 3, 207–218. https://doi.org/10.1016/S1567-1348(03)00089-3
Marangi, M., Giangaspero, A., Lacasella, V., Lonigro, A., Gasser, R.B., 2015. Multiplex PCR for the detection and quantification of zoonotic taxa of *Giardia*, *Cryptosporidium* and *Toxoplasma* in wastewater and mussels. Mol. Cell. Probes 29, 122–125. https://doi.org/10.1016/j.mcp.2015.01.001
Marquis, N.D., Record, N.R., Fernández Robledo, J.A., Robledo, F.J.A., 2015. Survey for protozoan parasites in Eastern oysters (*Crassostrea virginica*) from the Gulf of Maine using PCR-based assays. Parasitol. Int. 64, 299–302. https://doi.org/10.1016/J.PARINT.2015.04.001
Mayne, D.J., Ressler, K.-A., Smith, D., Hockey, G., Botham, S.J., Ferson, M.J., 2011. A community outbreak of cryptosporidiosis in Sydney associated with a public swimming facility: a case-control study. Interdiscip. Perspect. Infect. Dis. 2011. https://doi.org/10.1155/2011/341065
McClung, R.P., Roth, D.M., Vigar, M., Roberts, V.A., Kahler, A.M., Cooley, L.A., Hilborn, E.D., Wade, T.J., Fullerton, K.E., Yoder, J.S., Hill, V.R., 2018. Waterborne disease outbreaks associated with environmental and undetermined exposures to water — United States, 2013- 2014. Am. J. Transplant. 18, 262–267. https://doi.org/10.1111/ajt.14607
McKerr, C., Adak, G.K., Nichols, G., Gorton, R., Chalmers, R.M., Kafatos, G., Cosford, P., Charlett, A., Reacher, M., Pollock, K.G., Alexander, C.L., Morton, S., 2015. An outbreak of *Cryptosporidium parvum* across England and Scotland associated with consumption of fresh pre-cut salad leaves, May 2012. PLoS One 10, 1–13. https://doi.org/10.1371/journal.pone.0125955
McRoberts, K.M., Meloni, B.P., Morgan, U.M., Marano, R., Binz, N., Erlandsen, S.L., Halse, A., Thompson, R.C.A., 1996. Morphological and molecular characterization of *Giardia* isolated from the straw-necked ibis (*Threskiornis spinicollis*) in western Australia. J. Parasitol. 82, 711–718.
Meyer, W.T., 1973. Epidemic giardiasis. A continued elusive entity. Rocky Mt. Med. J. 70, 48–49.
Millard, P.S., Gensheimer, K.F., Addiss, D.G., Sosin, D.M., Beckett, G.A., Houck-Jankoski, A., Hudson, A., 1994. An outbreak of cryptosporidiosis from fresh-pressed apple cider. JAMA J. Am. Med. Assoc. 272, 1592–1596. https://doi.org/10.1001/jama.1994.03520200048034
Minetti, C., Chalmers, R.M., Beeching, N.J., Probert, C., Lamden, K., 2016. Giardiasis. BMJ 355, 1–9. https://doi.org/10.1136/bmj.i5369
Minetti, C., Taweenan, W., Hogg, R., Featherstone, C., Randle, N., Latham, S.M., Wastling, J.M., 2014. Occurrence and diversity of *Giardia duodenalis* assemblages in livestock in the UK. Transbound. Emerg. Dis. 61, e60–e67. https://doi.org/10.1111/tbed.12075
Mintz, E.D., Hudson-Wragg, M., Mshar, P., Cartter, M.L., Hadler, J.L., 1993. Foodborne giardiasis in a corporate office setting. J. Infect. Dis. 167, 250–253. https://doi.org/10.1093/infdis/167.1.250
Mohamed, M.A., Siddig, E.E., Elaagip, A.H., Edris, A.M.M., Nasr, A.A., 2016. Parasitic contamination of fresh vegetables sold at central markets in Khartoum state, Sudan. Ann. Clin. Microbiol. Antimicrob. 15, 5–11. https://doi.org/10.1186/s12941-016-0133-5
Monis, P.T., Andrews, R.H., Mayrhofer, G., Ey, P.L., 2003. Genetic diversity within the

morphological species *Giardia intestinalis* and its relationship to host origin. Infect. Genet. Evol. 3, 29–38. https://doi.org/10.1016/S1567-1348(02)00149-1

Monis, P.T., Andrews, R.H., Mayrhofer, G., Ey, P.L., 1999. Molecular systematics of the parasitic protozoan *Giardia intestinalis*. Mol. Biol. Evol. 16, 1135–1144. https://doi.org/10.1093/oxfordjournals.molbev.a026204

Mons, C., Dumètre, A., Gosselin, S., Galliot, C., Moulin, L., 2009. Monitoring of *Cryptosporidium* and *Giardia* river contamination in Paris area. Water Res. 43, 211–217. https://doi.org/10.1016/j.watres.2008.10.024

Mørch, K., Hanevik, K., Rortveit, G., Wensaas, K.A., Eide, G.E., Hausken, T., Langeland, N., 2009a. Severity of *Giardia* infection associated with post-infectious fatigue and abdominal symptoms two years after. BMC Infect. Dis. 9, 1–9. https://doi.org/10.1186/1471-2334-9-206

Mørch, K., Hanevik, K., Rortveit, G., Wensaas, K.A., Langeland, N., 2009b. High rate of fatigue and abdominal symptoms 2 years after an outbreak of giardiasis. Trans. R. Soc. Trop. Med. Hyg. 103, 530–532. https://doi.org/10.1016/j.trstmh.2009.01.010

Morgan, U.M., Deplazes, P., Forbes, D.A., Spano, F., Hertzberg, H., Sargent, K.D., Elliot, A., Thompson, R.C.A., 1999. Sequence and PCR-RFLP analysis of the internal transcribed spacers of the rDNA repeat unit in isolates of *Cryptosporidium* from different hosts. Parasitology 118, 49–58. https://doi.org/10.1017/S0031182098003412

Morrison, L.J., Mallon, M.E., Smith, H. V., MacLeod, A., Xiao, L., Tait, A., 2008. The population structure of the *Cryptosporidium parvum* population in Scotland: a complex picture. Infect. Genet. Evol. 8, 121–129. https://doi.org/10.1016/j.meegid.2007.10.010

Mossallam, S.F., 2010. Detection of some intestinal protozoa in commercial fresh juices. J. Egypt. Soc. Parasitol. 40, 135–149.

Muhsen, K., Levine, M.M., 2012. A systematic review and meta-analysis of the association between *Giardia lamblia* and endemic pediatric diarrhea in developing countries. Clin. Infect. Dis. 55. https://doi.org/10.1093/cid/cis762

Muthusamy, D., Rao, S.S., Ramani, S., Monica, B., Banerjee, I., Abraham, O.C., Mathai, D.C., Primrose, B., Muliyil, J., Wanke, C.A., Ward, H.D., Kang, G., 2006. Multilocus genotyping of *Cryptosporidium* sp. isolates from human immunodeficiency virus-infected individuals in South India. J. Clin. Microbiol. 44, 632–634. https://doi.org/10.1128/JCM.44.2.632-634.2006

Nader, J.L., Mathers, T.C., Ward, B.J., Pachebat, J.A., Swain, M.T., Robinson, G., Chalmers, R.M., Hunter, P.R., van Oosterhout, C., Tyler, K.M., 2019. Evolutionary genomics of anthroponosis in *Cryptosporidium*. Nat. Microbiol. 4, 826–836. https://doi.org/10.1038/s41564-019-0377-x

Nash, T.E., Herrington, D.A., Losonsky, G.A., Levine, M.M., 1987. Experimental human infections with *Giardia lamblia*. J. Infect. Dis. 156, 974–984. https://doi.org/10.1093/infdis/156.6.974

Nazemalhosseini-Mojarad, E., Feng, Y., Xiao, L., 2012. The importance of subtype analysis of *Cryptosporidium* spp. in epidemiological investigations of human cryptosporidiosis in Iran and other Mideast countries. Gastroenterol. Hepatol. from Bed to Bench 5, 67–70. https://doi.org/10.22037/ghfbb.v5i2.254

Newell, D.G., Koopmans, M., Verhoef, L., Duizer, E., Aidara-Kane, A., Sprong, H., Opsteegh, M., Langelaar, M., Threfall, J., Scheutz, F., ven der Giessen, J., Kruse, H., 2010. Food-borne diseases - The challenges of 20 years ago still persist while new ones continue to emerge. Int. J. Food Microbiol. 139, S3–S15. https://doi.org/10.1016/j.ijfoodmicro.2010.01.021

Ng-Hublin, J.S.Y., Hargrave, D., Combs, B., Ryan, U., 2015. Investigation of a swimming pool-associated cryptosporidiosis outbreak in the Kimberley region of Western Australia. Epidemiol. Infect. 143, 1037–1041. https://doi.org/10.1017/S095026881400106X

Ng, J., Yang, R., Whiffin, V., Cox, P., Ryan, U., 2011. Identification of zoonotic *Cryptosporidium* and *Giardia* genotypes infecting animals in Sydney's water catchments. Exp. Parasitol. 128, 138–144. https://doi.org/10.1016/j.exppara.2011.02.013

Ng, J.S.Y., Eastwood, K., Walker, B., Durrheim, D.N., Massey, P.D., Porigneaux, P., Kemp, R., McKinnon, B., Laurie, K., Miller, D., Bramley, E., Ryan, U., 2012. Evidence of *Cryptosporidium* transmission between cattle and humans in northern New South Wales. Exp. Parasitol. 130, 437–441. https://doi.org/10.1016/j.exppara.2012.01.014

Nichols, G., Chalmers, R., Lake, I., Sopwith, W., Regan, M., Hunter, P., Grenfell, P., Harrison, F., Lane, C., 2006. Cryptosporidiosis: A report on the surveillance and epidemiology of *Cryptosporidium* infection in England and Wales, Drinking Water Directorate Contract Number DWI 70/2/201. U. K. https://doi.org/08/11/2006

Nydam, D. V., Wade, S.E., Schaaf, S.L., Mohammed, H.O., 2001. Number of *Cryptosporidium parvum* oocysts or *Giardia* cysts shed by dairy calves after natural infection. Am. J. Vet. Res. 62, 1612–1615. https://doi.org/10.2460/ajvr.2001.62.1612

Nygård, K., Schimmer, B., Søbstad, Ø., Walde, A., Tveit, I., Langeland, N., Hausken, T., Aavitsland, P., 2006. A large community outbreak of waterborne giardiasis- delayed detection in a non-endemic urban area. BMC Public Health 6. https://doi.org/10.1186/1471-2458-6-141

O'Handley, R.M., Olson, M.E., 2006. Giardiasis and cryptosporidiosis in ruminants. Vet. Clin. North Am. - Food Anim. Pract. 22, 623–643. https://doi.org/10.1016/j.cvfa.2006.07.002

Okhuysen, P.C., Chappell, C.L., Crabb, J.H., Sterling, C.R., DuPont, H.L., 1999. Virulence of three distinct *Cryptosporidium parvum* isolates for healthy adults. J. Infect. Dis. 180, 1275–1281. https://doi.org/10.1086/315033

Olson, M.E., Goh, J., Phillips, M., Guselle, N., McAllister, T.A., 1999. *Giardia* cyst and *Cryptosporidium* oocyst survival in water, soil, and cattle feces. J. Environ. Qual. 28, 1991–1996. https://doi.org/10.2134/jeq1999.00472425002800060040x

Olson, M.E., Thorlakson, C.L., Deselliers, L., Morck, D.W., McAllister, T.A., 1997. *Giardia* and *Cryptosporidium* in Canadian farm animals. Vet. Parasitol. 68, 375–381. https://doi.org/10.1016/S0304-4017(96)01072-2

Omarova, A., Tussupova, K., Berndtsson, R., Kalishev, M., Sharapatova, K., 2018. Protozoan parasites in drinking water: A system approach for improved water, sanitation and hygiene in developing countries. Int. J. Environ. Res. Public Health 15, 1–18. https://doi.org/10.3390/ijerph15030495

Ortega, Y.R., Adam, R.D., 1997. *Giardia*: overview and update. Clin. Infect. Dis. 25, 545–549. https://doi.org/10.1086/513745

Osterholm, M.T., Forfang, J.C., Ristinen, T.L., Dean, A.G., Washburn, J.W., Godes, J.R., Rude, R.A., McCullough, J.G., 1981. An outbreak of foodborne giardiasis. N. Engl. J. Med. 304, 24–28. https://doi.org/10.1056/NEJM198101013040106

Pagoso, E.J.A., Rivera, W.L., 2017. *Cryptosporidium* species from common edible bivalves in Manila Bay, Philippines. Mar. Pollut. Bull. 119, 31–39. https://doi.org/10.1016/j.marpolbul.2017.03.005

Painter, J.E., Hlavsa, M.C., Collier, S.A., Xiao, L., Yoder, J.S., 2015. Cryptosporidiosis surveillance - United States, 2011-2012. Morb. Mortal. Wkly. Rep. 64, 1–13.

Pallant, L., Barutzki, D., Schaper, R., Thompson, R.C.A., 2015. The epidemiology of infections

with *Giardia* species and genotypes in well cared for dogs and cats in Germany. Parasit Vectors 8, 1–14. https://doi.org/10.1186/s13071-014-0615-2

Palmer, C.S., Traub, R.J., Robertson, I.D., Devlin, G., Rees, R., Thompson, R.C.A., 2008. Determining the zoonotic significance of *Giardia* and *Cryptosporidium* in Australian dogs and cats. Vet. Parasitol. 154, 142–147. https://doi.org/10.1016/j.vetpar.2008.02.031

Pan, W., Wang, M., Abdullahi, A.Y., Fu, Y., Yan, X., Yang, F., Shi, X., Zhang, P., Hang, J., Li, G., 2018. Prevalence and genotypes of *Giardia lamblia* from stray dogs and cats in Guangdong, China. Vet. Parasitol. Reg. Stud. Reports 13, 30–34. https://doi.org/10.1016/j.vprsr.2018.03.012

Papini, R., Cardini, G., Paoletti, B., Giangaspero, A., 2007. Detection of *Giardia* assemblage A in cats in Florence, Italy. Parasitol. Res. 100, 653–656. https://doi.org/10.1007/s00436-006-0290-0

Parsons, M.B., Travis, D., Lonsdorf, E. V., Lipende, I., Roellig, D.M.A., Kamenya, S., Zhang, H., Xiao, L., Gillespie, T.R., 2015. Epidemiology and molecular characterization of *Cryptosporidium* spp. in humans, wild primates, and domesticated animals in the greater gombe ecosystem, Tanzania. PLoS Negl. Trop. Dis. 9, 1–13. https://doi.org/10.1371/journal.pntd.0003529

Peeters, J.E., Mazas, E.A., Masschelein, W.J., Dematurana, I.V.M., Debacker, E., 1989. Effect of disinfection of drinking water with ozone or chlorine dioxide on survival of *Cryptosporidium parvum* oocysts. Appl. Environ. Microbiol. 55, 1519–1522.

Pelayo, L., Nuñez, F.A., Rojas, L., Furuseth Hansen, E., Gjerde, B., Wilke, H., Mulder, B., Robertson, L., 2008. *Giardia* infections in Cuban children: the genotypes circulating in a rural population. Ann. Trop. Med. Parasitol. 102, 585–595. https://doi.org/10.1179/136485908X355247

Peng, M.M., Meshnick, S.R., Cunliffe, N.A., Thindwa, D.M., Hart, A.C., Broadhead, R.L., Xiao, L., 2003. Molecular epidemiology of cryptosporidiosis in children in Malawi. J. Eukaryot. Microbiol. 50, 557–559. https://doi.org/10.3389/fmicb.2017.01701

Peng, X., Murphy, T., Holden, N.M., 2008. Evaluation of the effect of temperature on the die-off rate for *Cryptosporidium parvum* oocysts in water, soils, and feces. Appl. Environ. Microbiol. 74, 7101–7107. https://doi.org/10.1128/AEM.01442-08

Pérez-Cordón, G., Robinson, G., Nader, J., Chalmers, R.M., 2016. Discovery of new variable number tandem repeat loci in multiple *Cryptosporidium parvum* genomes for the surveillance and investigation of outbreaks of cryptosporidiosis. Exp. Parasitol. 169, 119–128. https://doi.org/10.1016/j.exppara.2016.08.003

Petersen, H.H., Jianmin, W., Katakam, K.K., Mejer, H., Thamsborg, S.M., Dalsgaard, A., Olsen, A., Enemark, H.L., 2015. *Cryptosporidium* and *Giardia* in Danish organic pig farms: seasonal and age-related variation in prevalence, infection intensity and species/genotypes. Vet. Parasitol. 214, 29–39. https://doi.org/10.1016/j.vetpar.2015.09.020

Petersen, L.R., Cartter, M.L., Halder, L.L., 1988. A food-borne outbreak of *Giardia lamblia*. J. Infec 157, 846–848. https://doi.org/10.1097/00152193-200404000-00056

Pipiková, J., Papajová, I., Majláthová, V., Šoltys, J., Bystrianska, J., Schusterová, I., Vargová, V., 2020. First report on *Giardia duodenalis* assemblage F in Slovakian children living in poor environmental conditions. J. Microbiol. Immunol. Infect. 53, 148–156. https://doi.org/10.1016/j.jmii.2018.04.007

Plutzer, J., Ongerth, J., Karanis, P., 2010. *Giardia* taxonomy, phylogeny and epidemiology: Facts and open questions. Int. J. Hyg. Environ. Health 213, 321–333.

https://doi.org/10.1016/j.ijheh.2010.06.005
Porter, J.D.H., Gaffney, C., Heymann, D., Parkin, W., 1990. Food-borne outbreak of *Giardia lamblia*. Am. J. Public Health 80, 1259–1260. https://doi.org/10.2105/AJPH.80.10.1259
Prystajecky, N., Huck, P.M., Schreier, H., Isaac-Renton, J.L., 2014. Assessment of *Giardia* and *Cryptosporidium* spp. as a microbial source tracking tool for surface water: Application in a mixed-use watershed. Appl. Environ. Microbiol. 80, 2328–2336. https://doi.org/10.1128/AEM.02037-13
Putignani, L., Menichella, D., 2010. Global distribution, public health and clinical impact of the protozoan pathogen *Cryptosporidium*. Interdiscip. Perspect. Infect. Dis. https://doi.org/10.1155/2010/753512
Qi, M., Zhou, H., Wang, H., Wang, R., Xiao, L., Arrowood, M.J., Li, J., Zhang, L., 2015. Molecular identification of *Cryptosporidium* spp and *Giardia duodenalis* in grazing horses from Xinjiang, China. Vet. Parasitol. 209, 169–172. https://doi.org/10.1016/j.vetpar.2015.02.030
Quiroz, E.S., Bern, C., MacArthur, J.R., Xiao, L., Fletcher, M., Arrowood, M.J., Shay, D.K., Levy, M.E., Glass, R.I., Lal, A., 2000. An outbreak of cryptosporidiosis linked to a foodhandler. J. Infect. Dis. 181, 695–700. https://doi.org/10.1086/315279
Rafael, K., Marchioro, A.A., Colli, C.M., Tiyo, B.T., Evangelista, F.F., Bezagio, R.C., Falavigna-Guilherme, A.L., 2017. Genotyping of *Giardia duodenalis* in vegetables cultivated with organic and chemical fertilizer from street markets and community vegetable gardens in a region of Southern Brazil. Trans. R. Soc. Trop. Med. Hyg. 111, 540–545. https://doi.org/10.1093/trstmh/try013
Rai, A.K., Chakravorty, R., Paul, J., 2008. Detection of *Giardia*, *Entamoeba*, and *Cryptosporidium* in unprocessed food items from northern India. World J. Microbiol. Biotechnol. 24, 2879–2887. https://doi.org/10.1007/s11274-008-9824-1
Ralston, B.J., McAllister, T.A., Olson, M.E., 2003. Prevalence and infection pattern of naturally acquired giardiasis and cryptosporidiosis in range beef calves and their dams. Vet. Parasitol. 114, 113–122. https://doi.org/10.1016/S0304-4017(03)00134-1
Ramirez-Martinez, M.L., Olmos-Ortiz, L.M., Barajas-Mendiola, M.A., Giono Cerezo, S., Avila, E.E., Cuellar-Mata, P., 2015. A PCR procedure for the detection of *Giardia intestinalis* cysts and *Escherichia coli* in lettuce. Lett. Appl. Microbiol. 60, 517–523. https://doi.org/10.1111/lam.12402
Rašková, V., Květoňová, D., Sak, B., McEvoy, J., Edwinson, A., Stenger, B., Kváč, M., 2013. Human cryptosporidiosis caused by *Cryptosporidium tyzzeri* and *C. parvum* isolates presumably transmitted from wild mice. J. Clin. Microbiol. 51, 360–362. https://doi.org/10.1128/JCM.02346-12
Read, C.M., Monis, P.T., Thompson, R.C.A., 2004. Discrimination of all genotypes of *Giardia duodenalis* at the glutamate dehydrogenase locus using PCR-RFLP. Infect. Genet. Evol. 4, 125–130. https://doi.org/10.1016/j.meegid.2004.02.001
Reboredo-Fernández, A., Gómez-Couso, H., Martínez-Cedeira, J.A., Cacciò, S.M., Ares-Mazás, E., 2014. Detection and molecular characterization of *Giardia* and *Cryptosporidium* in common dolphins (*Delphinus delphis*) stranded along the Galician coast (Northwest Spain). Vet. Parasitol. 202, 132–137. https://doi.org/10.1016/j.vetpar.2014.03.018
Rehn, M., Wallensten, A., Widerström, M., Lilja, M., Grunewald, M., Stenmark, S., Kark, M., Lindh, J., 2015. Post-infection symptoms following two large waterborne outbreaks of *Cryptosporidium hominis* in Northern Sweden, 2010-2011. BMC Public Health 15, 529. https://doi.org/10.1186/s12889-015-1871-6

Rendtorff, R.C., 1954. The experimental transmission of human intestinal protozoan parasites II. *Giardia lambia* cysts given in capsules. Am. J. Epidemiol. 59, 209–220. https://doi.org/10.1093/oxfordjournals.aje.a119725

Rimšelienė, G., Vold, L., Nygård, K., Robertson, L., Nelke, C., Søli, K., Johansen, Ø.H., Thrana, F.S., 2011. An outbreak of gastroenteritis among schoolchildren staying in a wildlife reserve: thorough investigation reveals Norway's largest cryptosporidiosis outbreak. Scand. J. Public Health 39, 287–295. https://doi.org/10.1177/1403494810396557

Robertson, L.., Campbell, A.., Smith, H. V., 1992. Survival of *Cryptosporidium parvum* oocysts under various environmental pressures. Appl. Environ. Microbiol. 58, 3494–3500.

Robertson, L., Gjerde, B., Hansen, E.F., Stachurska-Hagen, T., 2009. A water contamination incident in Oslo, Norway during October 2007; a basis for discussion of boil-water notices and the potential for post-treatment contamination of drinking water supplies. J. Water Health 7, 55–56. https://doi.org/10.2166/wh.2009.014

Robertson, L.J., 2014. *Cryptosporidium* as a Foodborne Pathogen. Springer, New York.

Robertson, L.J., 2013. *Giardia* as a Foodborne Pathogen. Springer, New York.

Robertson, L.J., 2009. *Giardia* and *Cryptosporidium* infections in sheep and goats: a review of the potential for transmission to humans via environmental contamination. Epidemiol. Infect. 137, 913–921. https://doi.org/10.1017/S0950268809002295

Robertson, L.J., 2007. The potential for marine bivalve shellfish to act as transmission vehicles for outbreaks of protozoan infections in humans: A review. Int. J. Food Microbiol. 120, 201–216. https://doi.org/10.1016/j.ijfoodmicro.2007.07.058

Robertson, L.J., Casaert, S., Valdez-Nava, Y., Ehsan, M.A., Claerebout, E., 2014a. Drying of *Cryptosporidium* oocysts and *Giardia* cysts to slides abrogates use of vital dyes for viability staining. J. Microbiol. Methods 96, 68–69. https://doi.org/10.1016/j.mimet.2013.11.001

Robertson, L.J., Chalmers, R.M., 2013. Foodborne cryptosporidiosis: Is there really more in Nordic countries? Trends Parasitol. 29, 3–9. https://doi.org/10.1016/j.pt.2012.10.003

Robertson, L.J., Clark, C.G., Debenham, J.J., Dubey, J.P., Kváč, M., Li, J., Ponce-Gordo, F., Ryan, U., Schares, G., Su, C., Tsaousis, A.D., 2019a. Are molecular tools clarifying or confusing our understanding of the public health threat from zoonotic enteric protozoa in wildlife? Int. J. Parasitol. Parasites Wildl. 9, 323–341. https://doi.org/10.1016/j.ijppaw.2019.01.010

Robertson, L.J., Forberg, T., Hermansen, L., Gjerde, B.K., Alvsvåg, J.O., Langeland, N., 2006a. *Cryptosporidium parvum* infections in Bergen, Norway, during an extensive outbreak of waterborne giardiasis in autumn and winter 2004. Appl. Environ. Microbiol. 72, 2218–2220. https://doi.org/10.1128/AEM.72.3.2218-2220.2006

Robertson, L.J., Gjerde, B., 2008. Development and use of a pepsin digestion method for analysis of shellfish for *Cryptosporidium* oocysts and *Giardia* cysts. J. Food Prot. 71, 959–966. https://doi.org/10.4315/0362-028X-71.5.959

Robertson, L.J., Gjerde, B., 2001a. Occurrence of parasites on fruits and vegetables in Norway. J. Food Prot. 64, 1793–1798. https://doi.org/10.4315/0362-028X-64.11.1793

Robertson, L.J., Gjerde, B., 2001b. Factors affecting recovery efficiency in isolation of *Cryptosporidium* oocysts and *Giardia* cysts from vegetables for standard method development. J. Food Prot. 64, 1799–1805. https://doi.org/10.4315/0362-028X-64.11.1799

Robertson, L.J., Gjerde, B., 2000. Isolation and enumeration of *Giardia* cysts, *Cryptosporidium* oocysts, and *Ascaris* eggs from fruits and vegetables. J. Food Prot. 63, 775–778.

https://doi.org/10.4315/0362-028X-63.6.775
Robertson, L.J., Gjerde, B.K., 2006. Fate of *Cryptosporidium* oocysts and *Giardia* cysts in the Norwegian aquatic environment over winter. Microb. Ecol. 52, 597–602. https://doi.org/10.1007/s00248-006-9005-4
Robertson, L.J., Hanevik, K., Escobedo, A.A., Mørch, K., Langeland, N., 2010. Giardiasis - why do the symptoms sometimes never stop? Trends Parasitol. 26, 75–82. https://doi.org/10.1016/j.pt.2009.11.010
Robertson, L.J., Hermansen, L., Gjerde, B.K., 2006b. Occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in sewage in Norway. Appl. Environ. Microbiol. 72, 5297–5303. https://doi.org/10.1128/AEM.00464-06
Robertson, L.J., Hermansen, L., Gjerde, B.K., Strand, E., Alvsvåg, J.O., Langeland, N., 2006c. Application of genotyping during an extensive outbreak of waterborne giardiasis in Bergen, Norway, during autumn and winter 2004. Appl. Environ. Microbiol. 72, 2212–2217. https://doi.org/10.1128/AEM.72.3.2212-2217.2006
Robertson, L.J., Johannessen, G.S., Gjerde, B.K., Loncarevic, S., 2002. Microbiological analysis of seed sprouts in Norway. Int. J. Food Microbiol. 75, 119–126. https://doi.org/10.1016/S0168-1605(01)00738-3
Robertson, L.J., Lim, A.L., 2011. Waterborne and environmentally-borne giardiasis, in: Luján, H.D., Svärd, S. (Eds.), Giardia: A Model Organism. New York, pp. 29–69.
Robertson, L.J., Sprong, H., Ortega, Y.R., van der Giessen, J.W.B., Fayer, R., 2014b. Impacts of globalisation on foodborne parasites. Trends Parasitol. 30, 37–52. https://doi.org/10.1016/j.pt.2013.09.005
Robertson, L.J., Temesgen, T.T., Tysnes, K.R., Eikås, J.E., 2019b. An apple a day: an outbreak of cryptosporidiosis in Norway associated with self-pressed apple juice. Epidemiol. Infect. 147, 1–3. https://doi.org/10.1017/S0950268819000232
Rosa, L.A.G., Gomes, M.A., Mundim, A. V., Mundim, M.J.S., Pozzer, E.L., Faria, E.S.M., Viana, J.C., Cury, M.C., 2007. Infection of dogs by experimental inoculation with human isolates of *Giardia duodenalis*: clinical and laboratory manifestations. Vet. Parasitol. 145, 37–44. https://doi.org/10.1016/j.vetpar.2006.11.016
Rose, J.B., Slifko, T.R., 1999. *Giardia*, *Cryptosporidium*, and *Cyclospora* and their impact on foods: a review. J. Food Prot. 62, 1059–1070. https://doi.org/10.4315/0362-028X-62.9.1059
Rosenthal, M., Pedersen, R., Leibsle, S., Hill, V., Carter, K., Roellig, D.M., 2015. Cryptosporidiosis asscociated with consumption of unpasteurized goat milk - Idaho, 2014, Morbidity and Mortality Weekly Report. https://doi.org/10.1111/ajt.13333
Rousseau, A., La Carbona, S., Dumètre, A., Robertson, L.J., Gargala, G., Escotte-Binet, S., Favennec, L., Villena, I., Gérard, C., Aubert, D., 2018. Assessing viability and infectivity of foodborne and waterborne stages (cysts/oocysts) of *Giardia duodenalis*, *Cryptosporidium* spp., and *Toxoplasma gondii*: a review of methods. Parasite 25. https://doi.org/10.1051/parasite/2018009
Ryan, U., Cacciò, S.M., 2013. Zoonotic potential of *Giardia*. Int. J. Parasitol. 43, 943–956. https://doi.org/10.1016/j.ijpara.2013.06.001
Ryan, U., Fayer, R., Xiao, L., 2014. *Cryptosporidium* species in humans and animals: current understanding and research needs. Parasitology 141, 1667–1685. https://doi.org/10.1017/S0031182014001085
Ryan, U., Hijjawi, N., Feng, Y., Xiao, L., 2019. *Giardia*: an under-reported foodborne parasite. Int. J. Parasitol. 49, 1–11. https://doi.org/10.1016/j.ijpara.2018.07.003

Ryan, U., Hijjawi, N., Xiao, L., 2018. Foodborne cryptosporidiosis. Int. J. Parasitol. 48, 1–12. https://doi.org/10.1016/j.ijpara.2017.09.004

Ryan, U., Lawler, S., Reid, S., 2017. Limiting swimming pool outbreaks of cryptosporidiosis – the roles of regulations, staff, patrons and research. J. Water Health 15, 1–16. https://doi.org/10.2166/wh.2016.160

Ryan, U., Paparini, A., Monis, P., Hijjawi, N., 2016a. It's official – *Cryptosporidium* is a gregarine: What are the implications for the water industry? Water Res. 105, 305–313. https://doi.org/10.1016/j.watres.2016.09.013

Ryan, U., Xiao, L., Read, C., Zhou, L., Lal, A.A., Pavlasek, I., 2003. Identification of novel *Cryptosporidium* genotypes from the Czech Republic. Appl. Environ. Microbiol. 69, 4302–4307. https://doi.org/10.1128/AEM.69.7.4302-4307.2003

Ryan, U., Zahedi, A., 2019. Molecular epidemiology of giardiasis from a veterinary perspective, 1st ed, Advances in Parasitology. Elsevier Ltd. https://doi.org/10.1016/bs.apar.2019.07.002

Ryan, U., Zahedi, A., Paparini, A., 2016b. *Cryptosporidium* in humans and animals—a one health approach to prophylaxis. Parasite Immunol. 38, 535–547. https://doi.org/10.1111/pim.12350

Ryan, U.M., Monis, P., Enemark, H.L., Sulaiman, I., Samarasinghe, B., Read, C., Buddle, R., Robertson, I., Zhou, L., Thompson, R.C.A., Xiao, L., 2004. *Cryptosporidium suis* n. sp. (Apicomplexa: Cryptosporidiidae) in pigs (*Sus scrofa*). J. Parasitol. 90, 769–773. https://doi.org/10.1645/ge-202r1

Saeed, H.A., Hamid, H.H., 2010. Bacteriological and parasitological assessment of food handlers in the Omdurman area of Sudan. J. Microbiol. Immunol. Infect. 43, 70–73. https://doi.org/10.1016/S1684-1182(10)60010-2

Sahagún, J., Clavel, A., Goñi, P., Seral, C., Llorente, M.T., Castillo, F.J., Capilla, S., Arias, A., Gómez-Lus, R., 2008. Correlation between the presence of symptoms and the *Giardia duodenalis* genotype. Eur. J. Clin. Microbiol. Infect. Dis. Off. Publ. Eur. Soc. Clin. Microbiol. 27, 81–83. https://doi.org/10.1007/s10096-007-0404-3

Sakkas, H., Economou, V., Bozidis, P., Gousia, P., Papadopoulou, C., Karanis, P., 2020. Detection of *Cryptosporidium* and *Giardia* in foods of plant origin in North-Western Greece. J. Water Health 18, 574–578. https://doi.org/10.2166/wh.2020.037

Samie, A., Tanih, N.F., Seisa, I., Seheri, M., Mphahlele, J., ElBakri, A., Mbati, P., 2020. Prevalence and genetic characterization of *Giardia lamblia* in relation to diarrhea in Limpopo and Gauteng provinces, South Africa. Parasite Epidemiol. Control 9, e00140. https://doi.org/10.1016/j.parepi.2020.e00140

Santín, M., 2020. *Cryptosporidium* and *Giardia* in ruminants. Vet. Clin. North Am. - Food Anim. Pract. 36, 223–238. https://doi.org/10.1016/j.cvfa.2019.11.005

Santín, M., 2013. Clinical and subclinical infections with *Cryptosporidium* in animals. N. Z. Vet. J. 61, 1–10. https://doi.org/10.1080/00480169.2012.731681

Santín, M., Cortés Vecino, J.A., Fayer, R., 2013. A large scale molecular study of *Giardia duodenalis* in horses from Colombia. Vet. Parasitol. 196, 31–36. https://doi.org/10.1016/j.vetpar.2013.02.006

Santín, M., Trout, J.M., Fayer, R., 2008. A longitudinal study of cryptosporidiosis in dairy cattle from birth to 2 years of age. Vet. Parasitol. 155, 15–23. https://doi.org/10.1016/j.vetpar.2008.04.018

Santos, A.L., de Oliveira, L.T.F., Souza, A.L.A., Hauser-Davis, R.A., De Simone, S.G., 2018. *Cryptosporidium* spp. contamination in *Perna perna* mussels destined for human

consumption in Southeastern Rio de Janeiro, Brazil. Bull. Environ. Contam. Toxicol. 100, 240–244. https://doi.org/10.1007/s00128-017-2223-2

Savioli, L., Smith, H. V., Thompson, A., 2006. *Giardia* and *Cryptosporidium* join the "Neglected Diseases Initiative." Trends Parasitol. 22, 203–208. https://doi.org/10.1016/j.pt.2006.02.015

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R. V., Widdowson, M.A., Roy, S.L., Jones, J.L., Griffin, P.M., 2011. Foodborne illness acquired in the United States-major pathogens. Emerg. Infect. Dis. 17, 7–15. https://doi.org/10.3201/eid1701.P11101

Schets, F.M., van den Berg, H.H.J.L., de Roda Husman, A.M., 2013. Determination of the recovery efficiency of *Cryptosporidium* oocysts and *Giardia* cysts from seeded bivalve mollusks. J. Food Prot. 76, 93–98. https://doi.org/10.4315/0362-028X.JFP-12-326

Schets, F.M., van den Berg, H.H.J.L., Engels, G.B., Lodder, W.J., de Roda Husman, A.M., 2007. *Cryptosporidium* and *Giardia* in commercial and non-commercial oysters (*Crassostrea gigas*) and water from the Oosterschelde , the Netherlands. Int. J. Food Microbiol. 113, 189–194. https://doi.org/10.1016/j.ijfoodmicro.2006.06.031

Schubnell, F., von Ah, S., Graage, R., Sydler, T., Sidler, X., Hadorn, D., Basso, W., 2016. Occurrence, clinical involvement and zoonotic potential of endoparasites infecting Swiss pigs. Parasitol. Int. 65, 618–624. https://doi.org/10.1016/j.parint.2016.09.005

Scorza, V., Tangtrongsup, S., 2010. Update on the diagnosis and management of *Cryptosporidium* spp infections in dogs and cats. Top. Companion Anim. Med. 25, 163–169. https://doi.org/10.1053/j.tcam.2010.07.007

Semenza, J.C., Herbst, S., Rechenburg, A., Suk, J.E., Höser, C., Schreiber, C., Kistemann, T., 2012. Climate change impact assessment of food- and waterborne diseases. Crit. Rev. Environ. Sci. Technol. 42, 857–890. https://doi.org/10.1080/10643389.2010.534706

Semenza, J.C., Nichols, G., 2007. Cryptosporidiosis surveillance and water-borne outbreaks in Europe. Eurosurveillance 12, 120–123.

Shapiro, K., Kim, M., Rajal, V.B., Arrowood, M.J., Packham, A., Aguilar, B., Wuertz, S., 2019. Simultaneous detection of four protozoan parasites on leafy greens using a novel multiplex PCR assay. Food Microbiol. 84, 103252. https://doi.org/10.1016/j.fm.2019.103252

Sischo, W.M., Atwill, E.R., Lanyon, L.E., George, J., 2000. Cryptosporidia on dairy farms and the role these farms may have in contaminating surface water supplies in the northeastern United States. Prev. Vet. Med. 43, 253–267. https://doi.org/10.1016/S0167-5877(99)00107-5

Slifko, T.R., Smith, H. V., Rose, J.B., 2000. Emerging parasite zoonoses associated with water and food. Int. J. Parasitol. 30, 1379–1393. https://doi.org/10.1016/S0020-7519(00)00128-4

Smith-DeWaal, C., Barlow, K., Alderton, L., Jacobson, M.F., 2001. Outbreak Alert., in: Center for Science in the Public Interest. Washington, DC, USA.

Smith, A., Reacher, M., Smerdon, W., Adak, G.K., Nichols, G., Chalmers, R.M., 2006. Outbreaks of waterborne infectious intestinal disease in England and Wales, 1992-2003. Epidemiol. Infect. 134, 1141–1149. https://doi.org/10.1017/S0950268806006406

Smith, H. V., Cacciò, S.M., Cook, N., Nichols, R.A.B., Tait, A., 2007. *Cryptosporidium* and *Giardia* as foodborne zoonoses. Vet. Parasitol. 149, 29–40. https://doi.org/10.1016/j.vetpar.2007.07.015

Smith, H. V., Cacciò, S.M., Tait, A., McLauchlin, J., Thompson, R.C.A., 2006. Tools for investigating the environmental transmission of *Cryptosporidium* and *Giardia* infections

in humans. Trends Parasitol. 22, 160–7. https://doi.org/10.1016/j.pt.2006.02.009
Smith, H. V., Nichols, R.A.B., 2010. *Cryptosporidium*: detection in water and food. Exp. Parasitol. 124, 61–79. https://doi.org/10.1016/j.exppara.2009.05.014
Soave, R., Ruiz, J., Garcia-Saucedo, V., Garrocho, C., Kean, B.H., 1989. Cryptosporidiosis in a rural community in Central Mexico. J. Infect. Dis. 159, 1160–1162.
Soliman, R.H., Fuentes, I., Rubio, J.M., 2011. Identification of a novel Assemblage B subgenotype and a zoonotic Assemblage C in human isolates of *Giardia intestinalis* in Egypt. Parasitol. Int. 60, 507–511. https://doi.org/10.1016/j.parint.2011.09.006
Souza, S.L.P., Gennari, S.M., Richtzenhain, L.J., Pena, H.F.J., Funada, M.R., Cortez, A., Gregori, F., Soares, R.M., 2007. Molecular identification of *Giardia duodenalis* isolates from humans, dogs, cats and cattle from the state of São Paulo, Brazil, by sequence analysis of fragments of glutamate dehydrogenase (gdh) coding gene. Vet. Parasitol. 149, 258–264. https://doi.org/10.1016/j.vetpar.2007.08.019
Sponseller, J.K., Griffiths, J.K., Tzipori, S., 2014. The evolution of respiratory cryptosporidiosis: evidence for transmission by inhalation. Clin. Microbiol. Rev. 27, 575–586. https://doi.org/10.1128/CMR.00115-13
Sprong, H., Cacciò, S.M., Van Der Giessen, J.W.B., Partners, on behalf of the ZOOPNET network and partners, 2009. Identification of zoonotic genotypes of *Giardia duodenalis*. PLoS Negl. Trop. Dis. 3, 1–12. https://doi.org/10.1371/journal.pntd.0000558
Squire, S.A., Ryan, U., 2017. *Cryptosporidium* and *Giardia* in Africa: current and future challenges. Parasit Vectors 10, 1–32. https://doi.org/10.1186/s13071-017-2111-y
Stiff, R.E., Davies, A.P., Mason, B.W., Hutchings, H.A., Chalmers, R.M., 2017. Long-term health effects after resolution of acute *Cryptosporidium parvum* infection: a 1-year follow-up of outbreak-associated cases. J. Med. Microbiol. 66, 1607–1611. https://doi.org/10.1099/jmm.0.000609
Stokdyk, J.P., Spencer, S.K., Walsh, J.F., de Lambert, J.R., Firnstahl, A.D., Anderson, A.C., Rezania, L.W., Borchardt, M.A., 2019. *Cryptosporidium* incidence and surface water influence of groundwater supplying public water systems in Minnesota, USA. Environ. Sci. Technol. 53, acs.est.8b05446. https://doi.org/10.1021/acs.est.8b05446
Štrkolcová, G., Madár, M., Hinney, B., Goldová, M., Mojžišová, J., Halánová, M., 2015. Dog's genotype of *Giardia duodenalis* in human: first evidence in Europe. Acta Parasitol. 60, 796–799. https://doi.org/10.1515/ap-2015-0113
Sulaiman, I.M., Fayer, R., Bern, C., Gilman, R.H., Trout, J.M., Schantz, P.M., Das, P., Lal, A.A., Xiao, L., 2003. Triosephosphate isomerase gene characterization and potential zoonotic transmission of *Giardia duodenalis.* Emerg Infect Dis. 9, 144–1452. https://doi.org/10.3201/eid0911.030084
Sulaiman, I.M., Hira, P.R., Zhou, L., Al-Ali, F.M., Al-Shelahi, F.A., Shweiki, H.M., Iqbal, J., Khalid, N., Xiao, L., 2005. Unique endemicity of cryptosporidiosis in children in Kuwait. J. Clin. Microbiol. 43, 2805–2809. https://doi.org/10.1128/JCM.43.6.2805-2809.2005
Sulaiman, I.M., Lal, A.A., Xiao, L., 2002. Molecular phylogeny and evolutionary relationships of *Cryptosporidium* parasites at the actin locus. J. Parasitol. 88, 388–394. https://doi.org/10.1645/0022-3395(2002)088[0388:MPAERO]2.0.CO;2
Sulaiman, I.M., Lal, A.A., Xiao, L., 2001. A population genetic study of the *Cryptosporidium parvum* human genotype parasites. J. Eukaryot. Microbiol. 48, 24s-27s. https://doi.org/10.1111/j.1550-7408.2001.tb00441.x
Sulaiman, I.M., Morgan, U.M., Thompson, R.C.A., Lal, A.A., Xiao, L., 2000. Phylogenetic relationships of *Cryptosporidium* parasites based on the 70- kilodalton heat shock

protein (HSP70) gene. Appl. Environ. Microbiol. 66, 2385–2391. https://doi.org/10.1128/AEM.66.6.2385-2391.2000

Sunnotel, O., Snelling, W.J., Xiao, L., Moule, K., Moore, J.E., Millar, B.C., Dooley, J.S.G., Lowery, C.J., 2006. Rapid and sensitive detection of single *Cryptosporidium* oocysts from archived glass slides. J. Clin. Microbiol. 44, 3285–3291. https://doi.org/10.1128/JCM.00541-06

Taghipour, A., Olfatifar, M., Bahadory, S., Godfrey, S.S., Abdoli, A., Khatami, A., Javanmard, E., Shahrivar, F., 2020. The global prevalence of *Cryptosporidium* infection in dogs: a systematic review and meta-analysis. Vet. Parasitol. 281, 109093. https://doi.org/10.1016/j.vetpar.2020.109093

Takizawa, M. das G.M.H., Falavigna, D.L.M., Gomes, M.L., 2009. Enteroparasitosis and their ethnographic relationship to food handlers in a tourist and economic center in Paraná, southern Brazil. Rev. Inst. Med. Trop. Sao Paulo 51, 31–35. https://doi.org/10.1590/S0036-46652009000100006

Tangtrongsup, S., Scorza, A.V., Reif, J.S., Ballweber, L.R., Lappin, M.R., Salman, M.D., 2020. Seasonal distributions and other risk factors for *Giardia duodenalis* and *Cryptosporidium* spp. infections in dogs and cats in Chiang Mai, Thailand. Prev. Vet. Med. 174, 104820. https://doi.org/10.1016/j.prevetmed.2019.104820

Tangtrongsup, S., Scorza, V., 2010. Update on the diagnosis and management of *Giardia* spp infections in dogs and cats. Top. Companion Anim. Med. 25, 155–162. https://doi.org/10.1053/j.tcam.2010.07.003

Tei, F.F., Kowalyk, S., Reid, J.A., Presta, M.A., Yesudas, R., Mayer, D.C.G., 2016. Assessment and molecular characterization of human intestinal parasites in bivalves from Orchard Beach, NY, USA. Int. J. Environ. Res. Public Health 381. https://doi.org/10.3390/ijerph13040381

Teunis, P.F.M., van der Heijden, O.G., van der Giessen, J.W.B., Havelaar, A.H., 1996. The dose-response relation in human volunteers for gastro-intestinal pathogens. The Netherlands.

Thompson, R.C.A., 2004. The zoonotic significance and molecular epidemiology of *Giardia* and giardiasis. Vet. Parasitol. 126, 15–35. https://doi.org/10.1016/j.vetpar.2004.09.008

Thompson, R.C.A., Monis, P.T., 2004. Variation in *Giardia*: Implications for taxonomy and epidemiology. Adv. Parasitol. 58, 69–137. https://doi.org/10.1016/S0065-308X(04)58002-8

Thompson, R.C.A., Olson, M.E., Zhu, G., Enomoto, S., Abrahamsen, M.S., Hijjawi, N.S., 2005. *Cryptosporidium* and cryptosporidiosis. Adv. Parasitol. 59, 77–158. https://doi.org/10.1016/S0065-308X(05)59002-X

Thompson, R.C.A., Palmer, C.S., O'Handley, R., 2008. The public health and clinical significance of *Giardia* and *Cryptosporidium* in domestic animals. Vet. J. 177, 18–25. https://doi.org/10.1016/j.tvjl.2007.09.022

Tiyo, R., De Souza, C.Z., Arruda Piovesani, A.F., Tiyo, B.T., Colli, C.M., Marchioro, A.A., Gomes, M.L., Falavigna-Guilherme, A.L., 2016. Predominance of *Giardia duodenalis* assemblage AII in fresh leafy vegetables from a market in Southern Brazil. J. Food Prot. 79, 1036–1039. https://doi.org/10.4315/0362-028X.JFP-15-306

Torgerson, P.R., Devleesschauwer, B., Praet, N., Speybroeck, N., Willingham, A.L., Kasuga, F., Rokni, M.B., Zhou, X.N., Fèvre, E.M., Sripa, B., Gargouri, N., Fürst, T., Budke, C.M., Carabin, H., Kirk, M.D., Angulo, F.J., Havelaar, A., de Silva, N., 2015. World Health Organization estimates of the global and regional disease burden of 11 foodborne parasitic diseases, 2010: A data synthesis. PLoS Med. 12, 1–22. https://doi.org/10.1371/journal.pmed.1001920

Traub, R., Wade, S., Read, C., Thompson, A., Mohammed, H., 2005. Molecular characterization of potentially zoonotic isolates of *Giardia duodenalis* in horses. Vet. Parasitol. 130, 317–321. https://doi.org/10.1016/j.vetpar.2005.02.011

Traversa, D., Otranto, D., Milillo, P., Latrofa, M.S., Giangaspero, A., Di Cesare, A., Paoletti, B., 2012. *Giardia duodenalis* sub-assemblage of animal and human origin in horses. Infect. Genet. Evol. 12, 1642–1646. https://doi.org/10.1016/j.meegid.2012.06.014

Tryland, I., Myrmel, M., Østensvik, Ø., Wennberg, A.C., Robertson, L.J., 2014. Impact of rainfall on the hygienic quality of blue mussels and water in urban areas in the Inner Oslofjord, Norway. Mar. Pollut. Bull. 85, 42–49. https://doi.org/10.1016/j.marpolbul.2014.06.028

Tysnes, K.R., Skancke, E., Robertson, L.J., 2014. Subclinical *Giardia* in dogs: a veterinary conundrum relevant to human infection. Trends Parasitol. 30, 520–527. https://doi.org/10.1016/j.pt.2014.08.007

Tyzzer, E.E., 1910. An extracellular Coccidium, *Cryptosporidium muris* (Gen. Et Sp. Nov.), of the gastric glands of the common mouse. J. Med. Res. 23, 487-510.3.

Tyzzer, E.E., 1907. A sporozoan found in the peptic glands of the common mouse. Sci. Proc. Soc. Exp. Biol. Med. 5, 12–13.

Ulloa-Stanojlović, F.M., Aguiar, B., Jara, L.M., Sato, M.I.Z., Guerrero, J.A., Hachich, E., Matté, G.R., Dropa, M., Matté, M.H., de Araújo, R.S., 2016. Occurrence of *Giardia intestinalis* and *Cryptosporidium* sp. in wastewater samples from São Paulo State, Brazil, and Lima, Peru. Environ. Sci. Pollut. Res. https://doi.org/10.1007/s11356-016-7537-9

Upton, S.J., Zien, C.A., 1997. Description of a *Giardia varani*-like flagellate from a water monitor, *Varanus salvator*, from Malaysia. J. Parasitol. 83, 970–971. https://doi.org/10.2307/3284303

Ursini, T., Moro, L., Requena-Méndez, A., Bertoli, G., Buonfrate, D., 2020. A review of outbreaks of cryptosporidiosis due to unpasteurized milk. Infection 1–5. https://doi.org/10.1007/s15010-020-01426-3

US EPA 2005. Method 1623: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA. U.S Environmental Protection Agency, Office of Water, Washington, D.C., USA

US EPA 2012. Method 1623.1: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA. U.S Environmental Protection Agency, Office of Water, Washington, D.C., USA

Utaaker, K.S., Kumar, A., Joshi, H., Chaudhary, S., Robertson, L.J., 2017a. Checking the detail in retail: occurrence of *Cryptosporidium* and *Giardia* on vegetables sold across different counters in Chandigarh, India. Int. J. Food Microbiol. 263, 1–8. https://doi.org/10.1016/j.ijfoodmicro.2017.09.020

Utaaker, K.S., Skjerve, E., Robertson, L.J., 2017b. Keeping it cool: survival of *Giardia* cysts and *Cryptosporidium* oocysts on lettuce leaves. Int. J. Food Microbiol. 255, 51–57. https://doi.org/10.1016/j.ijfoodmicro.2017.05.009

van de Venter, E.C., Oliver, I., Stuart, J.M., 2015. Timeliness of epidemiological outbreak investigations in peer-reviewed European publications, January 2003 to August 2013. Eurosurveillance 20, 1–10. https://doi.org/10.2807/1560-7917.ES2015.20.6.21035

van Keulen, H., Macechko, P.T., Wade, S., Schaaf, S., Wallis, P.M., Erlandsen, S.L., 2002. Presence of human *Giardia* in domestic, farm and wild animals, and environmental samples suggests a zoonotic potential for giardiasis. Vet. Parasitol. 108, 97–107. https://doi.org/10.1016/S0304-4017(02)00181-4

Veazie, L., 1969. Epidemic giardiasis. N. Engl. J. Med. 281.

Veronesi, F., Passamonti, F., Cacciò, S., Diaferia, M., Piergili Fioretti, D., 2010. Epidemiological survey on equine *Cryptosporidium* and *Giardia* infections in Italy and molecular

characterization of isolates. Zoonoses Public Health 57, 510–517. https://doi.org/10.1111/j.1863-2378.2009.01261.x

Vetterling, J.M., Takeuchi, A., Madden, P.A., 1971. Ultrastructure of *Cryptosporidium* wrairi from the guinea pig. J. Protozool. 18, 248–260. https://doi.org/10.1111/j.1550-7408.1971.tb03316.x

Wagnerová, P., Sak, B., McEvoy, J., Rost, M., Sherwood, D., Holcomb, K., Kváč, M., 2016. *Cryptosporidium parvum* and *Enterocytozoon bieneusi* in American mustangs and chincoteague ponies. Exp. Parasitol. 162, 24–27. https://doi.org/10.1016/j.exppara.2015.12.004

Waldron, L.S., Ferrari, B.C., Cheung-Kwok-Sang, C., Beggs, P.J., Stephens, N., Power, M.L., 2011. Molecular epidemiology and spatial distribution of a waterborne cryptosporidiosis outbreak in Australia. Appl. Environ. Microbiol. 77, 7766–7771. https://doi.org/10.1128/AEM.00616-11

Wang, Y., Gonzalez-Moreno, O., Roellig, D.M., Oliver, L., Huguet, J., Guo, Y., Feng, Y., Xiao, L., 2019. Epidemiological distribution of genotypes of *Giardia duodenalis* in humans in Spain. Parasit Vectors 12, 6–15. https://doi.org/10.1186/s13071-019-3692-4

Wei, Z., Liu, Q., Zhao, W., Jiang, X., Zhang, Y., Zhao, A., Jing, B., Lu, G., Qi, M., 2019. Prevalence and diversity of *Cryptosporidium* spp. in bamboo rats (*Rhizomys sinensis*) in South Central China. Int. J. Parasitol. Parasites Wildl. 9, 312–316. https://doi.org/10.1016/j.ijppaw.2019.06.010

WHO, 2004. The Global Burden of Disease, 2004 update, World Health Organization, Geneva, Switzerland. https://doi.org/10.1038/npp.2011.85

WHO, 1996. The World Health Report 1996. Fighting disease Foresting development.

WHO, 1979. Parasitic zoonoses. Report of a WHO expert committee with the participation of FAO., World Health Organization, Geneva. Technical Report Series No. 637.

Widerström, M., Schönning, C., Lilja, M., Lebbad, M., Ljung, T., Allestam, G., Ferm, M., Björkholm, B., Hansen, A., Hiltula, J., Långmark, J., Löfdahl, M., Omberg, M., Reuterwall, C., Samuelsson, E., Widgren, K., Wallensten, A., Lindh, J., 2014. Large outbreak of *Cryptosporidium hominis* infection transmitted through the public water supply, Sweden. Emerg. Infect. Dis. 20, 581–589.

Widmer, G., 2009. Meta-analysis of a polymorphic surface glycoprotein of the parasitic protozoa *Cryptosporidium parvum* and *Cryptosporidium hominis*. Epidemiol. Infect. 137, 1800–1808. https://doi.org/10.1017/S0950268809990215.

Wielinga, P.R., de Vries, A., van der Goot, T.H., Mank, T., Mars, M.H., Kortbeek, L.M., van der Giessen, J.W.B., 2008. Molecular epidemiology of *Cryptosporidium* in humans and cattle in The Netherlands. Int. J. Parasitol. 38, 809–817. https://doi.org/10.1016/j.ijpara.2007.10.014

Willis, J.E., McClure, J.T., Davidson, J., McClure, C., Greenwood, S.J., 2013. Global occurrence of *Cryptosporidium* and *Giardia* in shellfish: Should Canada take a closer look? Food Res. Int. 52, 119–135. https://doi.org/10.1016/j.foodres.2013.02.020

Winiecka-Krusnell, J., Linder, E., 1998. Cysticidal effect of chlorine dioxide on *Giardia intestinalis* cysts. Acta Trop. 70, 369–372. https://doi.org/10.1016/S0001-706X(98)00036-9

Xiao, L., 2010. Molecular epidemiology of cryptosporidiosis: An update. Exp. Parasitol. 124, 80–89. https://doi.org/10.1016/j.exppara.2009.03.018

Xiao, L., 1994. *Giardia* infection in farm animals. Parasitol. Today 10, 436–438. https://doi.org/10.1016/0169-4758(94)90178-3

Xiao, L., Escalante, L., Yang, C., Sulaiman, I., Escalante, A.A., Montali, R.J., Fayer, R., Lal, A.A., 1999. Phylogenetic analysis of *Cryptosporidium* parasites based on the small- subunit rRNA gene locus. Appl. Environ. Microbiol. 65, 1578–1583. https://doi.org/10.1128/aem.65.4.1578-1583.1999

Xiao, L., Fayer, R., 2008. Molecular characterisation of species and genotypes of *Cryptosporidium* and *Giardia* and assessment of zoonotic transmission. Int. J. Parasitol. 38, 1239–1255. https://doi.org/10.1016/j.ijpara.2008.03.006

Xiao, L., Feng, Y., 2017. Molecular epidemiologic tools for waterborne pathogens *Cryptosporidium* spp. and *Giardia duodenalis*. Food Waterborne Parasitol. 8–9, 14–32. https://doi.org/10.1016/j.fawpar.2017.09.002

Xiao, L., Feng, Y., 2008. Zoonotic cryptosporidiosis. FEMS Immunol. Med. Microbiol. 52, 309–323. https://doi.org/10.1111/j.1574-695X.2008.00377.x

Xiao, L., Herd, R.P., 1994. Epidemiology of equine *Cryptosporidium* and *Giardia* infections. Equine Vet. J. 26, 14–17. https://doi.org/10.1111/j.2042-3306.1994.tb04323.x

Xiao, L., Limor, J., Morgan, U.M., Sulaiman, I.M., Thompson, R.C.A., Lal, A.A., 2000. Sequence differences in the diagnostic target region of the oocyst wall protein gene of *Cryptosporidium* parasites. Appl. Environ. Microbiol. 66, 5499–5502. https://doi.org/10.1128/AEM.66.12.5499-5502.2000

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

Xie, F., Zhang, Z., Zhao, A., Jing, B., Qi, M., Wang, R., 2019. Molecular characterization of *Cryptosporidium* and *Enterocytozoon bieneusi* in Père David's deer (*Elaphurus davidianus*) from Shishou, China. Int. J. Parasitol. Parasites Wildl. 10, 184–187. https://doi.org/10.1016/j.ijppaw.2019.09.001

Xu, F., Jerlström-Hultqvist, J., Andersson, J.O., 2012. Genome-wide analyses of recombination suggest that *Giardia intestinalis* assemblages represent different species. Mol. Biol. Evol. 29, 2895–2898. https://doi.org/10.1093/molbev/mss107

Yang, R., Paparini, A., Monis, P., Ryan, U., 2014. Comparison of next-generation droplet digital PCR (ddPCR) with quantitative PCR (qPCR) for enumeration of *Cryptosporidium* oocysts in faecal samples. Int. J. Parasitol. 44, 1105–1113. https://doi.org/10.1016/j.ijpara.2014.08.004

Yoder, J.S., Beach, M.J., 2007. Giardiasis surveillance - United States, 2003-2005. Morb. Mortal. Wkly. Rep. 56, 11–18.

Yoshida, H., Matsuo, M., Miyoshi, T., Uchino, K., Nakaguchi, H., Fukumoto, T., Teranaka, Y., Tanaka, T., 2007. An outbreak of cryptosporidiosis suspected to be related to contaminated food, October 2006, Sakai City, Japan. Jpn. J. Infect. Dis. 60, 405–407.

Yui, T., Nakajima, T., Yamamoto, N., Kon, M., Abe, N., Matsubayashi, M., Shibahara, T., 2014. Age-related detection and molecular characterization of *Cryptosporidium suis* and *Cryptosporidium scrofarum* in pre- and post-weaned piglets and adult pigs in Japan. Parasitol. Res. 113, 359–365. https://doi.org/10.1007/s00436-013-3662-2

Zaglool, D.A., Khodari, Y.A., Othman, R.A.M., Farooq, M.U., 2011. Prevalence of intestinal parasites and bacteria among food handlers in a tertiary care hospital. Niger. Med. J. 52, 266–270. https://doi.org/10.4103/0300-1652.93802

Zahedi, A., Field, D., Ryan, U., 2017. Molecular typing of *Giardia duodenalis* in humans in Queensland – first report of assemblage E. Parasitology 114, 1154–1161. https://doi.org/10.1017/S0031182017000439

Zahedi, A., Greay, T.L., Paparini, A., Linge, K.L., Joll, C.A., Ryan, U.M., 2019. Identification of eukaryotic microorganisms with 18S rRNA next-generation sequencing in wastewater treatment plants, with a more targeted NGS approach required for *Cryptosporidium* detection. Water Res. 158, 301–312. https://doi.org/10.1016/j.watres.2019.04.041

Zahedi, A., Monis, P., Aucote, S., King, B., Paparini, A., Jian, F., Yang, R., Oskam, C., Ball, A., Robertson, I., Ryan, U., 2016a. Zoonotic *Cryptosporidium* species in animals inhabiting Sydney water catchments. PLoS One 11, 1–18. https://doi.org/10.1371/journal.pone.0168169

Zahedi, A., Paparini, A., Jian, F., Robertson, I., Ryan, U., 2016b. Public health significance of zoonotic *Cryptosporidium* species in wildlife: Critical insights into better drinking water management. Int. J. Parasitol. Parasites Wildl. 5, 88–109. https://doi.org/10.1016/j.ijppaw.2015.12.001

Zanzani, S.A., Gazzonis, A.L., Scarpa, P., Berrilli, F., Manfredi, M.T., 2014. Intestinal parasites of owned dogs and cats from metropolitan and micropolitan areas: prevalence, zoonotic risks, and pet owner awareness in northern Italy. Biomed Res. Int. 2014. https://doi.org/10.1155/2014/696508

Zhang, X., Wang, L., Lan, X., Dan, J., Ren, Z., Cao, S., Shen, L., Deng, J., Zuo, Z., Yu, S., Wang, Ya, Ma, X., Liu, H., Zhou, Z., Hu, Y., Fu, H., He, C., Geng, Y., Gu, X., Peng, G., Wang, Yufei, WangZhong, Z., 2020. Occurrence and multilocus genotyping of *Giardia duodenalis* in captive non-human primates from 12 zoos in China. PLoS One 15, 1–11. https://doi.org/10.1371/journal.pone.0228673

Objectives

Cryptosporidium and *Giardia* are important parasitic protozoa, causing gastrointestinal disease in both humans and animals. Both parasites are considered to be zoonotic (Thompson, 2004; Xiao, 2010), however the importance of their zoonotic transmission remains under debate, especially for *Giardia*, mainly due to the use of new molecular genotyping tools (Cacciò et al., 2018; Ryan and Cacciò, 2013; Thompson and Ash, 2019). The most common route of transmission is through contaminated water and/or food, and they are responsible for many waterborne (sometimes also foodborne) outbreaks of disease worldwide, having an important impact on public health (Baldursson and Karanis, 2011; Efstratiou et al., 2017b; Karanis et al., 2007; McClung et al., 2018; Ryan et al., 2019, 2018; Semenza and Nichols, 2007). Thus, identifying and understanding transmission cycles of these parasites is an important issue that needs to be investigated thoroughly, as information on their transmission dynamics is vital for the prevention of outbreaks and minimization of the public health risk.

Contamination of water sources (and consequently of foodstuff) with both protozoa may have many sources, including runoffs from agricultural areas, drainage from manure storage, wastewaters overflows or improper sewage systems (Bodley-Tickell et al., 2002; Cacciò et al., 2003; Daniels et al., 2016; Kistemann et al., 2012; Robertson et al., 2006; Sischo et al., 2000; Slifko et al., 2000; Swaffer et al., 2018). Also climate changes and, specifically, extreme rainfall events have been associated with elevated levels of water contamination with *Cryptosporidium* and/or *Giardia* (Atherholt et al., 1998; Daniels et al., 2016; Dias et al., 2018; Fayer et al., 2002; Mellor et al., 2016; Mons et al., 2009; Semenza et al., 2012; Sischo et al., 2000). However, investigating these parasites in an aquatic environment is challenging, due to costly, time consuming techniques that need to be performed by experienced personnel (Efstratiou et al., 2017a). As a result, alternative markers (e.g. microbiological, physicochemical and weather-related) that are easier and cheaper to measure in water matrices have been investigated in order to correlate with the presence of these parasites (Cizek et al., 2008; Duris et al., 2013; Levantesi et al., 2010; Tolouei et al., 2019; Wilkes et al., 2011, 2009; Young et al., 2015), although with little success due to the heterogeneity among their relationships (e.g. different source, size, fate, density, frequency and levels of excretion) (Brookes et al., 2005, 2004; Davies et al., 2003; Levantesi et al., 2010; Wilkes et al., 2009). This

constitutes another limitation on management and control of these parasitic protozoa in water resources.

Greece is considered to be a relatively safe country as concerns the contamination of water with *Cryptosporidium* and/or *Giardia*, since up to date only one giardiasis outbreak due to waterborne transmission has been reported (Hadjichristodoulou et al., 1998; Hardie et al., 1999, 1997) and waterborne disease outbreaks are rare (https://eody.gov.gr). Although lack of surveillance and underreporting could be a reason for the absence of other reported outbreaks, parasite contamination levels in Greek water sources were quite low (<0.5 *Cryptosporidium* oocysts/L and <1 *Giardia* cysts/L) in the few studies that are available (Economou et al., 2013; Karanis et al., 2005, 2002; Papadopoulou et al., 2008; Spanakos et al., 2015).

However, even if the presence of *Cryptosporidium* and *Giardia* in surface waters in Greece seems to be relatively low, it is not safe to underestimate the risk for future waterborne outbreaks, taking into consideration that: a) both parasites are highly abundant in different animal species (both potentially zoonotic and species-specific genotypes/assemblages identified) (Arsenopoulos et al., 2017; Giadinis et al., 2015; Haralabidis et al., 1988; Kostopoulou et al., 2017, 2015; Papanikolopoulou et al., 2018; Papazahariadou et al., 2007; Symeonidou et al., 2017; Tzanidakis et al., 2014) and were isolated from humans (the potentially zoonotic *G. duodenalis* sub-assemblage AII has been detected; lack of information on *Cryptosporidium* species detected) (Kostopoulou et al., 2020; Maltezou et al., 2001; Printza et al., 2013) and food (only *Cryptosporidium* oocysts) (Sakkas et al., 2020), b) surface water sources are commonly used for production of drinking water in Greece and often livestock farms are found in close proximity, and c) Greece, due to its geographical location, shares common water bodies with other Balkan countries (i.e. Bulgaria and North Macedonia), where confirmed cases of cryptosporidiosis and giardiasis have been documented (Plutzer et al., 2018). Therefore, understanding waterborne transmission of *Cryptosporidium* and *Giardia* and determining their zoonotic nature in aquatic resources is important in order to estimate the public health risk for disease outbreaks and to generate appropriate preventive measures.

For the current thesis, Northern Greece and precisely the Thessaloniki greater area and the northern part of Thermaikos Gulf has been selected as an interesting case scenario to study the dynamics of waterborne transmission of *Cryptosporidium* and *Giardia*. The area is

characterised by: (i) an urban environment – the city of Thessaloniki (> 1,100,000 inhabitants) is built around Thermaikos Gulf; (ii) an agricultural environment – the area around the city is a dense farming area with numerous cattle and sheep farms; (iii) many surface water sources – there are 4 rivers, i.e. Gallikos, Axios, Loudias and Aliakmonas, that form a large deltaic complex on the northern and western parts of the gulf; (iv) a water production company using water from Aliakmonas river for the production of drinking water for Thessaloniki; (v) 3 wastewater treatment plants that treat human, agricultural and industrial waste, which are either re-used for irrigation or discharged into the gulf (mostly); (vi) the most intensive mussel farming of the Mediterranean mussel, *Mytilus galloprovincialis*, located close to the deltaic complex of the rivers and wastewater discharges, totaling 80-90% of the whole mussel production in Greece (Theodorou et al., 2011).

Specifically, the objectives of this Ph.D. thesis were:

1. To monitor the presence of *Cryptosporidium* and *Giardia* in different water sources in Northern Greece, including surface water, drinking water and treated waste waters.
2. To monitor the presence of *Cryptosporidium* and *Giardia* in sheep and cattle farms in the vicinity of surface water (rivers), as potential sources of water contamination.
3. To determine the *Cryptosporidium* species/genotypes and *Giardia* (sub)assemblages, through the performance of molecular analyses, in order to evaluate their zoonotic potential and to identify potential sources of water contamination and the risk of waterborne and foodborne infections.
4. To develop a risk assessment model to identify parameters that can predict the risk of surface water contamination with *Cryptosporidium* and/or *Giardia*.
5. To monitor the presence of *Cryptosporidium* and *Giardia* in mussels cultured in Thermaikos Gulf.

References

Arsenopoulos, K., Theodoridis, A., Papadopoulos, E., 2017. Effect of colostrum quantity and quality on neonatal calf diarrhoea due to *Cryptosporidium* spp. infection. Comp. Immunol. Microbiol. Infect. Dis. 53, 50–55. https://doi.org/10.1016/j.cimid.2017.07.005

Atherholt, T.B., LeChevallier, M.W., Norton, W.D., Rosen, J.S., 1998. Effect of rainfall on *Giardia* and *Crypto*. J. Am. Water Work. Assoc. 90, 66–80. https://doi.org/10.1002/j.1551-8833.1998.tb08499.x

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

Bodley-Tickell, A.T., Kitchen, S.E., Sturdee, A.P., 2002. Occurrence of *Cryptosporidium* in agricultural surface waters during an annual farming cycle in lowland UK. Water Res. 36, 1880–1886.

Brookes, J.D., Antenucci, J., Hipsey, M., Burch, M.D., Ashbolt, N.J., Ferguson, C., 2004. Fate and transport of pathogens in lakes and reservoirs. Environ. Int. 30, 741–759. https://doi.org/10.1016/j.envint.2003.11.006

Brookes, J.D., Hipsey, M.R., Burch, M.D., Regel, R.H., Linden, L.G., Ferguson, C.M., Antenucci, J.P., 2005. Relative value of surrogate indicators for detecting pathogens in lakes and reservoirs. Environ. Sci. Technol. 39, 8614–8621. https://doi.org/10.1021/es050821+

Cacciò, S.M., De Giacomo, M., Aulicino, F.A., Pozio, E., 2003. *Giardia* cysts in wastewater treatment plants in Italy. Appl. Environ. Microbiol. 69, 3393–3398. https://doi.org/10.1128/AEM.69.6.3393-3398.2003

Cacciò, S.M., Lalle, M., Svärd, S.G., 2018. Host specificity in the *Giardia duodenalis* species complex. Infect. Genet. Evol. 66, 335–345. https://doi.org/10.1016/j.meegid.2017.12.001

Cizek, A.R., Characklis, G.W., Krometis, L.-A., Hayes, J.A., Simmons, O.D., Di Lonardo, S., Alderisio, K.A., Sobsey, M.D., 2008. Comparing the partitioning behavior of *Giardia* and *Cryptosporidium* with that of indicator organisms in stormwater runoff. Water Res. 42, 4421–4438. https://doi.org/10.1016/j.watres.2008.06.020

Daniels, M.E., Smith, W.A., Schmidt, W.-P., Clasen, T., Jenkins, M.W., 2016. Modeling *Cryptosporidium* and *Giardia* in ground and surface water sources in rural India: associations with latrines, livestock, damaged wells, and rainfall patterns. Environ. Sci. Technol. 50, 7498–7507. https://doi.org/10.1021/acs.est.5b05797

Davies, C.M., Kaucner, C., Deere, D., Ashbolt, N.J., 2003. Recovery and enumeration of *Cryptosporidium parvum* from animal fecal matrices. Appl. Environ. Microbiol. 69, 2842–2847. https://doi.org/10.1128/AEM.69.5.2842-2847.2003

Dias, A.P., Calegar, D., Carvalho-Costa, F.A., Alencar, M. de F.L., Ignacio, C.F., da Silva, M.E., Henrique, A., de Moraes Neto, A.H.A., 2018. Assessing the influence of water management and rainfall seasonality on water quality and intestinal parasitism in rural northeastern Brazil. J. Trop. Med. 2018. https://doi.org/10.1155/2018/8159354

Duris, J.W., Reif, A.G., Krouse, D.A., Isaacs, N.M., 2013. Factors related to occurrence and distribution of selected bacterial and protozoan pathogens in Pennsylvania streams. Water Res. 47, 300–314. https://doi.org/10.1016/j.watres.2012.10.006

Economou, V., Gousia, P., Kansouzidou, A., Sakkas, H., Karanis, P., Papadopoulou, C., 2013. Prevalence, antimicrobial resistance and relation to indicator and pathogenic microorganisms of *Salmonella enterica* isolated from surface waters within an

agricultural landscape. Int. J. Hyg. Environ. Health 216, 435–444. https://doi.org/10.1016/j.ijheh.2012.07.004

Efstratiou, A., Ongerth, J., Karanis, P., 2017a. Evolution of monitoring for *Giardia* and *Cryptosporidium* in water. Water Res. 123, 96–112. https://doi.org/10.1016/j.watres.2017.06.042

Efstratiou, A., Ongerth, J.E., Karanis, P., 2017b. Waterborne transmission of protozoan parasites: Review of worldwide outbreaks - An update 2011–2016. Water Res. 114, 14–22. https://doi.org/10.1016/j.watres.2017.01.036

Fayer, R., Trout, J.., Lewis, E.., Xiao, L., Lal, A., Jenkins, M.., Graczyk, T.., 2002. Temporal variability of *Cryptosporidium* in the Chesapeake Bay. Parasitol. Res. 88, 998–1003. https://doi.org/10.1007/s00436-002-0697-1

Giadinis, N.D., Papadopoulos, E., Lafi, S.Q., Papanikolopoulou, V., Karanikola, S., Diakou, A., Vergidis, V., Xiao, L., Ioannidou, E., Karatzias, H., 2015. Epidemiological observations on cryptosporidiosis in diarrheic goat kids in Greece. Vet. Med. Int. 2015, 4–7. https://doi.org/10.1155/2015/764193

Hadjichristodoulou, C., Gkikas, A., Pediatidis, Y., Tselentis, Y., 1998. Outbreak of giardiasis among English tourists in Crete. Lancet 351, 65–66. https://doi.org/10.1016/s0140-6736(05)78057-0

Haralabidis, S.T., Papazachariadou, M.G., Koutinas, A.F., Rallis, T.S., 1988. A survey on the prevalence of gastrointestinal parasites of dogs in the area of Thessaloniki, Greece. J. Helminthol. 62, 45–49. https://doi.org/10.1017/S0022149X00011196

Hardie, R.M., Bardham, M., Wall, P.G., 1997. Unwanted holiday souvenirs: who should pay? J. Infect. Dis. 350, 964.

Hardie, R.M., Wall, P.G., Gott, P., Bardhan, M., Bartlett, C.L.R., 1999. Infectious diarrhea in tourists staying in a resort hotel. Emerg. Infect. Dis. 5, 168–171. https://doi.org/10.3201/eid0501.990123

Karanis, P., Chronis, I., Zakas, G., Kourenti, C., Sotiriadou, I., Papadopoulou, C., 2005. A preliminary survey of the level of microbiological pollution of major rivers in Northern Greece. Acta Hydrochim. Hydrobiol. 33, 346–354. https://doi.org/10.1002/aheh.200400581

Karanis, P., Kourenti, C., Smith, H. V., 2007. Waterborne transmission of protozoan parasites: A worldwide review of outbreaks and lessons learnt. J. Water Health 5, 1–38. https://doi.org/10.2166/wh.2006.002

Karanis, P., Papadopoulou, C., Kimura, A., Economou, E., Kourenti, C., Sakkas, H., 2002. *Cryptosporidium* and *Giardia* in natural, drinking, and recreational water of Northwestern Greece. Acta Hydrochim. Hydrobiol. 30, 49–58. https://doi.org/10.1002/1521-401x(200207)30:1<49::aid-aheh49>3.0.co;2-r

Kistemann, T., Rind, E., Koch, C., Claßen, T., Lengen, C., Exner, M., Rechenburg, A., 2012. Effect of sewage treatment plants and diffuse pollution on the occurrence of protozoal parasites in the course of a small river. Int. J. Hyg. Environ. Health 215, 577–583. https://doi.org/10.1016/j.ijheh.2011.12.008

Kostopoulou, D., Casaert, S., Tzanidakis, N., van Doorn, D., Demeler, J., von Samson-Himmelstjerna, G., Saratsis, A., Voutzourakis, N., Ehsan, A., Doornaert, T., Looijen, M., De Wilde, N., Sotiraki, S., Claerebout, E., Geurden, T., 2015. The occurrence and genetic characterization of *Cryptosporidium* and *Giardia* species in foals in Belgium, The Netherlands, Germany and Greece. Vet. Parasitol. 211, 170–174. https://doi.org/10.1016/j.vetpar.2015.04.018

Kostopoulou, D., Claerebout, E., Arvanitis, D., Ligda, P., Casaert, S., Sotiraki, S., 2020. Identifying human enteric parasitic infections in Greece, with focus on *Giardia* and *Cryptosporidium*. Exp. Parasitol. 211, 107864. https://doi.org/10.1016/j.exppara.2020.107864

Kostopoulou, D., Claerebout, E., Arvanitis, D., Ligda, P., Voutzourakis, N., Casaert, S., Sotiraki, S., 2017. Abundance, zoonotic potential and risk factors of intestinal parasitism amongst dog and cat populations: The scenario of Crete, Greece. Parasit Vectors 10. https://doi.org/10.1186/s13071-017-1989-8

Levantesi, C., La Mantia, R., Masciopinto, C., Böckelmann, U., Ayuso-Gabella, M.N., Salgot, M., Tandoi, V., Van Houtte, E., Wintgens, T., Grohmann, E., 2010. Quantification of pathogenic microorganisms and microbial indicators in three wastewater reclamation and managed aquifer recharge facilities in Europe. Sci. Total Environ. 408, 4923–4930. https://doi.org/10.1016/j.scitotenv.2010.07.042

Maltezou, H.C., Zafiropoulou, A., Mavrikou, M., Bozavoutoglou, E., Liapi, G., Foustoukou, M., Kafetzis, D.A., 2001. Acute diarrhoea in children treated in an outpatient setting in Athens, Greece. J. Infect. 43, 122–127. https://doi.org/10.1053/jinf.2001.0844

McClung, R.P., Roth, D.M., Vigar, M., Roberts, V.A., Kahler, A.M., Cooley, L.A., Hilborn, E.D., Wade, T.J., Fullerton, K.E., Yoder, J.S., Hill, V.R., 2018. Waterborne disease outbreaks associated with environmental and undetermined exposures to water — United States, 2013- 2014. Am. J. Transplant. 18, 262–267. https://doi.org/10.1111/ajt.14607

Mellor, J., Kumpel, E., Ercumen, A., Zimmerman, J., 2016. Systems approach to climate, water, and diarrhea in Hubli-Dharwad, India. Environ. Sci. Technol. 50, 13042–13051. https://doi.org/10.1021/acs.est.6b02092

Mons, C., Dumètre, A., Gosselin, S., Galliot, C., Moulin, L., 2009. Monitoring of *Cryptosporidium* and *Giardia* river contamination in Paris area. Water Res. 43, 211–217. https://doi.org/10.1016/j.watres.2008.10.024

Papadopoulou, C., Economou, V., Sakkas, H., Gousia, P., Giannakopoulos, X., Dontorou, C., Filioussis, G., Gessouli, H., Karanis, P., Leveidiotou, S., 2008. Microbiological quality of indoor and outdoor swimming pools in Greece: Investigation of the antibiotic resistance of the bacterial isolates. Int. J. Hyg. Environ. Health 211, 385–397. https://doi.org/10.1016/j.ijheh.2007.06.007

Papanikolopoulou, V., Baroudi, D., Guo, Y., Wang, Y., Papadopoulos, E., Lafi, S.Q., Abd El-Tawab, M.M., Diakou, A., Giadinis, N.D., Feng, Y., Xiao, L., 2018. Genotypes and subtypes of *Cryptosporidium* spp. in diarrheic lambs and goat kids in northern Greece. Parasitol. Int. 67, 472–475. https://doi.org/10.1016/j.parint.2018.04.007

Papazahariadou, M., Founta, A., Papadopoulos, E., Chliounakis, S., Antoniadou-Sotiriadou, K., Theodorides, Y., 2007. Gastrointestinal parasites of shepherd and hunting dogs in the Serres Prefecture, Northern Greece. Vet. Parasitol. 148, 170–173. https://doi.org/10.1016/j.vetpar.2007.05.013

Plutzer, J., Lassen, B., Jokelainen, P., Djurković-Djaković, O., Kucsera, I., Dorbek-Kolin, E., Šoba, B., Sréter, T., Imre, K., Omeragić, J., Nikolić, A., Bobić, B., Živičnjak, T., Lučinger, S., Stefanović, L.L., Kučinar, J., Sroka, J., Deksne, G., Keidāne, D., Kváč, M., Hůzová, Z., Karanis, P., 2018. Review of *Cryptosporidium* and *Giardia* in the eastern part of Europe, 2016. Eurosurveillance 23, 1–23. https://doi.org/10.2807/1560-7917.ES.2018.23.4.16-00825

Printza, N., Sapountzi, E., Dotis, J., Papachristou, F., 2013. Hemolytic uremic syndrome related to *Cryptosporidium* infection in an immunocompetent child. Pediatr. Int. 55, 788–790.

https://doi.org/10.1111/ped.12127
Robertson, L.J., Hermansen, L., Gjerde, B.K., 2006. Occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in sewage in Norway. Appl. Environ. Microbiol. 72, 5297–5303. https://doi.org/10.1128/AEM.00464-06
Ryan, U., Cacciò, S.M., 2013. Zoonotic potential of *Giardia*. Int. J. Parasitol. 43, 943–956. https://doi.org/10.1016/j.ijpara.2013.06.001
Ryan, U., Hijjawi, N., Feng, Y., Xiao, L., 2019. *Giardia*: an under-reported foodborne parasite. Int. J. Parasitol. 49, 1–11. https://doi.org/10.1016/j.ijpara.2018.07.003
Ryan, U., Hijjawi, N., Xiao, L., 2018. Foodborne cryptosporidiosis. Int. J. Parasitol. 48, 1–12. https://doi.org/10.1016/j.ijpara.2017.09.004
Sakkas, H., Economou, V., Bozidis, P., Gousia, P., Papadopoulou, C., Karanis, P., 2020. Detection of *Cryptosporidium* and *Giardia* in foods of plant origin in North-Western Greece. J. Water Health 18, 574–578. https://doi.org/10.2166/wh.2020.037
Semenza, J.C., Nichols, G., 2007. Cryptosporidiosis surveillance and water-borne outbreaks in Europe. Eurosurveillance 12, 120–123.
Semenza, J.C., Herbst, S., Rechenburg, A., Suk, J.E., Höser, C., Schreiber, C., Kistemann, T., 2012. Climate change impact assessment of food- and waterborne diseases. Crit. Rev. Environ. Sci. Technol. 42, 857–890. https://doi.org/10.1080/10643389.2010.534706
Sischo, W.M., Atwill, E.R., Lanyon, L.E., George, J., 2000. Cryptosporidia on dairy farms and the role these farms may have in contaminating surface water supplies in the northeastern United States. Prev. Vet. Med. 43, 253–267. https://doi.org/10.1016/S0167-5877(99)00107-5
Slifko, T.R., Smith, H. V., Rose, J.B., 2000. Emerging parasite zoonoses associated with water and food. Int. J. Parasitol. 30, 1379–1393. https://doi.org/10.1016/S0020-7519(00)00128-4
Spanakos, G., Biba, A., Mavridou, A., Karanis, P., 2015. Occurrence of *Cryptosporidium* and *Giardia* in recycled waters used for irrigation and first description of *Cryptosporidium parvum* and *C. muris* in Greece. Parasitol. Res. 114, 1803–1810. https://doi.org/10.1007/s00436-015-4366-6
Sponseller, J.K., Griffiths, J.K., Tzipori, S., 2014. The evolution of respiratory cryptosporidiosis: evidence for transmission by inhalation. Clin. Microbiol. Rev. 27, 575–586. https://doi.org/10.1128/CMR.00115-13
Swaffer, B., Abbott, H., King, B., van der Linden, L., Monis, P., 2018. Understanding human infectious *Cryptosporidium* risk in drinking water supply catchments. Water Res. 138, 282–292. https://doi.org/10.1016/j.watres.2018.03.063
Symeonidou, I., Gelasakis, A., Arsenopoulos, K. V., Schaper, R., Papadopoulos, E., 2017. Regression models to assess the risk factors of canine gastrointestinal parasitism. Vet. Parasitol. 248, 54–61. https://doi.org/10.1016/j.vetpar.2017.10.019
Theodorou, J.A., Viaene, J., Sorgeloos, P., Tzovenis, I., 2011. Production and marketing trends of the cultured Mediterranean Mussel *Mytilus galloprovincialis* Lamarck 1819, in Greece. J. Shellfish Res. 30, 859–874. https://doi.org/10.2983/035.030.0327
Thompson, R.C.A., 2004. The zoonotic significance and molecular epidemiology of *Giardia* and giardiasis. Vet. Parasitol. 126, 15–35. https://doi.org/10.1016/j.vetpar.2004.09.008
Thompson, R.C.A., Ash, A., 2019. Molecular epidemiology of *Giardia* and *Cryptosporidium* infections - What's new? Infect. Genet. Evol. 75. https://doi.org/10.1016/j.meegid.2019.103951
Tolouei, S., Burnet, J.B., Autixier, L., Taghipour, M., Bonsteel, J., Duy, S.V., Sauvé, S., Prévost,

M., Dorner, S., 2019. Temporal variability of parasites, bacterial indicators, and wastewater micropollutants in a water resource recovery facility under various weather conditions. Water Res. 148, 446–458. https://doi.org/10.1016/j.watres.2018.10.068

Tzanidakis, N., Sotiraki, S., Claerebout, E., Ehsan, A., Voutzourakis, N., Kostopoulou, D., Stijn, C., Vercruysse, J., Geurden, T., 2014. Occurrence and molecular characterization of *Giardia duodenalis* and *Cryptosporidium* spp. in sheep and goats reared under dairy husbandry systems in Greece. Parasite 21. https://doi.org/10.1051/parasite/2014048

Wilkes, G., Edge, T., Gannon, V., Jokinen, C., Lyautey, E., Medeiros, D., Neumann, N., Ruecker, N., Topp, E., Lapen, D.R., 2009. Seasonal relationships among indicator bacteria, pathogenic bacteria, *Cryptosporidium* oocysts, *Giardia* cysts, and hydrological indices for surface waters within an agricultural landscape. Water Res. 43, 2209–2223. https://doi.org/10.1016/j.watres.2009.01.033

Wilkes, G., Edge, T.A., Gannon, V.P.J., Jokinen, C., Lyautey, E., Neumann, N.F., Ruecker, N., Scott, A., Sunohara, M., Topp, E., Lapen, D.R., 2011. Associations among pathogenic bacteria, parasites, and environmental and land use factors in multiple mixed-use watersheds. Water Res. 45, 5807–5825. https://doi.org/10.1016/j.watres.2011.06.021

Xiao, L., 2010. Molecular epidemiology of cryptosporidiosis: An update. Exp. Parasitol. 124, 80–89. https://doi.org/10.1016/j.exppara.2009.03.018

Young, I., Smith, B.A., Fazil, A., 2015. A systematic review and meta-analysis of the effects of extreme weather events and other weather-related variables on *Cryptosporidium* and *Giardia* in fresh surface waters. J. Water Health 13, 1–17. https://doi.org/10.2166/wh.2014.079

Chapter 2

Based on:

Cryptosporidium and *Giardia* in surface water and drinking water: animal sources and towards the use of a machine-learning approach as a tool for predicting contamination

P. Ligda, E. Claerebout, D. Kostopoulou, A. Zdragas, S. Casaert, L. J. Robertson, S. Sotiraki

In: Environmental Pollution. 2020;264:114766.
doi: 10.1016/j.envpol.2020.114766

Introduction

Cryptosporidium spp. and *Giardia duodenalis* are globally recognised as important waterborne parasites, with many documented outbreaks worldwide (Baldursson and Karanis, 2011; Efstratiou et al., 2017; Fayer, 2004; Karanis et al., 2007; McClung et al., 2018; Semenza and Nichols, 2007). This is mainly due to their significant impact on human health, as, for example, expressed in 2,159,331 Disability-Adjusted Life Years (DALYs) for cryptosporidiosis and 171,100 DALYs for giardiasis for 2010 (Kirk et al., 2015).

Cryptosporidium oocysts and *Giardia* cysts are shed with in faeces of infected hosts and are transmitted either directly (i.e., contact with faeces from infected hosts) or indirectly (i.e., ingestion of contaminated water or food), potentially causing gastrointestinal disease (Chalmers, 2012; Ryan et al., 2014). The parasites have prolonged survival in the environment, and are also highly resistant to common disinfectants, such as chlorine (Korich et al., 1990; Peeters et al., 1989; Winiecka-Krusnell and Linder, 1998). Furthermore, their infectious dose is low (DuPont et al., 1995; Okhuysen et al., 1999; Rendtorff, 1954). Both parasites are considered to be a significant, often-neglected, public health risk (Certad et al., 2017).

Many waterborne cryptosporidiosis and giardiasis outbreaks have been reported worldwide (Efstratiou et al., 2017), with an increasing number reported since the early 2000s. Water contamination can be of human origin (sewage effluent) or of animal origin (e.g., runoff from contaminated fields), and these contamination sources may contain human, zoonotic, or host-specific parasite species (Cacciò et al., 2003; Gibson et al., 1998; Kistemann et al., 2012, 2002; McCuin and Clancy, 2006; Robertson et al., 2006). However, investigating water contamination by these parasites is quite challenging, requiring the application of costly, time consuming techniques performed by experienced personnel. As a result, systematic monitoring of drinking water sources is not common practice, and performed routinely in only a few countries (e.g., UK, Netherlands, USA, some provinces of Canada and Australia). In Greece, monitoring of these two parasites in water for human consumption is not mandatory, as set by the Greek Common Ministerial Decision No 67322/2017 (Greek Government Gazette 3282/Β΄/19.09.2017).

To address the above difficulties in determining contamination of water sources, several attempts have been made to identify alternative markers, that are easier and cheaper to

measure, and that could be used to predict the presence of these parasites in water samples. Studies have attempted to correlate the presence of these parasites in aquatic environments with: (i) presence of faecal indicator bacteria (i.e., *Escherichia coli, Clostridium perfringens,* bacteriophages, *Enterococci*, total and faecal coliforms) and pathogenic bacteria (e.g., *Salmonella* spp. and *Campylobacter* spp.) (Cizek et al., 2008; Duris et al., 2013; Levantesi et al., 2010; Tolouei et al., 2019; Wilkes et al., 2011, 2009), (ii) weather-related parameters (e.g., temperature, turbidity, flow rate, rainfall, and tributary discharge) (Cizek et al., 2008; Tolouei et al., 2019; Wilkes et al., 2009; Young et al., 2015), and (iii) wastewater micropollutants (e.g., caffeine, carbamazepine) (Tolouei et al., 2019). However, to date, no single parameter has been entirely suitable for all environmental systems. This reflects the heterogeneity in the relationships between these indicators and the presence of *Cryptosporidium* and/or *Giardia*, which are affected by differences in frequencies and levels of excretion at the source of the faecal pollution (Davies et al.,2003), differences in size, density, fate, and transport in a catchment (Brookes et al., 2005, 2004), and also by the different water sources (i.e., surface, wastewater, purified) and sampling sites (Levantesi et al., 2010; Wilkes et al., 2009).

In Greece, one waterborne outbreak of parasitic infection has been reported; a giardiasis outbreak affecting 224 tourists at a hotel in Crete, resulting from sewage contamination of the hotel's water supply system (Hadjichristodoulou et al., 1998; Hardie et al., 1999, 1997). Since then, investigations on *Cryptosporidium* and *Giardia* in water matrices in Greece have been quite limited, but include: i) three studies on surface waters (Economou et al., 2013; Karanis et al., 2005, 2002), where both parasites were detected in different locations in north-western Greece (Karanis et al., 2005, 2002), ii) two studies on wastewaters, with *Cryptosporidium* and *Giardia* identified in both of them (Ligda et al., 2020; Spanakos et al., 2015), and iii) two studies on drinking water and swimming pools (Karanis et al., 2002; Papadopoulou et al., 2008), where *Cryptosporidium* oocysts were reported once in each matrix, in relatively low numbers (Karanis et al., 2002). Genotyping data are only available for the positive wastewater samples, where the zoonotic *C. parvum*, *C. muris* (Spanakos et al., 2015), and *G. duodenallis* assemblage AII were detected (Ligda et al., 2020).

Cryptosporidium and/or *Giardia* infections have been reported in different animal species in Greece, e.g. lambs (Papanikolopoulou et al., 2018; Tzanidakis et al., 2014), goat kids (Giadinis et al., 2015; Papanikolopoulou et al., 2018; Tzanidakis et al., 2014), calves

(Arsenopoulos et al., 2017), foals (Kostopoulou et al., 2015), dogs (Haralabidis et al., 1988; Kostopoulou et al., 2017; Papazahariadou et al., 2007; Symeonidou et al., 2017) and cats (Kostopoulou et al., 2017; Symeonidou et al., 2018). Although low infection rates with potentially zoonotic species of both parasites were detected, the risk of zoonotic infection cannot be disregarded (Kostopoulou et al., 2017, 2015; Tzanidakis et al., 2014). Regarding human infections with these parasites in Greece, very few data are available, probably due to misdiagnosis or underreporting of cases rather than absence of infection (Kostopoulou et al., 2020; Maltezou et al., 2001; Printza et al., 2013).

The presence of both *Cryptosporidium* and *Giardia* in different animal species and aquatic environments in Greece, despite the sparse information regarding their prevalence and genetic classification in the latter, highlights the need for research on the abundance of these parasites in water matrices and estimation of possible health risks due to consumption of contaminated water. Therefore, the aims of this study were to: a) investigate contamination of surface and drinking water by *Cryptosporidium* spp. and *G. duodenalis* in Northern Greece; b) undertake molecular characterisation of parasites detected in order to determine their zoonotic potential; c) identify potential sources of contamination of water sources where the parasites are detected; and d) develop risk assessment models to identify interactions with biological, physicochemical, and environmental factors. To address these objectives, a 2-year longitudinal study was designed, which included repeated sampling at monthly intervals at several locations in a potentially high-risk area (dense livestock and human populations and several surface-water sources).

Materials and Methods

Study area

The study was carried out in the northern part of Thermaikos Gulf, in Northern Greece (Fig. 1). This area is characterised by: (i) urban environment - the city of Thessaloniki (>1,100,000 inhabitants); (ii) surface water - there are four rivers, Gallikos, Axios, Loudias, and Aliakmonas, which form a large deltaic complex in the northern and western parts of Thermaikos Gulf. Aliakmonas river is used for production of drinking water for Thessaloniki; (iii) agricultural environment - the area around the city is a dense farming area, with numerous cattle and

sheep farms in close proximity to the rivers. Moreover, a water production company and a wastewater treatment plant are located in the area.

At the water production company, multi-level water cleaning procedures are applied, including flocculant and sedimentation processes, filtration in rapid charge gravity filtration sand beds, ozonation, filtration via granular activated carbon beds, chlorination, and pH correction (EYATH, 2019).

The sewage at the wastewater treatment plant undergoes a double sedimentation process, before the effluent is chlorinated, and the treated wastewaters are discharged into the Axios river. During the summer, the wastewater effluent is used for crop irrigation.

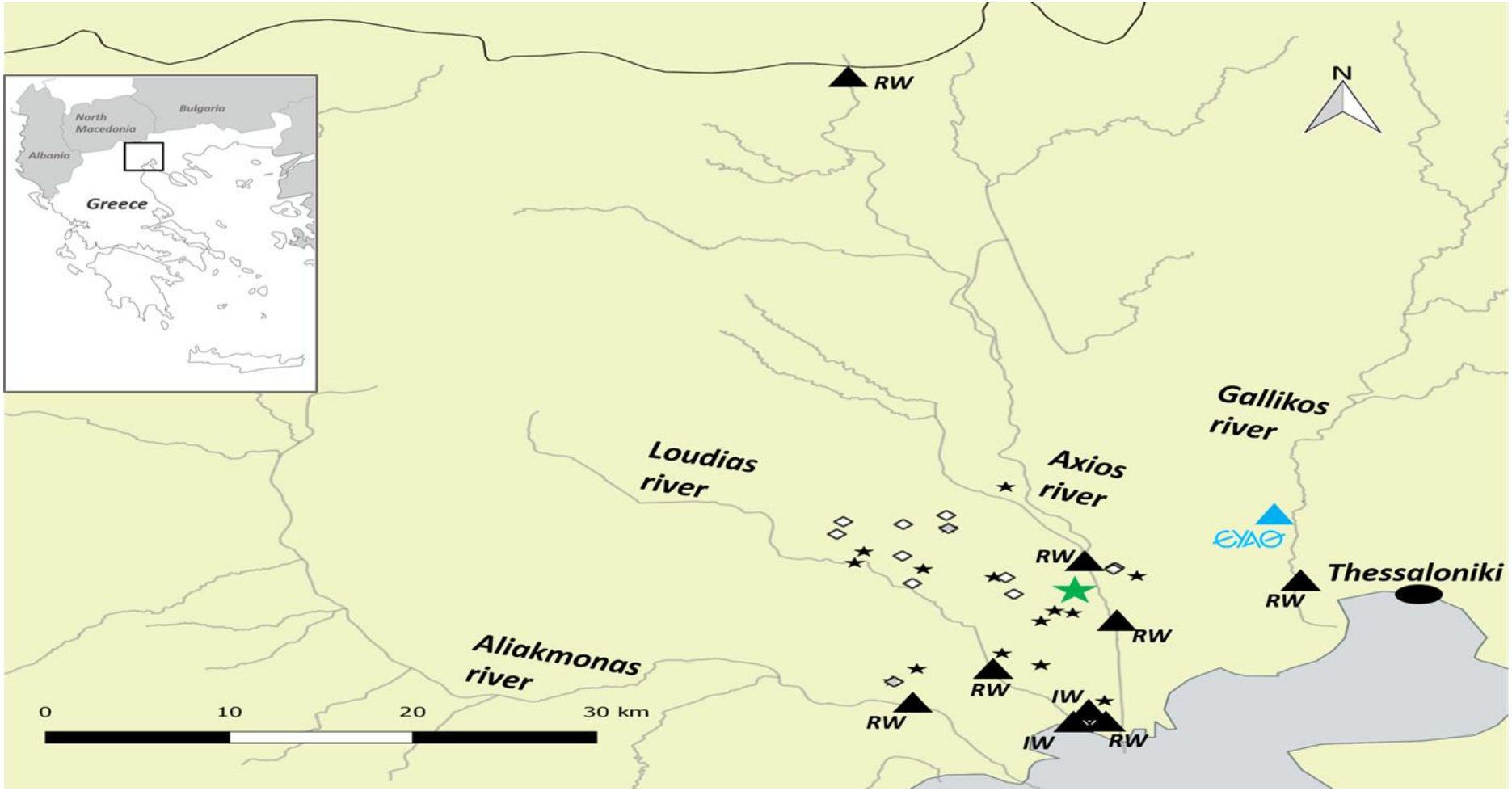


Fig 1. Map of the study area. Black triangles indicate the sampling points at the four rivers (RW) and irrigational canals (IW). The location of the water production company is indicated with a blue triangle (named EYAΘ). The green asterisk indicates the treated wastewater source point. The black asterisks indicate the cattle farms and rhombi indicate the sheep farms sampled.

Sample collection

Water

From February 2015 to January 2017, water samples were collected monthly from 12 sampling points, including the four rivers, the water production company, and irrigation canals. Sampling points were selected based on location, considering risk factors such as proximity to farms, upstream and downstream of the wastewater treatment plant, and accessibility (Fig. 1).

Nine points were sampled throughout the study on a monthly basis (suppl. 1), including one sampling point of raw surface water (RW) per river (except for Axios where 2 points of RW were included, upstream and downstream of a treated wastewater point source), one RW sampling point from the estuary of the rivers Axios and Loudias, and 3 points from the water production company, i.e. raw intake water (RIW), filtered water (FW) after filtration but prior to chlorination, and purified drinking water (DW). Moreover, two sampling points from irrigation canals (IW) were sampled monthly during their operational period (June to August). One extra point at the entry of the Axios river into Greece was sampled monthly from October 2016 to January 2017, as Axios river originates in the neighbouring country of North Macedonia (former FYROM).

For RW samples (including RIW and IW) 15 L of water was sampled and 20 L for FW and DW. The samples were collected into plastic containers, transported to the lab immediately after collection, and filtered on the day of sampling. Plastic containers were washed thoroughly with detergent and chlorine solution and autoclaved after each sampling.

Animals

In an effort to estimate the contribution of farms to contamination of surface water, approximately 10 faecal samples per farm were collected directly from the rectum of young animals (< 6 months old), from 15 cattle farms and 12 sheep farms located in close proximity to the sampling points (Fig. 1), in January and February 2017 (immediately after the lambing period).

Meteorological data

Temperature data on the day of sampling (T) and mean of the week (MeanTW) (defined as 6 days prior to the sampling plus the day of sample collection), as well as mean temperature of sampling month (MeanTM) were recorded from nearby weather stations of the Institute for Environmental Research and Sustainable Development of the National Observatory of Athens.

Likewise, and from the same source, rainfall data were recorded for day of sampling (RainDS), 1-3 days before sampling (Rain1DBS-Rain2DBS-Rain3DBS), total rainfall of sampling week (TotalRainW), mean rainfall of week (MeanRainW), total rainfall of month (TotalRainM), and mean rainfall of month (MeanRainM).

Sample analyses

Detection of Cryptosporidium oocysts and Giardia cysts in water samples

For the water sample analyses the International Standard Method (ISO) 15553 (ISO 15553, 2006) and U.S. Environmental Protection Agency Method 1623.1 (USEPA, 2012) for detection of *Cryptosporidium* oocysts and *G. duodenalis* cysts were followed. After collection, samples were filtered through Filta-Max® Filter Modules (IDEXX Laboratories, Inc., Westbrook, ME, USA) using a peristaltic pump (flow rate 2 L/min). The Filta-Max® Filter Modules were washed using the Filta-Max® Manual Wash Station, according to the manufacturer's instructions. The eluate was centrifuged and the pellet volume measured. Between 0.5 ml (FW and DW) and 2 ml (RW, RIW and IW) of sediment was used for immunomagnetic separation (IMS) using Dynabeads™ GC-Combo (Applied Biosystems™). For larger pellets, the pellets were split into 0.5 ml sub-pellets for IMS. The resulting eluates were equally divided in two 50 µl aliquots. One aliquot was stored at -20 °C until DNA extraction, or used for viability staining, and the other aliquot was air-dried onto well slides and stained with fluorescein isothiocyanate (FITC)-conjugated anti-*Cryptosporidium* and anti-*Giardia* monoclonal antibodies (mAbs) (Aqua-Glo™ G/C Direct, Waterborne™, Inc.). The slides were examined using an Olympus fluorescence microscope at 200x or 400x magnification using a FITC fluorescence filter (Chroma technology corp., Bellows Falls, USA). (Oo)cyst counts were multiplied by 2, as 50% of the total eluate from the IMS was examined, and expressed as number of (oo)cysts per litre.

Recovery efficiency measurement

In order to measure the recovery efficiency of the method, spiking experiments were performed with 21 water samples. A total of 13 RW samples (15 L), 4 FW samples (15 L), and 4 DW samples (20 L) were spiked with 100 inactivated *Cryptosporidium* oocysts and 100 inactivated *Giardia* cysts, permanently labelled with red fluorescent dye (ColorSeed™, BTF Pty Ltd., Sydney, Australia). These were added to the water samples prior to filtration to estimate the recovery efficiency of the method. The spiked samples were processed as described above, but examined using also a Texas Red fluorescence filter (Chroma technology corp., Bellows Falls, USA) to distinguish ColorSeed™ (oo)cysts (that fluoresce red under the Texas Red filter) from natural (oo)cysts (which fluoresce bright green under the FITC filter). Results were expressed as recovery percentage of the (oo)cysts.

Viability assessment of the (oo)cysts recovered from water samples

During the first year of the study, a vital dye inclusion/exclusion assay was performed on the 50 µl sample from the IMS, in order to estimate the viability of *Cryptosporidium* oocysts and *Giardia* cysts recovered from the water samples. (Oo)cysts were stained with 4',6-diamidino-2-phenylidone (DAPI) and propidium iodide (PI) in suspension, with FITC-labelled monoclonal antibodies used to aid identification, as previously described (Campbell et al., 1992). Microscopy was performed on wet mounts and the (oo)cysts were scored according to DAPI and PI inclusion/exclusion and morphology with differential interference contrast microscopy (DIC), in order to categorise them as either viable or nonviable/dead (Robertson and Gjerde, 2006).

Detection of Cryptosporidium oocysts and Giardia cysts in faecal samples

Faecal samples were examined for *Cryptosporidium* oocysts and *Giardia* cysts with a quantitative direct immunofluorescence assay (IFAT) using the MERIFLUOR *Cryptosporidium/Giardia* kit (Meridian Diagnostics Inc., Cincinnati, Ohio) and following the manufacturer's instructions. Positive faecal samples were stored at -20 °C for molecular analysis.

Molecular analyses

DNA was extracted from positive non-spiked water samples and from thawed positive faecal samples for genotyping of *Cryptosporidium* spp. and/or *Giardia duodenalis*. Genomic DNA was extracted from the (oo)cysts of water samples that were purified with IMS concentration (either from the 50 µl of the eluate stored in -20 °C or by scraping the slides to collect the (oo)cysts (Robertson et al., 2009; Sunnotel et al., 2006)) and from animal faecal samples using the QIAamp Fast DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, incorporating an initial step of 3 freeze-thaw cycles (freezing in liquid nitrogen for 5 min and heating at 95 °C for 5 min) for water samples and 5 cycles for faecal samples, into the protocol to maximize disruption of the (oo)cysts.

For *Cryptosporidium*, PCR protocols targeting the 18S ribosomal DNA (18S rDNA) gene (Ryan et al., 2003), heat-shock protein 70 (HSP-70) gene (Morgan et al., 2001), and 60-kilodalton glycoprotein (GP60) gene (Peng et al., 2001), were used. The β-giardin gene (Lalle et al., 2005), glutamate dehydrogenase (GDH) gene (Read et al., 2004), and triose phosphate isomerase (TPI) gene (Sulaiman et al., 2003) were amplified for multi-locus molecular characterisation of *Giardia.* Assemblage specific (A-F) primers targeting the TPI gene were also used with a *G. duodenalis* assemblage A and E PCR according to Geurden et al. (2008), for assemblage B according to Levecke et al. (2009), for assemblage C according to Levecke et al. (2011), for assemblage D according to Lebbad et al. (2010), and for assemblage F according to Ligda et al. (2020). Positive controls (purified positive DNA from: a) *Giardia* cysts (assemblages A – F) isolated from different animal faecal samples, and b) *Cryptosporidium parvum* oocysts isolated from cattle) and a negative control (PCR water) were included in each PCR run. Amplification products were visualized on 1.5% agarose gels stained with ethidium bromide.

Purification and sequencing on both strands (Sanger sequencing) of PCR products was performed by GATC Biotech. Sequences were assembled with Seqman 8.1 Software (Lasergene DNASTAR) and aligned using the Basic Local Alignment Search Tool (BLAST) and compared with reference sequences using MegAlign (Lasergene DNASTAR).

Microbiological and physicochemical analysis in water samples

Faecal indicator bacteria were quantified in all water samples. *Escherichia coli* and coliforms were quantified in 100 ml water samples according to ISO 9308-2 (2012).

Enterococci and *Clostridium perfringens* were also detected and quantified following the ISO 7899-1 (1998) and ISO 6461-2 (1986), respectively. Total colony counts at 22 °C/37 °C were counted according the ISO 6222 (1999), with a detection limit of 1 CFU/100 ml. The microbiological analyses were performed within 12 h after sampling.

Turbidity of the water samples was measured immediately after sampling using a portable turbidity meter (HI 98713 ISO Portable Turbidimeter, HANNA® Instruments Ltd Eden Way) and the results expressed as Formazin Nephelometric units (FNU). The pH, Electrical Conductivity (EC), and Total Dissolved Solids (TDS) of each sample were also measured, with a portable pH/EC/TDS meter (HI 991300, HANNA® Instruments Ltd. Eden Way).

Statistical analyses

Descriptive statistical analyses were performed to obtain range (R), median (Mdn), mean (M), and standard deviation (SD) values for *Cryptosporidium* oocyst counts and *Giardia* cyst counts from monthly water samples and spiked water samples. R, M and SD values were also calculated for microbiological and physicochemical parameters.

Mixed effect zero-inflated negative binomial models for repeated measurements (Atkins et al., 2013; Garay et al., 2011; Hilbe, 2011; Mwalili et al., 2008) were used, due to the excess of zero counts, in order to evaluate relationships of microbiological, physicochemical, and meteorological parameters with protozoan concentrations.

Linear discriminant function analysis (LDFA) aims to find functions of the data that optimally discriminate between two or more groups, in order to build a predictive model for group membership (Brown and Wicker, 2000; Fisher, 1936; Jia et al., 2019; McLachlan, 2004; Simarmat et al., 2018). The model is composed of a discriminant function based on linear combinations of predictor variables. Those predictor variables provide the best discrimination between groups. The discriminant function equations are defined as: $LDF_i = b_1\, x_1 + b_2\, x_2 + \cdots + b_n\, x_n$ (*LDF_i: the predicted score, i.e. the discriminant function score; ; x_i: the predictors; b_j: the discriminant coefficients (j=1,..,n)*) and they were used to classify cases. In our study, LDFA was used to classify *Cryptosporidium* oocysts/L and *Giardia* cysts/L into categories, according to (oo)cyst counts, using microbiological, physicochemical, and meteorological parameters as predictors. LDFA was used as a machine-learning classification method and its

main purpose was to predict (oo)cyst concentrations from selected microbiological, physicochemical, and meteorological parameters. The parameters were selected using the stepwise method and their statistical significance was evaluated. The accuracy of the model was evaluated using the leave-one-out cross validation, whereas the validity of the predictive model was tested using the proportional chance criterion (PCC) (Erechtchoukova et al., 2013; McGarigal et al., 2000; Morrison, 1969; White, 2013).

Statistical analyses were conducted in R (R Core Team, 2017), using the glmmADMB package for zero-inflated mixed effects models and the SPSS v22 software for Linear Discriminant Analysis. The statistical significance in all tests was defined as α = 5%.

Results

Recovery efficiency measurement

For both parasites, variable recovery rates were obtained, with higher recoveries for *Cryptosporidium* (up to 44%) than for *Giardia* (up to 30%). Typical recovery ranges (33-100% for *Cryptosporidium* and 22-100% for *Giardia*) as referenced by USEPA (2012), were measured for *Giardia* in all sample types, but for *Cryptosporidium* in DW samples only (Table 1).

Table 1
Mean recovery rates ± standard deviation for 100 spiked *Cryptosporidium* oocysts and 100 spiked *Giardia* cysts in raw surface water (RW, n = 13), filtered water (FW, n = 4) and purified drinking water (DW, n = 4).

Type of matrix	*Cryptosporidium* oocysts (%)	*Giardia* cysts (%)
RW	32 (± 23)	28 (± 28)
FW	27 (± 19)	30 (± 10)
DW	44 (± 11)	22 (± 23)

Cryptosporidium oocysts and Giardia cysts in water samples

Of the 136 RW samples examined during the 2-year sampling period, *Cryptosporidium* oocysts were detected in 64 (47.1%) and *Giardia* cysts in 90 (66.2%) (Figs. 2 and 3).

Among the four rivers, Axios river was the most frequently contaminated (57.8% *Cryptosporidium* and 84.6% *Giardia* positive samples). At the river's entry into Greece, it was

contaminated with *Cryptosporidium* on all 4 sampling occasions and with *Giardia* on three occasions. The sampling point downstream of the wastewater treatment plant was more frequently (not significantly different) contaminated with *Cryptosporidium* (58.3%) than at the point upstream (50.0%), and vice versa for *Giardia* (79.2% and 91.7%, respectively). Loudias river was the second-most contaminated river, with 58.3% samples positive for *Cryptosporidium* and 70.8% *Giardia*. In addition, *Cryptosporidium* (45.8% positive samples) and *Giardia* (70.8% positive samples) (oo)cysts were commonly found at the Axios/Loudias estuary. Aliakmonas (29.2% *Cryptosporidium* and 29.2% *Giardia* positive samples) and Gallikos rivers (16.7% *Cryptosporidium* and 41.7% *Giardia* positive samples) showed the lowest contamination frequency. Results of Gallikos river refer to 12 RW samples, as in the second year of the study, sampling here was precluded by insufficient water flow.

In positive RW samples, *Cryptosporidium* counts ranged between 0.1 and 13.5 oocysts per L (Mdn: 0.4, M: 0.8, SD: 1.9) and *Giardia* cyst counts ranged from 0.1 to 48.6 per L (Mdn: 1.6, M: 4.7, SD: 8.4). The highest contamination intensity for both parasites was recorded in Axios (0.1–5.4 *Cryptosporidium* oocysts/L and 0.1–48.6 *Giardia* cysts/L) and Loudias (0.1–13.5 *Cryptosporidium* oocysts/L and 0.1–7.9 *Giardia* cysts/L). Aliakmonas river had the lowest counts for *Cryptosporidium* (0.1–0.3 oocysts/L) and the estuary of Axios and Loudias the lowest counts for *Giardia* (0.1–5.2 cysts/L) (Figs. 2 and 3).

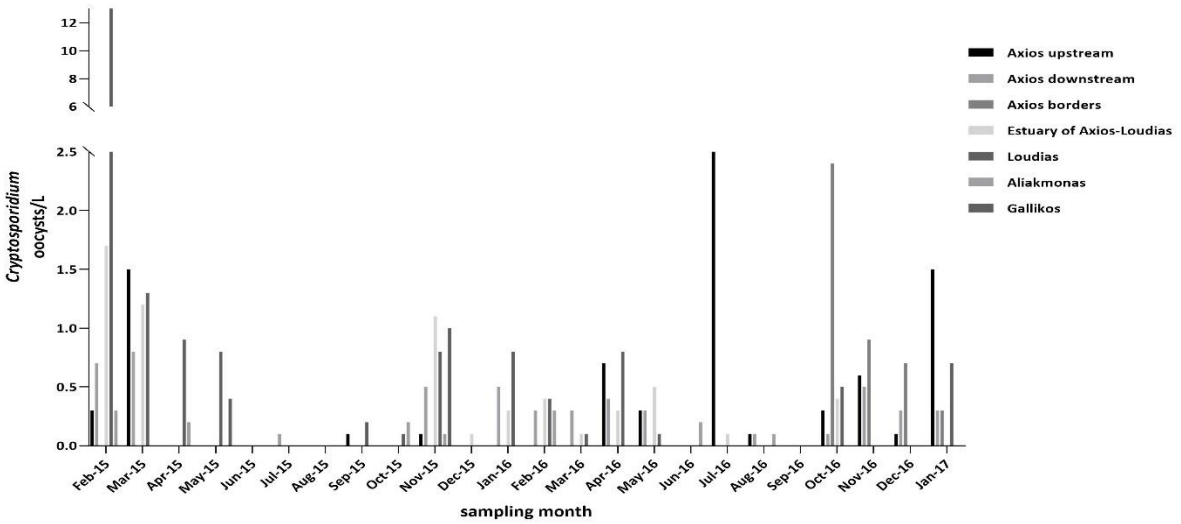
Water samples collected from the water production company, including FW and DW, were contaminated with both parasites between January and May 2016 (Figs. 2 and 3). *Cryptosporidium* oocyst counts in RIW samples ranged between 0.1 and 0.5 per L (Mdn: 0.2, M: 0.2, SD: 0.2), in FW from 0.2 to 1.1 oocysts per L (Mdn: 0.4, M: 0.5, SD: 0.3) and in DW from 0.1 to 0.9 oocysts per L (Mdn: 0.3, M: 0.4, SD: 0.3). *Giardia* cyst counts ranged from 0.1 to 5.3 per L (Mdn: 0.3, M: 1.1, SD: 1.7) in RIW, from 0.1 to 0.9 cysts per L (Mdn: 0.5, M: 0.5, SD: 0.4) in FW and from 0.1 to 0.3 per L (Mdn: 0.1, M: 0.2, SD: 0.1) in DW. Outside this period, low numbers of (oo)cysts were occasionally detected, only in RW samples. Overall, during the 2-year study period, 20.8% and 16.7% of DW samples were contaminated with *Cryptosporidium* and *Giardia,* respectively (Figs. 2 and 3).

During the summer months, in IW samples both parasites were detected (25% *Cryptosporidium* oocysts and 50% *Giardia* cysts). Parasitic loads were low, with 0.2 to 1.3 *Cryptosporidium* oocysts per L (Mdn: 0.9, M: 0.8, SD: 0.6) and *Giardia* counts ranging from 0.1

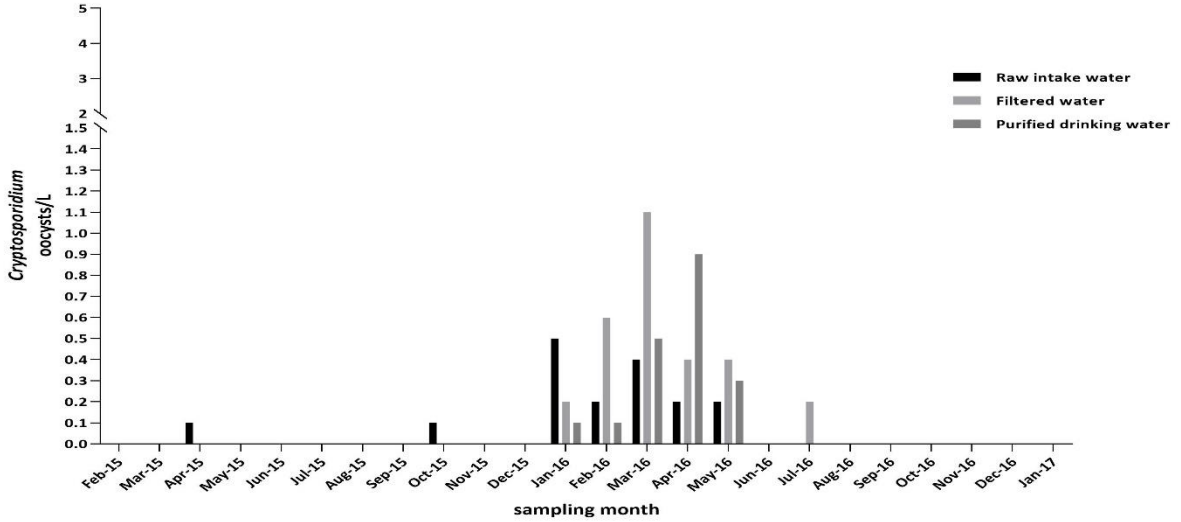
to 1.2 cysts per L (Mdn: 0.2, M: 0.5, SD: 0.5).

(It should be noted that the data presented are the actual counts, and not extrapolated according to the recovery efficiency data.)

2a.



2b.



2c.

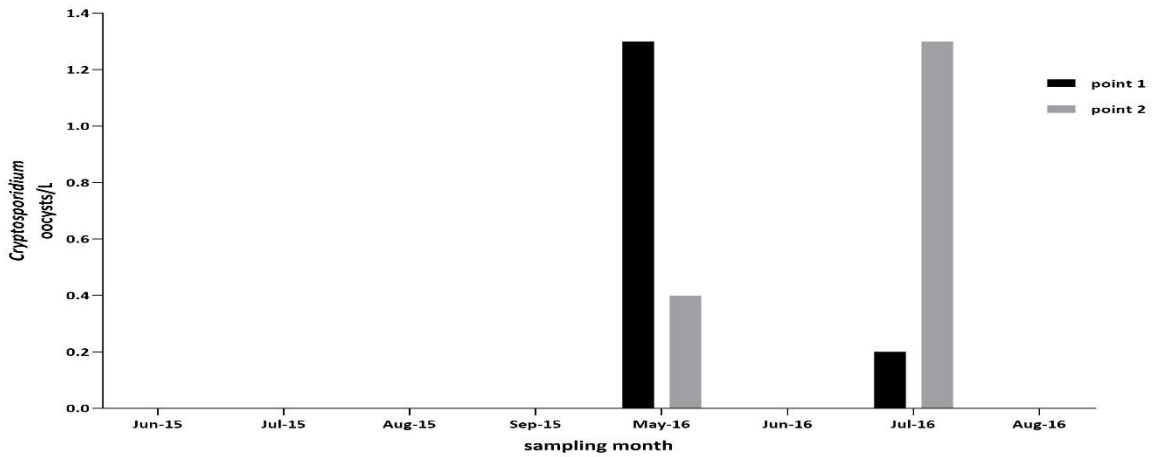
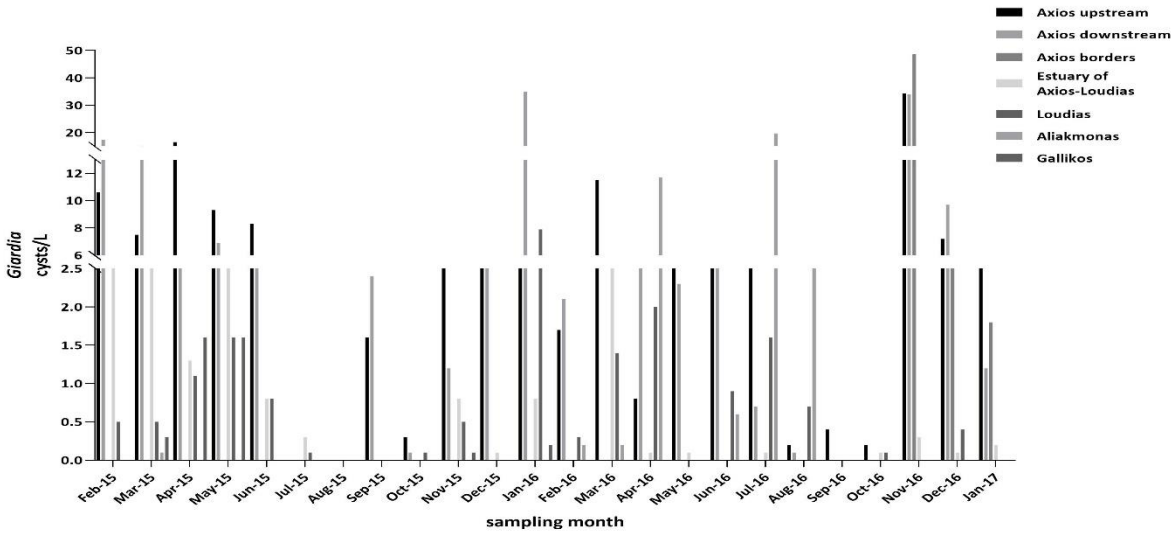
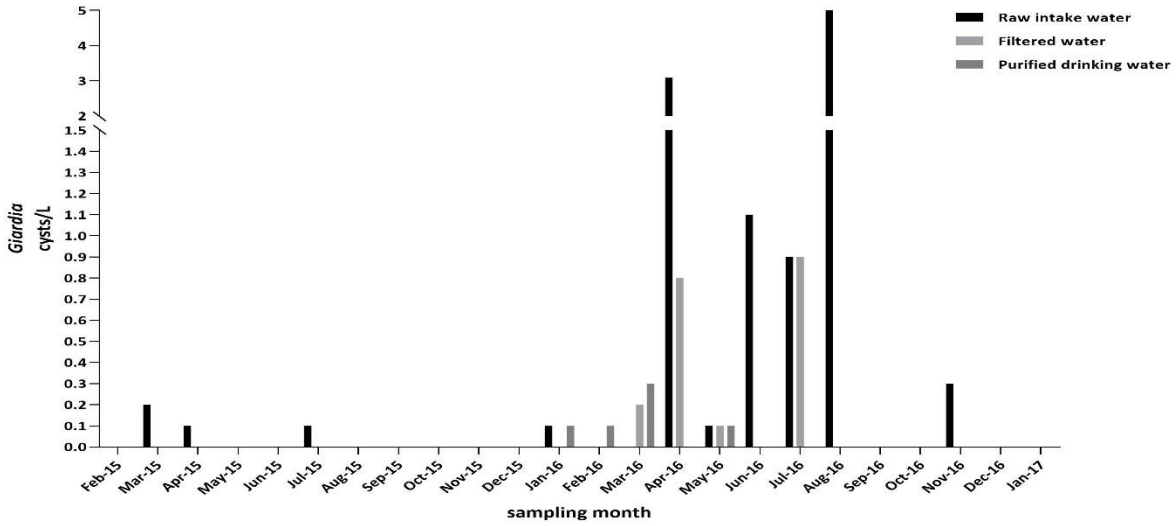


Fig. 2. *Cryptosporidium* oocysts/L in *(a)* raw surface water samples, *(b)* samples collected from the water production company (raw intake, filtered and purified drinking water) and *(c)* water samples collected from irrigation canals from February 2015 to January 2017.

3a.



3b.



3c.

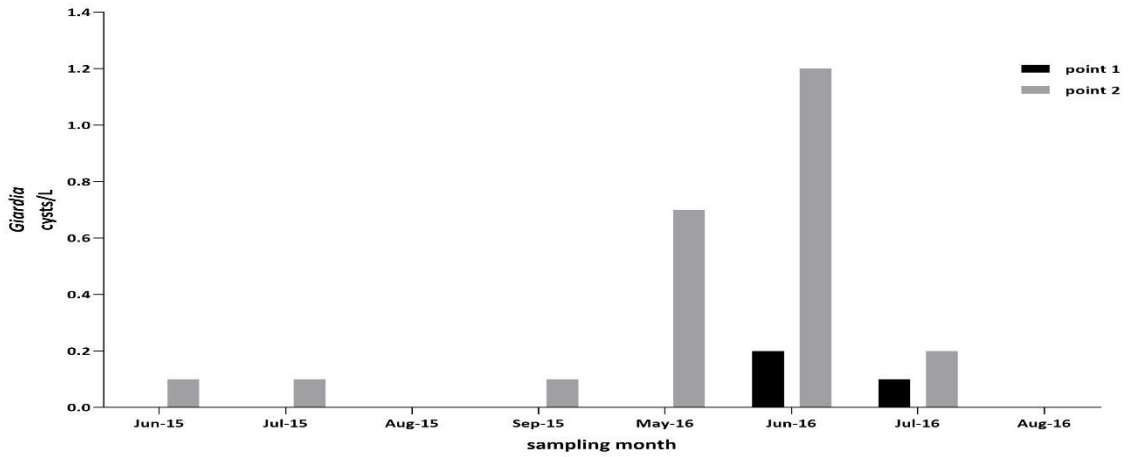


Fig. 3. *Giardia* cysts/L in *(a)* raw surface water samples, *(b)* samples collected from the water production company (raw intake, filtered and purified drinking water) and *(c)* water samples collected from irrigation canals from February 2015 to January 2017.

Viability of the (oo)cysts recovered from water samples

For some samples, *Cryptosporidium* oocysts were lost during the vital dye assay, and recovered only from RW of Loudias river and RIW in January 2016. Recovered *Cryptosporidium* oocysts from RW and RIW were 100% viable. *Giardia* cysts in RW were 79% viable (range: 11.1 – 100%, SD: 29.3%). From FW, DW, and IW samples, (oo)cysts could not be recovered for evaluation via the vital dye assay.

Microbiological indicators of faecal contamination and physicochemical parameters

E. coli, coliforms, Enterococci, and *Clostridia* were detected in all samples of RW and IW (suppl. 2a), with higher contamination levels during winter months (results not shown). Low numbers of coliforms (up to 3/100 ml) and *Clostridia* (up to 10/100 ml) were also found in DW samples. Turbidity measurements were higher in RW (up to 92.7 FNU) than DW (up to 4.6 FNU) samples (suppl. 2b). Furthermore, turbidity measurements were higher during rainy months (data not shown). Values of pH were up to 8.4 for RW and IW, and up to 7.9 for DW samples. Low values of EC (up to 536 µS/cm) and TDS (up to 269 ppm) were recorded in DW. Paired data of parasite concentrations with microbiological and physicochemical parameters are presented in Suppl. 3.

Animal shedding of Cryptosporidium oocysts and Giardia cysts

All farms in the area of the sampling points owned sheep or cattle that were infected with both *Cryptosporidium* and *Giardia*. Of 254 faecal samples examined, 16.7% of the calf samples and 17.2% of the lamb samples were positive for *Cryptosporidium* oocysts, and 41.3% of the calves and 43.1% of the lambs were shedding *Giardia* cysts.

In cattle, *Cryptosporidium* oocyst excretion rates were higher, but within the same range (300 – 117,000 oocysts/g) as *Giardia* cyst counts (100 – 94,000 cysts/g). However, in sheep *Cryptosporidium* oocyst counts were lower (100 – 55,000 oocysts/g) than those of *Giardia* cyst counts (100 – 515,000 cysts/g).

Molecular identification of parasites recovered from water and animal faecal samples

Sequencing results for positive water and faecal samples are presented in Table 2.

Cryptosporidium sequences were obtained from only one positive water sample (RW from Loudias river), and indicated contamination with *C. andersoni* (NCBI accession number: MK414209 – 18S rDNA gene) and *C. parvum* IIaA15G2R1 (NCBI accession number: MK4141210 – GP60 gene) (suppl. 4a). *Giardia* sequences were obtained from 36 out of 115 (31.3%) positive water samples, and generated 43 sequences. Most sequences were from RW and IW and the majority were sub-assemblage AII (*n* = 36 out of 43) based on sequences from the β-giardin, GDH, and TPI genes, with one assemblage E (NCBI accession number: MK414190 – β-giardin gene) sample detected in RW of Axios river. Two sequences (both sub-assemblage AII based on the amplicon sequences from the β-giardin gene) were obtained from water samples from the water production company, one from RIW (NCBI accession number: MK414192) and one from DW (NCBI accession number: MK414191).

Of 42 positive animal faecal samples, 28 (66.7%) were sequenced successfully for *Cryptosporidium*. Most sequences, from both calves and lambs, were *C. parvum* (*n* = 29) based on sequences from amplicons of the 18S rDNA and HSP-70 genes. Sequencing of the GP60 gene revealed the presence of subtypes IIaA15G2R1 (*n* = 3 in calves & *n* = 3 in lambs) and IIaA17G2R1 (*n* = 2 in calves) for *C. parvum*. In calves there were also mixed infections of *C. bovis* together with *C. andersoni,* and of *C. bovis* together with *C. parvum*. In addition, one *C. ryanae* (NCBI accession number: MK501765; suppl. 4b) isolate was detected in calves. Of 107 animal faecal samples positive for *Giardia*, 79 (73.8%) were sequenced successfully. The predominant genotype in both calves and lambs was sub-assemblage AII (*n* = 70). Assemblage E and mixed infections of sub-assemblages AI together with AII and sub-assemblage AII together with E were also detected (Table 2).

Table 2

Cryptosporidium species and *Giardia duodenalis* assemblages (genotypes) recovered from water samples (RW, IW, RIW, DW) and animal faecal samples.

	Cryptosporidium							*Giardia duodenalis*						
								assemblage						
	C. andersoni	*C. parvum*	*C. ryanae*	*C. ubiquitum*	*C.andersoni + C. bovis*	*C. andersoni + C. parvum*	*C. bovis + C. parvum*	A	AI	AII	A4	AI+AII	E	AII + E
water samples	-	-	-	-	-	100%*	-	8.3%	2.8%	80.6%	2.8%	2.8%	2.8%	-
RW	-	-	-	-	-	*100%**	-	*10%*	*3.3%*	*3.3%*	*3.3%*	*3.3%*	*3.3%*	-
IW	-	-	-	-	-	-	-	-	-	*100%*	-	-	-	-
RIW	-	-	-	-	-	-	-	-	-	*100%*	-	-	-	-
DW	-	-	-	-	-	-	-	-	-	*100%*	-	-	-	-
animal faecal samples	3.6%	64.3%	3.6%	14.3%	7.1%	-	7.1%	2.5%	5%	68.4%	-	1.3%	16.5%	6.3%
cattle	*6.7%*	*60%*	*6.7%*	-	*13.3%*	-	*13.3%*	*2.2%*	-	*76.1%*	-	*2.2%*	*13%*	*6.5%*
sheep	-	*69.2%*	-	*30.8%*	-	-	-	*3%*	*12.1%*	*57.6%*	-	-	*21.2%*	*6.1%*

**one sample (corresponding to RW) sequenced*

RW: raw surface water; IW: water from the irrigational canals; RIW: raw intake water from the water production company; DW: purified drinking water

Zero-inflated negative binomial models

No statistically significant correlation was found between: i) *Cryptosporidium* and *Giardia* (oo)cyst counts, ii) faecal indicator bacteria and *Cryptosporidium* or *Giardia* (oo)cyst counts, and iii) physicochemical or meteorological parameters and *Giardia* cyst counts. However, a statistically significant positive correlation was observed between *Cryptosporidium* oocyst counts and pH (*p = 0.0043*) and TDS (*p = 0.0021*), and a negative correlation with EC (*p = 0.0023*). Moreover, *Cryptosporidium* oocyst counts were positively correlated with T (*p = 0.0336*) and negatively with MeanTM (*p = 0.0024*). *Cryptosporidium* oocyst counts were positively correlated with Rain2DBS (*p = 0.0092*) and TotalRainW (*p = 0.0106*), but negatively correlated with Rain1DBS (*p = 0.0216*) and MeanRainW (*p = 0.0105*).

Machine Learning – Linear Discriminant Function Analysis

Classification of *Cryptosporidium* was as follows: Category 1 = none (0 – 0.1 oocysts/L), Category 2 = low (0.1 – 4.6 oocysts/L), Category 3 = moderate (4.6 – 9 oocysts/L), and Category 4 = high (9 – max oocysts/L). The most informative variables using the stepwise method were *Clostridia*/100 ml, colony count at 22 °C, MeanTM, and TotalRainW. These variables were appropriate to discriminate between the four categories of *Cryptosporidium*, as they presented statistically significant differences between the group means according to tests of equality of group means, having *p-values* lower than 0.05 (Suppl. 5a). According to the Wilk's Lambda test (*p-value = 0.0 < 0.05*), the model was a good fit for the data and better than a model that separates the groups by chance. A model classifying cases to one of the categories described was built, using these variables (Suppl. 6a). The results of the classification model (Table 3a) using leave-one-out classification, indicated that under field conditions, estimates of the values of those four parameters at any given sampling point can predict, with approximately 75% accuracy, the intensity of *Cryptosporidium* contamination. The hit ratio (*i.e.* percentages of either original (75.7%) or cross-validated (75.2%) correctly classified cases) was accepted, since it was 25% higher than that of the proportional chance criterion (*PCC = 0.5605; 1.25*PCC=0.7006*) and therefore the validity of LDFA was satisfactory. Percentage of accuracy indicates no overfitting of the model to the data.

Classification of *Giardia* was as follows: Category 1 = none (0 – 0.1 cysts/L), Category 2 = low (0.2 – 16.3 cysts/L), Category 3 = moderate (16.4 – 32.4 cysts/L), and Category 4 = high

(32.5 – max cysts/L). The most informative variables using the stepwise method were coliforms/100 ml, *E. coli*/100 ml, and turbidity (FNU). These variables were appropriate to discriminate between the four categories of *Giardia*, since they presented statistically significant differences between the group means according to tests of equality of group means, having *p-values* lower than 0.05 (Suppl. 5b). According to the Wilk's Lambda test (*p-value = 0.0 < 0.05*), the model was a good fit for the data and better than a model that separates the groups by chance. A model classifying cases to one of the categories described was built, using these variables (Suppl. 6b). The results of the classification model (Table 3b) using leave-one-out classification indicated that under field conditions, estimates of the values of those three parameters at any given sampling point can predict, with approximately 69% accuracy, the intensity of *Giardia* contamination. The hit ratio (*i.e.* percentages of either original (69.2%) or cross-validated (68.8%) correctly classified cases) was accepted, since it was 25% higher than that of proportional chance criterion (*PCC = 0.4945; 1.25*PCC=0.6181*) and therefore the validity of LDFA was satisfactory. The percentage of accuracy indicates no overfitting of the model to the data.

Table 3

Classification results for *(a) Cryptosporidium* and *(b) Giardia* (oo)cysts/L categories using the leave-one-out classification.

(a)

		Cryptosporidium oocysts/L category	Predicted Group Membership				Total
			none	low	moderate	high	
Original	Count	none	145	10	0	0	155
		low	42	22	0	1	65
		moderate	1	0	0	0	1
		high	0	0	0	1	1
	%	none	93.5	6.5	.0	.0	100.0
		low	64.6	33.8	.0	1.5	100.0
		moderate	100.0	.0	.0	.0	100.0

		high	.0	.0	.0	100.0	100.0
Cross-validated	Count	none	145	10	0	0	155
		low	42	22	0	1	65
		moderate	1	0	0	0	1
		high	0	1	0	0	1
	%	none	93.5	6.5	.0	.0	100.0
		low	64.6	33.8	.0	1.5	100.0
		moderate	100.0	.0	.0	.0	100.0
		high	.0	100.0	.0	.0	100.0

- 75.7% of original grouped cases correctly classified.

- 75.2% of cross-validated grouped cases correctly classified.

(b)

		Giardia cysts/L category	Predicted Group Membership				Total
			none	low	moderate	high	
Original	Count	none	122	10	0	1	133
		low	49	29	3	3	84
		moderate	1	1	1	0	3
		high	1	0	0	3	4
	%	none	91.7	7.5	.0	0.8	100.0
		low	58.3	34.5	3.6	3.6	100.0
		moderate	33.3	33.3	33.3	.0	100.0
		high	25.0	.0	.0	75.0	100.0
Cross-validated	Count	none	122	10	0	1	133

	low	49	29	3	3	84
	moderate	1	2	0	0	3
	high	1	0	0	3	4
%	none	91.7	7.5	.0	0.8	100.0
	low	58.3	34.5	3.6	3.6	100.0
	moderate	33.3	66.7	.0	.0	100.0
	high	25.0	.0	.0	75.0	100.0

- 69.2% of original grouped cases correctly classified.

- 68.8% of cross-validated grouped cases correctly classified.

Discussion

Our results show that *Cryptosporidium* spp. and *G. duodenalis* occur commonly, and at relatively high concentrations, in surface waters in Greece. These data confirm previous reports in which, however, the detection levels tended to be lower (Karanis et al., 2005, 2002). The contamination may be even underestimated in the current study, since (oo)cyst recovery efficiencies were quite variable. However, these variabilities were within the acceptance criteria of the USEPA (2012) for *Giardia* in all matrices and for *Cryptosporidium* in drinking water, and were in agreement with results from previous studies (Carmena et al., 2007; Ehsan et al., 2015b; McCuin and Clancy, 2003). Although the abundance of the parasites and the (oo)cyst counts in these Greek rivers are higher than reported from previous studies, the levels of contamination are in the same range as in most rivers in Europe (Ongerth et al., 2018). Among the four rivers, Axios and Loudias were the two most contaminated, with considerably higher (oo)cyst counts than in previous records (0-0.086 *Cryptosporidium* oocysts/L & 0-0.424 *Giardia* cysts/L for Axios and 0.008 *Cryptosporidium* oocysts/L & 0.004 *Giardia* cysts/L for Loudias; Karanis et al., 2005). Such an abundance could be explained by the fact that both rivers flow in an area characterised by a high population density and farming activity. Moreover, the Axios river, which rises in North Macedonia, was already heavily contaminated when entering Greece, indicating (an)other important source(s) of

contamination upstream of the border. In contrast, Aliakmonas river, which is used for drinking water production, had the lowest contamination levels, especially for *Cryptosporidium*, although in higher concentrations (up to 0.3 oocysts/L) than previously described (up to 0.002 oocysts/L; Karanis et al., 2005). There is less farming activity close to the banks of Aliakmonas, and therefore the probability that this river is exposed to contamination with these parasites is lower than Axios and Loudias.

Interestingly, there was a similar seasonal pattern for both years, with greater likelihood of contamination and higher concentrations of parasites in winter and spring. Similar findings have also been reported in previous studies (Ajonina et al., 2013, 2012; Ehsan et al., 2015b; Helmi et al., 2011; Isaac-Renton et al., 1996; Keeley and Faulkner, 2008; Van Dyke et al., 2012). Contamination peaks were recorded mainly during winter (rainy months) and this may be explained by a higher persistence of the (oo)cysts at low temperatures (Fayer, 2004; Robertson et al., 1992), indicating, furthermore, that surface runoff probably contributes to water contamination (Fayer, 2004; Slifko et al., 2000). To investigate this, farm animals were screened for these parasites, and excretion of high numbers of (oo)cysts into the environment was confirmed. It is noteworthy that the same species and (sub)assemblages were identified in some of the water and animal samples, with predominantly *G. duodenalis* sub-assemblage AII and the zoonotic *C. parvum* subtype IIaA15G2R1 (in water and animal faecal samples), which has previously been implicated in waterborne and foodborne outbreaks (Blackburn et al., 2006; DeSilva et al., 2015). Genetic characterization of *Cryptosporidium* oocysts was successful for only one water sample (raw surface water from Loudias river). Extraction of the DNA from the (oo)cyts detected was performed with a kit commonly used for water matrices (Hawash et al., 2015; Ng-Hublin et al., 2015; Traub et al., 2005), with quite a high efficiency, as shown in comparative studies (Adamska et al., 2011, 2010). However, the low success of the genetic characterization of oocysts recovered could be due to the generally low number of (oo)cysts recovered, the presence of PCR inhibitors in environmental samples (Schrader et al, 2012), and the fact that the oocysts detected could have been walls only, lacking nuclei and thus DNA to extract or amplify, due to age or environmental pressures. It should be noted that *G. duodenalis* assemblage AII is considered to be of zoonotic importance (Sprong et al., 2009). However, contamination of the rivers with parasites of human origin cannot be excluded; *C. parvum* and *G. duodenalis* AII are commonly found in humans (Ryan et al., 2014;

Xiao, 2010), including in Greece (Kostopoulou et al., 2020), and *Cryptosporidium* and *Giardia* have previously been detected in wastewater in Greece (Spanakos et al., 2015). Although our results indicated that the wastewater treatment plant did not contribute substantially to river contamination with *Cryptosporidium* and *Giardia,* other human (point) sources of contamination could have been overlooked.

C. parvum and *G. duodenalis* AII in surface water that is used for the production of drinking water poses a notable health risk to consumers. Importantly, similar to previous studies (Helmi et al., 2011; Karanis et al., 2006; Pignata et al., 2019; Ramo et al., 2017), both parasites were present in water intended for human consumption. These were identified in samples taken following the filtration process as well as in the final purified water intended for the distribution network. Such contamination was recorded in five consecutive months and we have considered two possible hypotheses to explain the causes of these events: a) there was an occasional system flaw, or b) the purification system used is inadequate to prevent contamination by those parasites. Supporting the first scenario, we have recorded abnormalities of the filtration system at other time points as well, such as individual occasions of extreme turbidity values (*i.e.* 4.2 FNU in November 2016). The majority of turbidity values recorded were below 1 FNU, as suggested by the WHO guidelines (WHO, 2017). If the second scenario is the correct one, the public health risk is unfortunately much higher, as when the (oo)cysts were identified in the drinking water, the contamination load of Aliakmonas river (the source of the drinking water) was relatively low. Thus, an extreme event of river contamination with high numbers of (oo)cysts could result in a waterborne outbreak of cryptosporidiosis and/or giardiasis. Assessment of the viability of the (oo)cysts, which is critical for full evaluation of the public health risk, was not possible in our study. Although high counts of viable (oo)cysts were recorded in surface water and raw intake water at the water company, for filtered and drinking water viability assessment was not possible, probably due to the low number of (oo)cysts present. Despite the absence of viability data, the repeated observation of drinking water contamination in Thessaloniki implies a significant public health risk, particularly considering the low infective dose of these parasites (DuPont et al., 1995; Okhuysen et al., 1999; Rendtorff, 1954) and the huge amount of water produced daily by the company (up to 260,000 m^3) (EYATH, 2019). Taking into account the increased rainfall erosivity that is associated with foreseen climate changes until 2050 (Panagos et al.,

2017), the risk of such parasitic waterborne outbreaks may even increase. Studies that investigated the risk for consumers of this water to become infected, based on the (oo)cyst concentrations, water consumption data, and the infection rates of the parasites would therefore be of interest (Ehsan et al., 2015a; Helmi et al., 2011; Schets et al., 2008). It should be noted that no cases or outbreaks of cryptosporidiosis or giardiasis among consumers were reported in the catchment area during the contamination period. This could be because parasites in the water supply were inactivated by water treatment (ozonation can inactivate these parasites, and subsequent chlorination may have a synergistic effect, although this is limited in more alkaline waters; Biswas et al., 2003), or could reflect lack of diagnosis and/or lack of reporting of diagnosed infections.

Given the public health risk associated with water contamination and the challenging diagnostic techniques, alternative methods for determining the occurrence of *Cryptosporidium* and *Giardia* in water samples would be advantageous. Here different statistical approaches were used to correlate the presence of the parasites with various microbiological, physicochemical, and environmental parameters to identify appropriate indicators. As a first approach, zero-inflated negative binomial models were used. No association was found between faecal indicator bacteria and *Cryptosporidium* or *Giardia* (oo)cyst counts, as has been reported in previous studies that have tried to correlate *C. perfringens* or other faecal indicator bacteria with *Cryptosporidium* and *Giardia* (oo)cyst counts (Briancesco and Bonadonna, 2005; Ehsan et al., 2015b; Masina et al., 2019; Xiao et al., 2018). These data suggest that no single bacterial indicator organism can safely predict contamination with these parasites, due partly to the heterogeneity of the relationships among faecal indicator bacteria and (oo)cyst counts, with many more zero counts in the parasitological data than in the bacterial counts (Levantesi et al., 2010; Wilkes et al., 2009). The presence of *E. coli* is routinely monitored in drinking water, according to guidelines for safe drinking water (WHO, 2017). Although a useful indicator of faecal contamination, *E. coli*, as with other faecal indicator bacteria, is known to be of negligible importance for predicting contamination with *Cryptosporidium* or *Giardia* and its absence in drinking water does not necessarily indicate freedom from these parasites, as also shown by our results.

Regarding abiotic factors, *Giardia* was not correlated with any of those investigated, whereas *Cryptosporidium* was either positively or negatively correlated with various

physicochemical (e.g., pH and EC) and meteorological parameters (e.g., rainfall and temperature). That rainfall parameters were sometimes positively (Rain2DBS, TotalRainW) and sometimes negatively (Rain1DBS, MeanRainW) correlated with *Cryptosporidium* contamination could be attributed to surface conditions (vegetation, roughness, slope gradient of riverbank etc.) and soil type, which can influence runoff properties (Duris et al., 2013; Mavimbela et al., 2019; Zhang et al., 2018). We have insufficient data to explain why specific parameters influenced (either positively or negatively) contamination with *Cryptosporidium,* but had no apparent association with *Giardia* contamination. However, our findings indicate the need for further sampling and analyses; event-based sampling, for instance during or after rainfall events, may provide further useful information regarding this aspect.

The machine-learning approach (Linear Discriminant Function Analysis) indicated that the different indicators (microbiological, physicochemical, meteorological) could be informative when used in combination. This also suggests that the interactions are complicated and related to various environmental characteristics of the sampling site. The use of the LDFA, when complex interactions of the ecosystem have to be taken into account, has also been supported by other studies (Ballesté et al., 2020; Duran et al., 2006; Mustapha et al., 2012). Our results suggested that the contamination intensity at a sampling point with *Cryptosporidium* can be predicted (with an accuracy of 75%) by measuring *Clostridia*/100 ml, colony at 22 ^{o}C, mean temperature of the relevant month, and total rainfall of the week, and with *Giardia* (with an accuracy of 69%) by measuring coliforms/100 ml, *E. coli*, and turbidity. As these models are based on a combination of different parameters at any given sampling point, they avoid the problem of lack of reliability of a single parameter. However, in future studies, the parameters to be included in such combinations should be carefully selected based on specific characteristics. Should such parameters be used as replacements for determining the actual (oo)cyst loads, then the methods required should be accurate, rapid, and cost efficient. For example, determining *Clostridia*/100 ml and colony counts at 22 ^{o}C is relatively cheap, but the long sample-to-result time for these parameters precludes their use as highly efficient predictors.

Rainfall and temperature seem to be informative parameters for predicting the presence of *Cryptosporidium* in water sources, being important factors in both models. A correlation

between *Cryptosporidium* water contamination and rainfall has been previously documented (Cizek et al., 2008; Duris et al., 2013; Tolouei et al., 2019; Wilkes et al., 2009; Young et al., 2015), and is also supported by the seasonal contamination peaks noted in the current study. To our knowledge, similar data suggesting possible correlations with temperature are unavailable and still need to be validated. However, whether the prediction accuracies of 75% for *Cryptosporidium* and 69% for *Giardia* are adequate from a public health perspective should be determined by risk managers. Even if insufficient, they could be used to augment and inform risk-based sampling plans.

Conclusions

There is a high abundance of potentially zoonotic *Cryptosporidium* species and *G. duodenalis* (sub)assemblages in all four main rivers of Northern Greece, in the irrigation canals, and, most importantly, in treated drinking water of Thessaloniki. Run-off from farms may contribute to water contamination. Although machine-learning modelling approaches seem to provide promising results to predict contamination, the accuracy was sub-optimal and further studies are needed to test these models in different environmental settings. They could be used to inform risk-based sampling plans and to augment current knowledge. Based on the current data, to avoid the risk of waterborne transmission, it is suggested that water intended for human consumption or recreational purposes should be tested for contamination for those parasites during rainy winter months, upgrading of the treatment processes of the drinking water supply company should be considered (i.e., inclusion of efficient filters, ultraviolet purification), and, if the latter is not possible, a programme of testing the purified drinking water for parasite contamination should be implemented.

Acknowledgements

We would like to thank all people mentioned below for their support during sample collection: the personnel of VRI, especially Mr Dionisis Birbilis, Dr Anastasios Saratsis, Mr Georgios Vafeas; the personnel of EYATH company and especially Ms Georgia Seretoudi; the personnel of SWRI and especially Dr Andreas Panagopoulous and Ms Anastasia Kassapi.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declarations of interest

None.

Appendix A. Supplementary data

Suppl. 1. Gantt chart representing monthly sampling from different rivers and sampling points.

Suppl. 2. Descriptive statistics of *(a)* microbiological and *(b)* physicochemical parameters of each water sample, collected monthly from February 2015 to January 2017.

Suppl. 3. *Cryptosporidium* and *Giardia* (occurrence and concentrations) paired with range values of faecal indicator bacteria (total coliforms, *E. coli*, enterococci, clostridia) and turbidity data for waters samples at the various sampling points, collected and analysed monthly from February 2015 to January 2017.

Suppl. 4. NCBI accession numbers for *Cryptosporidium* species and *Giardia duodenalis* assemblages (genotypes) detected in *(a)* water samples (RW, IW, RIW, DW) and *(b)* animal faecal samples.

Suppl. 5. (a) Standardized Canonical Discriminant Function Coefficients of the variables selected for discrimination between the four categories of *Cryptosporidium* oocysts/L and their Tests of Equality of Group Means. **(b)** Standardized Canonical Discriminant Function Coefficients of the variables selected for discrimination between the four categories of *Giardia* cysts/L and their Tests of Equality of Group Means.

Suppl. 6. (a) Linear Discriminant Function model for *Cryptosporidium*. **(b)** Linear Discriminant Function model for *Giardia*.

References

Adamska, M., Leońska-Duniec, A., Maciejewska, A., Sawczuk, M., Skotarczak, B., 2011. PCR and real time PCR for the detection of *Cryptosporidium parvum* oocyst DNA. Folia Biol. 59, 25–30. https://doi.org/10.3409/fb59_3-4.115-120

Adamska, M., Leońska-Duniec, A., Maciejewska, A., Sawczuk, M., Skotarczak, B., 2010. Comparison of efficiency of various DNA extraction methods from cysts of *Giardia intestinalis* measured by PCR and TaqMan real time PCR. Parasite 17, 299–305. https://doi.org/10.1051/parasite/2010174299

Ajonina, C., Buzie, C., Ajonina, I.U., Basner, A., Reinhardt, H., Gulyas, H., Liebau, E., Otterpohl, R., 2012. Occurrence of *Cryptosporidium* in a wastewater treatment plant in North Germany. J. Toxicol. Environ. Heal. - Part A Curr. Issues 75, 1351–1358. https://doi.org/10.1080/15287394.2012.721167

Ajonina, C., Buzie, C., Otterpohl, R., 2013. The detection of *Giardia* cysts in a large-scale wastewater treatment plant in Hamburg, Germany. J. Toxicol. Environ. Heal. - Part A Curr. Issues 76, 509–514. https://doi.org/10.1080/15287394.2013.785208

Arsenopoulos, K., Theodoridis, A., Papadopoulos, E., 2017. Effect of colostrum quantity and quality on neonatal calf diarrhoea due to *Cryptosporidium* spp. infection. Comp. Immunol. Microbiol. Infect. Dis. 53, 50–55. https://doi.org/10.1016/j.cimid.2017.07.005

Atkins, D.C., Baldwin, S.A., Zheng, C., Gallop, R.J., Neighbors, C., 2013. A tutorial on count regression and zero-altered count models for longitudinal addictions data. Psychol. Addict. Behav. 27, 166- 177. https://doi.org/10.1037/a0029508

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

Ballesté, E., Belanche-Muñoz, L.A., Farnleitner, A.H., Linke, R., Sommer, R., Santos, R., Monteiro, S., Maunula, L., Oristo, S., Tiehm A, A., Stange, C., Blanch, A.R., 2020. Improving the identification of the source of faecal pollution in water using a modelling approach: from multi-source to aged and diluted samples. Water Res. 171, 115392. https://doi.org/10.1016/j.watres.2019.115392

Biswas, K., Craik, S., Smith, D.W., Belosevic, M., 2003. Synergistic inactivation of *Cryptosporidium parvum* using ozone followed by free chlorine in natural water. Water Res. 37, 4737-4747. https://doi.org/10.1016/S0043-1354(03)00435-4

Blackburn, B.G., Mazurek, J.M., Hlavsa, M., Park, J., Tillapaw, M., Parrish, M.K., Salehi, E., Franks, W., Koch, E., Smith, F., Xiao, L., Arrowood, M., Hill, V., Da Silva, A., Johnston, S., Jones, J.L., 2006. Cryptosporidiosis associated with ozonated apple cider. Emerg. Infect. Dis. 12, 684–686. https://doi.org/10.3201/eid1204.050796

Briancesco, R., Bonadonna, L., 2005. An Italian study on *Cryptosporidium* and *Giardia* in wastewater, fresh water and treated water. Environ. Monit. Assess. 104, 445–457. https://doi.org/10.1007/s10661-005-2282-4

Brookes, J.D., Antenucci, J., Hipsey, M., Burch, M.D., Ashbolt, N.J., Ferguson, C., 2004. Fate and transport of pathogens in lakes and reservoirs. Environ. Int. 30, 741–759. https://doi.org/10.1016/j.envint.2003.11.006

Brookes, J.D., Hipsey, M.R., Burch, M.D., Regel, R.H., Linden, L.G., Ferguson, C.M., Antenucci, J.P., 2005. Relative value of surrogate indicators for detecting pathogens in lakes and reservoirs. Environ. Sci. Technol. 39, 8614–8421. https://doi.org/10.1021/es050821+

Brown, M.T., Wicker, L.R., 2000. Discriminant Analysis, in: Handbook of Applied Multivariate

Statistics and Mathematical Modeling. Academic Press, pp. 209–235.
Campbell, A.T., Robertson, L.J., Smith, H. V., 1992. Viability of *Cryptosporidium parvum* oocysts: Correlation of in vitro excystation with inclusion or exclusion of fluorogenic vital dyes. Appl. Environ. Microbiol. 58, 3488–3493.
Cacciò, S.M., De Giacomo, M., Aulicino, F.A., Pozio, E., 2003. *Giardia* cysts in wastewater treatment plants in Italy. Appl. Environ. Microbiol. 69, 3393–3398. https://doi.org/10.1128/AEM.69.6.3393-3398.2003
Carmena, D., Aguinagalde, X., Zigorraga, C., Fernández-Crespo, J.C., Ocio, J.A., 2007. Presence of *Giardia* cysts and *Cryptosporidium* oocysts in drinking water supplies in northern Spain. J. Appl. Microbiol. 102, 619–629. https://doi.org/10.1111/j.1365-2672.2006.03193.x
Certad, G., Viscogliosi, E., Chabé, M., Cacciò, S.M., 2017. Pathogenic mechanisms of *Cryptosporidium* and *Giardia*. Trends Parasitol. 33, 561–576. https://doi.org/10.1016/j.pt.2017.02.006
Chalmers, R.M., 2012. Waterborne outbreaks of cryptosporidiosis. Ann. Ist. Super. Sanita 48, 429–446. https://doi.org/10.4415/ANN_12_04_10
Cizek, A.R., Characklis, G.W., Krometis, L.-A., Hayes, J.A., Simmons, O.D., Di Lonardo, S., Alderisio, K.A., Sobsey, M.D., 2008. Comparing the partitioning behavior of *Giardia* and *Cryptosporidium* with that of indicator organisms in stormwater runoff. Water Res. 42, 4421–4438. https://doi.org/10.1016/j.watres.2008.06.020
Davies, C.M., Kaucner, C., Deere, D., Ashbolt, N.J., 2003. Recovery and enumeration of *Cryptosporidium parvum* from animal fecal matrices. Appl. Environ. Microbiol. 69, 2842–2847. https://doi.org/10.1128/AEM.69.5.2842-2847.2003
DeSilva, M.B., Schafer, S., Scott, M.K., Robinson, B., Hills, A., Buser, G.L., Salis, K., Gargano, J., Yoder, J., Hill, V., Xiao, L., Roellig, D., Hedberg, K., 2015. Communitywide cryptosporidiosis outbreak associated with a surface water-supplied municipal water system – Baker City, Oregon, 2013. Epidemiol. Infect. 144, 274–284. https://doi.org/10.1017/S0950268815001831
DuPont, H.L., Chappell, C.L., Sterling, C.R., Okhuysen, P.C., Rose, J.B., Jakubowski, W., 1995. The infectivity of *Cryptosporidium parvum* in healthy volunteers. N. Engl. J. Med. 332, 855–859. https://doi.org/10.1056/NEJM199503303321304
Duran, M., Haznedaroğlu, B.Z., Zitomer, D.H., 2006. Microbial source tracking using host specific FAME profiles of fecal coliforms. Water Res. 40, 67–74. https://doi.org/10.1016/j.watres.2005.10.019
Duris, J.W., Reif, A.G., Krouse, D.A., Isaacs, N.M., 2013. Factors related to occurrence and distribution of selected bacterial and protozoan pathogens in Pennsylvania streams. Water Res. 47, 300–314. https://doi.org/10.1016/j.watres.2012.10.006
Economou, V., Gousia, P., Kansouzidou, A., Sakkas, H., Karanis, P., Papadopoulou, C., 2013. Prevalence, antimicrobial resistance and relation to indicator and pathogenic microorganisms of *Salmonella enterica* isolated from surface waters within an agricultural landscape. Int. J. Hyg. Environ. Health 216, 435–444. https://doi.org/10.1016/j.ijheh.2012.07.004
Efstratiou, A., Ongerth, J.E., Karanis, P., 2017. Waterborne transmission of protozoan parasites: Review of worldwide outbreaks - An update 2011–2016. Water Res. 114, 14–22. https://doi.org/10.1016/j.watres.2017.01.036
Ehsan, A., Casaert, S., Levecke, B., Van Rooy, L., Pelicaen, J., Smis, A., De Backer, J., Vervaeke, B., De Smedt, S., Schoonbaert, F., Lammens, S., Warmoes, T., Geurden, T., Claerebout,

E., 2015a. *Cryptosporidium* and *Giardia* in recreational water in Belgium. J. Water Health 13, 870–878. https://doi.org/10.2166/wh.2015.268

Ehsan, A., Geurden, T., Casaert, S., Paulussen, J., De Coster, L., Schoemaker, T., Chalmers, R., Grit, G., Vercruysse, J., Claerebout, E., 2015b. Occurrence and potential health risk of *Cryptosporidium* and *Giardia* in different water catchments in Belgium. Environ. Monit. Assess. 187. https://doi.org/10.1007/s10661-014-4157-z

Erechtchoukova, M.G., Khaiter, P.A., Golinska-Dawson, P. (Eds.), 2013. Sustainability appraisal: Quantitative methods and mathematical techniques for environmental performance evaluation. Springer.

EYATH (Thessaloniki Water Supply & Sewerage Co S.A.), 2019. https://www.eyath.gr/our-water-sources/?lang=en

Fayer, R., 2004. *Cryptosporidium*: a water-borne zoonotic parasite. Vet. Parasitol. 126, 37–56. https://doi.org/10.1016/J.VETPAR.2004.09.004

Fisher, R.A., 1936. The use of multiple measurements in taxonomic problem. Ann. Eugenics. 7, 179 – 188. https://doi.org/10.1111/j.1469-1809.1936.tb02137.x

Garay, A.M., Hashimoto, E.M., Ortega, E.M.M., Lachos, V.H., 2011. On estimation and influence diagnostics for zero-inflated negative binomial regression models. Comput. Stat. Data Anal. 55, 1304–1318. https://doi.org/10.1016/j.csda.2010.09.019

Geurden, T., Geldhof, P., Levecke, B., Martens, C., Berkvens, D., Casaert, S., Vercruysse, J., Claerebout, E., 2008. Mixed *Giardia duodenalis* assemblage A and E infections in calves. Int. J. Parasitol. 38, 259–264. https://doi.org/10.1016/j.ijpara.2007.07.016

Giadinis, N.D., Papadopoulos, E., Lafi, S.Q., Papanikolopoulou, V., Karanikola, S., Diakou, A., Vergidis, V., Xiao, L., Ioannidou, E., Karatzias, H., 2015. Epidemiological observations on cryptosporidiosis in diarrheic goat kids in Greece. Vet. Med. Int. 2015, 4–7. https://doi.org/10.1155/2015/764193

Gibson, C.J., Stadterman, K.L., States, S., Sykorat, J., 1998. Combined sewer overflows: a source of *Cryptosporidium* and *Giardia*? Water Sci. Technol. 38, 67–72. https://doi.org/10.1016/s0273-1223(98)00802-6

Hadjichristodoulou, C., Gkikas, A., Pediatidis, Y., Tselentis, Y., 1998. Outbreak of giardiasis among English tourists in Crete. Lancet 351, 65–66. https://doi.org/10.1016/s0140-6736(05)78057-0

Haralabidis, S.T., Papazachariadou, M.G., Koutinas, A.F., Rallis, T.S., 1988. A survey on the prevalence of gastrointestinal parasites of dogs in the area of Thessaloniki, Greece. J. Helminthol. 62, 45–49. https://doi.org/10.1017/S0022149X00011196

Hardie, R.M., Bardham, M., Wall, P.G., 1997. Unwanted holiday souvenirs: who should pay? J. Infect. Dis. 350, 964.

Hardie, R.M., Wall, P.G., Gott, P., Bardhan, M., Bartlett, C.L.R., 1999. Infectious diarrhea in tourists staying in a resort hotel. Emerg. Infect. Dis. 5, 168–171. https://doi.org/10.3201/eid0501.990123

Hawash, Y., Ghonaim, M., Hussein, Y., Alhazmi, A., Alturkistani, A., 2015. Identification of *Giardia lamblia* and the human infectious-species of *Cryptosporidium* in drinking water resources in Western Saudi Arabia by nested-PCR assays. Trop. Biomed. 32, 216–224.

Helmi, K., Skraber, S., Burnet, J.-B., Leblanc, L., Hoffmann, L., Cauchie, H.-M., 2011. Two-year monitoring of *Cryptosporidium parvum* and *Giardia lamblia* occurrence in a recreational and drinking water reservoir using standard microscopic and molecular biology techniques. Environ. Monit. Assess. 179, 163–175. https://doi.org/10.1007/s10661-010-1726-7

Hilbe, J.M., 2011. Negative Binomial Regression, 2nd edition. ed. Cambridge University Press. https://doi.org/10.1017/CBO9780511973420

Isaac-Renton, J., Moorehead, W., Ross, A., 1996. Longitudinal studies of *Giardia* contamination in two community drinking water supplies: cyst levels, parasite viability, and health impact. Appl. Environ. Microbiol. 62, 47–54.

ISO, 1986. ISO 6461-2: Water quality - Detection and enumeration of the spores of sulphite-reducing anaerobes (*Clostridia*) - part 2: Method by membrane filtration and conform WAC/V/A/007. International Organization for Standardization, Geneva, Switzerland.

ISO, 1998. ISO 7899-1: Water quality – Detection and enumeration of intestinal enterecocci – part 1: Miniaturized method (Most probable number) for surface and waste water. International Organization for Standardization, Geneva, Switzerland.

ISO, 1999. ISO 6222: Water quality - Enumeration of culturable micro-organisms - Colony Count by inoculation in a nutrient agar culture medium and conform WAC/V/A/001. International Organization for Standardization, Geneva, Switzerland.

ISO, 2006. ISO 15553: Water quality - Isolation and identification of *Cryptosporidium* oocysts and *Giardia* cysts from water. International Organization for Standardization, Geneva, Switzerland.

ISO, 2012. ISO 9308-2: Water quality – Enumeration of *Escherichia coli* and coliform bacteria – part 2: Most probable number method and conform WAC/V/A/002. International Organization for Standardization, Geneva, Switzerland.

Jia, W., Deng, Y., Xin, C., Liu, X., 2019. A classification algorithm with Linear Discriminant Analysis and Axiomatic Fuzzy Sets. Math. Found. Comput. 2, 73–81. https://doi.org/10.3934/mfc.2019006

Karanis, P., Chronis, I., Zakas, G., Kourenti, C., Sotiriadou, I., Papadopoulou, C., 2005. A preliminary survey of the level of microbiological pollution of major rivers in Northern Greece. Acta Hydrochim. Hydrobiol. 33, 346–354. https://doi.org/10.1002/aheh.200400581

Karanis, P., Kourenti, C., Smith, H. V., 2007. Waterborne transmission of protozoan parasites: A worldwide review of outbreaks and lessons learnt. J. Water Health 5, 1–38. https://doi.org/10.2166/wh.2006.002

Karanis, P., Papadopoulou, C., Kimura, A., Economou, E., Kourenti, C., Sakkas, H., 2002. *Cryptosporidium* and *Giardia* in natural, drinking, and recreational water of northwestern Greece. Acta Hydrochim. Hydrobiol. 30, 49–58. https://doi.org/10.1002/1521-401X(200207)30:1<49::AID-AHEH49>3.0.CO;2-R

Karanis, P., Sotiriadou, I., Kartashev, V., Kourenti, C., Tsvetkova, N., Stojanova, K., 2006. Occurrence of *Giardia* and *Cryptosporidium* in water supplies of Russia and Bulgaria. Environ. Res. 102, 260–271. https://doi.org/10.1016/j.envres.2006.05.005

Keeley, A., Faulkner, B.R., 2008. Influence of land use and watershed characteristics on protozoa contamination in a potential drinking water resources reservoir. Water Res. 42, 2803–2813. https://doi.org/10.1016/j.watres.2008.02.028

Kirk, M.D., Pires, S.M., Black, R.E., Caipo, M., Crump, J.A., Devleesschauwer, B., Döpfer, D., Fazil, A., Fischer-Walker, C.L., Hald, T., Hall, A.J., Keddy, K.H., Lake, R.J., Lanata, C.F., Torgerson, P.R., Havelaar, A.H., Angulo, F.J., 2015. World Health Organization Estimates of the Global and Regional Disease Burden of 22 Foodborne Bacterial, Protozoal, and Viral Diseases, 2010: A Data Synthesis. PLoS Med. 12, 1–21. https://doi.org/10.1371/journal.pmed.1001921

Kistemann, T., Claßen, T., Koch, C., Dangendorf, F., Fischeder, R., Gebel, J., Vacata, V., Exner,

M., 2002. Microbial load of drinking water reservoir tributaries during extreme rainfall and runoff. Appl. Environ. Microbiol. 68, 2188–2197. https://doi.org/10.1128/AEM.68.5.2188-2197.2002

Kistemann, T., Rind, E., Koch, C., Claßen, T., Lengen, C., Exner, M., Rechenburg, A., 2012. Effect of sewage treatment plants and diffuse pollution on the occurrence of protozoal parasites in the course of a small river. Int. J. Hyg. Environ. Health 215, 577–583. https://doi.org/10.1016/j.ijheh.2011.12.008

Korich, D.G., Mead, J.R., Madore, M.S., Sinclair, N.A., Sterling, C.R., 1990. Effects of ozone, chlorine dioxide, chlorine, and monochloramine on *Cryptosporidium parvum* oocyst viability. Appl. Environ. Microbiol. 56, 1423–1428.

Kostopoulou, D., Casaert, S., Tzanidakis, N., van Doorn, D., Demeler, J., von Samson-Himmelstjerna, G., Saratsis, A., Voutzourakis, N., Ehsan, A., Doornaert, T., Looijen, M., De Wilde, N., Sotiraki, S., Claerebout, E., Geurden, T., 2015. The occurrence and genetic characterization of *Cryptosporidium* and *Giardia* species in foals in Belgium, The Netherlands, Germany and Greece. Vet. Parasitol. 211, 170–174. https://doi.org/10.1016/j.vetpar.2015.04.018

Kostopoulou, D., Claerebout, E., Arvanitis, D., Ligda, P., Casaert, S., Sotiraki, S., 2020. Identifying human enteric parasitic infections in Greece, with focus on *Giardia* and *Cryptosporidium*. Exp. Parasitol. 211, 107864. https://doi.org/10.1016/j.exppara.2020.107864

Kostopoulou, D., Claerebout, E., Arvanitis, D., Ligda, P., Voutzourakis, N., Casaert, S., Sotiraki, S., 2017. Abundance, zoonotic potential and risk factors of intestinal parasitism amongst dog and cat populations: The scenario of Crete, Greece. Parasit Vectors 10. https://doi.org/10.1186/s13071-017-1989-8

Lalle, M., Pozio, E., Capelli, G., Bruschi, F., Crotti, D., Cacciò, S.M., 2005. Genetic heterogeneity at the β-giardin locus among human and animal isolates of *Giardia duodenalis* and identification of potentially zoonotic subgenotypes. Int. J. Parasitol. 35, 207–213. https://doi.org/10.1016/j.ijpara.2004.10.022

Lebbad, M., Mattsson, J.G., Christensson, B., Ljungström, B., Backhans, A., Andersson, J.O., Svärd, S.G., 2010. From mouse to moose: Multilocus genotyping of *Giardia* isolates from various animal species. Vet. Parasitol. 168, 231–239. https://doi.org/10.1016/j.vetpar.2009.11.003

Levantesi, C., La Mantia, R., Masciopinto, C., Böckelmann, U., Ayuso-Gabella, M.N., Salgot, M., Tandoi, V., Van Houtte, E., Wintgens, T., Grohmann, E., 2010. Quantification of pathogenic microorganisms and microbial indicators in three wastewater reclamation and managed aquifer recharge facilities in Europe. Sci. Total Environ. 408, 4923–4930. https://doi.org/10.1016/j.scitotenv.2010.07.042

Levecke, B., Geldhof, P., Claerebout, E., Dorny, P., Vercammen, F., Cacciò, S.M., Vercruysse, J., Geurden, T., 2009. Molecular characterisation of *Giardia duodenalis* in captive non-human primates reveals mixed assemblage A and B infections and novel polymorphisms. Int. J. Parasitol. 39, 1595 – 1601. https://doi.org/10.1016/j.ijpara.2009.05.013

Levecke, B., Meulemans, L., Dalemans, T., Casaert, S., Claerebout, E., Geurden, T., 2011. Mixed *Giardia duodenalis* assemblage A, B, C and E infections in pet chinchillas (*Chinchilla lanigera*) in Flanders (Belgium). Vet. Parasitol. 177, 166–170. https://doi.org/10.1016/j.vetpar.2010.11.027

Ligda, P., Claerebout, E., Casaert, S., Robertson, L.J., Sotiraki, S., 2020. Investigations from Northern Greece on mussels cultivated in areas proximal to wastewater discharges, as

a potential source for human infection with *Giardia* and *Cryptosporidium.* Exp. Parasitol. 210, 107848. https://doi.org/10.1016/j.exppara.2020.107848

Maltezou, H.C., Zafiropoulou, A., Mavrikou, M., Bozavoutoglou, E., Liapi, G., Foustoukou, M., Kafetzis, D.A., 2001. Acute diarrhoea in children treated in an outpatient setting in Athens, Greece. J. Infect. 43, 122–127. https://doi.org/10.1053/jinf.2001.0844

Masina, S., Shirley, J., Allen, J., Sargeant, J.M., Guy, R.A., Wallis, P.M., Weese, J.S., Cunsolo, A., Bunce, A., Harper, S.L., 2019. Weather, environmental conditions, and waterborne *Giardia* and *Cryptosporidium* in Iqaluit, Nunavut. J. Water Health 17, 84–97. https://doi.org/10.2166/wh.2018.323

Mavimbela, S.S.W., Dlamini, P., van Rensburg, L.D., 2019. Infiltration-excess runoff properties of dryland floodplain soil types under simulated rainfall conditions. Arid L. Res. Manag. 33, 235–254. https://doi.org/10.1080/15324982.2018.1531441

McClung, R.P., Roth, D.M., Vigar, M., Roberts, V.A., Kahler, A.M., Cooley, L.A., Hilborn, E.D., Wade, T.J., Fullerton, K.E., Yoder, J.S., Hill, V.R., 2018. Waterborne disease outbreaks associated with environmental and undetermined exposures to water — United States, 2013-2014. Am. J. Transplant. 18, 262–267. https://doi.org/10.1111/ajt.14607

McCuin, R.M., Clancy, J.L., 2006. Occurrence of *Cryptosporidium* oocysts in US wastewaters. J. Water Health 4, 437–452. https://doi.org/10.2166/wh.2006.019

McCuin, R.M., Clancy, J.L., 2003. Modifications to United States Environmental Protection Agency Methods 1622 and 1623 for detection of *Cryptosporidium* oocysts and *Giardia* cysts in water. Appl. an 69, 267–274. https://doi.org/10.1128/AEM.69.1.267–274.2003

McGarigal, K., Cushman, S.A., Stafford, S., 2000. Multivariate Statistics for Wildlife and Ecology Research. Springer.

McLachlan, G.J., 1993. Discriminant analysis and statistical pattern recognition. p. 5012. https://doi.org/10.1016/S0016-7037(05)80008-0

Morgan, U.M., Monis, P.T., Xiao, L., Limor, J., Sulaiman, I., Raidal, S., O'Donoghue, P., Gasser, R., Murray, A., Fayer, R., Blagburn, B.L., Lal, A.A., Thompson, R.C.A., 2001. Molecular and phylogenetic characterisation of *Cryptosporidium* from birds. Int. J. Parasitol. 31, 289–296. https://doi.org/10.1016/s0020-7519(00)00164-8

Morrison, D.G., 1969. On the of interpretation of discriminant analysis. J. Mark. Res. 6, 156–163.

Mustapha, A., Aris, A.Z., Ramli, M.F., Juahir, H., 2012. Temporal aspects of surface water quality variation using robust statistical tools. Sci. World J. 2012. https://doi.org/10.1100/2012/294540

Mwalili, S.M., Lesaffre, E., Declerck, D., 2008. The zero-inflated negative binomial regression model with correction for misclassification: an example in caries research. Stat. Methods Med. Res. 17, 123–139. https://doi.org/10.1177/0962280206071840

Ng-Hublin, J.S.Y., Hargrave, D., Combs, B., Ryan, U., 2015. Investigation of a swimming pool-associated cryptosporidiosis outbreak in the Kimberley region of Western Australia. Epidemiol. Infect. 143, 1037–1041. https://doi.org/10.1017/S095026881400106X

Okhuysen, P.C., Chappell, C.L., Crabb, J.H., Sterling, C.R., DuPont, H.L., 1999. Virulence of three distinct *Cryptosporidium parvum* isolates for healthy adults. J. Infect. Dis. 180, 1275–1281. https://doi.org/10.1086/315033

Ongerth, J.E, Plutzer, J., Karanis, P., 2018. *Cryptosporidium* and *Giardia* – levels and distribution in surface water throughout Europe. Proceedings 2, 690. https://doi.org/10.3390/proceedings2110690

Panagos, P. Ballabio, C., Meusburger, K., Spinoni, J., Alewell, C., Borrelli, P., 2017. Towards

estimated of future rainfall erosivity in Europe based on REDES and WorldClim datashets. J. Hydrol. 548. 251–262. https://doi.org/j.hydrol.2017.03.006

Papadopoulou, C., Economou, V., Sakkas, H., Gousia, P., Giannakopoulos, X., Dontorou, C., Filioussis, G., Gessouli, H., Karanis, P., Leveidiotou, S., 2008. Microbiological quality of indoor and outdoor swimming pools in Greece: Investigation of the antibiotic resistance of the bacterial isolates. Int. J. Hyg. Environ. Health 211, 385–397. https://doi.org/10.1016/j.ijheh.2007.06.007

Papanikolopoulou, V., Baroudi, D., Guo, Y., Wang, Y., Papadopoulos, E., Lafi, S.Q., Abd El-Tawab, M.M., Diakou, A., Giadinis, N.D., Feng, Y., Xiao, L., 2018. Genotypes and subtypes of *Cryptosporidium* spp. in diarrheic lambs and goat kids in northern Greece. Parasitol. Int. 67, 472–475. https://doi.org/10.1016/j.parint.2018.04.007

Papazahariadou, M., Founta, A., Papadopoulos, E., Chliounakis, S., Antoniadou-Sotiriadou, K., Theodorides, Y., 2007. Gastrointestinal parasites of shepherd and hunting dogs in the Serres Prefecture, Northern Greece. Vet. Parasitol. 148, 170–173. https://doi.org/10.1016/j.vetpar.2007.05.013

Peeters, J.E., Mazas, E.A., Masschelein, W.J., Dematurana, I.V.M., Debacker, E., 1989. Effect of disinfection of drinking water with ozone or chlorine dioxide on survival of *Cryptosporidium parvum* oocysts. Appl. Environ. Microbiol. 55, 1519–1522.

Peng, M.M., Matos, O., Gatei, W., Das, P., Stantic-Pavlinic, M., Bern, C., Sulaiman, I.M., Glaberman, S., Lal, A.A., Xiao, L., 2001. A comparison of *Cryptosporidium* subgenotypes from several geographic regions. J. Eukaryot. Microbiol. Suppl, 28S-31S. https://doi.org/10.1111/j.1550-7408.2001.tb00442.x

Pignata, C., Bonetta, Silvia, Bonetta, Sara, Cacciò, S.M., Sannella, A.R., Gilli, G., Carraro, E., 2019. *Cryptosporidium* oocyst contamination in drinking water: a case study in Italy. Int. J. Environ. Res. Public Health 16, 1–10. https://doi.org/10.3390/ijerph16112055

Printza, N., Sapountzi, E., Dotis, J., Papachristou, F., 2013. Hemolytic uremic syndrome related to *cryptosporidium* infection in an immunocompetent child. Pediatr. Int. 55, 788–790. https://doi.org/10.1111/ped.12127

Ramo, A., Del Cacho, E., Sánchez-Acedo, C., Quílez, J., 2017. Occurrence of *Cryptosporidium* and *Giardia* in raw and finished drinking water in north-eastern Spain. Sci. Total Environ. 580, 1007–1013. https://doi.org/10.1016/j.scitotenv.2016.12.055

R Core Team, 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Read, C.M., Monis, P.T., Thompson, R.C.A., 2004. Discrimination of all genotypes of *Giardia duodenalis* at the glutamate dehydrogenase locus using PCR-RFLP. Infect. Genet. Evol. 4, 125–130. https://doi.org/10.1016/j.meegid.2004.02.001

Rendtorff, R.C., 1954. The experimental transmission of human intestinal protozoan parasites II. *Giardia lambia* cysts given in capsules. Am. J. Epidemiol. 59, 209–220. https://doi.org/10.1093/oxfordjournals.aje.a119725

Robertson, L., Campbell, A.., Smith, H. V., 1992. Survival of *Cryptosporidium parvum* oocysts under various environmental pressures. Appl. Environ. Microbiol. 58, 3494–3500.

Robertson, L., Gjerde, B., Hansen, E.F., Stachurska-Hagen, T., 2009. A water contamination incident in Oslo, Norway during October 2007; a basis for discussion of boil-water notices and the potential for post-treatment contamination of drinking water supplies. J. Water Health 7, 55–56. https://doi.org/10.2166/wh.2009.014

Robertson, L.J., Gjerde, B.K., 2006. Fate of *Cryptosporidium* oocysts and *Giardia* cysts in the Norwegian aquatic environment over winter. Microb. Ecol. 52, 597–602.

https://doi.org/10.1007/s00248-006-9005-4
Robertson, L.J., Hermansen, L., Gjerde, B.K., 2006. Occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in sewage in Norway. Appl. Environ. Microbiol. 72, 5297–5303. https://doi.org/10.1128/AEM.00464-06
Ryan, U., Fayer, R., Xiao, L., 2014. *Cryptosporidium* species in humans and animals: current understanding and research needs. Parasitology 141, 1667–1685. https://doi.org/10.1017/S0031182014001085
Ryan, U., Xiao, L., Read, C., Zhou, L., Lal, A.A., Pavlasek, I., 2003. Identification of novel *Cryptosporidium* genotypes from the Czech Republic. Appl. Environ. Microbiol. 69, 4302–4307. https://doi.org/10.1128/AEM.69.7.4302-4307.2003
Schets, F.M., van Wijnen, J.H., Schijven, J.F., Schoon, H., de Roda Husman, A.M., 2008. Monitoring of waterborne pathogens in surface waters in Amsterdam, the Netherlands, and the potential health risk associated with exposure to *Cryptosporidium* and *Giardia* in these waters. Appl. Environ. Microbiol. 74, 2069–2078. https://doi.org/10.1128/AEM.01609-07
Schrader, C., Schielke, A., Ellerbroek, L., Johne, R., 2012. PCR inhibitors – occurrence, properties and removal. J. Appl. Microbiol. 113, 1014–1026. https://doi.org/10.1111/j.1365-2672.2012.05384.x
Semenza, J.C., Nichols, G., 2007. Cryptosporidiosis surveillance and water-borne outbreaks in Europe. Eurosurveillance 12, 120–123
Simarmata, J.E., Sutarman, Mardiningsih, 2018. Object classification with classical Linear Discriminant Analysis and robust Linear Discriminant Analysis. Int. J. Res. Appl. Sci. Eng. Technol. 6, 84–91. https://doi.org/10.22214/ijraset.2018.5012
Slifko, T.R., Smith, H.V., Rose, J.B., 2000. Emerging parasite zoonoses associated with water and food. Int. J. Parasitol. 30, 1379-1393. https://doi.org/10.1016/S0020-7519(00)00128-4
Spanakos, G., Biba, A., Mavridou, A., Karanis, P., 2015. Occurrence of *Cryptosporidium* and *Giardia* in recycled waters used for irrigation and first description of *Cryptosporidium parvum* and *C. muris* in Greece. Parasitol. Res. 114, 1803–1810. https://doi.org/10.1007/s00436-015-4366-6
Sprong, H., Cacciò, S.M., van der Giessen, J.W.B., on behalf of the ZOOPNET network and partners., 2009. Identification of Zoonotic Genotypes of *Giardia duodenalis.* PLoS Negl. Trop. Dis. 3(12): e558. https://doi.org/10.1371/journal.pntd.0000558
Sulaiman, I.M., Fayer, R., Bern, C., Gilman, R.H., Trout, J.M., Schantz, P.M., Das, P., Lal, A.A., Xiao, L., 2003. Triosephosphate isomerase gene characterization and potential zoonotic transmission of *Giardia duodenalis*. Emerg. Infect. Dis. 9, 1444–1452. https://doi.org/10.3201/eid0911.030084
Sunnotel, O., Snelling, W.J., Xiao, L., Moule, K., Moore, J.E., Millar, B.C., Dooley, J.S.G., Lowery, C.J., 2006. Rapid and sensitive detection of single *Cryptosporidium* oocysts from archived glass slides. J. Clin. Microbiol. 44, 3285–3291. https://doi.org/10.1128/JCM.00541-06
Symeonidou, I., Gelasakis, A., Arsenopoulos, K. V., Schaper, R., Papadopoulos, E., 2017. Regression models to assess the risk factors of canine gastrointestinal parasitism. Vet. Parasitol. 248, 54–61. https://doi.org/10.1016/j.vetpar.2017.10.019
Symeonidou, I., Gelasakis, A.I., Arsenopoulos, K., Angelou, A., Beugnet, F., Papadopoulos, E., 2018. Feline gastrointestinal parasitism in Greece: emergent zoonotic species and associated risk factors. Parasit Vectors 11, 1–13. https://doi.org/10.1186/s13071-018-2812-x

Tolouei, S., Burnet, J.B., Autixier, L., Taghipour, M., Bonsteel, J., Duy, S.V., Sauvé, S., Prévost, M., Dorner, S., 2019. Temporal variability of parasites, bacterial indicators, and wastewater micropollutants in a water resource recovery facility under various weather conditions. Water Res. 148, 446–458. https://doi.org/10.1016/j.watres.2018.10.068

Traub, R., Wade, S., Read, C., Thompson, A., Mohammed, H., 2005. Molecular characterization of potentially zoonotic isolates of *Giardia duodenalis* in horses. Vet. Parasitol. 130, 317–321. https://doi.org/10.1016/j.vetpar.2005.02.011

Tzanidakis, N., Sotiraki, S., Claerebout, E., Ehsan, A., Voutzourakis, N., Kostopoulou, D., Casaert, S., Vercruysse, J., Geurden, T., 2014. Occurrence and molecular characterization of *Giardia duodenalis* and *Cryptosporidium* spp. in sheep and goats reared under dairy husbandry systems in Greece. Parasite 21. https://doi.org/10.1051/parasite/2014048

USEPA 1623.1, 2012. *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA. Protocol, EPA 816-R-12-001, Office of Water, U.S Environmental Protection Agency, Washington, D.C.

Van Dyke, M.I., Ong, C.S.L., Prystajecky, N.A., Isaac-Renton, J.L., Huck, P.M., 2012. Identifying host sources, human health risk and indicators of *Cryptosporidium* and *Giardia* in a Canadian watershed influenced by urban and rural activities. J. Water Health 10, 311–323. https://doi.org/10.2166/wh.2012.131

White, J.L., 2013. Logistic regression model effectiveness: proportional chance criteria and proportional reduction in error. J. Contemp. Water Res. Educ. 2, 4–10.

WHO, 2017. Guidelines for drinking-water quality: fourth edition incorporating the first addendum. World Health Organization, Geneva. Licence: CC BY-NL-SA 3.0 IGO

Wilkes, G., Edge, T., Gannon, V., Jokinen, C., Lyautey, E., Medeiros, D., Neumann, N., Ruecker, N., Topp, E., Lapen, D.R., 2009. Seasonal relationships among indicator bacteria, pathogenic bacteria, *Cryptosporidium* oocysts, *Giardia* cysts, and hydrological indices for surface waters within an agricultural landscape. Water Res. 43, 2209–2223. https://doi.org/10.1016/j.watres.2009.01.033

Wilkes, G., Edge, T.A., Gannon, V.P.J., Jokinen, C., Lyautey, E., Neumann, N.F., Ruecker, N., Scott, A., Sunohara, M., Topp, E., Lapen, D.R., 2011. Associations among pathogenic bacteria, parasites, and environmental and land use factors in multiple mixed-use watersheds. Water Res. 45, 5807–5825. https://doi.org/10.1016/j.watres.2011.06.021

Winiecka-Krusnell, J., Linder, E., 1998. Cysticidal effect of chlorine dioxide on *Giardia intestinalis* cysts. Acta Trop. 70, 369–372. https://doi.org/10.1016/S0001-706X(98)00036-9

Xiao, L., 2010. Molecular epidemiology of cryptosporidiosis: An update. Exp. Parasitol. 124, 80–89. https://doi.org/10.1016/j.exppara.2009.03.018

Xiao, S., Zhang, Y., Zhao, X., Sun, L., Hu, S., 2018. Presence and molecular characterization of *Cryptosporidium* and *Giardia* in recreational lake water in Tianjin, China: A preliminary study. Sci. Rep. 8, 1–8. https://doi.org/10.1038/s41598-018-20902-3

Young, I., Smith, B.A., Fazil, A., 2015. A systematic review and meta-analysis of the effects of extreme weather events and other weather-related variables on *Cryptosporidium* and *Giardia* in fresh surface waters. J. Water Health 13, 1–17. https://doi.org/10.2166/wh.2014.079

Zhang, R., Li, M., Yuan, X., Pan, Z., 2018. Influence of rainfall intensity and slope on suspended solids and phosphorus losses in runoff. Environ. Sci. Pollut. Res. 1–13. https://doi.org/10.1007/s11356-018-2999-6

Supplementary data

Supl. 1 Gantt chart representing monthly sampling from different rivers and sampling points.

			2015			2016		
Rivers	***Sampling points***	***Type of matrix***	Feb-May	Jun-Sept	Oct-Jan	Feb-May	Jun-Sept	Oct-Jan
Gallikos		raw water						
Axios	upstream wastewater treatment plant	raw water						
	downstream wastewater treatment plant	raw water						
	entrance point from North Macedonia	raw water						
Loudias		raw water						
	estuary of Axios-Loudias	raw water						
	irrigation canals	mixed raw/treated water						
Aliakmonas	water production company	raw water						
		raw intake water						
		filtered water						
		purified drinking water						

Suppl. 2

Descriptive statistics of *(a)* microbiological and *(b)* physicochemical parameters of each water sample, collected monthly from February 2015 to January 2017.

(a)

Water samples	Total *Coliforms*/100 ml			*E.coli*/100 ml			*Enterococci*/100 ml			*Clostridia*/100 ml		
	R	**M**	**SD**	**R**	**M**	**SD**	**R**	**M**	**SD**	**R**	**M**	**SD**
Axios upstream	23 - 12 x 10^4	19457	32974	23 - 6 x 10^4	7661	13546	0 - 2.4 x 10^4	1699	13546	0 - 9 x 10^3	606	1820
Axios downstream	23 - 8.8 x 10^4	12708	22316	20 - 5 x 10^4	6251	11385	0 - 3.6 x 10^3	878	1038	0 - 6 x 10^3	479	1209
Axios borders	6.4 x 10^3 - 15 x 10^4	81350	59146	4.7 x 10^2 - 6.5 x 10^4	18243	31213	7.7 x 10^2 - 1.8 x 10^4	1243	431	1 - 8.4 x 10^2	238	402
Estuary of Axios-Loudias	23 - 4.3 x 10^4	6570	12796	10 - 2.4 x 10^4	5015	11030	1 - 5 x 10^3	699	1422	6 - 1 x 10^4	622	2013
Loudias	23 - 2.4 x 10^4	6386	7849	7.4 - 2.4 x 10^4	1501	4812	0 - 1.7 x 10^3	275	393	10 - 3. 6 x 10^3	467	767
Aliakmonas	0 - 9.8 x 10^3	757	2036	0 - 2.4 x 10^3	270	670	0 - 2.3 x 10^2	28	54	0 - 50	8	12
Gallikos	23 - 11 x 10^4	14761	31246	3 - 2.1 x 10^4	3650	6303	0 - 4.6 x 10^3	611	1288	0 - 6 x 10^2	219	200
Raw intake water	0 - 2.8 x 10^3	458	829	0 - 2.4 x 10^3	296	683	0 - 4 x 10^2	33	92	0 - 12	3	4
Filtered water	0 - 2.3 x 10^2	30	51	0 - 2.3 x 10^2	16	48	0 – 2	0.2	0.5	0 - 11	1	3
Purified drinking water	0 - 3	0.3	0.8	0	0	0	0	0	0	0 - 10	1.4	3.4
Irrigation water (1)	1.3 x 10^3 - 2.1 x 10^4	6098	6734	0 - 2.1 x 10^4	3531	7253	0 - 2.9 x 10^3	550	969	70 - 1.6 x 10^3	784	649
Irrigation water (2)	1.1 x 10^3 - 2.4 x 10^4	4725	8650	0 - 2.4 x 10^4	4725	8650	23 - 1.9 x 10^3	387	628	100 - 1.6 x 10^3	714	544

R range, *M* mean, *SD* standard deviation

(b)

Water samples	pH			turbidity (FNU)			EC (μS/cm)			TDS (ppm)		
	R	M	SD	R	M	SD	R	M	SD	R	M	SD
Axios upstream	7.5 - 8.3	7.9	0.2	7.9 - 92.7	39.2	31.8	364 - 562	438	67	147 - 317	222	39
Axios downstream	7.5 - 8.4	7.8	0.2	8.7 - 62.5	28.4	16.1	309 - 638	445	92	154 - 322	223	46
Axios borders	7.7 - 7.9	7.8	0.1	6.9 - 42.1	19.4	16.2	283 - 358	335	35	175 - 195	182	9
Estuary of Axios-Loudias	7.4 - 8.2	7.6	0.3	7.1 - 70.8	25.7	20	401 - 3999	3441	1287	196 - 2000	1720	645
Loudias	6.5 - 7.9	7.5	0.3	2.5 - 48	18.5	12.8	490 - 3999	1724	1346	248 - 2000	863	673
Aliakmonas	7.4 - 8.4	7.9	0.3	0.8 - 10.5	3.5	2.2	368 - 454	403	27	183 - 234	203	14
Gallikos	7.5 - 8.2	7.9	0.2	5.5 - 53.6	23.5	17.5	321 - 1210	857	215	148 - 561	417	101
Raw intake water	6.9 - 7.9	7.4	0.2	0.6 - 10.7	3.8	2.5	375 - 568	431	50	188 - 277	215	24
Filtered rater	6.9 - 7.8	7.4	0.2	0.1 - 4.6	1.2	1.2	377 - 593	428	49	142 - 269	212	26
Purified drinking water	7.0 - 7.9	7.5	0.2	0.1 - 4.2	0.8	0.9	391 - 536	455	44	194 - 266	228	23
Irrigation water (1)	7.2 - 7.5	7.3	0.1	2.2 - 15.3	7.5	5.5	374 - 3999	1814	1022	187 - 2000	906	510
Irrigation water (2)	7.3 - 7.9	7.6	0.2	5.8 - 56.3	18.1	17.1	610 - 1440	844	282	305 - 719	424	139

R range, *M* mean, *SD* standard deviation, *EC* electrical conductivity, *TDS* total dissolved solids

Suppl. 3

Cryptosporidium and *Giardia* (occurrence and concentrations) paired with range values of faecal indicator bacteria (total coliforms, *E. coli*, enterococci, clostridia) and turbidity data for waters samples at the various sampling points, collected and analysed monthly from February 2015 to January 2017.

Water samples	***Cryptosporidium***		***Giardia***		Total *Coliforms*/100 ml	*E.coli*/100 ml	*Enterococci*/100 ml	*Clostridia*/100 ml	turbidity (FNU)
	prevalence	oocysts/L	prevalence	cysts/L					
Axios upstream	50%	0 - 5.4	83.3%	0 - 34.2	23 - 12 x 10^4	23 - 6 x 10^4	0 - 2,4 x 10^4	0 - 9 x 10^3	7.9 - 92.7
Axios downstream	58.3%	0 - 0.8	79.2%	0 - 34.9	23 - 8,8 x 10^4	20 - 5 x 10^4	0 - 3,6 x 10^3	0 - 6 x 10^3	8.7 - 62.5
Axios borders	100%	0.3 - 2.4	75%	0 - 48.6	6,4 x 10^3 - 15 x 10^4	4,7 x 10^2 - 6,5 x 10^4	7,7 x 10^2 - 1,8 x 10^4	1 - 8,4 x 10^2	6.9 - 42.1
Estuary of Axios-Loudias	45.8%	0 - 1.7	70.8%	0 - 5.2	23 - 4,3 x 10^4	10 - 2,4 x 10^4	1 - 5 x 10^3	6 - 1 x 10^4	7.1 - 70.8
Loudias	58.3%	0 - 13.5	70.8%	0 - 7.9	23 - 2,4 x 10^4	7,4 - 2,4 x 10^4	0 - 1,7 x 10^3	10 - 3, 6 x 10^3	2.5 - 48
Aliakmonas	29.2%	0 - 0.3	29.2%	0 - 19.7	0 - 9,8 x 10^3	0 - 2,4 x 10^3	0 - 2,3 x 10^2	0 - 50	0.8 - 10.5
Gallikos	16.7%	0 - 1	41.7%	0 - 1.6	23 - 11 x 10^4	3 - 2,1 x 10^4	0 - 4,6 x 10^3	0 - 6 x 10^2	5.5 - 53.6
Raw intake water	29.2%	0 - 0.5	41.7%	0 - 5.3	0 - 2,8 x 10^3	0 - 2,4 x 10^3	0 - 4 x 10^2	0 - 12	0.6 - 10.7
Filtered water	25%	0 - 1.1	16.7%	0 - 0.9	0 - 2,3 x 10^2	0 - 2,3 x 10^2	0 - 2	0 - 11	0.1 - 4.6
Purified drinking water	20.8%	0 - 0.9	16.7%	0 - 0.3	0 - 3	0	0	0 - 10	0.1 - 4.2
Irrigation water (1)	25%	0 - 1.3	25%	0 - 0.2	1,3 x 10^3 - 2,1 x 10^4	0 - 2,1 x 10^4	0 - 2,9 x 10^3	70 - 1,6 x 10^3	2.2 - 15.3
Irrigation water (2)	25%	0 - 1.3	75%	0 - 0.7	1,1 x 10^3 - 2,4 x 10^4	0 - 2,4 x 10^4	23 - 1,9 x 10^3	100 - 1,6 x 10^3	5.8 - 56.3

Suppl. 4

NCBI accession numbers for *Cryptosporidium* species and *Giardia duodenalis* assemblages (genotypes) detected in *(a)* water samples (RW, IW, RIW, DW) and *(b)* animal faecal samples.

(a)

	Species/assemblage	*n*	NCBI accession numbers
Cryptosporidium	*C. andersoni*	1	MK414209
	C. parvum (IIaA15G2R1)	1	MK414210
G. duodenalis	A	2	MK414179, MK414184
	AI	2	MK414187, MK414196
	AII	36	MK414175 - MK414178, MK414180 - MK414183, MK414185 - MK414186, MK414188 - MK414189, MK414191 - MK414195, MK414197 - MK414208, MK574032, MK574034 - MK574037, MK585274
	A4	1	MK574033
	E	1	MK414190

RW: raw surface water; IW: water from the irrigational canals; RIW: raw intake water from the water production company; DW: purified drinking water
accession numbers:MK414176, MK414180, MK414194 & MK414200 (all G. duodenalis AII) correspond to IW; MK414192 (G. duodenalis AII) corresponds to RIW; MK414191 (G. duodenalis AII) corresponds to DW; all the other sequences correspond to RW

(b)

	Species/assemblage	***n***	***NCBI accession numbers***
Cryptosporidium	*C. andersoni*	3	MK533139, MK533140, MK533142
	C. bovis	4	MK501766, MK501768, MK501770, MK501771
	C. parvum	35	MK492588 - MK492595, MK501764, MK501767, MK501769, MK501772 - MK501774, MK501778 - MK501780, MK533137, MK533138, MK533141, MK561311 - MK561318, MK561320, MK561321, MK561324, MK561326 - MK561329
	C. ryanae	1	MK501765
	C. ubiquitum	7	MK501775 - MK501777, MK561319, MK561322, MK561323, MK561325
Giardia duodenalis	A	2	MK452901, MK452903
	AI	11	MK452864, MK452869, MK452870, MK452872, MK452887 - MK452890, MK473861, MK561333, MK574021

AII	70	MK452835, MK452836, MK452838 - MK452845, MK452847 - MK452852, MK452854, MK452855, MK452857 - MK452862, MK452865 - MK452868, MK452871, MK452873, MK452875 - MK452878, MK452881 - MK452883, MK452886, MK452895 - MK452900, MK452902, MK452904, MK452905, MK561330 - MK561332, MK561334 - MK561341, MK561345, MK561346, MK561348, MK561351, MK561352, MK561355, MK561357, MK561358, MK574023, MK574027, MK574028, MK574030
E	35	MK452837, MK452846, MK452853, MK452856, MK452863, MK452874, MK452879, MK452880, MK452884, MK452885, MK452891 - MK452894, MK473859, MK473860, MK473862 - MK473865, MK561342 - MK561344, MK561347, MK561349, MK561350, MK561353, MK561354, MK561356, MK574022, MK574024 - MK574026, MK574029, MK574031

Suppl. 5

(a) Standardized Canonical Discriminant Function Coefficients of the variables selected for discrimination between the four categories of *Cryptosporidium* oocysts/L and their Tests of Equality of Group Means.

Variables Selected	Function			p-value
	1	2	3	
Clostridia/100 ml	0.415	-0.264	0.629	0.032
Colony count 22 °C	0.559	0.815	0.144	0.000
MeanTM	-0.641	0.351	0.661	0.000
TotalRainW	0.460	-0.385	0.345	0.006

(b) Standardized Canonical Discriminant Function Coefficients of the variables selected for discrimination between the four categories of *Giardia* cysts/L and their Tests of Equality of Group Means.

Variables Selected	Function			p-value
	1	2	3	
Coliforms/100 ml	1.000	-1.268	-0.025	0.000
E. coli/100 ml	-0.626	1.116	1.009	0.047
Turbidity (FNU)	0.822	0.497	-0.333	0.000

Suppl. 6

(a) Linear Discriminant Function model for *Cryptosporidium*.

$$LDF_1 = 0.415 * \frac{Clostridia}{100ml} + 0.559 * \ Colony\ Count\ 22°C - 0.641 * Mean\ Temperature\ of\ Actual\ Month\ (^oC) + 0.46 * Total\ Rain\ of\ Week\ (mm)$$

$$LDF_2 = -0.264 * \frac{Clostridia}{100ml} + 0.815 * \ Colony\ Count\ 22°C + 0.351 * Mean\ Temperature\ of\ Actual\ Month\ (^oC) - 0.385 * Total\ Rain\ of\ Week\ (mm)$$

$$LDF_3 = 0.629 * \frac{Clostridia}{100ml} + 0.144 * \ Colony\ Count\ 22°C + 0.661 * Mean\ Temperature\ of\ Actual\ Month\ (^oC) + 0.345 * Total\ Rain\ of\ Week\ (mm)$$

The first function (LDF_1) maximizes the difference between the values of the dependent variable (Cryptosporidium oocysts/l). The second function (LDF_2) maximizes the difference between the values of the dependent variable, while controlling the first function. The third function (LDF_3) maximizes the difference between the values of the dependent variable, while controlling the first and the second function. The first function is the most powerful differentiating dimension. The signs (+ or -) indicate a positive or negative relationship with the dependent variable. The linear discriminant function coefficients indicate the partial contribution of each variable to the discriminate function controlling for all other variables in the equation. They can be used to assess each independent variables' unique contribution to the discriminate function and therefore provide information on the relative importance of each variable.

(b) Linear Discriminant Function model for *Giardia*.

$$LDF_1 = 1.0 * \frac{Coliforms}{100ml} - 0.626 * \frac{E.\ coli}{100ml} + 0.822 * turbidity\ (FNU)$$

$$LDF_2 = -1.268 * \frac{Coliforms}{100ml} + 1.116 * \frac{E.\ coli}{100ml} + 0.497 * turbidity\ (FNU)$$

$$LDF_3 = -0.025 * \frac{Coliforms}{100ml} + 1.009 * \frac{E.\ coli}{100ml} - 0.333 * turbidity\ (FNU)$$

The first function (LDF_1) maximizes the difference between the values of the dependent variable (Giardia cysts/l). The second function (LDF_2) maximizes the difference between the values of the dependent variable, while controlling the first function. The third function (LDF_3) maximizes the

difference between the values of the dependent variable, while controlling the first and the second function. The first function is the most powerful differentiating dimension. The signs (+ or -) indicate a positive or negative relationship with the dependent variable. The linear discriminant function coefficients indicate the partial contribution of each variable to the discriminate function controlling for all other variables in the equation. They can be used to assess each independent variables' unique contribution to the discriminate function and therefore provide information on the relative importance of each variable.

Chapter 3

Based on:

Protocol standardization for the detection of *Giardia* cysts and *Cryptosporidium* oocysts in Mediterranean mussels (*Mytilus galloprovincialis*)

P. Ligda, E. Claerebout, L. J. Robertson, S. Sotiraki

In: International Journal of Food Microbiology. 2019;298:31-38.
doi: 10.1016/j.ijfoodmicro.2019.03.009

Introduction

Foodborne parasitic diseases have become of increasing relevance during recent decades, affecting not only people's health, but also having serious economic consequences. Potential causes of the emergence and re-emergence of foodborne parasitic diseases include climate change and the rising human population that has led to implementation of new food production systems and increased global trade of foodstuff, as well as new dietary habits and trends, with increased consumption of raw or undercooked animal products, such as fish, meat, and shellfish (Broglia and Kapel, 2011). Moreover, improved diagnostic tools and better reporting systems (as, for example, WHO reports), have resulted in increased diagnosis of these diseases worldwide (Dorny et al., 2009).

Marine bivalve shellfish are considered to act as possible carriers of foodborne infections. They can filter between 20 and 100 L of sea water every 24 h and have the ability to accumulate pollutants and microorganisms in their tissues (gills, glands, and digestive tracts). These pathogens can remain in the tissues of the shellfish and may cause infection to humans, as people often eat them lightly cooked or even raw (Robertson, 2007).

During recent years there have been several reports indicating the presence of viruses (e.g., norovirus) and bacteria (e.g., *Escherichia coli*) in mussels that were ready for consumption (Rees et al., 2015; Souza et al., 2018; Strubbia et al., 2016). Protozoan parasites, including *Giardia* duodenalis and *Cryptosporidium* spp., have also been recovered from shellfish (e.g., *Mytilus galloprovincialis*) in several countries around the Mediterranean basin, such as Italy (Giangaspero et al., 2007, 2014;) and Spain (Freire-Santos et al., 2000; Gomez-Bautista et al., 2000; Gómez-Couso et al., 2003a, 2003b, 2004, 2005a, 2005b, 2006b), as well as from other European countries (Chalmers et al., 1997; Graczyk et al., 2004; Li et al., 2006; Lowery et al., 2001; Robertson and Gjerde, 2008; Schets et al., 2007; Tryland et al., 2014), the USA (Adell et al., 2014; Tei et al., 2016), Brazil (Santos et al., 2018), Canada (Willis et al., 2013), New Zealand (Coupe et al., 2018) and Asia (Pagoso and Rivera, 2017; Srisuphanunt et al., 2009).

There are several reports of outbreaks of viral and bacterial infections associated with the consumption of shellfish (Alfano-Sobsey et al., 2012; Cho et al., 2016; Dewey-Mattia et al., 2018; Le Mennec et al., 2017; Lunestad et al., 2016; Rippey, 1994; Woods et al., 2016),

but, to our knowledge, there are no data about similar parasitic foodborne outbreaks. Although *G. duodenalis* and *Cryptosporidium* spp. have been associated with foodborne outbreaks worldwide, involving different food matrices (e.g., salads, milk, apple juice, meat) as implicated transmission vehicles (Robertson and Chalmers, 2013), there are no reports of mussels or other shellfish being responsible for such an outbreak. The reasons for this are multiple, including: a) that the consumption of shellfish is not commonly recognised as a potential source of infection with these two parasites (Robertson, 2007), and b) there are no adequate surveillance systems to detect parasitic contamination in those organisms and, as a result, most of them are not notifiable to the authorities (Dorny et al., 2009; Ryan et al., 2018).

In addition, there is no standard validated method to detect *Giardia* cysts and *Cryptosporidium* oocysts in mussels. The direct immunofluorescence assay (IFAT) has been considered to be the gold standard method for the detection of these parasites in other samples (e.g., faeces and water) (Geurden et al., 2004; Gotfred-Rasmussen et al., 2016; ISO 15553, 2006; Scorza and Tangtrongsup, 2010; Tangtrongsup and Scorza, 2010; USEPA 1623.1, 2012), but there is no standardized protocol available for the analytical workup of mussel matrices before applying this technique. Different approaches have been used by different authors concerning the type of sample to be tested, e.g. individual whole mussels (Schets et al., 2013; Tei et al., 2016), pooled whole mussels (Adell et al., 2014; Robertson and Gjerde, 2008; Tryland et al., 2014), specific organs (gills, glands, intestinal tracts) or haemolymph (Aksoy et al., 2014; Gómez-Couso et al., 2003a, 2004, 2005a, 2006b). Moreover, researchers have used different approaches for the preparation of the matrices before detection by immunofluorescence, such as extraction of the lipids using diethyl ether (Gómez-Couso et al., 2003a; Tedde et al., 2013), removal of coarse particles by sieving (Schets et al., 2013), or artificial digestion of the whole sample using a pepsin digestion solution (Robertson and Gjerde, 2008). In addition, immunomagnetic separation (IMS) for the concentration of the (oo)cysts has been included in the protocols applied by some research groups (Lowery et al., 2001; MacRae et al., 2005; Miller et al., 2006; Schets et al., 2013, 2007), but not by others (Gomez-Bautista et al., 2000; Gómez-Couso et al., 2003a, 2003b, 2005a, 2005b, 2006a, 2006b; Lucy et al., 2008).

Although many of the techniques have been widely used in other types of matrices (e.g., faeces, surface water or wastewater samples) for the detection of *Giardia* and

Cryptosporidium (oo)cysts and have been proven to be efficient using spiking experiments, most have not been experimentally tested for their sensitivity when used for analysis of shellfish (Robertson and Gjerde, 2008).

Adding to the complexity of the above, the type of the shellfish tested in the above studies varies, with different species of mussels, clams, oysters being analysed. This makes the need of a standardized technique, that could potentially be applied to different species of mussels even stronger. For example, Mediterranean mussels (*M. galloprovincialis*) and blue mussels (*M. edulis*) are smaller (Hepper, 1957; Seed, 1968) and more difficult to handle (e.g., for extracting haemolymph) than bigger ones (*M. californianus*) (Dehnel, 1956).

For the above-mentioned reasons, a comparative study between most commonly applied methods was performed in an effort to identify and characterize the most sensitive technique to detect *Giardia* cysts and *Cryptosporidium* oocysts in Mediterranean mussels, and that can be used as reference technique in future epidemiological studies. Two different experiments with experimentally spiked mussels with *Giardia* cysts and *Cryptosporidium* oocysts were performed. The first experiment was performed in order to determine the most sensitive method to detect the (oo)cysts of the parasites in mussel samples, and in the second experiment the analytical sensitivity of this method was assessed.

Materials and methods

Shellfish and parasites

Mediterranean farmed mussels, *M. galloprovincialis*, purchased from local markets in the city of Thessaloniki, in Northern Greece were used for all the spiking experiments. The mussels were immediately transported refrigerated at 5 °C to the Laboratory of Parasitology of the Veterinary Research Institute of Thessaloniki (HAO-DEMETER) and then processed accordingly to the experimental protocol. *Giardia* cysts and *Cryptosporidium* oocysts were commercially supplied (AccuSpike™-IR, Waterborne™, INC.) and stored refrigerated at 4 °C, according to the manufacturer's instructions.

Experiment 1

Five whole commercial Mediterranean mussels were opened and the entire contents, including the animal and the intravalvular liquid, were pooled and homogenized for 2 min using a laboratory blender (8010EG Waring®, Christison Particle Technologies, Gateshead, UK). From the total amount of the homogenate six sub-samples were taken, each weighing 1 g. Each sub-sample was spiked with 1000 *Giardia* cysts and 1000 *Cryptosporidium* oocysts. The volume of 1 g was chosen as a reference quantity to express our results (cysts/oocysts per gram). Different approaches were followed for preparation of the matrix before the IFAT, with treatment with either: (i) only coarse-sieving, (ii) diethyl ether, or (iii) pepsin digestion solution (Fig. 1).

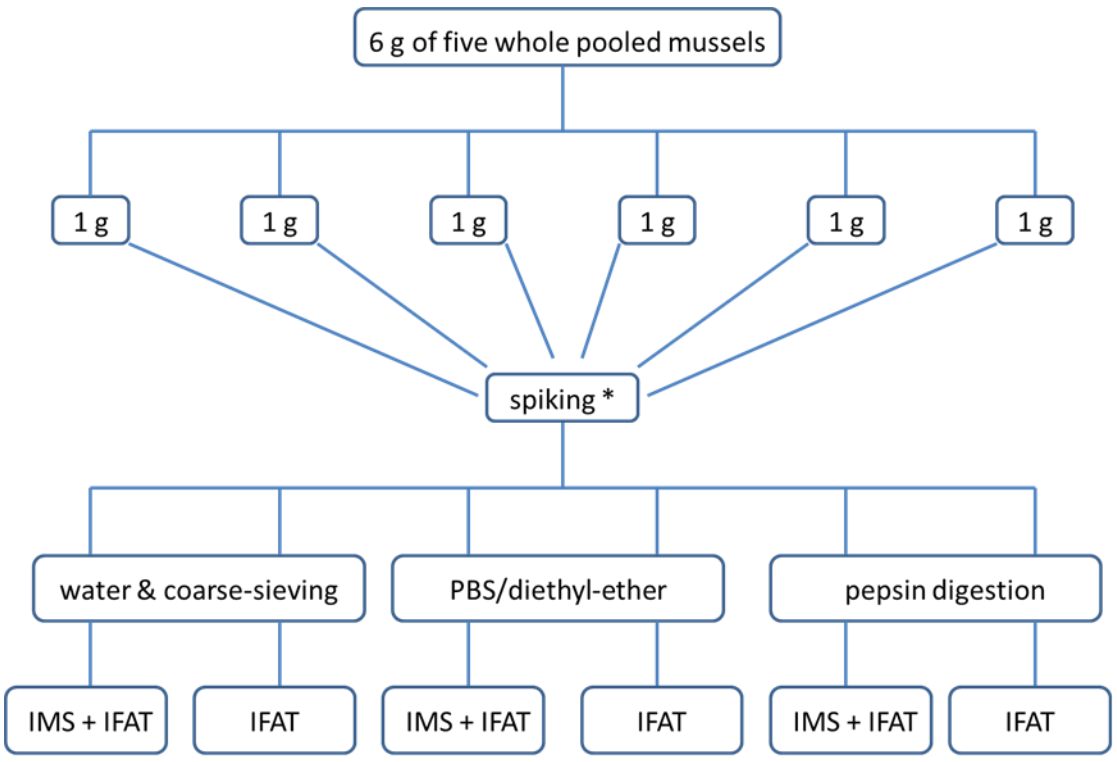
i. Water and coarse-sieving treatment: Distilled water up to 10 ml was added in the matrix, mixed thoroughly by vortexing and then it was sieved through a stainless-steel grid with a pore size of approximately 0.05mm to remove coarse particles. The collected filtrate was then centrifuged at 1170×g for 10 min (Schets et al., 2013).

ii. Diethyl ether treatment for lipid removal: The matrix after being sieved (according to the procedure described above) was suspended in PBS/diethyl ether (2:1) solution, thoroughly mixed, and concentrated by centrifugation at 1250×g for 5 min. Then it was washed once with PBS and twice with distilled water (Gómez-Couso et al., 2003a).

iii. Pepsin digestion: The matrix was mixed with pepsin solution (20 ml of 1M HCl, 80 ml of distilled water and 1 g of pepsin [1:10,000 NF]), that was prepared immediately before use for each experiment, and incubated at 37 °C for 1 h, with intervals of vortexing every 15 min. Then, the homogenate was concentrated by centrifugation at 13,000×g for 1 min, washed once with phosphate buffered saline (PBS), and twice with distilled water (Robertson and Gjerde, 2008).

After the different approaches for sample preparation, each sample was further processed, either with IMS for the concentration of the (oo)cysts using the commercial Dynabeads™ GC-Combo (Applied Biosystems™), following the manufacturer's instructions, or not. Finally, the parasites were detected and enumerated by means of a quantitative IFAT based on the commercial MERIFLUOR *Cryptosporidium*/*Giardia* (Meridian Diagnostics Inc., Cincinnati, Ohio) kit. For samples that were processed directly with IFAT (without IMS), the sediment was resuspended in distilled water up to volume of 1 ml. After thorough vortexing,

two aliquots of 50 µl each were pipetted onto treated IFA slides. For samples that were processed with IMS, the resulting eluate (100 µl) was pipetted onto two treated IFA-slides of 50 µl each. After drying, fixing, and staining on the slide, as instructed by the manufacturer, the entire smear was examined at a 400× magnification under a fluorescence microscope. For samples that were not processed with IMS, the total number of *Giardia* cysts and *Cryptosporidium* oocysts was obtained by multiplying the total number of (oo)cysts on the two smears by 10. For those samples that were processed with IMS, the total number of (oo)cysts was the total number of (oo)cysts on the two smears. In total, 5 repetitions of the entire experiment were performed. For each replicate, negative controls (homogenates "spiked" with PBS) were also included and processed using the protocols described above.



*spiking with 1000 *Giardia* cysts & 1000 *Cryptosporidium* oocysts/g or with 1 ml PBS/g (negative controls)

Fig. 1. Experimental design for comparison of methods to detect *Giardia* cysts and *Cryptosporidium* oocysts in spiked mussel samples.

Experiment 2

To determine the detection threshold of the method that had been proven to be more sensitive based on the results of Experiment 1, a new spiking experiment was designed. Five whole commercial *M. galloprovincialis* mussels were opened and the entire contents, including the intravalvular liquid, were pooled and homogenized using a laboratory blender as previously. Four sub-samples of the homogenate, each weighing 1 g, were weighed into tubes, and then each was spiked with either 1, 10, 100, or 1000 *Giardia* cysts and *Cryptosporidium* oocysts. The homogenate was then processed with the method that shown to be more sensitive in the first experiment. The parasites were detected and enumerated by IFAT using the commercial MERIFLUOR *Cryptosporidium*/*Giardia* kit as previously described. In total, 5 repetitions of the experiment were performed. For each replicate, negative controls (homogenates "spiked" with PBS) were also included.

Statistical analysis

Descriptive statistical analyses were performed in order to investigate the distribution of the recovery rates for each protocol that was used and boxplots of the data were generated. The non-parametric Kruskal-Wallis test was performed in order to investigate differences in the recovery rates of the (oo)cysts between the different sample preparation methods (coarse sieving, diethyl ether, or pepsin). In addition, the non-parametric Mann-Whitney test was used to investigate the effect of the IMS on the protocol. Also, the two-way non-parametric Scheirer-Ray-Hare test (Sokal and Rohlf, 1995) was performed to investigate whether the interaction between the different sample preparation methods (coarse sieving, diethyl ether, pepsin digestion) and isolation-detection methods (IMS or not) was statistically significant or not. Data were analysed using the SPSS statistics software (version 19.0).

Results

Experiment 1

The recovery efficiency of each technique applied for each parasite is shown in Fig. 2. The artificial digestion of the whole mussel homogenates using pepsin digestion solution in combination with IMS of the (oo)cysts was the most effective protocol to detect both parasites: 32.1% (SD: 21.1) of *Giardia* cysts and 61.4% (SD: 26.2) *Cryptosporidium* oocysts

were detected with this method (Fig. 2). When only the pepsin solution was used, without the IMS concentration step, 16.2% (SD: 19.0) of *Giardia* cysts and 8.9% (SD: 10.1) of *Cryptosporidium* oocysts were detected by IFAT.

Only 0.8% (SD: 1.4) of *Giardia* cysts were recovered when diethyl ether was used for lipid removal in combination with IMS concentration, but 32.3% (SD: 25.3) of *Cryptosporidium* oocysts were detected with the same protocol. When the IMS concentration step was not performed, no *Giardia* cysts were recovered and 26.8% (SD: 35.3) *Cryptosporidium* oocysts were counted.

The sieving of the homogenates only with water resulted in the detection of 4.0% (SD: 6.5) and 1.0% (SD: 2.2) of *Giardia* and *Cryptosporidium* (oo)cysts, respectively. With the inclusion of the IMS concentration step in the protocol, higher recovery rates were achieved, with 13.2% (SD: 12.3) *Giardia* cysts and 15.7% (SD: 13.2) of *Cryptosporidium* oocysts recovered.

Samples that were spiked only with PBS (negative controls) were found negative with all protocols.

For the recovery of *Giardia* cysts there was a statistically significant difference for the sample preparation method (H=13.333, p=0.001), with mean rank 16.25 for “water and coarse sieving”, 8.20 for “diethyl ether” and 22.05 for “pepsin digestion”. On the other hand, there was no statistically significant difference for the isolation-detection method (U=67.5, p=0.054) with a mean rank 18.50 for “IMS+IFAT” and 12.50 for “IFAT”.

For *Cryptosporidium* oocysts there was no statistically significant difference for the sample preparation method (H=5.104, p=0.078), with mean rank 10.40 for “water and coarse sieving”, 18.00 for “diethyl ether” and 18.10 for “pepsin digestion”. But there was a statistically significant difference for the isolation-detection methods (U=43.5, p=0.004), with a mean rank 20.10 for “IMS+IFAT” and 10.90 for “IFAT”.

According to the Scheirer-Ray-Hare test, the interaction between the different sample preparation methods and the isolation-detection methods was not statistically significant for the recoveries of both *Giardia* (H=0.4844, p=0.78491) and *Cryptosporidium* (H=2.2316, p=0.32766) (oo)cysts.

Experiment 2

Based on the results from Experiment 1, pepsin digestion solution for the treatment of the matrix, followed by IMS for the concentration of *Giardia* cysts and *Cryptosporidium* oocysts, was considered to provide the best results. Therefore, the detection limits of that protocol were explored as shown in Fig. 3.

When the homogenates were spiked with 1000 *Giardia* cysts and 1000 *Cryptosporidium* oocysts per 1 g of matrix, the recovery efficiency of the protocol was similar to the first part of the trial: 42.8% (SD: 11.4) of *Giardia* cysts and 45.7% (SD: 14.5) of *Cryptosporidium* oocysts were recovered. The recovery rates were also high, 34.0% (SD: 9.5) and 28.4% (SD: 10.7) for *Giardia* cysts and *Cryptosporidium* oocysts respectively, when the mussel homogenates were spiked with 100 (oo)cysts/g. The lowest concentration of (oo)cysts that could be detected with this protocol, was 10 (oo)cysts/g with 30.0% (SD: 13.2) for *Giardia* cysts and 5.0% (SD: 11.2) recovery efficiency for *Cryptosporidium* oocysts. No (oo)cysts were detected when the mussel homogenates were spiked with 1 (oo)cyst/g and in the negative control samples.

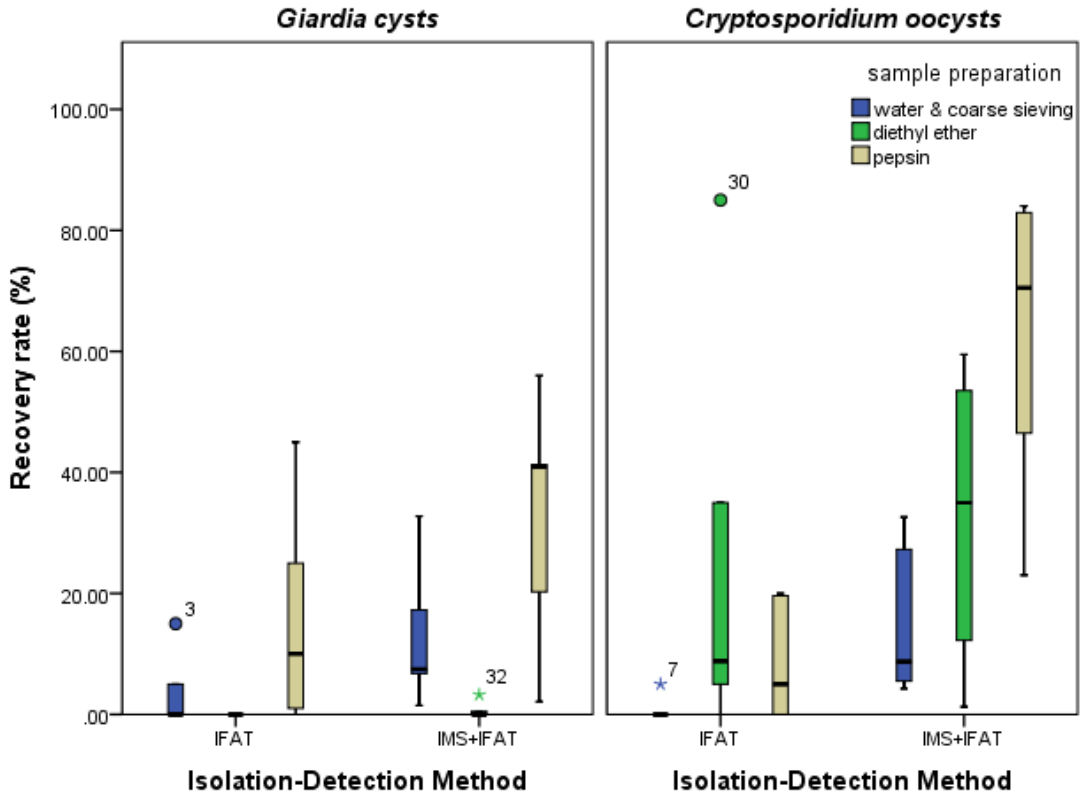


Fig. 2. Boxplots representing the range of the recovery rates (through the 5 repetitions) of *Giardia* cysts and *Cryptosporidium* oocysts after the use of different protocols for the preparation of the matrix (coarse sieving only with water, diethyl ether lipid removal or pepsin digestion) and the isolation-detection method (IMS+IFAT or only IFAT) in mussel homogenates experimentally contaminated with known concentrations of (oo)cysts. Dots and stars indicate the outliers.

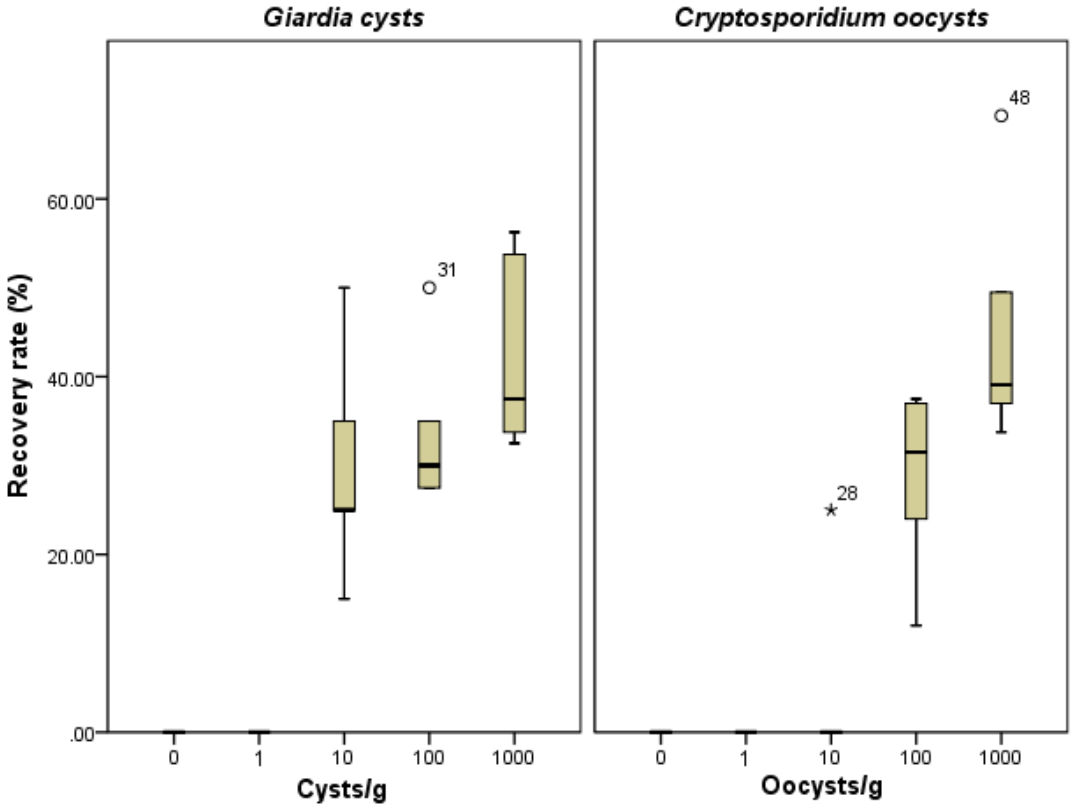


Fig. 3. Boxplots representing the range of the recovery rates (through the 5 repetitions) of the different concentrations of *Giardia* cysts and *Cryptosporidium* oocysts, using pepsin digestion solution for the artificial digestion of the matrix, followed by IMS for the concentration of the (oo)cysts and detection by IFAT. Dots and stars indicate outliers.

Discussion

Although some previous studies have investigated contamination of the marine bivalves with *G. duodenalis* and *Cryptosporidium* spp., it is difficult to compare the results of these investigations, as different approaches and techniques are being used and no standardized protocol is followed (Robertson and Gjerde, 2008).

The occurrence of *Giardia* and *Cryptosporidium* is commonly investigated in faecal or water samples, and the gold standard detection method that is being used is the IFAT (Geurden et al., 2004; Gotfred-Rasmussen et al., 2016; ISO 15553, 2006; Scorza and Tangtrongsup, 2010; Tangtrongsup and Scorza, 2010; USEPA 1623.1, 2012). Although most studies investigating mussel samples also use IFAT to detect these parasites, the preparation of the sample before detection differs between studies. Sample preparation prior to detection is a very important step, and its efficacy impacts on the final analytical results,

regarding less of the sensitivity of the detection method. As mussels contain large amounts of proteins and lipids, with the fat: carbohydrate: protein ratios in mussels approximately, 1: 1: 4 (USDA, 2018), protein digestion or lipid removal prior to analysis may increase the sensitivity of the detection method.

The results of our study confirm that treatment of the mussels before detection of the (oo)cysts by IFAT is a crucial step, and that, depending on the technique applied, the sensitivity of the method varies. The processing of whole mussel homogenate with pepsin digestion solution improved the detection capacity of the IFAT assay, compared with only sieving of the matrix. A beneficial effect was also recorded, when lipids were removed from the homogenate using diethyl ether, but only for *Cryptosporidium* oocysts. The reason for this is not clear. Use of IMS for concentration of the (oo)cysts after the initial stages also improved the efficacy of the methods, even when a simple sieving of the matrix was performed, presumably due to the positive selection of these parasites against other debris and components of the mussels. Artificial digestion of the whole mussel homogenate using a pepsin digestion solution, as described previously (Robertson and Gjerde, 2008), followed by the IMS of the (oo)cysts was superior to the other protocols, resulting in a recovery of over 30% for *Giardia* and over 60% for *Cryptosporidium*. These recovery efficiencies were considered sufficient to proceed to the next trial.

As this is the first time that the whole procedure, including the pretreatment of the mussel matrix and the detection method, is characterized, our results can be only partly compared with the data generated by Robertson and Gjerde (2008), where the method with the pepsin digestion solution was investigated, but the effect of inclusion (or not) of the IMS step was not investigated.

Higher recovery efficiencies were achieved (70–81%) by Robertson and Gjerde (2008) than seen in our data, possibly because we used commercially obtained (oo)cysts (stored in formalin and Tween-20) rather than fresh (oo)cysts isolated directly from faecal samples (stored in PBS). (Oo)cyst quality may be relevant, since the ones we used tend to cluster in the spiking suspension (and also in the matrix) (results not shown). For both parasites, previous studies have reported higher recovery rates, but the mussels were examined individually without mentioning the exact recovery rates at different concentrations of (oo) cysts (Schets et al., 2013) and the proportion of homogenate examined was also either not

reported (MacRae et al., 2005) or only a small quantity of lipid-free mussel material was examined (Gómez-Couso et al., 2006a). Thus, results from such studies are not comparable with the results of our study, where the mussels were pooled and spiked with the (oo)cysts before proceeding with the analyses, all the material from the IMS (100 µl) was examined, and the recovery efficiencies refer to (oo)cysts/g. The minimum detection limit of 10 (oo)cysts per g of homogenized mussels in our study was similar to that from another study that used sieving of digestive glands with IMS; however, in that study only 10 µl digestive gland or 200 µl gills was analysed (Miller et al., 2006). In our opinion, standardization of the quantity and type of starting sample material is an important issue, especially when such methods will be used to investigate natural infections. Examining a pool of 5 mussels, rather than one individual mussel, is advantageous, as sample size is increased.

In general, natural contamination of mussels with *Giardia* reported to date has been below that of *Cryptosporidium* (Table 1). However, cyst counts in water samples from estuaries where mussels were harvested were quite high, ranging from 0.4 to 25.41×102 *Giardia* cysts (Gómez-Couso et al., 2005a). The low levels of *Giardia* contamination of shellfish could therefore be due to lower recovery efficiency of the protocols for *Giardia* cysts than *Cryptosporidium* oocysts, as observed in the present study.

Some of the low (oo)cyst counts in naturally contaminated bivalves are below the detection limit recorded for both parasites in this study. However, as most of these studies (Gomez-Bautista et al., 2000; Gómez-Couso et al., 2005a; Graczyk et al., 2004) did not define the recovery efficiency and detection limits of their method, the actual contamination level of shellfish is likely to be much higher. Thus, the protocol described here could be useful for investigating the natural contamination of shellfish with *Giardia* and *Cryptosporidium*.

The protocol in this study has been characterized throughout each of the individual steps. A key advantage is that artificial digestion is used in the initial step, ensuring examination of the whole organism. This provides the potential for the protocol described here to be further applied in studies on other species of marine bivalves, although it would be important to bear in mind the protein: fat ratio. For example, for Pacific oysters the fat: carbohydrate: protein ratios are approximately, 1: 2: 4, for scallops the corresponding ratios are 1: 9: 30 (USDA, 2018) indicating that for these molluscs, at least, protein digestion may be an appropriate first step. However, preliminary testing would be advised before proceeding

to full-scale analysis. Indeed, the differences in tissue composition between oysters and mussels may lead to different recovery efficiencies in oysters (4.0% for *Giardia* and 5.3% for *Cryptosporidium*) than mussels (82.0% for *Giardia* and 45.0% for *Cryptosporidium*), as previously recorded (Downey and Graczyk, 2007; Schets et al., 2013).

Regarding detection, although PCR has been used (Giangaspero et al., 2014; Marangi et al., 2015; Miller et al., 2006), and may be more specific and sensitive, limitations such as inhibitors in the matrices and the quality of the DNA, may make IFAT a more appropriate technique (Hohweyer et al., 2013). PCR also has the advantage of providing information on the presence of zoonotic species (by genotyping). Division of post-IMS into two sub-samples prior to IFAT could enable both immunofluorescence and PCR to be applied, although this could be at the expense of a lower sensitivity of the method. Alternatively, for positive samples post-IFAT, the parasites on the slides could be removed for DNA isolation and PCR, as has previously been done for positive water samples (e.g., Robertson et al., 2009; Sunnotel et al., 2006). This enables information of the parasitic load and genotypes of the (oo)cysts to be obtained (Downey and Graczyk, 2007).

Furthermore, as the pepsin digestion solution reduces the viability of the (oo)cysts by only 20.0% after 1 h exposure (Robertson and Gjerde, 2008), (oo)cysts that are recovered from the matrices using the protocol characterized in our study, could be further analysed using vital dyes for viability status. Although this method tends to overestimate viability, it is still considered a technique of relevance for the assessment of the viability of *Giardia* cysts and *Cryptosporidium* oocysts in food matrices (Rousseau et al., 2018).

Table 2
Surveys with natural contamination of different species of mussels with *Giardia* cysts and *Cryptosporidium* oocysts.

Mussel species	Country	No. of *Giardia* cysts detected	No. of *Cryptosporidium* oocysts detected	Analysis approach	Reference
Mytilus galloprovincialis (Mediterranean mussel)	Spain	ND	15 x 10^3 - 100 x 10^3/mussel	homogenized whole individual mussels	Gomez-Bautista et al., 2000
Dreissena polymorpha (zebra mussel)	Ireland	5 - 9/mussel	4 - 16/mussel	homogenized, sieved whole individual mussels	Graczyk et al., 2004
		ND	1 - 12/mussel	haemolymph of individual mussels	
Mytilus galloprovincialis (Mediterranean mussel)	Spain	1 - 19 /8 mussels	ND	homogenized, sieved gills & tracts of 8 pooled mussels - diethyl ether lipid removal	Gómez-Couso et al., 2005a
Mytilus galloprovincialis (Mediterranean mussel)	Spain	32.5 - 82.5/6 - 8 mussels (average)	ND	homogenized, sieved gills & tracts of 6-8 pooled mussels - diethyl ether lipid removal	Gómez-Couso et al., 2005b
	Spain	ND	6-300/6-8 mussels		

Mytilus galloprovincialis (Mediterranean mussel)				homogenized, sieved gills & tracts of 6-8 pooled mussels - diethyl ether lipid removal	Gómez-Couso et al., 2006a
Mytilus galloprovincialis (Mediterranean mussel)	Spain	ND	25-275/6-8 mussels	homogenized, sieved gills & tracts of 6-8 pooled mussels - diethyl ether lipid removal	Gómez-Couso et al., 2006b
Mytilus galloprovincialis (Mediterranean mussel)	France	ND	0.05 - 0.9/mussel 8-148/kg of mussels	pooled gill washing, gill homogenates, flesh washing, flesh homogenates, inner-shell water - 1 kg mussels/pool - flotation & IMS	Li et al., 2006
Dreissena polymorpha (zebra mussel)		ND	440/mussel		
Mylitus edulis (blue mussel)	Ireland	6/mussel (mean)	22/mussel (mean)	homogenized sediment of whole individual mussels	Lucy et al., 2008
Dreissena polymorpha (zebra mussel)		13/mussel (mean)	ND	homogenized sediment of whole individual mussels (with shells)	
Anodonta anatina (duck mussel)		97/mussel (mean)	ND		
Mytilus edulis (blue mussel)	Norway	0.4 - 4.5 /g	0.4 - 1/g	homogenized whole individual mussels - pepsin digestion & IMS	Robertson and Gjerde, 2008
Modiolus modiolus		0.3/g	ND		

(horse mussel)					
Mytilus edulis (blue mussel)	Netherlands	1/mussel	1 - 4 /mussel	homogenized, sieved whole individual mussels - IMS	Schets et al., 2013
Mytilus edulis (blue mussel)	Norway	< 0.2 - 4/g	0.3/g	homogenized whole individual mussels - pepsin digestion & IMS	Tryland et al., 2014
Mytilus galloprovincialis (Mediterranean mussel)	Italy	4 -78/5 µl genomic DNA	10 - 64/5 µl genomic DNA	homogenized, sieved gills & digestive glands of individual mussels & haemolymph	Marangi et al., 2015*
Mytilus galloprovincialis (Mediterranean mussel)	Tunisia	62 - 395/9 - 18 mussels	ND	homogenized, sieved 9 - 18 whole pooled mussels	Ghozzi et al., 2017*
Perna perna (brown mussel)					

*All surveys used IFAT for detection and enumeration of the (oo)cysts, apart from two (marked with *) that used qPCR.*

Conclusions

The present study characterises the individual steps of a protocol for the detection of *G. duodenalis* cysts and *Cryptosporidium* spp. oocysts in mussel matrices, using a pepsin solution for digestion of the whole organism, IMS for concentration of the (oo)cysts, and IFAT for their detection and enumeration. This detailed assessment, in which each step of the procedure has been evaluated, may provide the basis for adopting a common approach and define contamination limits in future studies to generate comparable results. However, as different sampling strategies (one mussel versus pool of several mussels) and various types of starting material (whole mussels, specific organs, haemolymph) may be used, it would be of value to investigate whether these factors affect the sensitivity of this protocol.

Acknowledgements

The authors acknowledge that collaboration within the framework of the European Network for Foodborne Parasites in Europe (EUROFBP), COST Action FA1408, facilitated this research study.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest

None.

References

Adell, A.D., Smith, W.A., Shapiro, K., Melli, A., Conrad, P.A., 2014. Molecular epidemiology of *Cryptosporidium* spp. and *Giardia* spp. in mussels (*Mytilus californianus*) and California sea lions (*Zalophus californianus*) from central California. Appl. Environ. Microbiol. 80, 7732–7740. https://doi.org/10.1128/AEM.02922-14

Aksoy, U., Marangi, M., Papini, R., Ozkoc, S., Delibas, S.B., Giangaspero, A., 2014. Detection of *Toxoplasma gondii* and *Cyclospora cayetanensis* in *Mytilus galloprovincialis* from Izmir Province coast (Turkey) by Real Time PCR/High-Resolution Melting analysis (HRM). Food Microbiol. 44, 128–135. https://doi.org/10.1016/j.fm.2014.05.012

Alfano-Sobsey, E., Sweat, D., Hall, A., Breedlove, F., Rodriguez, R., Greene, S., Pierce, A., Sobsey, M., Davies, M., Ledford, S.L., 2012. Norovirus outbreak associated with undercooked oysters and secondary household transmission. Epidemiol. Infect. 140, 276–282. https://doi.org/10.1017/S0950268811000665

Broglia, A., Kapel, C., 2011. Changing dietary habits in a changing world: Emerging drivers for the transmission of foodborne parasitic zoonoses. Vet. Parasitol. 182, 2–13. https://doi.org/10.1016/j.vetpar.2011.07.011

Chalmers, R.M., Sturdee, A.P., Mellors, P., Nicholson, V., Lawlor, F., Kenny, F., Timpson, P., 1997. *Cryptosporidium parvum* in environmental samples in the Sligo area, Republic of Ireland: a preliminary report. Lett. Appl. Microbiol. 25, 380–384. https://doi.org/10.1046/j.1472-765X.1997.00248.x

Cho, H.G., Lee, S.G., Lee, M.Y., Hur, E.S., Lee, J.S., Park, P.H., Park, Y.B., Yoon, M.H., Paik, S.Y., 2016. An outbreak of norovirus infection associated with fermented oyster consumption in South Korea, 2013. Epidemiol. Infect. 144, 2759–2764. https://doi.org/10.1017/S0950268816000170

Coupe, A., Howe, L., Burrows, E., Sine, A., Pita, A., Velathanthiri, N., Vallée, E., Hayman, D., Shapiro, K., Roe, W.D., 2018. First report of *Toxoplasma gondii* sporulated oocysts and *Giardia duodenalis* in commercial green-lipped mussels (*Perna canaliculus*) in New Zealand. Parasitol. Res. 117, 1453–1463. https://doi.org/10.1007/s00436-018-5832-8

Dehnel, P.A., 1956. Growth rates in latitudinally and vertically separated populations of *Mytilus californianus*. Biol. Bull. 110, 43–53.

Dewey-Mattia, D., Kisselburgh, H., Manikonda, K., Silver, R., Subramhanya, S., Sundaraman, P., Whitham, H., Crowe, S., 2018. Centers for Disease Control and Prevention (CDC). Surveillance for Foodborne Disease Outbreaks United States, 2016: Annual Report.

Dorny, P., Praet, N., Deckers, N., Gabriel, S., 2009. Emerging food-borne parasites. Vet. Parasitol. 163, 196–206. https://doi.org/10.1016/j.vetpar.2009.05.026

Downey, A.S., Graczyk, T.K., 2007. Maximizing recovery and detection of *Cryptosporidium parvum* oocysts from spiked eastern oyster (*Crassostrea virginica*) tissue samples. Appl. Environ. Microbiol. 73, 6910–6915. https://doi.org/10.1128/AEM.01027-07

Freire-Santos, F., Oteiza-López, A.M., Vergara-Castiblanco, C.A., Ares-Mazás, E., Álvarez-Suárez, E., García-Martín, O., 2000. Detection of *Cryptosporidium* oocysts in bivalve molluscs destined for human consumption. J. Parasitol. 86, 853–854. https://doi.org/10.1645/0022-3395(2000)086[0853:DOCOIB]2.0.CO;2

Geurden, T., Claerebout, E., Vercruysse, J., Berkvens, D., 2004. Estimation of diagnostic test characteristics and prevalence of *Giardia duodenalis* in dairy calves in Belgium using a Bayesian approach. Int. J. Parasitol. 34, 1121–1127. https://doi.org/10.1016/j.ijpara.2004.05.007

Ghozzi, K., Marangi, M., Papini, R., Lahmar, I., Houas, N., Ben, R., Normanno, G., Babba, H., Giangaspero, A., 2017. First report of Tunisian coastal water contamination by protozoan parasites using mollusk bivalves as biological indicators. Mar. Pollut. Bull. 117, 197–202. https://doi.org/10.1016/j.marpolbul.2017.01.057

Giangaspero, A., Berrilli, F., Brandonisio, O., 2007. *Giardia* and *Cryptosporidium* and public health: the epidemiological scenario from the Italian perspective. Parasitol. Res. 101, 1169–1182. https://doi.org/10.1007/s00436-007-0598-4

Giangaspero, A., Papini, R., Marangi, M., Koehler, A. V., Gasser, R.B., 2014. *Cryptosporidium parvum* genotype IIa and *Giardia duodenalis* assemblage A in *Mytilus galloprovincialis* on sale at local food markets. Int. J. Food Microbiol. 171, 62–67. https://doi.org/10.1016/j.ijfoodmicro.2013.11.022

Gomez-Bautista, M., Ortega-Mora, L.M., Tabares, E., Lopez-Rodas, V., Costas, E., 2000. Detection of infectious *Cryptosporidium parvum* oocysts in mussels (*Mytilus galloprovincialis*) and cockles (*Cerastoderma edule*). Appl. Environ. Microbiol. 66, 1866–1870. https://doi.org/10.1128/AEM.66.5.1866-1870.2000

Gómez-Couso, H., Freire-Santos, F., Martínez-Urtaza, J., García-Martín, O., Ares-Mazás, M.E., 2003a. Contamination of bivalve molluscs by *Cryptosporidium* oocysts: the need for new quality control standards. Int. J. Food Microbiol. 87, 97–105. https://doi.org/10.1016/S0168-1605(03)00057-6

Gómez-Couso, H., Freire-Santos, F., Ortega-Iñarrea, M.R., Castro-Hermida, J.A., Ares-Mazás, M.E., 2003b. Environmental dispersal of *Cryptosporidium parvum* oocysts and cross transmission in cultured bivalve molluscs. Parasitol. Res. 90, 140–142. https://doi.org/10.1007/s00436-002-0748-7

Gómez-Couso, H., Freire-Santos, F., Amar, C.F.L., Grant, K.A., Williamson, K., Ares-Mazás, M.E., McLauchlin, J., 2004. Detection of *Cryptosporidium* and *Giardia* in molluscan shellfish by multiplexed nested-PCR. Int. J. Food Microbiol. 91, 279–288. https://doi.org/10.1016/j.ijfoodmicro.2003.07.003

Gómez-Couso, H., Méndez-Hermida, F., Castro-Hermida, J.A., Ares-Mazás, E., 2005a. *Giardia* in shellfish-farming areas: Detection in mussels, river water and waste waters. Vet. Parasitol. 133, 13–18. https://doi.org/10.1016/j.vetpar.2005.04.043

Gómez-Couso, H., Méndez-Hermida, F., Castro-Hermida, J.A., Ares-Mazás, E., 2005b. Occurrence of *Giardia* cysts in mussels (*Mytilus galloprovincialis*) destined for human consumption. J. Food Prot. 68, 1702–1705.

Gómez-Couso, H., Méndez-Hermida, F., Ares-Mazás, E., 2006a. Levels of detection of *Cryptosporidium* oocysts in mussels (*Mytilus galloprovincialis*) by IFA and PCR methods. Vet. Parasitol. 141, 60–65. https://doi.org/10.1016/j.vetpar.2006.04.015

Gómez-Couso, H., Méndez-Hermida, F., Castro-Hermida, J.A., Ares-Mazás, E., 2006b. *Cryptosporidium* contamination in harvesting areas of bivalve molluscs. J. Food Prot. 69, 185–190. https://doi.org/10.4315/0362-028X-69.1.185

Gotfred-Rasmussen, H., Lund, M., Enemark, H.L., Erlandsen, M., Petersen, E., 2016. Comparison of sensitivity and specificity of 4 methods for detection of *Giardia duodenalis* in feces: immunofluorescence and PCR are superior to microscopy of concentrated iodine-stained samples. Diagn. Microbiol. Infect. Dis. 84, 187–190. https://doi.org/10.1016/j.diagmicrobio.2015.11.005

Graczyk, T.K., Conn, D.B., Lucy, F., Minchin, D., Tamang, L., Moura, L.N.S., Dasilva, A.J., 2004. Human waterborne parasites in zebra mussels (*Dreissena polymorpha*) from the Shannon River drainage area, Ireland. Parasitol. Res. 93, 385–391.

https://doi.org/10.1007/s00436-004-1142-4
Hepper, B.T., 1957. Notes on *Mytilus galloprovincialis* Lamarck in Great Britain. J. Mar. Biol. Assoc. United Kingdom 36, 33–40. https://doi.org/10.1017/S0025315400017045
Hohweyer, J., Dumètre, A., Aubert, D., Azas, N., Villena, I., 2013. Tools and methods for detecting and characterizing *Giardia*, *Cryptosporidium*, and *Toxoplasma* parasites in marine mollusks. J. Food Prot. 76, 1649–1657. https://doi.org/10.4315/0362-028X.JFP-13-002
ISO, 2006. ISO 15553: Water quality – isolation and identification of *Cryptosporidium* oocysts and *Giardia* cysts from water. International Organization for Standardization, Geneva, Switzerland.
Le Mennec, C., Parnaudeau, S., Rumebe, M., Le Saux, J.-C., Piquet, J.-C., Le Guyader, S.F., 2017. Follow-up of norovirus contamination in an oyster production area linked to repeated outbreaks. Food Environ. Virol. 9, 54–61. https://doi.org/10.1007/s12560-016-9260-6
Li, X., Guyot, K., Dei-Cas, E., Mallard, J., Ballet, J.J., Brasseur, P., 2006. *Cryptosporidium* oocysts in mussels (*Mytilus edulis*) from Normandy (France). Int. J. Food Microbiol. 108, 321–325. https://doi.org/10.1016/j.ijfoodmicro.2005.11.018
Lowery, C.J., Nugent, P., Moore, J.E., Millar, B.C., Xiru, X., Dooley, J.S.G., 2001. PCR – IMS detection and molecular typing of *Cryptosporidium parvum* recovered from a recreational river source and an associated mussel (*Mytilus edulis*) bed in Northern Ireland. Epidemiol. Infect. 127, 545–553. https://doi.org/10.1017/S0950268801006276
Lucy, F.E., Graczyk, T.K., Tamang, L., Miraflor, A., Minchin, D., 2008. Biomonitoring of surface and coastal water for *Cryptosporidium*, *Giardia*, and human-virulent microsporidia using molluscan shellfish. Parasitol. Res. 103, 1369–1375. https://doi.org/10.1007/s00436-008-1143-9
Lunestad, B.T., Maage, A., Roiha, I.S., Myrmel, M., Svanevik, C.S., Duinker, A., 2016. An outbreak of norovirus infection from shellfish soup due to unforeseen insufficient heating during preparation. Food Environ. Virol. 8, 231–234. https://doi.org/10.1007/s12560-016-9245-5
MacRae, M., Hamilton, C., Strachan, N.J.C., Wright, S., Ogden, I.D., 2005. The detection of *Cryptosporidium parvum* and *Escherichia coli* O157 in UK bivalve shellfish. J. Microbiol. Methods 60, 395–401. https://doi.org/10.1016/j.mimet.2004.10.017
Marangi, M., Giangaspero, A., Lacasella, V., Lonigro, A., Gasser, R.B., 2015. Multiplex PCR for the detection and quantification of zoonotic taxa of *Giardia*, *Cryptosporidium* and *Toxoplasma* in wastewater and mussels. Mol. Cell. Probes 29, 122–125. https://doi.org/10.1016/j.mcp.2015.01.001
Miller, W.A., Gardner, I.A., Atwill, E.R., Leutenegger, C.M., Miller, M.A., Hedrick, R.P., Melli, A.C., Barnes, N.M., Conrad, P.A., 2006. Evaluation of methods for improved detection of *Cryptosporidium* spp . in mussels (*Mytilus californianus*). J. Microbiol. Methods 65, 367–379. https://doi.org/10.1016/j.mimet.2005.08.011
Pagoso, E.J.A., Rivera, W.L., 2017. *Cryptosporidium* species from common edible bivalves in Manila Bay, Philippines. Mar. Pollut. Bull. 119, 31–39. https://doi.org/10.1016/j.marpolbul.2017.03.005
Rees, E.E., Davidson, J., Fairbrother, J.M., Hilaire, S. St., Saab, M., McClure, J.T., 2015. Occurrence and antimicrobial resistance of *Escherichia coli* in oysters and mussels from Atlantic Canada. Foodborne Pathog. Dis. 12, 164–169. https://doi.org/10.1089/fpd.2014.1840
Rippey, S.R., 1994. Infectious diseases associated with molluscan shellfish consumption. Clin.

Microbiol. Rev. 7, 419–425. https://doi.org/10.1128/CMR.7.4.419
Robertson, L.J., 2007. The potential for marine bivalve shellfish to act as transmission vehicles for outbreaks of protozoan infections in humans: A review. Int. J. Food Microbiol. 120, 201–216. https://doi.org/10.1016/j.ijfoodmicro.2007.07.058
Robertson, L.J., Gjerde, B., 2008. Development and use of a pepsin digestion method for analysis of shellfish for *Cryptosporidium* oocysts and *Giardia* cysts. J. Food Prot. 71, 959–966. https://doi.org/10.4315/0362-028X-71.5.959
Robertson, L., Gjerde, B., Hansen, E.F., Stachurska-Hagen, T., 2009. A water contamination incident in Oslo, Norway during October 2007; a basis for discussion of boil-water notices and the potential for post-treatment contamination of drinking water supplies. J. Water Health 7, 55–56. https://doi.org/10.2166/wh.2009.014
Robertson, L.J., Chalmers, R.M., 2013. Foodborne cryptosporidiosis: Is there really more in Nordic countries? Trends Parasitol. 29, 3–9. https://doi.org/10.1016/j.pt.2012.10.003
Rousseau, A., La Carbona, S., Dumètre, A., Robertson, L.J., Gargala, G., Escotte-Binet, S., Favennec, L., Villena, I., Gérard, C., Aubert, D., 2018. Assessing viability and infectivity of foodborne and waterborne stages (cysts/oocysts) of *Giardia duodenalis*, *Cryptosporidium* spp., and *Toxoplasma gondii*: a review of methods. Parasite 25. https://doi.org/10.1051/parasite/2018009
Ryan, U., Hijjawi, N., Xiao, L., 2018. Foodborne cryptosporidiosis. Int. J. Parasitol. 48, 1–12. https://doi.org/10.1016/j.ijpara.2017.09.004
Santos, A.L., de Oliveira, L.T.F., Souza, A.L.A., Hauser-Davis, R.A., De Simone, S.G., 2018. *Cryptosporidium* spp. contamination in *Perna perna* mussels destined for human consumption in Southeastern Rio de Janeiro, Brazil. Bull. Environ. Contam. Toxicol. 100, 240–244. https://doi.org/10.1007/s00128-017-2223-2
Schets, F.M., van den Berg, H.H.J.L., Engels, G.B., Lodder, W.J., de Roda Husman, A.M., 2007. *Cryptosporidium* and *Giardia* in commercial and non-commercial oysters (*Crassostrea gigas*) and water from the Oosterschelde , the Netherlands. Int. J. Food Microbiol. 113, 189–194. https://doi.org/10.1016/j.ijfoodmicro.2006.06.031
Schets, F.M., van den Berg, H.H.J.L., de Roda Husman, A.M., 2013. Determination of the recovery efficiency of *Cryptosporidium* oocysts and *Giardia* cysts from seeded bivalve mollusks. J. Food Prot. 76, 93–98. https://doi.org/10.4315/0362-028X.JFP-12-326
Scorza, V., Tangtrongsup, S., 2010. Update on the diagnosis and management of *Cryptosporidium* spp infections in dogs and cats. Top. Companion Anim. Med. 25, 163–169. https://doi.org/10.1053/j.tcam.2010.07.007
Seed, R., 1968. Factors influencing shell shape in the mussel *Mytilus edulis*. J. Mar. Biol. Assoc. United Kingdom, 48, 561-584. https://doi.org/10.1017/S0025315400019159
Sokal, R.R., Rohlf, F.J., 1995. Biometry: The Principles and Practice of Statistics in Biological Research., 3rd ed. W.H. Freeman and Co., New York.
Souza, D.S.M., Dominot, A.F.Á., Moresco, V., Barardi, C.R.M., 2018. Presence of enteric viruses, bioaccumulation and stability in *Anomalocardia brasiliana* clams (Gmelin, 1791). Int. J. Food Microbiol. 266, 363–371. https://doi.org/10.1016/j.ijfoodmicro.2017.08.004
Srisuphanunt, M., Saksirisampant, W., Karanis, P., 2009. Detection of *Cryptosporidium* oocysts in Green mussels (*Perna viridis*) from shell-fish markets of Thailand. Parasite 16, 235–239. https://doi.org/http://dx.doi.org/10.1051/parasite/2009163235
Strubbia, S., Lyons, B.P., Lee, R.J., 2016. Geographical and temporal variation of *E. coli* and norovirus in mussels. Mar. Pollut. Bull. 107, 66–70. https://doi.org/10.1016/j.marpolbul.2016.04.019

Sunnotel, O., Snelling, W.J., Xiao, L., Moule, K., Moore, J.E., Millar, B.C., Dooley, J.S.G., Lowery, C.J., 2006. Rapid and sensitive detection of single *Cryptosporidium* oocysts from archived glass slides. J. Clin. Microbiol. 44, 3285–3291. https://doi.org/10.1128/JCM.00541-06

Tangtrongsup, S., Scorza, V., 2010. Update on the diagnosis and management of *Giardia* spp infections in dogs and cats. Top. Companion Anim. Med. 25, 155–162. https://doi.org/10.1053/j.tcam.2010.07.003

Tedde, T., Piras, G., Salza, S., Nives, R.M., Sanna, G., Tola, S., Culurgioni, J., Piras, C., Merella, P., Garippa, G., Virgilio, S., 2013. Investigation into *Cryptosporidium* and *Giardia* in bivalve mollusks farmed in Sardinia region and destined for human consumption. Ital. J. Food Saf. 2, 91–93. https://doi.org/10.4081/ijfs.2013.e26

Tei, F.F., Kowalyk, S., Reid, J.A., Presta, M.A., Yesudas, R., Mayer, D.C.G., 2016. Assessment and molecular characterization of human intestinal parasites in bivalves from Orchard Beach, NY, USA. Int. J. Environ. Res. Public Health 381. https://doi.org/10.3390/ijerph13040381

Tryland, I., Myrmel, M., Østensvik, Ø., Wennberg, A.C., Robertson, L.J., 2014. Impact of rainfall on the hygienic quality of blue mussels and water in urban areas in the Inner Oslofjord, Norway. Mar. Pollut. Bull. 85, 42–49. https://doi.org/10.1016/j.marpolbul.2014.06.028

USDA, 2018. National Nutrient Database for Standard Reference Release Legacy. [WWW Document]. URL https://ndb.nal.usda.gov/ndb/search (accessed 12.20.18)

USEPA 1623.1, 2012. *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA. Protocol, EPA 816-R-12-001, Office of Water, U.S Environmental Protection Agency, Washington, D.C.

Willis, J.E., McClure, J.T., Davidson, J., McClure, C., Greenwood, S.J., 2013. Global occurrence of *Cryptosporidium* and *Giardia* in shellfish: Should Canada take a closer look? Food Res. Int. 52, 119–135. https://doi.org/10.1016/j.foodres.2013.02.020

Woods, J.W., Calci, K.R., Marchant-Tambone, J.G., Burkhardt III, W., 2016. Detection and molecular characterization of norovirus from oysters implicated in outbreaks in the US. Food Microbiol. 59, 76–84. https://doi.org/10.1016/j.fm.2016.05.009

Chapter 4

Based on:

Investigations from Northern Greece on mussels cultivated in areas proximal to wastewaters discharges, as a potential source for human infection with *Giardia* and *Cryptosporidium*

P. Ligda, E. Claerebout, S. Casaert, L. J. Robertson, S. Sotiraki

In: Experimental Parasitology. 2020;210:107848.
doi: 10.1016/j.exppara.2020.107848

Introduction

In Europe, bivalve production is approximately 600 thousand tonnes per year, of which mussels are the most important (500 thousand tonnes per year). With global production of marine bivalves for human consumption estimated to be more than 15 million tonnes per year (average for 2010-2015), most of it coming from aquaculture, it is clear that bivalves (and especially mussels) are popular worldwide (FAO, 2018; FEAP, 2016).

Marine bivalves, as filter-feeder organisms, have the capacity to filter large volumes of water, between 20 and 100 L of water a day depending on species. However, as pollutants and pathogenic microorganisms may occur in the environment, these may be filtered by the bivalves during the feeding process and thus concentrated in their tissues (gills, glands, and digestive tracts). Therefore, shellfish have been frequently considered as carriers of foodborne infections to humans, as, in addition to their risk factors for contamination, they are usually consumed lightly cooked or even raw (Robertson, 2007). The most commonly identified microorganisms are viruses (e.g., norovirus) and bacteria (e.g., *Escherichia coli*) (Rees et al., 2015; Souza et al., 2018; Strubbia et al., 2016).

Giardia duodenalis and *Cryptosporidium* spp. are protozoan parasites of public health importance, with species or genotypes that may be host-specific or zoonotic, and are commonly detected in aquatic environments due to disposal of human or animal effluents, either treated or untreated, and contaminated runoffs of agricultural areas (Fayer, 2004; Kistemann et al., 2012; Slifko et al., 2000). Furthermore, the (oo)cysts of the parasites are resistant to common disinfectants (e.g., chlorine) (Korich et al., 1990; Peeters et al., 1989; Winiecka-Krusnell and Linder, 1998) and can survive for prolonged periods under different environmental conditions (Feng and Xiao, 2011; Olson et al., 1999; Peng et al., 2008). Thus, environments may be under continuous contamination pressure.

As a consequence, several surveys have reported the presence of these protozoan parasites in several food sources including mussels, raising questions on their potential role as source of infections in cases of giardiasis or cryptosporidiosis, or even foodborne outbreaks. These surveys have reported contamination of mussels from the Mediterranean (i.e., France, Italy, and Spain) with *Giardia* and/or *Cryptosporidium* (Freire-Santos et al., 2000; Giangaspero et al., 2014, 2007; Gomez-Bautista et al., 2000; Gómez-Couso et al., 2006, 2005a,

2005b, 2004, 2003a, 2003b; Li et al., 2006), as well as other European regions (Chalmers et al., 1997; Graczyk et al., 2004; Lowery et al., 2001; Robertson and Gjerde, 2008; Schets et al., 2007; Tryland et al., 2014), and also USA (Adell et al., 2014; Tei et al., 2016), Brazil (Santos et al., 2018), Canada (Willis et al., 2013), New Zealand (Coupe et al., 2018), and Asia (Pagoso and Rivera, 2017; Srisuphanunt et al., 2009).

Mussels commonly thrive in sheltered marine environments, usually near-shore, shallow, estuarine waters where there is a high concentration of nutrients. Likewise, such environments (around river estuaries, wastewater discharges etc.) also favour the presence of several pathogenic microorganisms, raising the risk for mussels' contamination (Robertson, 2007).

Wastewater treatment plants (WWTP) are reservoirs for various microbiological pollutants, including protozoan parasites, and as they are primarily designed to remove organic load, solids, nitrogen, and phosphorus, do not usually accomplish complete removal of waterborne pathogens (Ajonina et al., 2012; Cacciò et al., 2003; Hachich et al., 2013; Kokkinos et al., 2015). Indeed, *Giardia* and *Cryptosporidium* have been previously detected in wastewaters (Ajonina et al., 2013, 2012; Cacciò et al., 2003; Hachich et al., 2013; Robertson et al., 2006, 2000; Spanakos et al., 2015).

Taking all the above into consideration, the main objective of this study was to investigate contamination of mussels with *Giardia* and *Cryptosporidium* spp., when cultivated in a potentially high-risk aquatic environment (dense farming area surrounded by river estuaries and WWTP discharges). A secondary objective was to investigate the presence of *Giardia* and *Cryptosporidium* in treated wastewater (TWW) samples, identify the assemblages/species present, and compare the results with those from the mussel analyses.

Materials and Methods

In the current study, we aimed to study the occurrence, contamination intensity, and to characterize the assemblages and species (to identify relevance to the ones found in terrestrial animals and zoonotic potential) over the duration of a whole mussel-farming period (6-months, from June to November). The extent of water contamination with *Giardia*

and *Cryptosporidium* was also investigated in TWWs that are discharged next to mussel farms, during the same time period.

Study area

The study was carried out in Thermaikos Gulf, in Northern Greece (Fig. 1). Intensive mussel farming of the Mediterranean mussel, *Mytilus galloprovincialis,* is the main marine activity of the gulf. The coasts of Thermaikos Gulf host the most extensive and productive mussel aquaculture of Greece, totaling 80-90% of the whole production (Theodorou et al., 2011). There are also 3 WWTPs in the area. The biggest of these, WWTP of Sindos (I), is located in the west and treats the wastewater of almost all Thessaloniki city and the surrounding area, which is densely populated with high farming activity. The other two WWTPs, of Thermi (II) and of Aggelochori (III), are smaller and located to the east and treat wastewaters of the eastern part of the area, which has a greater tourist-focus, with many hotels located in the region. At all 3 WWTPs, the sewage undergoes a sedimenation process twice, the effluent is chlorinated, and the TWWs are discharged into Thermaikos Gulf. Four large rivers of the area (Gallikos, Axios, Loudias and Aliakmonas) also drain into the gulf. The presence of both protozoa has been previously confirmed in all rivers and livestock (all highly contaminated/infected) of the area (Ligda et al., 2017).

Fig. 1 Map of the study area. Circles with *a, b, c and d* represent the mussel farming areas. Black triangles indicate the 3 wastewater treatment plants (WWTP I, WWTP II and WWTP III).

Sample collection

Mussels

During the 6-month mussel-farming period (June to November), 4 different mussel production areas (*a*, *b*, *c*, and *d*) of Thermaikos Gulf were sampled monthly (Fig. 1). Farmed Mediterranean mussels, *M. galloprovincialis,* of the same (commercial) size were collected from 3 different mussel farms in each area (*a*, *b*, and *c*), except for one area (*d*), where samples were obtained from the single existing mussel farm. In total, 10 mussel farms were sampled every month. Mussels were collected into plastic sterile bags and transported refrigerated at 5 °C to the lab. Mussels were processed immediately on the day of sampling.

Treated wastewaters

During the same time period, TWW samples were also collected monthly from the outlet of the 3 WWTPs. 20 L of TWW were collected in plastic sterile containers, using a

portable water pump, transported to the lab immediately after collection, and filtered on the day of sampling.

Sample processing and analyses

Detection of Giardia cysts and Cryptosporidium oocysts by immunofluorescence

Mussels

From each mussel farm, 2 pools consisting of 5 mussels each, were screened for the presence of *Giardia* and *Cryptosporidium* (oo)cysts. Mussels were approximately the same size throughout the study period (approximately 1 g per mussel). Mussels were opened and the entire contents, including the animal and the intravalvular liquid, were pooled, homogenized for 2 min using a laboratory blender (8010EG Waring®, Christison Particle Technologies, Gateshead, UK) and weighed. Homogenate weight varied from 5 g to 6 g, mean 5.5 g. The homogenate was treated with pepsin digestion solution and concentrated by immunomagnetic separation (IMS) using the commercial Dynabeads™ GC-Combo (Applied Biosystems™), as previously described (Ligda et al., 2019). The resulting eluate (100 µl) was screened by a quantitative immunofluorescence assay (IFAT) for the presence of *Giardia* and *Cryptosporidium* (oo)cysts using the commercial MERIFLUOR *Cryptosporidium/Giardia* (Meridian Diagnostics Inc., Cincinnati, Ohio) kit. See section "*Detection by immunofluorescent microscopy*" for detection.

Treated wastewater

TWW samples were processed based on the U.S. Environmental Protection Agency Method 1623.1 (US EPA, 2012) and International Standard Method 15553 (ISO, 2006) for the detection of *Giardia* cysts and *Cryptosporidium* oocysts in water. After collection, TWW samples were filtered through Filta-Max® Filter Modules (IDEXX Laboratories, Inc., Westbrook, ME, USA) with the aid of a peristaltic pump with recommended flow rate of 2 L/min. The Filta-Max® Filter Modules were washed with the Filta-Max® Manual Wash Station, according to the manufacturer's instructions. The eluate was centrifuged and the volume of the sediment was measured. Between 0.5 ml and 2 ml of sediment was used for the IMS of the (oo)cysts, using antibody-coated magnetic beads specific for *Giardia* and *Cryptosporidium* (Dynabeads™ GC-

Combo, Applied Biosystems™), following the manufacturer's instructions. The resulting eluate of the IMS was equally divided in two aliquots of 50 µl each. One aliquot was stored at -20 °C until DNA extraction, whereas the 50 µl left of the eluate was dried and methanol-fixed onto well slides and stained immediately with fluorescein isothiocyanate (FITC)-conjugated anti-*Giardia* and anti-*Cryptosporidium* monoclonal antibodies (MAbs) (Aqua-Glo™ G/C Direct, Waterborne™, Inc.) and processed according to the manufacturer's instructions. See section "*Detection by immunofluorescent microscopy*" for detection.

Detection by immunofluorescent microscopy

The stained slides, either from the mussel analyses or the TWW analyses, were examined using an Olympus fluorescence microscope at 200x or 400x magnification using a FITC fluorescence filter (400 - 650 nm Chroma technology corp., Bellows Falls, USA). *Giardia* cysts and *Cryptosporidium* oocysts were identified and counted based on their size, morphology, and fluorescence. Results were expressed as (oo)cyst counts per gram or per liter for mussels or TWWs, respectively.

Method validation for wastewater analyses

For the validation of the TWW protocol, spiking experiments were performed with one TWW sample from each of the WWTPs, before starting the monthly sampling. One hundred inactivated *Giardia* cysts and 100 inactivated *Cryptosporidium* oocysts, permanently labeled with red fluorescent dye (ColorSeed™, BTF Pty Ltd., Sydney, Australia), were added to 3 TWW samples (one for each WWTP), each of 20 L. The ColorSeed™ (oo)cysts were added to the samples prior to filtration to estimate the percent recovery of the (oo)cysts, according to the manufacturer's instructions. The spiked TWW samples were processed as described above, with the exception that the slides were scanned using also a Texas Red fluorescence filter (400 - 750 nm, Chroma technology corp., Bellows Falls, USA) to distinguish ColorSeed™ (oo)cysts (which fluoresce red with the Texas Red filter) from natural (oo)cysts (which fluoresce bright green under the FITC filter). Results were expressed as recovery percentage of the (oo)cysts.

Molecular analyses

DNA was extracted from all mussel samples and non-spiked IFAT-positive TWW samples for molecular characterisation of *Giardia* and/or *Cryptosporidium* spp. Genomic DNA was extracted from the homogenized mussel samples and from the 50 µl eluate purified with IMS concentration from the (oo)cysts of TWW samples using the QIAamp Fast DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, incorporating an initial step of 5 freeze-thaw cycles (freezing in liquid nitrogen for 5 min and heating at 95 °C for 5 min) for mussel samples and 3 cycles for TWW samples, in the protocol to maximize the disruption of the (oo)cysts for extraction of DNA.

For the amplification of *Giardia*, protocols targeting the 18S ribosomal DNA (rDNA18S) gene (Hopkins et al., 1997) and the β-giardin gene (Lalle et al., 2005) were used for mussel samples, whereas the β-giardin gene, the glutamate dehydrogenase (GDH) gene (Read et al., 2004) and the triose phosphate isomerase (TPI) gene (Sulaiman et al., 2003) were used for TWW samples. All TWW samples were also analyzed with nested PCR using assemblage specific (A-F) primers targeting the TPI gene. The amplification of *G. duodenalis* assemblage A and E was according to Geurden et al. (2008); for assemblage B the protocol described by Levecke et al. (2009) was used; amplification of assemblage C was according to Levecke et al. (2011); for assemblage D the protocol of Lebbad et al. (2010) was used. For the assemblage F-specific amplification, new primers (TpiF-forw: 5´-CTG CCG TGC ACC TGT CAA CA-3´ and TpiF-rev: 5´-GCA TCT TCG ACT CTC CAA GCT CCT-3´) were designed based on the sequencing results of the general TPI PCR as described by Sulaiman et al. (2003) and on a reference sequence from the NCBI GenBank (Accession No. EU781003). The first reaction of the nested PCR was performed as described at Sulaiman et al. (2003). The mixture of the secondary reaction consisted of 0.625 U of *Taq* DNA polymerase, 5 µl of 5x PCR-buffer, 0.2 µM of each of the new designed specific primers, 0.2 µM of each dNTP, 3 mM $MgCl_2$ and 2.5 µl of a 1/10 dilution of the first PCR product in a total volume of 25 µl. PCR conditions were identical with those used at the first reaction, with the exception that the annealing temperature was 62 °C. The 18S rDNA gene (Ryan et al., 2003) and the 60-kilodalton Glycoprotein (GP60) gene (Peng et al., 2001) were amplified for characterisation of *Cryptosporidium* in mussel samples, whereas for TWW samples both the 18S rDNA and GP60 genes were targeted and also the heat-shock protein 70 (HSP-70) gene (Morgan et al., 2001). Positive controls (purified positive DNA from *Giardia* cysts (assemblages A – F) isolated from different animal faecal samples)

and a negative control (PCR water) were included as relevant in each *Giardia* PCR run, whereas for *Cryptosporidium* PCR, a positive control (purified positive DNA from *Cryptosporidium parvum* oocysts isolated from cattle) and a negative control (PCR water) were included in each *Cryptosporidium* PCR. Amplification products were visualized on 1.5% agarose gels stained with ethidium bromide.

PCR products were sent to a commercial service for purification and sequencing on both strands (Sanger sequencing). The results were assembled with Seqman 8.1 Software (Lasergene DNASTAR). Assembled sequences were aligned using the Basic Local Alignment Tool (BLAST) and compared with reference sequences using the MegAlign (Lasergene DNASTAR).

Statistical analysis

Descriptive statistical analyses were performed in order to obtain range (R), median (Mdn), mean (M), and standard deviation (SD) values for *Giardia* cyst counts and *Cryptosporidium* oocyst counts from monthly TWW samples and spiked TWW samples.

Results

Method validation for wastewater analyses

For all TWW samples, higher recoveries were obtained for *Giardia* (up to 42%) than *Cryptosporidium* (up to 26%). Recovery standard rates (initial recovery rates of 22-100% for *Giardia* and 33-100% for *Cryptosporidium*) as set by US EPA (2012), were met for *Giardia* in samples from the WWTP I and II (Table 1). In addition, initial recovery rates of 15-100% for *Giardia* and 12-100% for *Cryptosporidium* as described for Wisconsin State Laboratory for Hygiene initial recovery rates by US EPA (2014) were met for both parasites in WWTP I and II.

Table 1

Recovery rates for *Giardia* and *Cryptosporidium* (oo)cysts after spiking (one sample per wastewater treatment plant (WWTP I, WWTP II and WWTP III)).

sampling point	*Giardia* cysts (%)	*Cryptosporidium* oocysts (%)
WWTP I	34%	26%
WWTP II	42%	20%
WWTP III	12%	10%

Detection of Giardia cysts and Cryptosporidium oocysts by immunofluorescence

Mussels

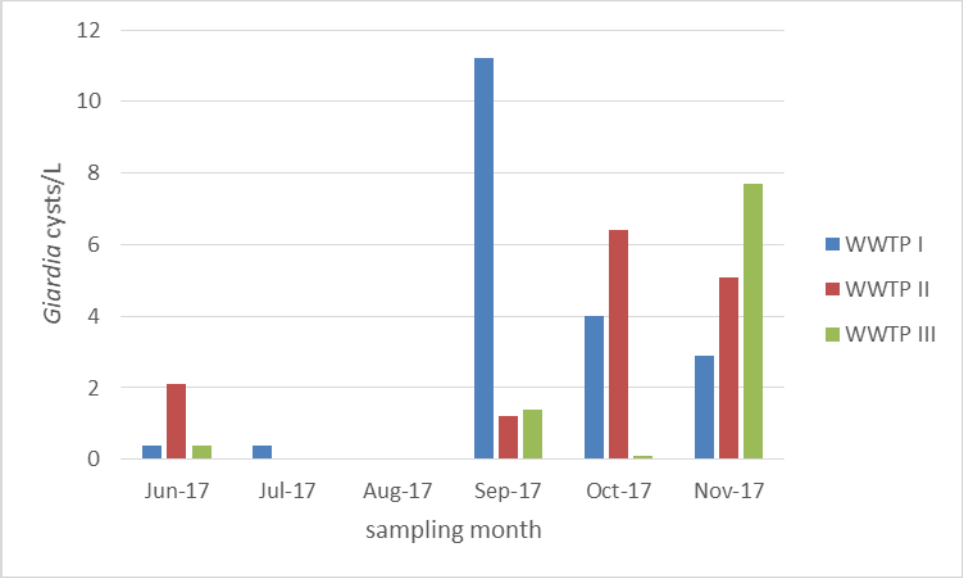
A total of 120 mussel pools were examined during the farming period, and *Giardia* cysts and *Cryptosporidium* oocysts were not recovered from any of the samples.

Treated wastewaters

A total of 18 TWW samples were examined, with *Giardia* cysts detected in 13 (72%) samples and *Cryptosporidium* oocysts in 4 (22%) samples (Fig. 2).

Among the 3 WWTPs, the most frequently contaminated was the WWTP I (83% positive samples) for *Giardia* and WWTP II (50% positive samples) for *Cryptosporidium*. Contamination with *Cryptosporidium* was not detected in WWTP I. In positive samples, *Giardia* cyst counts ranged from 0.4 to 11.2 cysts per L (Mdn: 2.1, mean: 3.3, SD: 3.4) and *Cryptosporidium* oocyst counts ranged from 0.4 to 0.9 oocysts per L (Mdn: 0.7, mean: 0.7, SD: 0.2). Similar to the prevalence rates, the highest contamination intensity for *Giardia* was recorded in WWTP I (0.4 – 11.2 *Giardia* cysts/L) and for *Cryptosporidium* in WWTP II (0.4 – 0.9 *Cryptosporidium* oocysts/L).

2a.



2b.

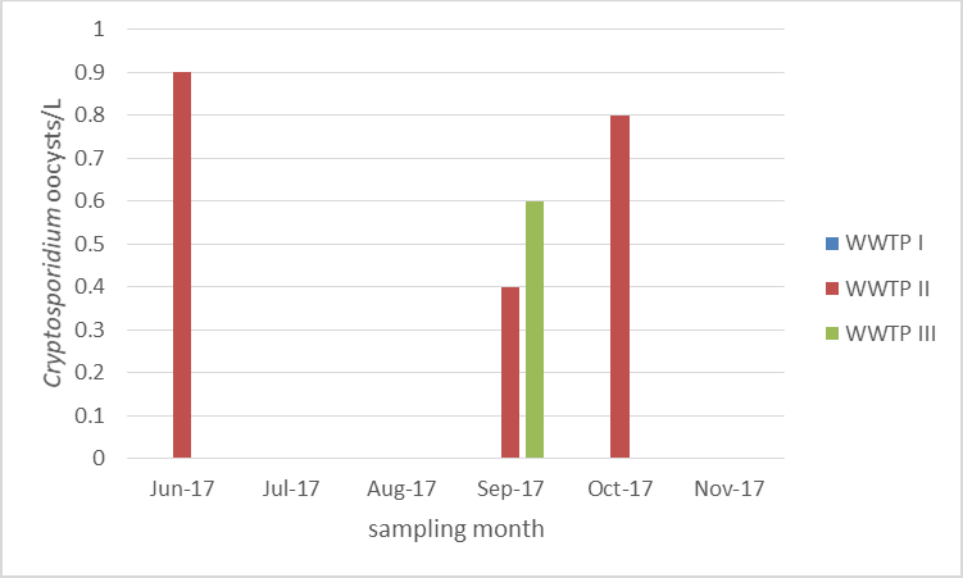


Fig. 2 *(a) Giardia* cysts/L and *(b) Cryptosporidium* oocysts/L recovered from the 3 wastewater treatment plants (WWTP I, WWTP II and WWTP III) according to sampling month.

Molecular identification of parasites

Mussels

Three out of 120 (2.5%) mussel samples, were apparently positive for *Cryptosporidium* contamination based on visualisation of PCR product of the appropriate size for the gene

GP60 (suppl. 1), however sequencing was not successful. Amplification of the 18S rDNA gene was not successful for those samples. *Giardia* contamination of mussel samples was not detected by PCR targeting any of the genes.

Treated wastewaters

Giardia sequences were obtained from 6 out of 13 (42.6%) positive TWW samples. All were found to be sub-assemblage AII for the genes β-giardin (NCBI accession numbers: MN483377-MN483378) and GDH (NCBI accession numbers: MN483373-MN483376). Out of 4 IFAT-positive TWW samples for *Cryptosporidium*, PCR products were obtained for 2 using PCR targeting the HSP-70 gene (suppl. 1). However, sequencing was not successful. Amplification of the TPI gene for *Giardia*, 18S rDNA and GP60 genes for *Cryptosporidium* was not successful.

Discussion

The hypothesis tested in the current study was that as mussels concentrate microorganisms in their tissues and may be also consumed lightly cooked or even raw, there could be a considerable risk of them being vehicles of infection for giardiasis and/or cryptosporidiosis.

The results of our study suggest that even when mussels are being farmed in a contaminated environment (rivers and wastewaters), they do not harbour high amounts of *Giardia* and/or *Cryptosporidium* (oo)cysts. Overall, the mussels tested in the current study were found negative for the presence of those protozoa by immunofluorescence, and only 3 samples (out of 120) were found positive by PCR for *Cryptosporidium*. However, even with these apparently PCR-positive samples, the lack of confirmation by sequencing and the lack of success with PCR targeting the multi-copy 18S rDNA gene means that this result should be treated with caution.

One reason for this apparent lack of contamination could be the low concentrations of parasites in the mussels, which remained undetected due to method limitations. The IFAT technique used in this study had previously been standardized showing a detection limit of 10 (oo)cysts per g of mussel homogenate. At this concentration, recovery efficiency was 30%

for *Giardia* cysts and 5% for *Cryptosporidium* oocysts (Ligda et al., 2019). Thus, should contamination be lower, then the parasites would be unlikely to be detected. Although previous reports have indicated that PCR is a more sensitive technique for detecting parasites in mussel matrices (Giangaspero et al., 2014; Marangi et al., 2015; Miller et al., 2006), limitations such as inhibitors in the matrices and the quality of the DNA, can influence the detection of the parasites (Adell et al., 2014; Hohweyer et al., 2013). We did not make any further attempts to improve the likelihood of amplification or avoiding inhibition, although approaches such as use of additional bovine serum albumin or trying different polymerases may have been relevant.

As the concentrations of those protozoa in the environment depend on various factors (e.g., health status of humans/animals, water use practices, rainfall etc.), which can vary seasonally and daily (Atherholt et al., 1998; Cizek et al., 2008; Tolouei et al., 2019; Wilkes et al., 2009), the results of our study may reflect a lower contamination pressure on the total environment during the sampling period (June-November) when total rainfall is generally low, as seen in previous studies (Adell et al., 2014). The lack of detection of any parasites in effluent during August may reflect lack of run off from agricultural land during mid-summer, but this is only speculation, and other reasons could be more important. In fact, contamination by those protozoa could be dynamic and affected by recent climatic conditions, as other studies have demonstrated that bivalves depurate the pathogens concentrated from surrounding water over the following days or weeks after ingestion (Miller et al., 2005). It should be noted that commercial mussels usually undergo a depuration process before released onto the markets, in controlled aquatic environments to purge their digestive tracts. Although this may result in loss of potentially infectious protozoa (Gómez-Bautista et al., 2000; Graczyk et al., 2006), investigations on the rate and extent of purging of protozoa have produced contrasting results (Robertson, 2007). Furthermore, such purging has the potential to enable cross-contamination between individual mussels.

Giardia and *Cryptosporidium s*pp. (oo)cyst counts recorded from all WWTPs sampled were relatively low compared with other published data where up to 20,000 parasites/L have been reported (Ajonina et al., 2013, 2012; Cacciò et al., 2003; Robertson et al., 2006; Zahedi et al., 2018), but similar to a previous study conducted in Greece (Spanakos et al., 2015). The lack of *Cryptosporidium* in any of the effluent samples from WWTP I is also surprising;

although methodological issues may be partly responsible as *Cryptosporidium* was detected from both the other WWTP effluents, it seems likely that this reflects catchment issues. Effluents of wastewaters undergo a process of chlorination, but this is unlikely to have any impact on the numbers or viability of any parasites. The absence of contaminated mussels in this study could be explained by the low concentration of (oo)cysts entering the WWTP effluents and from the rivers (Ligda et al., 2017), which were further diluted as water was discharged into the sea. Within the different WWTPs sampled, WWTP I was found to be the most contaminated with *Giardia* with high numbers of cysts recorded, which probably reflects that it is the largest of the 3 WWTPs included in the study, treating a vast amount of wastewaters, up to 150,000 m^3, compared with the other two that treat approximately up to 10,000 m^3, depending on the season. However, no *Cryptosporidium* oocysts have been recovered in WWTP I. In general, *Cryptosporidium* oocyst concentrations (up to 0.9/L) from WWTPs were lower than those of *Giardia* cysts (up to 11.2/L). This could be explained by detection limitations, as based on the spiking experiment results, the recovery rates especially for *Cryptosporidium* oocysts were relatively low (maximum of 26%). Although this is below the criteria described by US EPA (2012) for water, it should be noted that these criteria were set based on spiking of raw surface water samples. Sewage effluent samples are likely to be more complex, and these are also accepted as being likely to have lower recovery efficiencies (US EPA, 2012). With reference to US EPA (2014) for disinfected wastewater, the recovery rates at two of WWTPs were within the criteria for both parasites.

Regarding molecular identification of the *Giardia* cysts isolated, *G. duodenalis* AII was the only sub-assemblage recovered from TWW samples. *G. duodenalis* AII has been found both in humans and animals in Greece (Kostopoulou, 2018) and since it more commonly infects humans (Ryan and Cacciò, 2013), an anthropogenic contamination of wastewaters is more likely to occur.

Genetic characterisation of *Cryptosporidium* oocysts was not successful for the TWW samples in this study and this is possibly due to: a) the low numbers of oocysts recovered, due partly to methodological limitations; b) the presence of PCR inhibitors in environmental samples (Adell et al., 2014; Schrader et al., 2012); and c) the fact that the oocysts recovered could potentially lack nuclei due to age or environmental pressures. Furthermore, use of different tools, for example different primers for the 18S gene may have provided better

results. Nevertheless, the presence and circulation of zoonotic *Cryptosporidium* species in animals (Kostopoulou et al., 2017, 2015; Tzanidakis et al., 2014), surface waters (Ligda et al., 2017) and wastewaters (Spanakos et al., 2015) in Greece, highlights the risk of zoonotic infection.

Conclusions

To conclude, the results of our study suggest that under the described environmental conditions, mussels can be considered as a low-risk food source regarding potential contamination with *G. duodenalis* and *Cryptosporidium* spp. However, the risk of mussels to act as vehicles of foodborne transmission of these parasites should not be underestimated, as many factors affect the detection of the protozoa and the capacity of the (oo)cysts to cause disease to humans.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Panagiota Ligda: Conceptualization, Methodology, Investigation, Resources, Visualization, Writing - original draft, Writing - review & editing. **Edwin Claerebout**: Conceptualization, Project administration, Writing - review & editing, Supervision. **Stijn Casaert**: Resources, Data curation. **Lucy J. Robertson**: Conceptualization, Project administration, Writing - review & editing, Supervision. **Smaragda Sotiraki**: Conceptualization, Project administration, Writing - review & editing, Supervision.

Declarations of interest

None.

Acknowledgements

This article is based upon collaboration arising from COST Action FA1408 - A European Network for Foodborne Parasites in Europe (Euro-FBP), supported by COST (European Cooperation in Science and Technology; www.cost.eu). Moreover, we would like to acknowledge State Veterinary Authorities for allowing us to collect mussel samples and the personnel from each Wastewater Treatment Plants for their support.

Appendix A. Supplementary data

Picture 1. Gel electrophoresis of the *Cryptosporidium* GP60 gene for mussel samples.

Picture 2. Gel electrophoresis of the *Cryptosporidium* HSP-70 gene for wastewater samples.

References

Adell, A.D., Smith, W.A., Shapiro, K., Melli, A., Conrad, P.A., 2014. Molecular epidemiology of *Cryptosporidium* spp. and *Giardia* spp. in mussels (*Mytilus californianus*) and California sea lions (*Zalophus californianus*) from central California. Appl. Environ. Microbiol. 80, 7732–7740. https://doi.org/10.1128/AEM.02922-14

Ajonina, C., Buzie, C., Ajonina, I.U., Basner, A., Reinhardt, H., Gulyas, H., Liebau, E., Otterpohl, R., 2012. Occurrence of *Cryptosporidium* in a wastewater treatment plant in North Germany. J. Toxicol. Environ. Heal. - Part A Curr. Issues 75, 1351–1358. https://doi.org/10.1080/15287394.2012.721167

Ajonina, C., Buzie, C., Otterpohl, R., 2013. The detection of *Giardia* cysts in a large-scale wastewater treatment plant in Hamburg, Germany. J. Toxicol. Environ. Heal. - Part A Curr. Issues 76, 509–514. https://doi.org/10.1080/15287394.2013.785208

Atherholt, T.B., LeChevallier, M.W., Norton, W.D., Rosen, J.S., 1998. Effect of rainfall on *Giardia* and *Cryptosporidium*. J. / Am. Water Work. Assoc. 90, 66–80. https://doi.org/10.1002/j.1551-8833.1998.tb08499.x

Cacciò, S.M., De Giacomo, M., Aulicino, F.A., Pozio, E., 2003. *Giardia* cysts in wastewater treatment plants in Italy. Appl. Environ. Microbiol. 69, 3393–3398. https://doi.org/10.1128/AEM.69.6.3393-3398.2003

Chalmers, R.M., Sturdee, A.P., Mellors, P., Nicholson, V., Lawlor, F., Kenny, F., Timpson, P., 1997. *Cryptosporidium parvum* in environmental samples in the Sligo area, Republic of Ireland: a preliminary report. Lett. Appl. Microbiol. 25, 380–384. https://doi.org/10.1046/j.1472-765X.1997.00248.x

Cizek, A.R., Characklis, G.W., Krometis, L.-A., Hayes, J.A., Simmons, O.D., Di Lonardo, S., Alderisio, K.A., Sobsey, M.D., 2008. Comparing the partitioning behavior of *Giardia* and *Cryptosporidium* with that of indicator organisms in stormwater runoff. Water Res. 42, 4421–4438. https://doi.org/10.1016/j.watres.2008.06.020

Coupe, A., Howe, L., Burrows, E., Sine, A., Pita, A., Velathanthiri, N., Vallée, E., Hayman, D., Shapiro, K., Roe, W.D., 2018. First report of *Toxoplasma gondii* sporulated oocysts and *Giardia duodenalis* in commercial green-lipped mussels (*Perna canaliculus*) in New Zealand. Parasitol. Res. 117, 1453–1463. https://doi.org/10.1007/s00436-018-5832-8

FAO, 2018. Food and Agriculture Organization of the United Nations yearbook. Fishery and aquaculture statistics. 2016. Rome. 104pp.

Fayer, R., 2004. *Cryptosporidium*: a water-borne zoonotic parasite. Vet. Parasitol. 126, 37–56. https://doi.org/10.1016/J.VETPAR.2004.09.004

FEAP, 2016. Federation of European Aquaculture Producers. European Aquaculture Production Report 2007-2015. 46pp.

Feng, Y., Xiao, L., 2011. Zoonotic potential and molecular epidemiology of *Giardia* species and giardiasis. Clin. Microbiol. Rev. 24, 110–140. https://doi.org/10.1128/CMR.00033-10

Freire-Santos, F., Oteiza-López, A.M., Vergara-Castiblanco, C.A., Ares-Mazás, E., Álvarez-Suárez, E., García-Martín, O., 2000. Detection of *Cryptosporidium* oocysts in bivalve molluscs destined for human consumption. J. Parasitol. 86, 853–854. https://doi.org/10.1645/0022-3395(2000)086[0853:DOCOIB]2.0.CO;2

Geurden, T., Geldhof, P., Levecke, B., Martens, C., Berkvens, D., Casaert, S., Vercruysse, J., Claerebout, E., 2008. Mixed *Giardia duodenalis* assemblage A and E infections in calves. Int. J. Parasitol. 38, 259–264. https://doi.org/10.1016/j.ijpara.2007.07.016

Giangaspero, A., Berrilli, F., Brandonisio, O., 2007. *Giardia* and *Cryptosporidium* and public

health: the epidemiological scenario from the Italian perspective. Parasitol. Res. 101, 1169–1182. https://doi.org/10.1007/s00436-007-0598-4

Giangaspero, A., Papini, R., Marangi, M., Koehler, A. V., Gasser, R.B., 2014. *Cryptosporidium parvum* genotype IIa and *Giardia duodenalis* assemblage A in *Mytilus galloprovincialis* on sale at local food markets. Int. J. Food Microbiol. 171, 62–67. https://doi.org/10.1016/j.ijfoodmicro.2013.11.022

Gomez-Bautista, M., Ortega-Mora, L.M., Tabares, E., Lopez-Rodas, V., Costas, E., 2000. Detection of infectious *Cryptosporidium parvum* oocysts in mussels (*Mytilus galloprovincialis*) and cockles (*Cerastoderma edule*). Appl. Environ. Microbiol. 66, 1866–1870. https://doi.org/10.1128/AEM.66.5.1866-1870.2000

Gómez-Couso, H., Freire-santos, F., Amar, C.F.L., Grant, K.A., Williamson, K., Ares-Mazás, M.E., McLauchlin, J., 2004. Detection of *Cryptosporidium* and *Giardia* in molluscan shellfish by multiplexed nested-PCR. Int. J. Food Microbiol. 91, 279–288. https://doi.org/10.1016/j.ijfoodmicro.2003.07.003

Gómez-Couso, H., Freire-Santos, F., Martínez-Urtaza, J., García-Martín, O., Ares-Mazás, M.E., 2003a. Contamination of bivalve molluscs by *Cryptosporidium* oocysts: the need for new quality control standards. Int. J. Food Microbiol. 87, 97–105. https://doi.org/10.1016/S0168-1605(03)00057-6

Gómez-Couso, H., Freire-Santos, F., Ortega-Iñarrea, M.R., Castro-Hermida, J.A., Ares-Mazás, M.E., 2003b. Environmental dispersal of *Cryptosporidium parvum* oocysts and cross transmission in cultured bivalve molluscs. Parasitol. Res. 90, 140–142. https://doi.org/10.1007/s00436-002-0748-7

Gómez-Couso, H., Méndez-Hermida, F., Castro-Hermida, J.A., Ares-Mazás, E., 2006. *Cryptosporidium* contamination in harvesting areas of bivalve molluscs. J. Food Prot. 69, 185–190. https://doi.org/10.4315/0362-028X-69.1.185

Gómez-Couso, H., Méndez-Hermida, F., Castro-Hermida, J.A., Ares-Mazás, E., 2005a. *Giardia* in shellfish-farming areas: Detection in mussels, river water and waste waters. Vet. Parasitol. 133, 13–18. https://doi.org/10.1016/j.vetpar.2005.04.043

Gómez-Couso, H., Méndez-Hermida, F., Castro-Hermida, J.A., Ares-Mazás, E., 2005b. Occurrence of *Giardia* cysts in mussels (*Mytilus galloprovincialis*) destined for human consumption. J. Food Prot. 68, 1702–1705.

Graczyk, T.K., Girouard, A.S., Tamang, L., Nappier, S.P., Schwab, K.J., 2006. Recovery, bioaccumulation, and inactivation of human waterborne pathogens by the Chesapeake Bay nonnative oyster, *Crassostrea ariakensis*. Appl. Environ. Microbiol. 72, 3390-3395. https://doi.org/10.1128/AEM.72.5.3390–3395.2006

Graczyk, T.K., Conn, D.B., Lucy, F., Minchin, D., Tamang, L., Moura, L.N.S., Dasilva, A.J., 2004. Human waterborne parasites in zebra mussels (*Dreissena polymorpha*) from the Shannon River drainage area, Ireland. Parasitol. Res. 93, 385–391. https://doi.org/10.1007/s00436-004-1142-4

Hachich, E.M., Galvani, A.T., Padula, J.A., Stoppe, N.C., Garcia, S.C., Bonanno, V.M.S., Barbosa, M.R.F., Sato, M.I.Z., 2013. Pathogenic parasites and enteroviruses in wastewater: support for a regulation on water reuse. Water Sci. Technol. 67, 1512–1518. https://doi.org/10.2166/wst.2013.019

Hohweyer, J., Dumètre, A., Aubert, D., Azas, N., Villena, I., 2013. Tools and methods for detecting and characterizing *Giardia*, *Cryptosporidium*, and *Toxoplasma* parasites in marine mollusks. J. Food Prot. 76, 1649–1657. https://doi.org/10.4315/0362-028X.JFP-13-002

Hopkins, R.M., Meloni, B.P., Groth, D.M., Wetherall, J.D., Reynoldson, J.A., Thompson, R.C.A., 1997. Ribosomal RNA sequencing reveals differences between the genotypes of *Giardia* isolates recovered from humans and dogs living in the same locality. J. Parasitol. 83, 44–51. https://doi.org/10.2307/3284315

ISO, 2006. ISO 15553: Water quality - Isolation and identification of *Cryptosporidium* oocysts and *Giardia* cysts from water. International Organization for Standardization, Geneva, Switzerland.

Kistemann, T., Rind, E., Koch, C., Claßen, T., Lengen, C., Exner, M., Rechenburg, A., 2012. Effect of sewage treatment plants and diffuse pollution on the occurrence of protozoal parasites in the course of a small river. Int. J. Hyg. Environ. Health 215, 577–583. https://doi.org/10.1016/j.ijheh.2011.12.008

Kokkinos, P., Mandilara, G., Nikolaidou, A., Velegraki, A., Theodoratos, P., Kampa, D., Blougoura, A., Christopoulou, A., Smeti, E., Kamizoulis, G., Vantarakis, A., Mavridou, A., 2015. Performance of three small-scale wastewater treatment plants. A challenge for possible re use. Environ. Sci. Pollut. Res. 22, 17744–17752. https://doi.org/10.1007/s11356-015-4988-3

Korich, D.G., Mead, J.R., Madore, M.S., Sinclair, N.A., Sterling, C.R., 1990. Effects of ozone, chlorine dioxide, chlorine, and monochloramine on *Cryptosporidium* parvum oocyst viability. Appl. Environ. Microbiol. 56, 1423–1428.

Kostopoulou, D., 2018. Prevalence and zoonotic potential of *Cryptosporidium* spp and *Giardia duodenalis* in different host species in Greece. PhD thesis. Ghent University.

Kostopoulou, D., Casaert, S., Tzanidakis, N., van Doorn, D., Demeler, J., von Samson-Himmelstjerna, G., Saratsis, A., Voutzourakis, N., Ehsan, A., Doornaert, T., Looijen, M., De Wilde, N., Sotiraki, S., Claerebout, E., Geurden, T., 2015. The occurrence and genetic characterization of *Cryptosporidium* and *Giardia* species in foals in Belgium, The Netherlands, Germany and Greece. Vet. Parasitol. 211, 170–174. https://doi.org/10.1016/j.vetpar.2015.04.018

Kostopoulou, D., Claerebout, E., Arvanitis, D., Ligda, P., Voutzourakis, N., Casaert, S., Sotiraki, S., 2017. Abundance, zoonotic potential and risk factors of intestinal parasitism amongst dog and cat populations: The scenario of Crete, Greece. Parasit. Vectors 10. https://doi.org/10.1186/s13071-017-1989-8

Lalle, M., Pozio, E., Capelli, G., Bruschi, F., Crotti, D., Cacciò, S.M., 2005. Genetic heterogeneity at the β-giardin locus among human and animal isolates of *Giardia duodenalis* and identification of potentially zoonotic subgenotypes. Int. J. Parasitol. 35, 207–213. https://doi.org/10.1016/j.ijpara.2004.10.022

Lebbad, M., Mattsson, J.G., Christensson, B., Ljungström, B., Backhans, A., Andersson, J.O., Svärd, S.G., 2010. From mouse to moose: Multilocus genotyping of *Giardia* isolates from various animal species. Vet. Parasitol. 168, 231–239. https://doi.org/10.1016/j.vetpar.2009.11.003

Levecke, B., Geldhof, P., Claerebout, E., Dorny, P., Vercammen, F., Cacciò, S.M., Vercruysse, J., Geurden, T., 2009. Molecular characterisation of *Giardia duodenalis* in captive non-human primates reveals mixed assemblage A and B infections and novel polymorphisms. Int. J. Parasitol. 39, 1595–1601. https://doi.org/10.1016/j.ijpara.2009.05.013

Levecke, B., Meulemans, L., Dalemans, T., Casaert, S., Claerebout, E., Geurden, T., 2011. Mixed *Giardia duodenalis* assemblage A, B, C and E infections in pet chinchillas (*Chinchilla lanigera*) in Flanders (Belgium). Vet. Parasitol. 177, 166–170 . https://doi.org/10.1016/j.vetpar.2010.11.027

Li, X., Guyot, K., Dei-Cas, E., Mallard, J., Ballet, J.J., Brasseur, P., 2006. *Cryptosporidium* oocysts in mussels (*Mytilus edulis*) from Normandy (France). Int. J. Food Microbiol. 108, 321–325. https://doi.org/10.1016/j.ijfoodmicro.2005.11.018

Ligda, P., Zdragas, A., Claerebout, E., Sotiraki, S., 2017. *Cryptosporidium* and *Giardia* in different water catchments within a high dense farming area in Greece. In: Proceedings of the 26th International Conference of the World Association for the Advancement of the Veterinary Parasitology; 2017 Sep 4–8; Kuala Lumpur, Malaysia. Abstract No 4535

Ligda, P., Claerebout, E., Robertson, L.J., Sotiraki, S., 2019. Protocol standardization for the detection of *Giardia* cysts and *Cryptosporidium* oocysts in Mediterranean mussels (*Mytilus galloprovincialis*). Int. J. Food Microbiol. 298, 31–38. https://doi.org/10.1016/j.ijfoodmicro.2019.03.009

Lowery, C.J., Nugent, P., Moore, J.E., Millar, B.C., Xiru, X., Dooley, J.S.G., 2001. PCR – IMS detection and molecular typing of *Cryptosporidium parvum* recovered from a recreational river source and an associated mussel (*Mytilus edulis*) bed in Northern Ireland. Epidemiol. Infect. 127, 545–553. https://doi.org/10.1017/S0950268801006276

Marangi, M., Giangaspero, A., Lacasella, V., Lonigro, A., Gasser, R.B., 2015. Multiplex PCR for the detection and quantification of zoonotic taxa of *Giardia*, *Cryptosporidium* and *Toxoplasma* in wastewater and mussels. Mol. Cell. Probes 29, 122–125. https://doi.org/10.1016/j.mcp.2015.01.001

Miller, W.A., Atwill, E.R., Gardner, I.A., Miller, M.A., Fritz, H.M., Hedrick, R.P., Melli, A.C., Barnes, N.M., Conrad, P.A., 2005. Clams (*Corbicula fluminea*) as bioindicators of fecal contamination with *Cryptosporidium* and *Giardia* spp. in freshwater ecosystems in California. Int. J. Parasitol. 35, 673–684. https://doi.org/10.1016/j.ijpara.2005.01.002

Miller, W.A., Gardner, I.A., Atwill, E.R., Leutenegger, C.M., Miller, M.A., Hedrick, R.P., Melli, A.C., Barnes, N.M., Conrad, P.A., 2006. Evaluation of methods for improved detection of *Cryptosporidium* spp . in mussels (*Mytilus californianus*). J. Microbiol. Methods 65, 367–379. https://doi.org/10.1016/j.mimet.2005.08.011

Morgan, U.M., Monis, P.T., Xiao, L., Limor, J., Sulaiman, I., Raidal, S., O'Donoghue, P., Gasser, R., Murray, A., Fayer, R., Blagburn, B.L., Lal, A.A., Thompson, R.C.A., 2001. Molecular and phylogenetic characterisation of *Cryptosporidium* from birds. Int. J. Parasitol. 31, 289–296. https://doi.org/10.1016/s0020-7519(00)00164-8

Olson, M.E., Goh, J., Phillips, M., Guselle, N., McAllister, T.A., 1999. *Giardia* cyst and *Cryptosporidium* oocyst survival in water, soil, and cattle feces. J. Environ. Qual. 28, 1991–1996. https://doi.org/10.2134/jeq1999.00472425002800060040x

Pagoso, E.J.A., Rivera, W.L., 2017. *Cryptosporidium* species from common edible bivalves in Manila Bay, Philippines. Mar. Pollut. Bull. 119, 31–39. https://doi.org/10.1016/j.marpolbul.2017.03.005

Peeters, J.E., Mazas, E.A., Masschelein, W.J., Dematurana, I.V.M., Debacker, E., 1989. Effect of disinfection of drinking water with ozone or chlorine dioxide on survival of *Cryptosporidium parvum* oocysts. Appl. Environ. Microbiol. 55, 1519–1522.

Peng, M.M., Matos, O., Gatei, W., Das, P., Stantic-Pavlinic, M., Bern, C., Sulaiman, I.M., Glaberman, S., Lal, A.A., Xiao, L., 2001. A comparison of *Cryptosporidium* subgenotypes from several geographic regions. J. Eukaryot. Microbiol. Suppl, 28S-31S.

Peng, X., Murphy, T., Holden, N.M., 2008. Evaluation of the effect of temperature on the die-off rate for *Cryptosporidium parvum* oocysts in water, soils, and feces. Appl. Environ. Microbiol. 74, 7101–7107. https://doi.org/10.1128/AEM.01442-08

Read, C.M., Monis, P.T., Thompson, R.C.A., 2004. Discrimination of all genotypes of *Giardia*

duodenalis at the glutamate dehydrogenase locus using PCR-RFLP. Infect. Genet. Evol. 4, 125–130. https://doi.org/10.1016/j.meegid.2004.02.001

Rees, E.E., Davidson, J., Fairbrother, J.M., Hilaire, S. St., Saab, M., McClure, J.T., 2015. Occurrence and antimicrobial resistance of *Escherichia coli* in oysters and mussels from Atlantic Canada. Foodborne Pathog. Dis. 12, 164–169. https://doi.org/10.1089/fpd.2014.1840

Robertson, L.J., 2007. The potential for marine bivalve shellfish to act as transmission vehicles for outbreaks of protozoan infections in humans: A review. Int. J. Food Microbiol. 120, 201–216. https://doi.org/10.1016/j.ijfoodmicro.2007.07.058

Robertson, L.J., Gjerde, B., 2008. Development and use of a pepsin digestion method for analysis of shellfish for *Cryptosporidium* oocysts and *Giardia* cysts. J. Food Prot. 71, 959–966. https://doi.org/10.4315/0362-028X-71.5.959

Robertson, L.J., Hermansen, L., Gjerde, B.K., 2006. Occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in sewage in Norway. Appl. Environ. Microbiol. 72, 5297–5303. https://doi.org/10.1128/AEM.00464-06

Robertson, L.J., Paton, C.A., Campbell, A.T., Smith, P.G., Jackson, M.H., Gilmour, R.A., Black, S.E., Stevenson, D.A., Smith, H. V., 2000. *Giardia* cysts and *Cryptosporidium* oocysts at sewage treatment works in Scotland, UK. Water Res. 34, 2310–2322. https://doi.org/doi:10.1016/s0043-1354(99)00408-x

Ryan, U., Cacciò, S.M., 2013. Zoonotic potential of *Giardia*. Int. J. Parasitol. 43, 943–956. https://doi.org/10.1016/j.ijpara.2013.06.001

Ryan, U., Xiao, L., Read, C., Zhou, L., Lal, A.A., Pavlasek, I., 2003. Identification of novel *Cryptosporidium* genotypes from the Czech Republic. Appl. Environ. Microbiol. 69, 4302–4307. https://doi.org/10.1128/AEM.69.7.4302-4307.2003

Santos, A.L., de Oliveira, L.T.F., Souza, A.L.A., Hauser-Davis, R.A., De Simone, S.G., 2018. *Cryptosporidium* spp. contamination in *Perna perna* mussels destined for human consumption in Southeastern Rio de Janeiro, Brazil. Bull. Environ. Contam. Toxicol. 100, 240–244. https://doi.org/10.1007/s00128-017-2223-2

Schets, F.M., van den Berg, H.H.J.L., Engels, G.B., Lodder, W.J., de Roda Husman, A.M., 2007. *Cryptosporidium* and *Giardia* in commercial and non-commercial oysters (*Crassostrea gigas*) and water from the Oosterschelde , the Netherlands. Int. J. Food Microbiol. 113, 189–194. https://doi.org/10.1016/j.ijfoodmicro.2006.06.031

Schrader, C., Schielke, A., Ellerbroek, L., Johne, R., 2012. PCR inhibitors - occurrence, properties and removal. J. Appl. Microbiol. 113, 1014–1026. https://doi.org/10.1111/j.1365-2672.2012.05384.x

Slifko, T.R., Smith, H. V., Rose, J.B., 2000. Emerging parasite zoonoses associated with water and food. Int. J. Parasitol. 30, 1379–1393. https://doi.org/10.1016/S0020-7519(00)00128-4

Souza, D.S.M., Dominot, A.F.Á., Moresco, V., Barardi, C.R.M., 2018. Presence of enteric viruses, bioaccumulation and stability in *Anomalocardia brasiliana* clams (Gmelin, 1791). Int. J. Food Microbiol. 266, 363–371. https://doi.org/10.1016/j.ijfoodmicro.2017.08.004

Spanakos, G., Biba, A., Mavridou, A., Karanis, P., 2015. Occurrence of *Cryptosporidium* and *Giardia* in recycled waters used for irrigation and first description of *Cryptosporidium parvum* and *C. muris* in Greece. Parasitol. Res. 114, 1803–1810. https://doi.org/10.1007/s00436-015-4366-6

Srisuphanunt, M., Saksirisampant, W., Karanis, P., 2009. Detection of *Cryptosporidium* oocysts in Green mussels (*Perna viridis*) from shell-fish markets of Thailand. Parasite 16, 235–

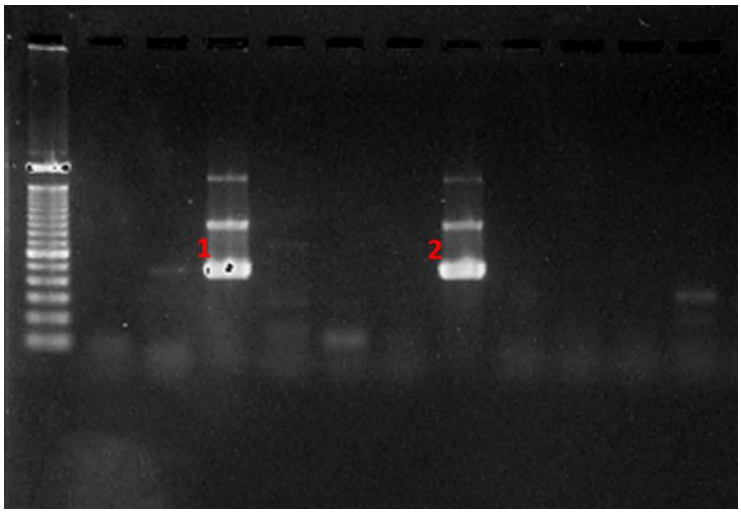
239. https://doi.org/http://dx.doi.org/10.1051/parasite/2009163235
Strubbia, S., Lyons, B.P., Lee, R.J., 2016. Geographical and temporal variation of *E. coli* and norovirus in mussels. Mar. Pollut. Bull. 107, 66–70. https://doi.org/10.1016/j.marpolbul.2016.04.019
Sulaiman, I.M., Fayer, R., Bern, C., Gilman, R.H., Trout, J.M., Schantz, P.M., Das, P., Lal, A.A., Xiao, L., 2003. Triosephosphate isomerase gene characterization and potential zoonotic transmission of *Giardia duodenalis*. Emerg. Infect. Dis. 9, 1444–1452. https://doi.org/10.3201/eid0911.030084
Tei, F.F., Kowalyk, S., Reid, J.A., Presta, M.A., Yesudas, R., Mayer, D.C.G., 2016. Assessment and molecular characterization of human intestinal parasites in bivalves from Orchard Beach, NY, USA. Int. J. Environ. Res. Public Health 381. https://doi.org/10.3390/ijerph13040381
Theodorou, J.A., Viaene, J., Sorgeloos, P., Tzovenis, I., 2011. Production and marketing trends of the cultured Mediterranean Mussel *Mytilus galloprovincialis* Lamarck 1819, in Greece. J. Shellfish Res. 30, 859–874. https://doi.org/10.2983/035.030.0327
Tolouei, S., Burnet, J.B., Autixier, L., Taghipour, M., Bonsteel, J., Duy, S.V., Sauvé, S., Prévost, M., Dorner, S., 2019. Temporal variability of parasites, bacterial indicators, and wastewater micropollutants in a water resource recovery facility under various weather conditions. Water Res. 148, 446–458. https://doi.org/10.1016/j.watres.2018.10.068
Tryland, I., Myrmel, M., Østensvik, Ø., Wennberg, A.C., Robertson, L.J., 2014. Impact of rainfall on the hygienic quality of blue mussels and water in urban areas in the Inner Oslofjord, Norway. Mar. Pollut. Bull. 85, 42–49. https://doi.org/10.1016/j.marpolbul.2014.06.028
Tzanidakis, N., Sotiraki, S., Claerebout, E., Ehsan, A., Voutzourakis, N., Kostopoulou, D., Stijn, C., Vercruysse, J., Geurden, T., 2014. Occurrence and molecular characterization of *Giardia duodenalis* and *Cryptosporidium* spp. in sheep and goats reared under dairy husbandry systems in Greece. Parasite 21. https://doi.org/10.1051/parasite/2014048
US EPA 2012. Method 1623.1: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA. U.S Environmental Protection Agency, Office of Water, Washington, D.C., USA
US EPA 2014. Method 1693: *Cryptosporidium* and *Giardia* in Disinfected Wastewater by Concentration/IMS/IFA. U.S Environmental Protection Agency, Office of Water, Washington, D.C., USA
Wilkes, G., Edge, T., Gannon, V., Jokinen, C., Lyautey, E., Medeiros, D., Neumann, N., Ruecker, N., Topp, E., Lapen, D.R., 2009. Seasonal relationships among indicator bacteria, pathogenic bacteria, *Cryptosporidium* oocysts, *Giardia* cysts, and hydrological indices for surface waters within an agricultural landscape. Water Res. 43, 2209–2223. https://doi.org/10.1016/j.watres.2009.01.033
Willis, J.E., McClure, J.T., Davidson, J., McClure, C., Greenwood, S.J., 2013. Global occurrence of *Cryptosporidium* and *Giardia* in shellfish: Should Canada take a closer look? Food Res. Int. 52, 119–135. https://doi.org/10.1016/j.foodres.2013.02.020
Winiecka-Krusnell, J., Linder, E., 1998. Cysticidal effect of chlorine dioxide on *Giardia intestinalis* cysts. Acta Trop. 70, 369–372. https://doi.org/10.1016/S0001-706X(98)00036-9
Zahedi, A., Gofton, A.W., Greay, T., Monis, P., Oskam, C., Ball, A., Bath, A., Watkinson, A., Robertson, I., Ryan, U., 2018. Profiling the diversity of *Cryptosporidium* species and genotypes in wastewater treatment plants in Australia using next generation sequencing. Sci. Total Environ. 644, 635–648. https://doi.org/10.1016/j.scitotenv.2018.07.024

Supplementary data

Picture 1

Gel electrophoresis of the *Cryptosporidium* GP60 gene for mussel samples.

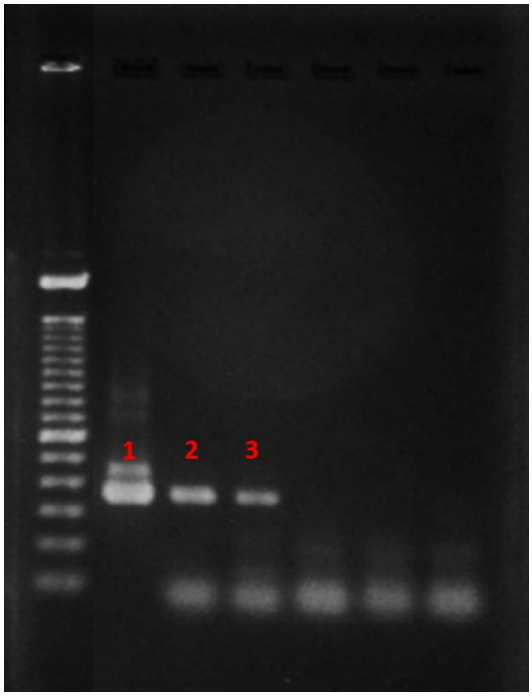
1 and 2: gel bands of the appropriate size (~450bp) of 2 positive mussel samples.



Picture 2

Gel electrophoresis of the Cryptosporidium HSP-70 gene for wastewater samples.

1: gel band of the positive control sample (product size: 325 bp); 2 and 3: gel bands of the 2 positive wastewater samples.



Chapter 5

General discussion

Introduction

Cryptosporidium and *Giardia* are of great concern, due to their impact on human and animal health. For this reason, research has focused on exploring their transmission routes and zoonotic importance, however there are several knowledge gaps still remaining to be filled, especially as concerns the zoonotic importance of *Giardia* (Cacciò et al., 2018; Ryan and Cacciò, 2013; Thompson and Ash, 2019).

The aim of the current thesis was to investigate waterborne and foodborne transmission of *Cryptosporidium* and *Giardia* in Greece, and more precisely to identify sources of water contamination, estimate the public health risk associated with contamination of water and food (mussels) and to improve the detection methods for *Cryptosporidium* and *Giardia* in water and mussels.

The Northern part of Greece, and specifically Thessaloniki greater area, was considered to be an ideal scenario to study the above, due to its geographical and environmental characteristics, such as the presence of multiple water sources, some of them originating from neighbouring countries (*i.e*. Axios river), in combination with the intense agriculture activity and the presence of wastewater treatment plants in the area, which allowed us to explore the impact of different factors to water contamination. Moreover, the use of surface waters for the production of drinking water, as well as for irrigation of the crops, and the presence of mussel production in the gulf that is fed by these surface waters, would allow us to investigate transmission cycles of the protozoa and identify potential public health risks.

Cryptosporidium and *Giardia* in water

Our results revealed the presence of both *Cryptosporidium* and *Giardia* in different water matrices in Greece, including treated wastewaters, surface water (rivers), irrigation water and purified drinking water (Chapters 2 and 4).

In general, relatively high levels of both protozoa were recorded in rivers, in accordance with data on other European rivers (Ongerth et al., 2018), following a seasonal pattern of contamination during the 2 years of monthly samplings, also observed in similar studies (Ajonina et al., 2013, 2012; Ehsan et al., 2015; Helmi et al., 2011; Isaac-Renton et al., 1996;

Keeley and Faulkner, 2008; Van Dyke et al., 2012). High precipitation levels and low temperatures during winter seemed to be associated with increased parasitic load in surface waters, as contamination peaks were recorded in those months. This may be associated with surface runoffs during rainfall events, from the surrounding agricultural area where the same parasitic species/genotypes were identified in farm animals (cattle and sheep). In fact, the two mostly contaminated rivers (Axios and Loudias) flow through a dense farming area.

As transmission cycles of *Cryptosporidium* and *Giardia* are particularly sensitive to environmental changes, it is expected that their epidemiology may be affected in the future by the global climatic change (Lal et al., 2013). Current climate change predictions for the area forecast periods of consecutive days of dryness, followed by extreme rainfall events (Forzieri et al., 2017; Panagos et al., 2017). Survival of both parasites is affected by temperature and solar radiation, with high temperatures forcing (oo)cyst degradation (DeRegnier et al., 1989; Feng and Xiao, 2011; King and Monis, 2007; Olson et al., 1999; Peng et al., 2008). However, droughts can lead to greater effluent parasite concentration in surface and groundwater sources, due to the accumulation of faecal material to the streams during such periods (Lal et al., 2013). Subsequent periods of heavy rainfall can result in increasing runoffs from these areas, together with resuspension of river bottom and storm drain sediment, causing resuspension of infectious (oo)cysts, contaminating drinking water pipelines, thus potentially resulting in disease outbreaks (Lal et al., 2013; Semenza, 2020; Semenza et al., 2012). However, we should acknowledge the uncertainty of climatic change predictions and the fact that it is a phenomenon that will affect multiple processes associated with (oo)cyst release, survival and transport, potentially affecting the parasites differentially with regional variation (Lal et al., 2013; Robertson and Gjerde, 2007).

In an attempt to investigate possible contamination sources of surface water, different sampling points of Axios river, one of the most frequently contaminated rivers, were tested, *i.e.* one sampling point upstream of the biggest wastewater treatment plant of the area and another sampling point downstream of the wastewater treatment plant. However, contamination levels were similar between these two sampling points, also without any difference among the species/genotypes isolated, indicating that wastewaters do not seem to significantly contribute to water contamination in Axios river. In fact, although treated wastewaters sampled were found contaminated with both parasites, contamination levels

were quite low (up to 0.9 *Cryptosporidium* oocysts/L and up to 11.2 *Giardia* cysts/L) compared to published records from several other locations (up to 20,000 (oo)cysts/L) (Ajonina et al., 2013, 2012; Cacciò et al., 2003; Robertson et al., 2006; Zahedi et al., 2018). On the other hand, Axios river that springs in and runs through North Macedonia, was already highly contaminated with the same species/genotypes when entering Greece. Even though sampling at the entrance point of the river was only possible for a limited period of time (4 months), the high (oo)cyst numbers, especially for *Giardia,* indicate the existence of sources of contamination across the border.

Our data combined with previously reported ones (Kostopoulou et al., 2020), revealed the circulation of the same, potentially zoonotic, species/genotypes in water, animals and humans. However, because it was not possible to perform multilocus genotyping analyses, zoonotic transmission of *Cryptosporidium* and *Giardia* could not be demonstrated.

The contamination of filtered and purified drinking water, even at relatively low levels (<1(oo)cysts/L) is of great concern for public health, especially since viable (oo)cysts of potentially zoonotic species/genotypes of *Cryptosporidium* and *Giardia* were isolated from surface water and raw intake water of the water company. Having also in mind that the source for the production of drinking water, Aliakmonas river, had quite low contamination levels, especially for *Cryptosporidium* (<0.3 oocysts/L), the risk for consumers may be even higher during an unexpected event of increased water contamination, *e.g.* after extreme rainfall. Overall, the purification system of the water company seems to be inadequate, as high turbidity values were also recorded occasionally during our study. Having all the above in mind, it is clear that monitoring of these parasites is an important aspect that needs to be seriously considered by the state authorities. Ideally, the filtration system should be also upgraded, for instance by adding UV filters. However, as such approaches are quite costly, a feasible approach would be to design a strategic sampling plan based on our findings (*e.g.* testing after extreme rainfall events, or regular testing during winter months). Future approaches based on more sophisticated data analyses, as we explain below, may further support strategic sampling systems providing more accurate proxies for *Cryptosporidium* and *Giardia*, in order to avoid disease outbreaks.

***Cryptosporidium* and *Giardia* in mussels**

To explore the possibility of contaminated food contributing to human infections with *Cryptosporidium* and/or *Giardia*, Mediterranean mussels *Mylitus galloprovincialis* were our target food product, as they are cultivated in high density in the Thermaikos gulf, around the estuaries of the contaminated rivers and proximal to discharges of contaminated wastewaters of human, animal and industrial origin. Therefore, they were considered to be a potentially contaminated food matrix, taking also into account their ability to filter large volumes of water and accumulate micropollutants in their tissues (Robertson, 2007). Even though mussel farming in Greece is quite widespread, studies about the possible role of mussels to act as carriers of parasitic infections have never been conducted before.

Challenges at this part of our study were obvious from the very beginning, as there is no standard validated method to detect these protozoa in shellfish and many different approaches have been used by different research groups. Even though immunofluorescence is most commonly preferred over PCR (Hohweyer et al., 2013), different analytical procedures have been used, without validated sensitivity and defined detection limits.

Method validation

In order to have a reference technique to investigate mussel contamination with *Cryptosporidium* and *Giardia* at Thermaikos gulf, we set up two different spiking experiments with known concentrations of the protozoa using commercial mussels (*M. galloprovincialis)* (Chapter 3). With the 1st experiment we proved that the most sensitive protocol (61.4% for *Cryptosporidium* and 32.1% for *Giardia* for 1000 (oo)cysts/g) among those commonly used is the pre-treatment of mussel homogenate (consisting of 5 whole pooled mussels) with pepsin digestion solution, followed by concentration of the (oo)cysts with immunomagnetic separation (IMS) and detection with a direct immunofluorescence assay. In the 2nd experiment the detection limit of this method was defined to be 10 (oo)cysts per g of mussel homogenate, although with low recovery efficiencies, especially for *Cryptosporidium* (5%). Recovery efficiencies for 1000 (oo)cysts/g of mussel homogenate were similar in both experiments, although *Cryptosporidium* recovery was a bit lower in the 2nd experiment (45.7% versus 61.4% in the 1st experiment), while for *Giardia* it was higher (42.8% versus 32.1% in the 1st experiment). Even though these recovery rates were considered acceptable, they were

lower than the ones achieved by Robertson and Gjerde (2008), where the same procedure was used.

A similar effort for identification of the most appropriate method for detection of *Cryptosporidium* oocysts in mussels (*M. edulis*) was published at the same time as our study (Kaupke et al., 2019). The effect of IMS concentration was only tested in mussels digested with pepsin solution and sieving was only tested in combination with IMS, while other lipid extraction methods, such as the use of diethyl ether, were not investigated. Interestingly, Kaupke et al. (2019) proved that sieving and use of IMS had the best recovery efficiencies (up to 100%) compared to the other 2 methods. Also with the combination of pepsin digestion and IMS, which was the most efficient method in Chapter 3, Kaupke et al. (2019) reported higher recovery efficiencies (up to 94% for 1000 oocysts) than we did. One possible limitation of our study that may have resulted in lower recoveries, could be the fact that we did not verify the homogeneity of (oo)cyst suspensions, as performed by Kaupke et al. (2019). In fact, while we tested the suspension samples, (oo)cysts tended to form clusters and this probably affected the equal distribution in the matrixes, resulting in lower recovery rates in our study.

There are many factors which may affect (oo)cyst recovery rates between different studies. Different studies may differ in i) the nature of spiking material (*e.g.,* freshly collected from faecal samples in Robertson and Gjerde (2008) versus commercially purchased in Kaupke et al. (2019) and in our study), ii) species of shellfish (*e.g. M. edulis* in Kaupke et al., 2019 vs. *M. galloprovincialis* in our study), iii) type of starting material (i.e. mussels examined individually or in pools) and iv) the amount of homogenate examined (*e.g.* only a small amount of lipid-free spiked homogenate, all the amount of the spiked homogenate or amount not specified) (Gómez-Couso et al., 2006a; MacRae et al., 2005; Schets et al., 2013).

The minimum detection limit of 10 (oo)cysts per g of mussel homogenate in our study was in accordance with other published studies (Kaupke et al., 2019; Miller et al., 2006), but this detection limit is higher than (o)cyst concentrations recorded in naturally contaminated shellfish (Gómez-Couso et al., 2006b, 2005; Graczyk et al., 2004; Li et al., 2006).

Risk for foodborne infection

Having a standardized technique, we proceeded with our aim to investigate the possibility of mussels farmed in close proximity to contaminated water sources to act as a possible source for human infections with *Cryptosporidium* and *Giardia* (Chapter 4).

All mussels (a total of 120 pools), corresponding to 10 mussel farms, tested negative for the presence of both protozoa with immunofluorescence. We had anticipated that the Thermaikos gulf would be a heavily contaminated environment, because all rivers and outlets from wastewater treatment plants feeding into the gulf were contaminated with both parasites (Chapters 2 and 4), and especially the rivers had high (oo)cyst concentrations (up to 13.5 *Cryptosporidium* oocysts and up to 48.6 *Giardia* cysts/L). Absence of contamination in the mussels could be explained by the fact that the contaminated water is diluted when entering into the sea. Thus, the (oo)cyst numbers that accumulated in the mussels may have been too low to be detected. In a similar study conducted in a geographically closed environment of Southern Italy, Mediterranean mussels were negative for *Cryptosporidium* and *Giardia* with both immunofluorescence and molecular testing, despite high contamination levels of both parasites in inflowing waters (Giangaspero et al., 2009). On the other hand, other studies reported contaminated mussels (up to 275 *Cryptosporidium* oocysts and up to 19 *Giardia* cysts per 6-8 mussels) farmed in a contaminated environment (treated sewage effluents with up to 520 *Cryptosporidium* oocysts/L and up to 1131 *Giardia* cysts/L) (Gómez-Couso et al., 2006b, 2005). Therefore, a low parasitic load in combination with the low sensitivity of the immunofluorescence protocol (5% for *Cryptosporidium* and 30% for *Giardia* for the detection limit of 10 (oo)cysts per g of mussel homogenate), could be an explanation for the lack of detection of these protozoa in the farmed mussels examined.

On the other hand, the capacity of mussels to expel those protozoa from their system in combination to low parasitic loads in mussels, as well as the effect of the salinity of the sea water on (oo)cysts could be responsible for the absence of contaminated mussels. However, studies have been demonstrated that *Cryptosporidium* (oo)cysts can survive long enough (up to 4 weeks) in different concentrations of salinity (up to 30 ppt) (Fayer et al., 1998) and for at least 1 year in artificial seawater (Tamburrini and Pozio, 1999), thus suggesting that sea water does not affect their infectivity. On the contrary, some others suggested that there might be a synergistic effect of salinity with the time that *Cryptosporidium* oocysts are present in such environments, affecting the infection intensity of the oocysts (Freire-Santos et al., 1999).

Moreover, trials conducted with oysters in static tank aquaria demonstrated that oysters held under high salinities (up to 29 ppt) were capable of gradually eliminate (oo)cysts over time, although chronic exposure to (oo)cysts may inhibit this ability (Willis et al., 2015, 2014). Interestingly, studies with *M. galloprovincialis* mussels proved that *Cryptosporidium* oocysts can remain in the intestinal tracts for up to 14 days and more importantly still retaining their infectivity.

As the sensitivity of our protocol was low at the detection limit of 10 (oo)cysts per gram, we further tested all the pooled mussel homogenates with molecular analyses targeting commonly used markers. Interestingly, we found *Cryptosporidium* DNA in 3 mussel samples from the eastern part of Thermaikos gulf, where the wastewater treatment plant with the highest parasitic load and largest volume of wastewater is located. These results suggest an effect of the surrounding environment to the potential of mussels to acquire contamination. However, this hypothesis needs to be further investigated, due to absence of positive results at other multi-copy loci (*i.e.* 18S rRNA gene) and sequencing data.

Overall it seems that mussels are of low risk to act as vehicles of foodborne transmission of these protozoa in the Thermaikos Gulf. Moreover, as commercial mussels commonly undergo a depuration process in controlled aquatic environments to purge their digestive tracts, the risk of infection due to consumption of mussels may be even lower. On the other hand, a possible increase of the contamination levels in the gulf in the future (*i.e.* due to surface runoffs caused by extreme rainfall events; Panagos et al., 2017), could result in an increase of the (oo)cyst levels accumulated by the mussels. Therefore, the risk of mussels to act as vehicles of foodborne transmission of *Cryptosporidium* and *Giardia* should not be neglected, as also a couple of small giardiasis outbreaks due to consumption of contaminated oysters have been reported in the past (Ryan et al., 2019; Smith-DeWaal et al., 2001).

Alternative markers for prediction of water contamination with *Cryptosporidium* and *Giardia*

Trying to achieve our last aim to propose alternative markers which could predict contamination of water with *Cryptosporidium* and *Giardia,* in order to improve their routine detection (Chapter 2), our first approach was not satisfactory. Our results, based on

commonly used statistics, were in agreement with previously published studies (Briancesco and Bonadonna, 2005; Ehsan et al., 2015; Masina et al., 2019; Xiao et al., 2018). Faecal indicator bacteria (FIB) (*i.e. Escherichia coli*, coliforms, *Clostridium perfringens* and enterococci) were not correlated with the presence of any of the two protozoa. Only *Cryptosporidium* was statistically correlated with some physicochemical (*i.e.* pH and EC) and meteorological (*i.e.* rainfall and temperature) factors, however without any strong evidence of a direct relationship that could be used as an alternative indicator of water contamination.

Thus, a more advanced approach, in terms of statistics, was taken in order to find reliable and useful alternative markers to predict (oo)cyst contamination of water matrices. Machine-learning modelling proved to be a promising tool, as it takes into account complex interactions and combines different parameters (in our case microbiological, physicochemical and meteorological parameters), to tackle the problem of the lack of reliability of a single parameter, as explained in Chapter 2. Based on our model, contamination intensity with *Cryptosporidium* could be predicted using a combination of different microbiological (*i.e*. *Clostridia*/100 ml, colony count at 22 °C) and meteorological (*i.e*. mean temperature of the relevant month, total rainfall of week) parameters, whereas for *Giardia* a combination of microbiological (*i.e*. coliforms/100 ml, *E. coli*) and physicochemical (*i.e*. turbidity) parameters proved efficient. Bearing in mind that our model was based on data that included several zero counts, the accuracy values achieved for both parasites (75% for *Cryptosporidium* and 69% for *Giardia*) are quite high and satisfactory. However, some of the markers included in the model are quite time consuming, such as *Clostridium* counts and colony counts at 22 °C. Therefore, additional markers (*e.g.* various potential micropollutants, such as caffeine and carbamazepine) that are cheap and timesaving at the same time, should also be tested for their reliability and efficiency in such models.

Conclusions

Based on our results and the data already published we conclude that the same potentially zoonotic *Cryptosporidium* species and *G. duodenalis* (sub)assemblages have been identified from different host species (humans and animals) and water sources (wastewater, raw surface water, irrigation water, filtered and treated drinking water) in Greece. However, due to the fact that multilocus genotyping was not possible and no outbreaks and only scarce

clinical cases were reported (according to official reported data, https://eody.gov.gr), we can only conclude that zoonotic species are circulating in the area, but we have no proof of zoonotic transmission and we cannot estimate their public health importance. Since our findings for surface waters are in accordance with other European studies suggesting that levels of circulating parasites are similar (Ongerth et al., 2018), the absence of reported outbreaks/cases in Greece could be due to lack of awareness and/or underreporting since those protozoa are not included in the differential diagnosis of cases with similar clinical symptoms. Animals were considered to be one of the sources of water contamination through run-off from the surrounding farms, whereas wastewater treatment plants seemed not to contribute significantly to water contamination. However, as the presence of these protozoa in humans in this area has been previously reported (Kostopoulou et al., 2020), other human point sources of contamination could have been overlooked. Finally, mussels farmed in the gulf where all these contaminated waters are discharged, seem to be a low risk food source for human infections with *Cryptosporidium* spp. and *G. duodenalis*.

Opportunities for future studies

In our opinion there are several research opportunities worth to be further pursued, such as the improvement of detection techniques and the identification of sources of human infection and risk assessment.

Improvement of detection techniques

Tracing back to the technical parts of our study and especially to the low recovery rates of *Cryptosporidium* and *Giardia* (oo)cysts, especially in treated sewage samples, future research should focus on the improvement of this part. Further improvement of the US EPA Method 1623.1 (US EPA, 2012), with additional steps for the removal of the algae that are present in water matrices, could be beneficial. Especially for the analysis of treated wastewaters, we followed the same protocol as with the other water matrices and this may have resulted in lower recoveries for these samples. Although the recoveries we achieved were within the acceptance criteria for disinfected treated wastewaters as proposed by the US EPA Method 1693 (US EPA, 2014), the use of sodium hexametaphosphate (NaHMP) prior to elution of the (oo)cysts from the filters as proposed by the Method 1693, could have decreased the impact

of the turbidity, thus increasing the recovery rates of the (oo)cysts. In fact the method for disinfected treated water follows the same procedure as the US EPA Method 1623.1 (US EPA, 2012) with some modifications, *i.e.*, different volume of water, concentration either by using filters or directly by centrifugation, and use of chemical compounds such as NaHMP or kaolin to reduce the effect of turbidity and fats that are present in such matrices (US EPA, 2014). Such modifications could be advantageous also for other types of waters, such as raw river samples where turbidity values are sometimes quite high.

However, as detection and quantification of these protozoa in water matrices are quite difficult and time-consuming, alternative methods may be worth to investigate. The machine-learning approach that can predict contamination intensity (Chapter 2), could be the basis for such studies, as it seemed to be a promising tool. However, it would be of value to test this model in other environments as well, and more importantly to explore the possible correlation with other biotic and abiotic parameters (*e.g.* chemical and biochemical compounds, environmental and pedoclimatic factors) that are more easily and time effective to be measured. Testing such models in different scenarios, e.g. other areas of Greece (*i.e.* Southern Greece where the climate is drier) or neighboring countries and specifically countries sharing common water bodies, such as Greece and North Macedonia, would be highly important in order to test the efficacy of such models to predict water contamination. Such approaches should ideally result in reliable and cost-effective strategic plans that could be used for surveillance and health risk management, in order to predict and minimize the risk for waterborne disease outbreaks.

As concerns the detection of *Cryptosporidium* and *Giardia* in mussels, we should try to further improve our developed mussel analyses protocol, in order to achieve higher recovery efficiencies. Moreover, it is crucial to perform interlaboratory testing of this technique, in order to confirm its reproducibility and efficiency to detect these protozoa. This then can become the basis for shellfish analyses to detect *Cryptosporidium* and *Giardia* (oo)cysts, and test its sensitivity using only specific tissues of shellfish, or different species of marine bivalves which may have different tissue composition in fat and proteins.

In order to better evaluate the public health risk, the zoonotic or anthroponotic nature of these protozoa needs to be clarified, with the use of more advanced molecular techniques. Ideally whole genome sequencing approaches (Ankarklev et al., 2018; Brynildsrud et al., 2018;

Durigan et al., 2018; Lecová et al., 2019) or the use of alternative markers, like variable number tandem repeats (VNTR) (Chalmers et al., 2017; Pérez-Cordón et al., 2016), should be included in epidemiological studies. On the other hand, in both environmental and food samples there are many PCR inhibitors that make the detection of *Cryptosporidium* and *Giardia* difficult. Thus, for routine analyses, apart from the adjustment of the PCR conditions (Robertson, 2014, 2013), other PCR-based methods that are not sensitive to inhibitors, such as the droplet digital PCR (ddPCR) or the loop mediated isothermal amplification (LAMP) could be more appropriate (Gallas-Lindemann et al., 2016; Yang et al., 2014). Moreover, the viability of the (oo)cysts detected in these matrices is crucial for risk assessment. However, for food samples there are no available appropriate protocols that can determine the viability of the (oo)cysts. Thus studies investigating appropriate protocols, that do not over- or underestimate the viability (*e.g.* combinations of commonly used methods based on DNA/RNA and vital dye assays) of the (oo)cysts detected in food matrixes are needed (Rousseau et al., 2018).

Identification of sources of human infection and risk assessment

Contaminated water matrices with *Cryptosporidium* and *G. duodenalis* have been recorded in a wide area of Northern Greece, where the second most populated (>1,100,000 inhabitants) city of Greece, Thessaloniki is located. Thessaloniki also attracts large numbers of tourists every year (>2.1 million international arrivals in 2018; https://www.statista.com/statistics/939435/international-air-arrivals-thessaloniki/). Having in mind that possible zoonotic species/genotypes of both protozoa are circulating in the environment, including in the river that is used for production of drinking water, as well as in the final drinking water, it would be of value to perform a Quantitative Microbial Risk Assessment (QMRA), in order to estimate the risk for consumers to get infected. Data of the current study regarding concentrations of *Cryptosporidium* and *Giardia* in surface waters could be used in combination with water consumption data and infection rates of the protozoa. Moreover, the presence of both parasites should also be investigated in recreational facilities, such as the swimming pools of the area and the coastal water at the beaches. In fact, swimming pools may be a hot-topic for Greece that really needs to be investigated, since most of the hotels have large, often several, swimming pools (some of which are improperly maintained) that are commonly over-crowed during the high touristic

summer season. Such studies could also serve as the beginning for the establishment of preventive measures and good-health practices that should be followed in recreational facilities. Ideally, at the same time period, it could be advantageous to include also humans in the study and to try to correlate their parasite infection status with the (oo)cyst counts recorded at the water matrixes during the same period.

In order to have a more holistic approach regarding the risk for consumers to get infected and in an effort to gain more knowledge regarding the foodborne transmission of these parasites, apart from the mussels that we have investigated, other products cultivated in the area could be investigated. Although in this area mainly cotton and rice are cultivated, corn and different vegetables can be also found. Vegetables could be of great interest, as it is known that they can easily get contaminated from irrigation to packaging or transport, and are often consumed raw, increasing the risk to act as vehicles of foodborne infections (Ryan et al., 2019, 2018). Also the sticky nature of the (oo)cysts in combination with the stomata on the surface of the green leaves, increase the possibility of contamination of these produce (Hohweyer et al., 2016; Macarisin et al., 2010). In fact, several outbreaks of cryptosporidiosis and giardiasis due to the consumption of raw vegetables have been reported in other areas (Åberg et al., 2015; Gherasim et al., 2012; Insulander et al., 2013, 2008; McKerr et al., 2015; Mintz et al., 1993; Rimšelienė et al., 2011; Robertson, 2013; Rose and Slifko, 1999; Ryan et al., 2018, 2019).

Our attempt to identify possible sources of water contamination could be expanded to the wildlife species inhabiting the area, as from many of them zoonotic (*i.e*. *C. parvum, G. duodenalis* assemblages A and B) and human specific (*i.e. C. hominis*) species of both parasites have been isolated (Feng and Xiao, 2011; Li et al., 2017; Robertson et al., 2019; Ryan and Zahedi, 2019; Zahedi et al., 2016). In fact, the area where the four rivers form a large deltaic complex is of great ecological importance and has been included in the Natura 2000 network of European ecological regions. The area is an ideal biotope for many species of wild mammals (*i.e.* ground squirrels, rodents, foxes, wolfs, wildcats), reptiles (*i.e.* Mediterranean tortoise) and birds (*i.e.* pygmy cormorant, Dalmatian pelican, black-headed seagull). The evaluation of the parasitism in these species may provide us more information regarding the transmission scenarios.

References

Åberg, R., Sjöman, M., Hemminki, K., Pirnes, A., Räsänen, S., Kalanti, A., Pohjanvirta, T., Cacciò, S.M., Pihlajasaari, A., Toikkanen, S., Huusko, S., Rimhanen-Finne, R., 2015. *Cryptosporidium parvum* caused a large outbreak linked to frisée salad in Finland, 2012. Zoonoses Public Health 62, 618–624. https://doi.org/10.1111/zph.12190

Ajonina, C., Buzie, C., Ajonina, I.U., Basner, A., Reinhardt, H., Gulyas, H., Liebau, E., Otterpohl, R., 2012. Occurrence of *Cryptosporidium* in a wastewater treatment plant in North Germany. J. Toxicol. Environ. Heal. - Part A Curr. Issues 75, 1351–1358. https://doi.org/10.1080/15287394.2012.721167

Ajonina, C., Buzie, C., Otterpohl, R., 2013. The detection of *Giardia* cysts in a large-scale wastewater treatment plant in Hamburg, Germany. J. Toxicol. Environ. Heal. - Part A Curr. Issues 76, 509–514. https://doi.org/10.1080/15287394.2013.785208

Ankarklev, J., Lebbad, M., Einarsson, E., Franzén, O., Ahola, H., Troell, K., Svärd, S.G., 2018. A novel high-resolution multilocus sequence typing of *Giardia intestinalis* assemblage A isolates reveals zoonotic transmission, clonal outbreaks and recombination. Infect. Genet. Evol. 60, 7–16. https://doi.org/10.1016/j.meegid.2018.02.012

Briancesco, R., Bonadonna, L., 2005. An Italian study on *Cryptosporidium* and *Giardia* in wastewater, fresh water and treated water. Environ. Monit. Assess. 104, 445–457. https://doi.org/10.1007/s10661-005-2282-4

Brynildsrud, O., Tysnes, K.R., Robertson, L.J., Debenham, J.J., 2018. *Giardia duodenalis* in primates: classification and host specificity based on phylogenetic analysis of sequence data. Zoonoses Public Health 65, 637–647. https://doi.org/10.1111/zph.12470

Cacciò, S.M., De Giacomo, M., Aulicino, F.A., Pozio, E., 2003. *Giardia* cysts in wastewater treatment plants in Italy. Appl. Environ. Microbiol. 69, 3393–3398. https://doi.org/10.1128/AEM.69.6.3393-3398.2003

Cacciò, S.M., Lalle, M., Svärd, S.G., 2018. Host specificity in the *Giardia duodenalis* species complex. Infect. Genet. Evol. 66, 335–345. https://doi.org/10.1016/j.meegid.2017.12.001

Chalmers, R.M., Robinson, G., Hotchkiss, E., Alexander, C., May, S., Gilray, J., Connelly, L., Hadfield, S.J., 2017. Suitability of loci for multiple-locus variable-number of tandem-repeats analysis of *Cryptosporidium parvum* for inter-laboratory surveillance and outbreak investigations. Parasitology 144, 37–47. https://doi.org/10.1017/S0031182015001766

DeRegnier, D.P., Cole, L., Schupp, D.G., Erlandsen, S.L., 1989. Viability of *Giardia* cysts suspended in lake, river, and tap water. Appl. Environ. Microbiol. 55, 1223–1229. https://doi.org/10.1128/aem.55.5.1223-1229.1989

Durigan, M., Cardoso-Silva, C.B., Ciampi-Guillardi, M., Toledo-Silva, G., Mori, G.M., Franco, R.M.B., Souza, A.P., 2018. Molecular genotyping, diversity studies and high-resolution molecular markers unveiled by microsatellites in *Giardia duodenalis*. PLoS Negl. Trop. Dis. 12, 1–18. https://doi.org/10.1371/journal.pntd.0006928

Ehsan, A., Geurden, T., Casaert, S., Paulussen, J., De Coster, L., Schoemaker, T., Chalmers, R., Grit, G., Vercruysse, J., Claerebout, E., 2015. Occurrence and potential health risk of *Cryptosporidium* and *Giardia* in different water catchments in Belgium. Environ. Monit. Assess. 187. https://doi.org/10.1007/s10661-014-4157-z

Fayer, R., Graczyk, T.K., Lewis, E.J., Trout, J.M., Parley, C.A., 1998. Survival of infectious *Cryptosporidium parvum* oocysts in seawater and Eastern oysters (*Crassostrea virginica*)

in the Chesapeake Bay. Appl. Environ. Microbiol. 64, 1070–1074.
Freire-Santos, F., Oteiza-López, A.M., Vergara-Castiblanco, C.A., Ares-Mazás, M.., 1999. Effect of salinity, temperature and storage time on mouse experimental infection by *Cryptosporidium parvum*. Vet. Parasitol. 87, 1–7.
Feng, Y., Xiao, L., 2011. Zoonotic potential and molecular epidemiology of *Giardia* species and giardiasis. Clin. Microbiol. Rev. 24, 110–140. https://doi.org/10.1128/CMR.00033-10
Forzieri, G., Cescatti, A., e Silva, F.B., Feyen, L., 2017. Increasing risk over time of weather-related hazards to the European population: a data-driven prognostic study. Lancet Planet. Heal. 1, e200–e208. https://doi.org/10.1016/S2542-5196(17)30082-7
Gallas-Lindemann, C., Sotiriadou, I., Plutzer, J., Noack, M.J., Mahmoudi, M.R., Karanis, P., 2016. *Giardia* and *Cryptosporidium* spp. dissemination during wastewater treatment and comparative detection via immunofluorescence assay (IFA), nested polymerase chain reaction (nested PCR) and loop mediated isothermal amplification (LAMP). Acta Trop. 158, 43–51. https://doi.org/10.1016/j.actatropica.2016.02.005
Gherasim, A., Lebbad, M., Insulander, M., Decraene, V., Kling, A., Hjertqvist, M., Wallensten, A., 2012. Two geographically separated food-borne outbreaks in Sweden linked by an unusual *Cryptosporidium parvum* subtype, October 2010. Eurosurveillance 17, 1–8. https://doi.org/10.2807/ese.17.46.20318-en
Giangaspero, A., Cirillo, R., Lacasella, V., Lonigro, A., Marangi, M., Cavallo, P., Berrilli, F., Di Cave, D., Brandonisio, O., 2009. *Giardia* and *Cryptosporidium* in inflowing water and harvested shellfish in a Lagoon in Southern Italy. Parasitol. Int. 58, 12–17. https://doi.org/10.1016/j.parint.2008.07.003
Gómez-Couso, H., Méndez-Hermida, F., Ares-Mazás, E., 2006a. Levels of detection of *Cryptosporidium* oocysts in mussels (*Mytilus galloprovincialis*) by IFA and PCR methods. Vet. Parasitol. 141, 60–65. https://doi.org/10.1016/j.vetpar.2006.04.015
Gómez-Couso, H., Méndez-Hermida, F., Castro-Hermida, J.A., Ares-Mazás, E., 2006b. *Cryptosporidium* contamination in harvesting areas of bivalve molluscs. J. Food Prot. 69, 185–190. https://doi.org/10.4315/0362-028X-69.1.185
Gómez-Couso, H., Méndez-Hermida, F., Castro-Hermida, J.A., Ares-Mazás, E., 2005. *Giardia* in shellfish-farming areas: Detection in mussels, river water and waste waters. Vet. Parasitol. 133, 13–18. https://doi.org/10.1016/j.vetpar.2005.04.043
Graczyk, T.K., Conn, D.B., Lucy, F., Minchin, D., Tamang, L., Moura, L.N.S., Dasilva, A.J., 2004. Human waterborne parasites in zebra mussels (*Dreissena polymorpha*) from the Shannon River drainage area, Ireland. Parasitol. Res. 93, 385–391. https://doi.org/10.1007/s00436-004-1142-4
Helmi, K., Skraber, S., Burnet, J.-B., Leblanc, L., Hoffmann, L., Cauchie, H.-M., 2011. Two-year monitoring of *Cryptosporidium parvum* and *Giardia lamblia* occurrence in a recreational and drinking water reservoir using standard microscopic and molecular biology techniques. Environ. Monit. Assess. 179, 163–175. https://doi.org/10.1007/s10661-010-1726-7
Hohweyer, J., Cazeaux, C., Travaille, E., Languet, E., Dumetre, A., Aubert, D., Terryn, C., Dubey, J.P., Azas, N., Houssin, M., Loïc, F., Villena, I., La Carbona, S., 2016. Simultaneous detection of the protozoan parasites *Toxoplasma*, *Cryptosporidium* and *Giardia* in food matrices and their persistence on basil leaves. Food Microbiol. 57, 36–44. https://doi.org/10.1016/j.fm.2016.01.002
Hohweyer, J., Dumètre, A., Aubert, D., Azas, N., Villena, I., 2013. Tools and methods for detecting and characterizing *Giardia*, *Cryptosporidium*, and *Toxoplasma* parasites in

marine mollusks. J. Food Prot. 76, 1649–1657. https://doi.org/10.4315/0362-028X.JFP-13-002

Insulander, M., de Jong, B., Svenungsson, B., 2008. A food-borne outbreak of cryptosporidiosis among guests and staff at a hotel restaurant in Stockholm county, Sweden, September 2008. Eurosurveillance 13.

Insulander, M., Silverlås, C., Lebbad, M., Karlsson, L., Mattsson, J.G., Svenungsson, B., 2013. Molecular epidemiology and clinical manifestations of human cryptosporidiosis in Sweden. Epidemiol. Infect. 141, 1009–1020. https://doi.org/10.1017/S0950268812001665

Isaac-Renton, J., Moorehead, W., Ross, A., 1996. Longitudinal studies of *Giardia* contamination in two community drinking water supplies: cyst levels, parasite viability, and health impact. Appl. Environ. Microbiol. 62, 47–54.

Kaupke, A., Osiński, Z., Rzeżutka, A., 2019. Comparison of *Cryptosporidium* oocyst recovery methods for their applicability for monitoring of consumer-ready fresh shellfish. Int. J. Food Microbiol. 296, 14–20. https://doi.org/10.1016/j.ijfoodmicro.2019.02.011

Keeley, A., Faulkner, B.R., 2008. Influence of land use and watershed characteristics on protozoa contamination in a potential drinking water resources reservoir. Water Res. 42, 2803–2813. https://doi.org/10.1016/j.watres.2008.02.028

King, B.J., Monis, P.T., 2007. Critical processes affecting *Cryptosporidium* oocyst survival in the environment. Parasitology 134, 309–323. https://doi.org/10.1017/S0031182006001491

Kostopoulou, D., Claerebout, E., Arvanitis, D., Ligda, P., Casaert, S., Sotiraki, S., 2020. Identifying human enteric parasitic infections in Greece, with focus on *Giardia* and *Cryptosporidium*. Exp. Parasitol. 211, 107864. https://doi.org/10.1016/j.exppara.2020.107864

Lal, A., Baker, M.G., Hales, S., French, N.P., 2013. Potential effects of global environmental changes on cryptosporidiosis and giardiasis transmission. Trends Parasitol. 29, 83–90. https://doi.org/10.1016/j.pt.2012.10.005

Lecová, L., Tůmová, P., Nohýnková, E., 2019. Clone-based haplotyping of *Giardia intestinalis* assemblage B human isolates. Parasitol. Res. 118, 355–361. https://doi.org/10.1007/s00436-018-6161-7 PROTOZOOLOGY

Li, W., Deng, L., Wu, K., Huang, X., Song, Y., Su, H., Hu, Y., Fu, H., Zhong, Z., Peng, G., 2017. Presence of zoonotic *Cryptosporidium scrofarum*, *Giardia duodenalis* assemblage A and *Enterocytozoon bieneusi* genotypes in captive Eurasian wild boars (*Sus scrofa*) in China: potential for zoonotic transmission. Parasites and Vectors 10, 1–8. https://doi.org/10.1186/s13071-016-1942-2

Li, X., Guyot, K., Dei-Cas, E., Mallard, J., Ballet, J.J., Brasseur, P., 2006. *Cryptosporidium* oocysts in mussels (*Mytilus edulis*) from Normandy (France). Int. J. Food Microbiol. 108, 321–325. https://doi.org/10.1016/j.ijfoodmicro.2005.11.018

Macarisin, D., Bauchan, G., Fayer, R., 2010. *Spinacia oleracea* L. leaf stomata harboring *Cryptosporidium parvum* oocysts: a potential threat to food safety. Appl. Environ. Microbiol. 76, 555–559. https://doi.org/10.1128/AEM.02118-09

MacRae, M., Hamilton, C., Strachan, N.J.C., Wright, S., Ogden, I.D., 2005. The detection of *Cryptosporidium parvum* and *Escherichia coli* O157 in UK bivalve shellfish. J. Microbiol. Methods 60, 395–401. https://doi.org/10.1016/j.mimet.2004.10.017

Masina, S., Shirley, J., Allen, J., Sargeant, J.M., Guy, R.A., Wallis, P.M., Weese, J.S., Cunsolo, A., Bunce, A., Harper, S.L., 2019. Weather, environmental conditions, and waterborne *Giardia* and *Cryptosporidium* in Iqaluit, Nunavut. J. Water Health 17, 84–97.

https://doi.org/10.2166/wh.2018.323
McKerr, C., Adak, G.K., Nichols, G., Gorton, R., Chalmers, R.M., Kafatos, G., Cosford, P., Charlett, A., Reacher, M., Pollock, K.G., Alexander, C.L., Morton, S., 2015. An outbreak of *Cryptosporidium parvum* across England and Scotland associated with consumption of fresh pre-cut salad leaves, May 2012. PLoS One 10, 1–13. https://doi.org/10.1371/journal.pone.0125955
Miller, W.A., Gardner, I.A., Atwill, E.R., Leutenegger, C.M., Miller, M.A., Hedrick, R.P., Melli, A.C., Barnes, N.M., Conrad, P.A., 2006. Evaluation of methods for improved detection of *Cryptosporidium* spp . in mussels (*Mytilus californianus*). J. Microbiol. Methods 65, 367–379. https://doi.org/10.1016/j.mimet.2005.08.011
Mintz, E.D., Hudson-Wragg, M., Mshar, P., Cartter, M.L., Hadler, J.L., 1993. Foodborne giardiasis in a corporate office setting. J. Infect. Dis. 167, 250–253. https://doi.org/10.1093/infdis/167.1.250
Olson, M.E., Goh, J., Phillips, M., Guselle, N., McAllister, T.A., 1999. *Giardia* cyst and *Cryptosporidium* oocyst survival in water, soil, and cattle feces. J. Environ. Qual. 28, 1991–1996. https://doi.org/10.2134/jeq1999.00472425002800060040x
Ongerth, J.E., Plutzer, J., Karanis, P., 2018. *Cryptosporidium* and *Giardia*—levels and distribution in surface water throughout Europe. Proceedings 2, 690. https://doi.org/10.3390/proceedings2110690
Panagos, P., Ballabio, C., Meusburger, K., Spinoni, J., Alewell, C., Borrelli, P., 2017. Towards estimates of future rainfall erosivity in Europe based on REDES and WorldClim datasets. J. Hydrol. 548, 251–262. https://doi.org/10.1016/j.jhydrol.2017.03.006
Peng, X., Murphy, T., Holden, N.M., 2008. Evaluation of the effect of temperature on the die-off rate for *Cryptosporidium parvum* oocysts in water, soils, and feces. Appl. Environ. Microbiol. 74, 7101–7107. https://doi.org/10.1128/AEM.01442-08
Pérez-Cordón, G., Robinson, G., Nader, J., Chalmers, R.M., 2016. Discovery of new variable number tandem repeat loci in multiple *Cryptosporidium parvum* genomes for the surveillance and investigation of outbreaks of cryptosporidiosis. Exp. Parasitol. 169, 119–128. https://doi.org/10.1016/j.exppara.2016.08.003
Rimšelienė, G., Vold, L., Nygård, K., Robertson, L., Nelke, C., Søli, K., Johansen, Ø.H., Thrana, F.S., 2011. An outbreak of gastroenteritis among schoolchildren staying in a wildlife reserve: thorough investigation reveals Norway's largest cryptosporidiosis outbreak. Scand. J. Public Health 39, 287–295. https://doi.org/10.1177/1403494810396557
Robertson, L.J., 2014. *Cryptosporidium* as a Foodborne Pathogen. Springer, New York.
Robertson, L.J., 2013. *Giardia* as a Foodborne Pathogen. Springer, New York.
Robertson, L.J., 2007. The potential for marine bivalve shellfish to act as transmission vehicles for outbreaks of protozoan infections in humans: A review. Int. J. Food Microbiol. 120, 201–216. https://doi.org/10.1016/j.ijfoodmicro.2007.07.058
Robertson, L.J., Clark, C.G., Debenham, J.J., Dubey, J.P., Kváč, M., Li, J., Ponce-Gordo, F., Ryan, U., Schares, G., Su, C., Tsaousis, A.D., 2019. Are molecular tools clarifying or confusing our understanding of the public health threat from zoonotic enteric protozoa in wildlife? Int. J. Parasitol. Parasites Wildl. 9, 323–341. https://doi.org/10.1016/j.ijppaw.2019.01.010
Robertson, L.J., Gjerde, B., 2008. Development and use of a pepsin digestion method for analysis of shellfish for *Cryptosporidium* oocysts and *Giardia* cysts. J. Food Prot. 71, 959–966. https://doi.org/10.4315/0362-028X-71.5.959
Robertson, L.J., Gjerde, B.K., 2007. *Cryptosporidium* oocysts: challenging adversaries? Trends

Parasitol. 23, 344–347. https://doi.org/10.1016/j.pt.2007.06.002
Robertson, L.J., Hermansen, L., Gjerde, B.K., 2006. Occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in sewage in Norway. Appl. Environ. Microbiol. 72, 5297–5303. https://doi.org/10.1128/AEM.00464-06
Rose, J.B., Slifko, T.R., 1999. *Giardia*, *Cryptosporidium*, and *Cyclospora* and their impact on foods: a review. J. Food Prot. 62, 1059–1070. https://doi.org/10.4315/0362-028X-62.9.1059
Rousseau, A., La Carbona, S., Dumètre, A., Robertson, L.J., Gargala, G., Escotte-Binet, S., Favennec, L., Villena, I., Gérard, C., Aubert, D., 2018. Assessing viability and infectivity of foodborne and waterborne stages (cysts/oocysts) of *Giardia duodenalis*, *Cryptosporidium* spp., and *Toxoplasma gondii*: a review of methods. Parasite 25. https://doi.org/10.1051/parasite/2018009
Ryan, U., Cacciò, S.M., 2013. Zoonotic potential of *Giardia*. Int. J. Parasitol. 43, 943–956. https://doi.org/10.1016/j.ijpara.2013.06.001
Ryan, U., Hijjawi, N., Feng, Y., Xiao, L., 2019. *Giardia*: an under-reported foodborne parasite. Int. J. Parasitol. 49, 1–11. https://doi.org/10.1016/j.ijpara.2018.07.003
Ryan, U., Hijjawi, N., Xiao, L., 2018. Foodborne cryptosporidiosis. Int. J. Parasitol. 48, 1–12. https://doi.org/10.1016/j.ijpara.2017.09.004
Ryan, U., Zahedi, A., 2019. Molecular epidemiology of giardiasis from a veterinary perspective, 1st ed, Advances in Parasitology. Elsevier Ltd. https://doi.org/10.1016/bs.apar.2019.07.002
Semenza, J.C., 2020. Cascading risks of waterborne diseases from climate change. Nat. Immunol. 21, 484–487. https://doi.org/10.1038/s41590-020-0631-7
Semenza, J.C., Herbst, S., Rechenburg, A., Suk, J.E., Höser, C., Schreiber, C., Kistemann, T., 2012. Climate change impact assessment of food- and waterborne diseases. Crit. Rev. Environ. Sci. Technol. 42, 857–890. https://doi.org/10.1080/10643389.2010.534706
Schets, F.M., van den Berg, H.H.J.L., de Roda Husman, A.M., 2013. Determination of the recovery efficiency of *Cryptosporidium* oocysts and *Giardia* cysts from seeded bivalve mollusks. J. Food Prot. 76, 93–98. https://doi.org/10.4315/0362-028X.JFP-12-326
Smith-DeWaal, C., Barlow, K., Alderton, L., Jacobson, M.F., 2001. Outbreak Alert., in: Center for Science in the Public Interest. Washington, DC, USA.
Tamburrini, A., Pozio, E., 1999. Long-term survival of *Cryptosporidium parvum* oocysts in seawater and in experimentally infected mussels (*Mytilus galloprovincialis*). Int. J. Parasitol. 29, 711–715. https://doi.org/10.1016/S0020-7519(99)00033-8
Thompson, R.C.A., Ash, A., 2019. Molecular epidemiology of *Giardia* and *Cryptosporidium* infections - What's new? Infect. Genet. Evol. 75. https://doi.org/10.1016/j.meegid.2019.103951
US EPA 2012. Method 1623.1: *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA. U.S Environmental Protection Agency, Office of Water, Washington, D.C., USA
US EPA 2014. Method 1693: *Cryptosporidium* and *Giardia* in Disinfected Wastewater by Concentration/IMS/IFA. U.S Environmental Protection Agency, Office of Water, Washington, D.C., USA
Van Dyke, M.I., Ong, C.S.L., Prystajecky, N.A., Isaac-Renton, J.L., Huck, P.M., 2012. Identifying host sources, human health risk and indicators of *Cryptosporidium* and *Giardia* in a Canadian watershed influenced by urban and rural activities. J. Water Health 10, 311–323. https://doi.org/10.2166/wh.2012.131
Willis, J.E., Mcclure, J.T., Mcclure, C., Spears, J., Davidson, J., Greenwood, S.J., 2015. Static

tank depuration and chronic short-term experimental contamination of Eastern oysters (*Crassostrea virginica*) with *Giardia duodenalis* cysts. Int. J. Food Microbiol. 192, 13–19. https://doi.org/10.1016/j.ijfoodmicro.2014.08.027

Willis, J.E., Mcclure, J.T., Mcclure, C., Spears, J., Davidson, J., Greenwood, S.J., 2014. Bioaccumulation and elimination of *Cryptosporidium parvum* oocysts in experimentally exposed Eastern oysters (*Crassostrea virginica*) held in static tank aquaria. Int. J. Food Microbiol. 173, 72–80. https://doi.org/10.1016/j.ijfoodmicro.2013.11.033

Xiao, S., Zhang, Y., Zhao, X., Sun, L., Hu, S., 2018. Presence and molecular characterization of *Cryptosporidium* and *Giardia* in recreational lake water in Tianjin, China: A preliminary study. Sci. Rep. 8, 1–8. https://doi.org/10.1038/s41598-018-20902-3

Yang, R., Paparini, A., Monis, P., Ryan, U., 2014. Comparison of next-generation droplet digital PCR (ddPCR) with quantitative PCR (qPCR) for enumeration of *Cryptosporidium* oocysts in faecal samples. Int. J. Parasitol. 44, 1105–1113. https://doi.org/10.1016/j.ijpara.2014.08.004

Zahedi, A., Gofton, A.W., Greay, T., Monis, P., Oskam, C., Ball, A., Bath, A., Watkinson, A., Robertson, I., Ryan, U., 2018. Profiling the diversity of *Cryptosporidium* species and genotypes in wastewater treatment plants in Australia using next generation sequencing. Sci. Total Environ. 644, 635–648. https://doi.org/10.1016/j.scitotenv.2018.07.024

Zahedi, A., Paparini, A., Jian, F., Robertson, I., Ryan, U., 2016. Public health significance of zoonotic *Cryptosporidium* species in wildlife: Critical insights into better drinking water management. Int. J. Parasitol. Parasites Wildl. 5, 88–109. https://doi.org/10.1016/j.ijppaw.2015.12.001

Summary

Cryptosporidium and *Giardia* are important parasitic protozoa, causing gastrointestinal disease in both humans and animals, and both parasites are considered to be zoonotic. The most common route of transmission is through contaminated water and/or food, and they are responsible for many waterborne (sometimes also foodborne) outbreaks of disease worldwide, having an important impact on public health. Contamination of water sources (and consequently of foodstuff) with both protozoa may have many sources, including runoffs from agricultural areas, drainage from manure storage, wastewaters overflows or improper sewage systems.

For the current thesis, the Thessaloniki greater area and the Thermaikos Gulf in Northern Greece has been selected as an interesting case scenario to study the dynamics of waterborne transmission of *Cryptosporidium* and *Giardia*. The area is characterised by: (i) an urban environment – the city of Thessaloniki (> 1,100,000 inhabitants) is built around Thermaikos Gulf; (ii) an agricultural environment – the area around the city is a dense farming area with numerous cattle and sheep farms; (iii) many surface water sources – there are 4 rivers, i.e. Gallikos, Axios, Loudias and Aliakmonas, that form a large deltaic complex on the northern and western parts of the gulf; (iv) a water production company using water from Aliakmonas river for the production of drinking water for Thessaloniki; (v) 3 wastewater treatment plants that treat human, agricultural and industrial waste, which are either re-used for irrigation or discharged into the gulf; (vi) the most intensive mussel farming of the Mediterranean mussel, *Mytilus galloprovincialis*, located close to the deltaic complex of the rivers and wastewater discharges, totaling 80-90% of the whole mussel production in Greece.

Specifically, the objectives of this Ph.D. thesis were:

6. To monitor the presence of *Cryptosporidium* and *Giardia* in different water sources in Northern Greece, including surface water, drinking water and treated waste waters.
7. To monitor the presence of *Cryptosporidium* and *Giardia* in sheep and cattle farms in the vicinity of surface water (rivers), as potential sources of water contamination.
8. To determine the *Cryptosporidium* species/genotypes and *Giardia* (sub)assemblages, through molecular analyses, in order to evaluate their zoonotic potential and to identify potential sources of water contamination.

9. To develop a risk assessment model to identify parameters that can predict the risk of surface water contamination with *Cryptosporidium* and/or *Giardia*.
10. To monitor the presence of *Cryptosporidium* and *Giardia* in mussels cultured in Thermaikos Gulf.

Chapter 1 provides a review of the recently published literature on some important aspects of *Cryptosporidium* and *Giardia*. This chapter describes the biology (taxonomy and life cycle) of the parasites, their presence in humans and animals and their zoonotic potential based on molecular data. The transmission routes of the parasites are described, focusing on waterborne and foodborne transmission. An overview of waterborne and foodborne disease outbreaks is also presented. Finally, the most widely used methods for their detection in water and on/in food matrices are briefly described.

In Greece, little information is available about water contamination with *Cryptosporidium* and *Giardia* and monitoring of drinking water for these parasites is not compulsory. Moreover, although many animal species are infected with both parasites, their role as potential sources of water contamination has not been investigated. The aim of **Chapter 2** was to investigate the presence and origin of *Cryptosporidium* and *Giardia* in different water sources in Northern Greece and to identify potential contamination sources of human and/or animal origin. Because the detection of (oo)cysts in water matrices is challenging, a second objective was to identify interactions between the presence of these parasites and biotic/abiotic factors, in order to develop risk-assessment models. During a 2-year period, using a longitudinal, repeated sampling approach, 12 locations in 4 rivers, irrigation canals, and a water production company, were monitored for *Cryptosporidium* and *Giardia,* using standard methods. Furthermore, 254 faecal samples from animals were collected from 15 cattle and 12 sheep farms located near the water sampling points and screened for both parasites, in order to estimate their potential contribution to water contamination. River-water samples were frequently contaminated with *Cryptosporidium* (47.1%) and *Giardia* (66.2%), with higher contamination rates during winter and spring. During a 5-month period, (oo)cysts were detected in drinking water (<1 /litre). Animals on all farms were infected by both parasites, with 16.7% of calves and 17.2% of lambs excreting *Cryptosporidium* oocysts and 41.3% of

calves and 43.1% of lambs excreting *Giardia* cysts. The most prevalent species identified in both water and animal samples were *C. parvum* and *G. duodenalis* assemblage AII. The presence of *G. duodenalis* assemblage AII in drinking water and *C. parvum* IIaA15G2R1 in surface water highlights the potential risk of waterborne infection. No correlation was found between (oo)cyst counts and faecal-indicator bacteria. Machine-learning models that can predict contamination intensity with *Cryptosporidium* (75% accuracy) and *Giardia* (69% accuracy), combining biological, physicochemical and meteorological factors, were developed. Although these prediction accuracies may be insufficient for public health purposes, they could be useful for augmenting and informing risk-based sampling plans.

Marine bivalve shellfish are of public health interest because they can accumulate pollutants in their tissues. As they are usually consumed raw or lightly cooked, they are considered to be a possible source of foodborne infections, including giardiosis and cryptosporidiosis. Although data indicating contamination of shellfish with *Giardia* cysts and *Cryptosporidium* oocysts have been published, comparing results from different studies is difficult, as there is no standardized protocol for the detection and quantification of these parasites in mussels, and different researchers have used different analytical approaches. The aim of **Chapter 3** was to identify and characterise the most sensitive protocol for the detection of *Giardia* cysts and *Cryptosporidium* oocysts in shellfish. In an effort to test the sensitivity and the detection limits of the protocol, every step of the process was investigated, from initial preparation of the mussel matrix through detection of the parasites. Comparative studies were conducted, including several methods previously applied by other researchers, on commercial mussels *Mytilus galloprovincialis* spiked with a known number of (oo)cysts of both parasites. The preparation of the mussel matrix plays an important role for the sensitivity of the method, thus different techniques were tested, including (ia) removal of the coarse particles from the matrix with sieving, (ib) extraction of the lipids with diethyl ether, and (ic) artificial digestion of the matrix with pepsin digestion solution, and (ii) the use or not of immunomagnetic separation (IMS) for the concentration of the (oo)cysts. Pre-treatment of the mussel homogenate with pepsin digestion solution, followed by IMS, then detection with a direct immunofluorescence assay, achieved the highest sensitivity: 32.1% (SD: 21.1) of *Giardia* cysts and 61.4% (SD: 26.2) *Cryptosporidium* oocysts were recovered, with a detection limit of 10 (oo)cysts per g of mussel homogenate. The outcome of the current study was the

standardization of a protocol, with defined detection limits, for the detection of those two protozoa in mussels, in order to be used as a reference technique in future studies. Further advantages of this protocol are that it uses the whole mussel as a starting material and does not require difficult handling procedures. The method has potential to be applied in larger surveys and, potentially, to other species of shellfish for the detection of these parasites.

In **Chapter 4**, we investigated the presence of *Cryptosporidium* and *Giardia* in Mediterranean mussels, *Mytilus galloprovincialis* that are cultivated in Thermaikos Gulf, North Greece, which is fed by the four rivers studied in Chapter 2, which were contaminated with both protozoa. Moreover, the occurrence of these protozoa was monitored in treated wastewaters from 3 treatment plants that discharge into the gulf. In order to identify potential sources of contamination and to estimate the risk for human infection, an attempt was made to genotype *Giardia* and *Cryptosporidium* in positive samples. Immunofluorescence was used for detection and molecular techniques were used for both detection and genotyping of the parasites. In total, 120 mussel samples, coming from 10 farms, were examined for the presence of both protozoa over the 6-month farming period. None of them were found positive by immunofluorescence microscopy for the presence of parasites. Only in 3 mussel samples, PCR targeting the GP60 gene detected *Cryptosporidium* spp. DNA, but sequencing was not successful. Thirteen out of 18 monthly samples collected from the 3 wastewater treatment plants, revealed the presence of *G. duodenalis* cysts belonging to sub-assemblage AII, at relatively low counts (up to 11.2 cysts/L). *Cryptosporidium* oocysts (up to 0.9 oocysts/L) were also detected in 4 out of 8 samples, although sequencing was not successful at any of the target genes. At the studied location and under the sampling conditions described, mussels tested were not found to be harboring *Giardia* cysts and the presence of *Cryptosporidium* was found only in few cases (by PCR detection only). Our results suggest that the likelihood that mussels from these locations act as vehicles of human infection for *Giardia* and *Cryptosporidium* seems low.

In **Chapter 5,** data presented in the current thesis are discussed and compared with recently published studies. In general, there is a high abundance of potentially zoonotic *Cryptosporidium* species and *G. duodenalis* (sub)assemblages in all water matrices and farm animals tested. A seasonal pattern of contamination has been revealed, with higher contamination rates during rainy months. Animals were considered to be a main source of

water contamination through run-off from the surrounding farms, whereas wastewater treatment plants seemed not to contribute to water contamination. Same potentially zoonotic species/genotypes are circulating in the environment and hosts, however lack of multilocus analyses and absence of reported human cases/outbreaks, do not allow us to safely conclude on their impact on public health. Machine learning modelling approaches seem to be a promising tool for the prediction of water contamination by these protozoa and could be used to augment and inform risk-based sampling plans. Finally, Mediterranean mussels farmed under the described contamination pressure of Thermaikos gulf, due to contaminated rivers and wastewaters discharged there, seem to be a low risk food source for human infections with *Cryptosporidium* spp. and *G. duodenalis*.

Samenvatting

Cryptosporidium en *Giardia* zijn belangrijke parasitaire protozoa, die gastro-intestinale aandoeningen veroorzaken bij zowel mens als dier, en beide parasieten worden als zoönotisch beschouwd. De meest voorkomende transmissieroute is via besmet water en/of voedsel, en ze zijn verantwoordelijk voor vele door water overgebrachte (soms ook via voedsel overgedragen) uitbraken van ziekten wereldwijd, die een belangrijke impact hebben op de volksgezondheid. Verontreiniging van water (en bijgevolg van voedsel) met beide protozoa kan verschillende bronnen hebben, waaronder afvloeiing uit landbouwgebieden, drainage uit mestopslag, overlopen van afvalwater of defecte riolering.

Voor het huidige proefschrift is het gebied van Thessaloniki en de Thermaikos-golf in Noord-Griekenland geselecteerd als een interessant casus om de dynamiek van de water-gedragen transmissie van *Cryptosporidium* en *Giardia* te bestuderen. Het gebied wordt gekenmerkt door: (i) een stedelijke omgeving - de stad Thessaloniki (> 1.100.000 inwoners) is gebouwd rond de Golf van Thermaikos; (ii) een landbouwomgeving - het gebied rond de stad is een dicht landbouwgebied met talrijke rundvee- en schapenboerderijen; (iii) veel oppervlaktewaters - er zijn 4 rivieren, d.w.z. Gallikos, Axios, Loudias en Aliakmonas, die een groot delta-complex vormen in het noord-westen van de golf; iv) een waterproductiebedrijf dat water uit de Aliakmonas-rivier gebruikt voor de productie van drinkwater voor Thessaloniki; (v) 3 afvalwaterzuiveringsinstallaties die menselijk, landbouwkundig en industrieel afval behandelen, die ofwel hergebruikt worden voor irrigatie of geloosd worden in de golf; (vi) intensieve mosselkweek van de mediterrane mossel, *Mytilus galloprovincialis*, gelegen nabij het delta-complex van de rivieren en de lozingen van afvalwater, goed voor 80-90% van de totale mosselproductie in Griekenland.

In het bijzonder zijn de doelstellingen van deze PhD thesis:

1. Monitoren van *Cryptosporidium* en *Giardia* in verschillende waterbronnen in Noord-Griekenland, waaronder oppervlaktewater, drinkwater en gezuiverd afvalwater.
2. Onderzoeken van de aanwezigheid van *Cryptosporidium* en *Giardia* op schapen- en rundveebedrijven in de buurt van oppervlaktewater (rivieren), als mogelijke bronnen van waterverontreiniging.

3. Bepalen van de *Cryptosporidium* soorten/genotypes en *Giardia* (sub)assemblages, door middel van moleculaire analyses, om hun zoönotisch potentieel te evalueren en potentiële bronnen van waterverontreiniging te identificeren.
4. Een risicobeoordelingsmodel ontwikkelen om parameters te identificeren die het risico op verontreiniging van het oppervlaktewater met *Cryptosporidium* en/of *Giardia* kunnen voorspellen.
5. De aanwezigheid van *Cryptosporidium* en *Giardia* in mosselen in de Thermaikos Golf opsporen.

Hoofdstuk 1 geeft een overzicht van de literatuur over enkele belangrijke aspecten van *Cryptosporidium* en *Giardia*. Dit hoofdstuk beschrijft de biologie (taxonomie en levenscyclus) van de parasieten, hun aanwezigheid bij mens en dier en hun zoönotisch potentieel op basis van moleculaire gegevens. De transmissieroutes van de parasieten worden beschreven, met de nadruk op transmissie via het water en via voedsel. Een overzicht van uitbraken van door water en door voedsel overgedragen ziekten wordt ook gepresenteerd. Ten slotte worden de meest gebruikte methoden voor hun detectie in water en op/in voedselmatrices kort beschreven.

In Griekenland is weinig informatie beschikbaar over waterverontreiniging met *Cryptosporidium* en *Giardia* en monitoring van drinkwater voor deze parasieten is niet verplicht. Hoewel veel diersoorten met beide parasieten zijn besmet, is hun rol als potentiële bron van waterverontreiniging niet onderzocht. Het doel van **Hoofdstuk 2** was om de aanwezigheid en oorsprong van *Cryptosporidium* en *Giardia* in verschillende waterbronnen in Noord-Griekenland te onderzoeken en potentiële besmettingsbronnen van menselijke en/of dierlijke oorsprong te identificeren. Omdat de detectie van (oö)cysten in watermatrices uitdagend is, was een tweede doelstelling het identificeren van interacties tussen de aanwezigheid van deze parasieten en biotische/abiotische factoren, om risicobeoordelings-modellen te ontwikkelen. Gedurende een periode van 2 jaar werden met behulp van een longitudinale, herhaalde bemonstering 12 locaties in 4 rivieren, irrigatiekanalen en een waterproductiebedrijf gecontroleerd op *Cryptosporidium* en *Giardia*, met behulp van standaardmethoden. Verder werden 254 fecale monsters van dieren verzameld van 15

runder- en 12 schapenboerderijen in de buurt van de waterbemonsteringspunten en gescreend op beide parasieten, om hun potentiële bijdrage aan waterverontreiniging in te schatten. Watermonsters van de rivieren waren vaak besmet met *Cryptosporidium* (47,1%) en *Giardia* (66,2%), met hogere besmettingspercentages in de winter en de lente. Gedurende een periode van 5 maanden werden ook (oö)cysten gedetecteerd in drinkwater (<1/liter). Dieren op alle bedrijven waren geïnfecteerd met beide parasieten, waarbij 16,7% van de kalveren en 17,2% van de lammeren *Cryptosporidium*-oöcysten uitscheiden en 41,3% van de kalveren en 43,1% van de lammeren *Giardia*-cysten uitscheiden. De meest voorkomende soorten die in zowel water- als diermonsters werden geïdentificeerd, waren *C. parvum* en *G. duodenalis* assemblage AII. De aanwezigheid van *G. duodenalis* assemblage AII in drinkwater en het *C. parvum* IIaA15G2R1 genotype in oppervlaktewater benadrukt het potentiële risico van infectie via het water. Er werd geen correlatie gevonden tussen (oö)cystentellingen en fecale indicatorbacteriën, maar er werden 'machine learning'-modellen ontwikkeld die de intensiteit van besmetting kunnen voorspellen voor *Cryptosporidium* (75% nauwkeurigheid) en *Giardia* (69% nauwkeurigheid), waarbij biologische, fysicochemische en meteorologische factoren werden gecombineerd. Hoewel deze voorspellingsnauwkeurigheden mogelijk onvoldoende zijn om direct bruikbaar te zijn voor de volksgezondheid, kunnen ze nuttig zijn voor het aanvullen en informeren van op risico gebaseerde steekproefplannen

Mariene tweekleppige schelpdieren zijn van belang voor de volksgezondheid omdat ze verontreinigende stoffen in hun weefsels kunnen ophopen. Omdat ze meestal rauw of licht gekookt worden gegeten, worden ze beschouwd als een mogelijke bron van door voedsel overgedragen infecties, waaronder giardiose en cryptosporidiose. Hoewel er gegevens zijn gepubliceerd over besmetting van schaaldieren met *Giardia*-cysten en *Cryptosporidium*-oöcysten, is het moeilijk om de resultaten van verschillende onderzoeken te vergelijken, aangezien er geen gestandaardiseerd protocol is voor de detectie en kwantificering van deze parasieten in mosselen, en verschillende onderzoekers hebben verschillende analytische benaderingen gebruikt. Het doel van **Hoofdstuk 3** was het identificeren en karakteriseren van het meest gevoelige protocol voor de detectie van *Giardia*-cysten en *Cryptosporidium*-oöcysten in schaaldieren. Om de gevoeligheid en de detectielimieten van het protocol te testen, werd elke stap van het proces onderzocht, van de eerste bereiding van het mosselhomogenaat tot de detectie van de parasieten. Er werden vergelijkende onderzoeken

uitgevoerd met verschillende methoden die eerder door andere onderzoekers waren toegepast, op commerciële *Mytilus galloprovincialis* mosselen, verrijkt met een bekend aantal (oö)cysten van beide parasieten. De bereiding van de mosselmatrix speelt een belangrijke rol voor de gevoeligheid van de methode, daarom werden verschillende technieken getest, waaronder (ia) het verwijderen van de grove deeltjes uit de matrix met zeven, (ib) extractie van de lipiden met diëthylether, en (ic) kunstmatige vertering van de matrix met een verteringsoplossing met pepsine, en (ii) het al dan niet gebruiken van immunomagnetische scheiding (IMS) voor de concentratie van de (oö)cysten. Voorbehandeling van het mosselhomogenaat met verteringsoplossing met pepsine, gevolgd door IMS en vervolgens detectie met een directe immunofluorescentietest, bereikte de hoogste gevoeligheid: 32,1% (SD: 21,1) van de *Giardia*-cysten en 61,4% (SD: 26,2) van de *Cryptosporidium*-oöcysten werden teruggevonden, met een detectiegrens van 10 (oö)cysten per g mosselhomogenaat. De uitkomst van de huidige studie was de standaardisatie van een protocol met gedefinieerde detectielimieten voor de detectie van deze twee protozoa in mosselen, om in toekomstige studies als referentietechniek te gebruiken. Verdere voordelen van dit protocol zijn dat het de hele mossel als uitgangsmateriaal gebruikt en geen moeilijke hanteringsprocedures vereist. De methode kan worden toegepast in grotere onderzoeken en mogelijk ook op andere soorten schaaldieren voor de detectie van deze parasieten.

In **Hoofdstuk 4** hebben we de aanwezigheid van *Cryptosporidium* en *Giardia* onderzocht in mediterrane mosselen, *Mytilus galloprovincialis,* die worden gekweekt in de Golf van Thermaikos, Noord-Griekenland, die wordt gevoed door de vier rivieren die zijn bestudeerd in hoofdstuk 2, en die besmet waren met beide protozoa. Bovendien werd de aanwezigheid van deze protozoa opgevolgd in behandeld afvalwater van 3 zuiveringsinstallaties die lozen in de golf. Om potentiële besmettingsbronnen te identificeren en het risico op infectie bij de mens in te schatten, werd geprobeerd *Cryptosporidium* en *Giardia* in positieve monsters te genotyperen. Immunofluorescentie werd gebruikt voor detectie en moleculaire technieken werden gebruikt voor zowel detectie als genotypering van de parasieten. In totaal zijn 120 mosselmonsters, afkomstig van 10 bedrijven, onderzocht op de aanwezigheid van beide protozoa gedurende de kweekperiode van 6 maanden. Geen van hen werd positief bevonden door immunofluorescentiemicroscopie. In 3 mosselmonsters werd *Cryptosporidium* DNA gedetecteerd met behulp van PCR, gericht op het GP60-gen, maar de sequentiebepaling was

niet succesvol. Dertien van de 18 maandelijkse monsters die werden verzameld bij de 3 afvalwaterzuiveringsinstallaties bevatten *G. duodenalis*-cysten van subassemblage AII, in relatief kleine aantallen (tot 11,2 cysten/L). *Cryptosporidium*-oöcysten (tot 0,9 oöcysten/l) werden ook gedetecteerd in 4 van de 8 monsters, hoewel de sequentiebepaling bij geen van de onderzochte genen succesvol was. Op de bestudeerde locatie en onder de beschreven bemonsteringsomstandigheden bleken de geteste mosselen geen *Giardia*-cysten te bevatten en de aanwezigheid van *Cryptosporidium* werd slechts in enkele gevallen gevonden (alleen door middel van PCR-detectie). Deze resultaten suggereren dat de kans dat mosselen van deze locaties fungeren als bron van menselijke infectie met *Giardia* en *Cryptosporidium* klein is.

In **Hoofdstuk 5** worden de data uit dit proefschrift besproken en vergeleken met recent gepubliceerde studies. Over het algemeen was er een groot aantal potentieel zoönotische *Cryptosporidium*-soorten en *G. duodenalis* (sub)assemblages aanwezig in alle geteste watermatrices en in landbouwhuisdieren. Er werd een seizoensgebonden besmettings-patroon gezien, met hogere besmettingspercentages tijdens de regenachtige maanden. Dieren werden beschouwd als de belangrijkste bron van waterverontreiniging door effluenten van de omliggende boerderijen, terwijl afvalwaterzuiveringsinstallaties niet leken bij te dragen aan waterverontreiniging. Dezelfde potentieel zoönotische soorten/genotypen circuleerden in het milieu en in verschillende gastheren, maar het gebrek aan multilocus-analyses en de afwezigheid van gemelde gevallen/uitbraken bij de mens stellen ons niet in staat om met zekerheid conclusies te trekken over hun impact op de volksgezondheid. Modelleermethoden met ‘machine learning’ lijken een veelbelovend instrument te zijn voor de voorspelling van waterverontreiniging door deze protozoa en kunnen worden gebruikt om risicogebaseerde bemonsteringsplannen op te stellen of aan te vullen. Tenslotte lijken mediterrane mosselen die gekweekt worden in het gecontamineerde milieu van de Thermaikos-golf, als gevolg van verontreinigde rivieren en afvalwater die daar worden geloosd, een voedselbron met een laag risico voor menselijke infecties met *Cryptosporidium* spp. en *G. duodenalis.*

Aknowledgements

Firstly, I would like to express my deep and sincere gratidute to both my supervisors Prof. Edwin Claerebout and Dr. Smaragda Sotiraki for their continuous trust and support all these years. Prof. Claerebout even though miles away, he was always there for me to encourange me and guide me in whatever difficulty I met. I really thank him for his patience and kindness during this chapter of my life. Dr. Sotiraki is the reason why I started this journey. A huge thank you isn't really enough to express her my graditute, not only for her constant help in the lab, but mainly for the mental support. She was always there to help me see the light at the end of the tunnel. I could not have imagined having better advisors and mentors!

Besides my promoters, I would like to thank the members of the examination committee: Prof. Lucy J. Robertson, Dr. Alexandros Stefanakis, Prof. Sarah Gabriel and Prof. Pierre Dorny for their valuable comments that assisted to the improvement of my thesis.

My sincere gratidute also goes to all the colleagues from the Veterinary Research Institute for their support and the best work environment I could have ever imagined, with special thanks to: Katerina Saratsi, Dr. Despoina Kostopoulou, Dr. Spyridon Vyzantinopoulos, Dr. Anastasios Saratsis and Dionisios Birbilis. Thank you for being more than colleagues to me.

My thanks also goes to UGENT personell and especially Stijn Casaert for his help and support with lab-stuff and technical support.

Last but not the least, I would like to thank my family for their unconditional love and support. I am grateful to my parents for all the sacrifices they have done for me and for giving me strength to reach for the stars and chase my dreams. My brother and my grandparents deserve my wholehearted thanks as well. Finally, this journey would not have been possible without the understanding and patience of my other half.