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

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ABSTRACT

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 this study was to identify and characterize 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. As preparation of the mussel matrix plays an important role in the sensitivity of the method, different techniques were tested. These included: (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 these two protozoan transmission stages 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. However, the composition (lipid to protein ratio) may be of relevance for detection efficiency for some other species of shellfish.

1. 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

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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.

2. Materials and methods

2.1. 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.

2.2. 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 0.05 mm 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 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 1 M 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

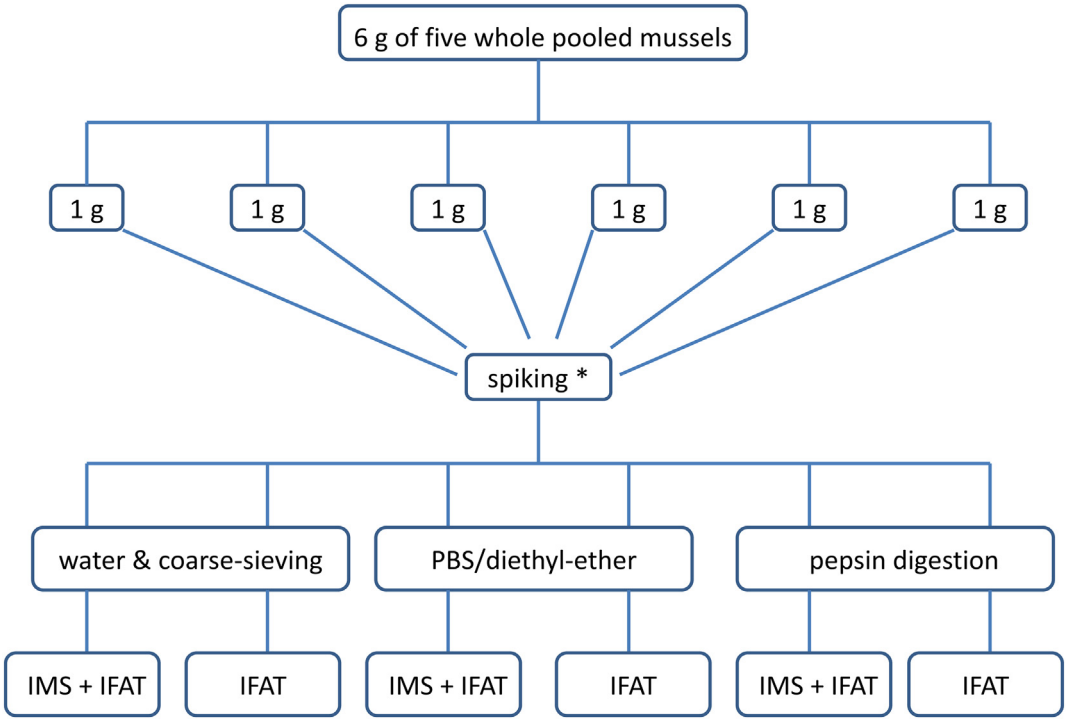

Fig. 1. Experimental design for comparison of methods to detect *Giardia* cysts and *Cryptosporidium* oocysts in spiked mussel samples.

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 re-suspended 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.

2.3. 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.

2.4. 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).

3. Results

3.1. 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

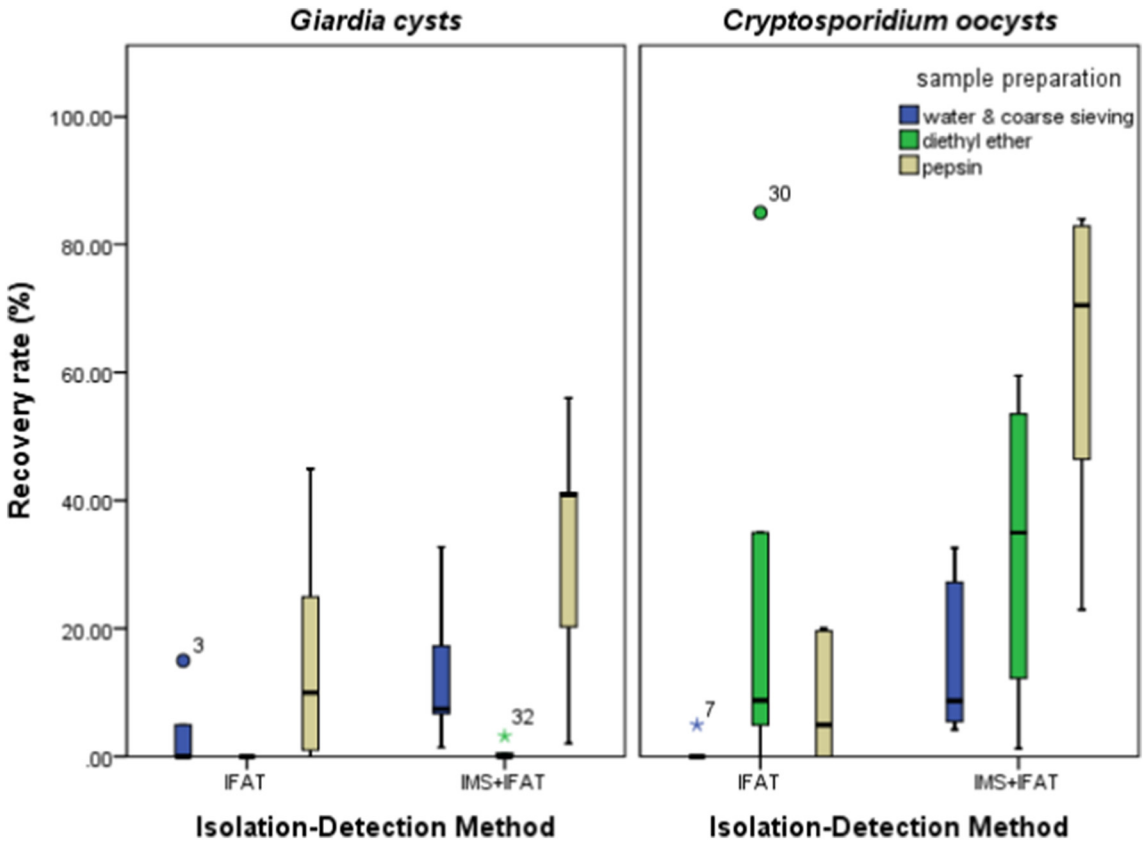

Fig. 2. Boxplots representing the range of the recovery rates (through the 5 repetitions) of *Giardia* cysts and *Cryptosporidium* oocysts after the use of different protocols for the preparation of the matrix (coarse sieving only with water, diethyl ether lipid removal or pepsin digestion) and the isolation-detection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

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.

3.2. 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 lower 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.

4. 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

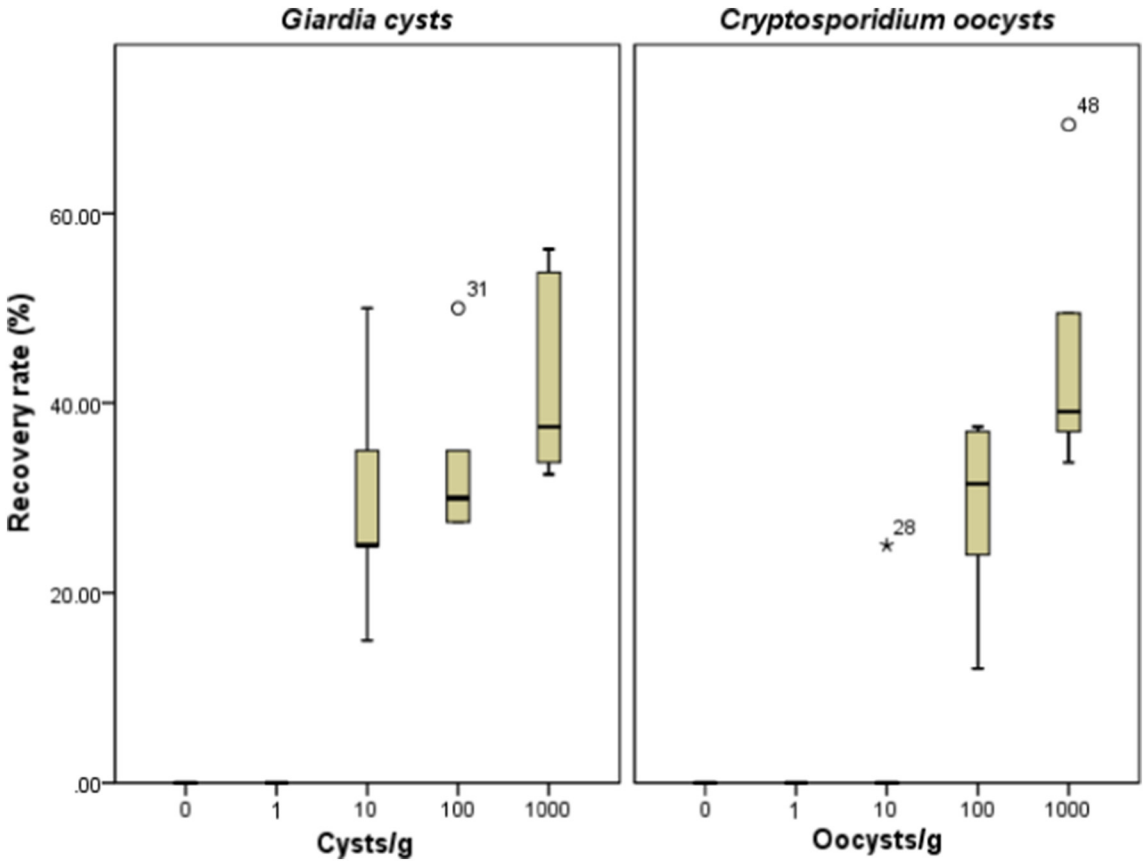

Fig. 3. Boxplots representing the range of the recovery rates (through the 5 repetitions) of the different concentrations of *Giardia* cysts and *Cryptosporidium* oocysts, using pepsin digestion solution for the artificial digestion of the matrix, followed by IMS for the concentration of the (oo)cysts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

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

Table 1
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	15×10^3–100×10^3/mussel	Homogenized whole individual mussels	Gomez-Bautista et al., 2000
Dreissena polymorpha (zebra mussel)	Ireland	5–9/mussel ND	4–16/mussel 1–12/mussel	Homogenized, sieved whole individual mussels Haemolymph of individual mussels	Graczyk et al., 2004
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
Mytilus galloprovincialis (Mediterranean mussel)	Spain	ND	6–300/6–8 mussels	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	Pooled gill washing, gill homogenates, flesh washing, flesh homogenates, inner-shell water - 1 kg mussels/pool - flotation & IMS	Li et al., 2006
Mylitus edulis (blue mussel) *Dreissena polymorpha* (zebra mussel) *Anodonta anatina* (duck mussel)	Ireland	6/mussel (mean) 13/mussel (mean) 97/mussel (mean)	22/mussel (mean) ND ND	Homogenized sediment of whole individual mussels Homogenized sediment of whole individual mussels (with shells)	Lucy et al., 2008
Mytilus edulis (blue mussel) *Modiolus modiolus* (horse mussel)	Norway	0.4–4.5/g 0.3/g	0.4–1/g ND	Homogenized whole individual mussels - pepsin digestion & IMS	Robertson and Gjerde, 2008
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) *Perna perna* (brown mussel)	Tunisia	62–395/9–18 mussels	ND	Homogenized, sieved 9–18 whole pooled mussels	Ghozzi et al., 2017*

All surveys used IFAT for detection and enumeration of the (oo)cysts, apart from two (marked with *) that used qPCR.

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 × 10^2 *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).

5. 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.

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Conflict of interest

None.

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