Toxoplasma gondii Oocyst Infectivity Assessed Using a Sporocyst-Based Cell Culture Assay Combined with Quantitative PCR for Environmental Applications

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Running title:

***Toxoplasma gondii* oocyst infectivity using cell culture-qPCR**

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ABSTRACT

Toxoplasma gondii is a ubiquitous foodborne protozoan that can infect humans at low dose and displays different prevalences among countries in the world. Ingestion of food or water contaminated with small amounts of *T. gondii* oocysts may result in human infection. However, there is no regulations for monitoring oocysts in food mainly because of a lack of standardized methods to detect them. The objectives of this study were (i) to develop a reliable method, applicable in biomonitoring, for the rapid detection of infectious oocysts by cell culture of their sporocysts combined with qPCR (sporocyst-CC-qPCR), and (ii) to adapt this method to blue and zebra mussels experimentally contaminated by oocysts with the objective to use these organisms as sentinels of aquatic environments. Combining mechanical treatment and bead beating leads to the release of 84 ± 14% of free sporocysts. The sporocyst-CC-qPCR can detect fewer than ten infectious oocysts in water within four days (one day of contact and three days of cell culture), compared to four weeks by mouse bioassay. For both mussel matrices, oocysts were pre-purified using a 30%-Percoll gradient and treated with sodium hypochlorite before cell culture of their sporocysts. This assay was able to detect as low as ten infective oocysts. This sporocyst-based CC-qPCR appears as a good alternative to mouse bioassay for monitoring infectious *T. gondii* oocysts directly in water but also using biosentinel mussel species. This method offers new perspective to assess the environmental risk for human health associated to this parasite.

Importance: The ubiquitous protozoan *Toxoplasma gondii* is the subject of renewed interest, due to the spread of oocysts in water and food causing endemic and epidemic outbreaks of toxoplasmosis in humans and animals worldwide. Displaying a sensitivity close to animal models, cell culture represents a real alternative, to assess the infectivity of oocysts in water and biosentinel mussels. This method opens interesting perspectives for evaluating human

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exposure to infectious *T. gondii* oocysts in the environment, where oocyst amounts is considered to be very low.

Keywords: *Toxoplasma gondii*, sporocysts, oocysts, *in vitro* cell culture, qPCR, infectivity, water, mussels, biomonitoring.

69 **Introduction**

70

71 The apicomplexan *Toxoplasma gondii*, an obligate intracellular parasite, can infect humans
72 and a wide range of warm-blooded vertebrates leading to toxoplasmosis. This generally
73 benign infection can cause severe life-threatening disease, particularly in
74 immunocompromised patients and congenitally infected children (1).
75 There are two major infective stages of the parasite that can infect humans: tissue cysts
76 (bradyzoites) found only in meat (2; 3), and oocysts (sporozoites), which are shed exclusively
77 by infected felids and contaminate soil, water or food (fruits, vegetables, mollusks) (4). One
78 infected cat can excrete up to 100 million oocysts, which become infective following
79 sporulation. Oocysts are known to resist to environmental conditions, most physical and / or
80 chemical treatments (5) and some industrial process applied to foods, such as high hydrostatic
81 pressure (6).
82 *Toxoplasma gondii* oocysts were responsible for 2% of parasitic protozoan outbreaks between
83 January 2004 and December 2010 (4, 7). Oocysts transmitted by water were associated with
84 21% of waterborne outbreaks between 1976 and 2009 (7-9). Several studies reported the
85 detection of the parasite in surface and drinking waters (10,11), and in fruits, vegetables and
86 mollusks exposed to contaminated waters (12,13).
87 Monitoring approach of waters quality are based upon punctual sampling (time and location)
88 and methods used to detect *T. gondii* oocysts in water require the filtration of large volumes of
89 water (up to 1 000 L) to concentrate parasites before their detection. Moreover, in aquatic
90 habitats, oocysts are subjected to dilution events and water characteristics such as salinity,
91 organic matter content and temperature can affect oocyst transport dynamics as well as their
92 spatial and temporal distributions (14). Using water for monitoring oocysts in water bodies
93 can thus lead to variable results depending on physico-chemical and meteorological

parameters, which are particularly important in the present context of global climate change. To circumvent the main drawbacks of water analyses, new alternative approaches to water analyses have recently emerged in water quality surveys using host-associated microorganisms as natural biosamplers (15). Special attention has been paid to bivalves because their intense filtering activity could lead to high accumulation of pathogens (15,16). Hence, studying bivalves can highlight pathogen contamination while water analysis results are negative (17). Laboratory studies have shown that marine and freshwater bivalves can concentrate waterborne protozoan parasites (18-20). Consistent with this, some studies have reported the detection of *T. gondii* oocysts in different marine (12, 21-23) or continental (24) bivalves, allowing the study of a large spatial scale (freshwater-seawater continuum).

Experiments have demonstrated that *T. gondii* oocysts can sporulate in seawater, be concentrated by mussels and remain infectious for laboratory mice (25-27). Usually, DNA-based methods are applied to detect protozoa in mollusks (23, 28, 29). However, DNA can persist for a long time in dead cells (30), thus preventing a distinction between viable and dead parasites. As only viable parasites are potentially infectious and can lead to illness, viability is a major feature for assessing the health risk. Methods to measure the viability and infectivity of protozoa including *T. gondii* have been recently reviewed (31). Among molecular techniques, Propidium MonoAzide based-PCR assays appeared not relevant to measure the viability of *T. gondii* oocysts (32) and RNA based-methods overestimated the exposure of humans to viable oocysts because of persistence of RNA in dead parasites (33, 34). Considering that all viable parasites are not necessarily infectious, i.e. able to replicate within host cells, the methods allowing the characterization of infectivity remained the most reliable ones.

Many authors have used animal models, the gold standard, to evaluate the infectiosity of *T. gondii* oocysts spiked on raspberries or blueberries (6, 35) or in naturally contaminated

mussels, oysters (12, 25) or in water (11). However, bioassays are time consuming, labor intensive, expensive and raise ethical concerns. Moreover, bioassays only provide a qualitative assessment of oocyst viability. It is therefore essential to develop complementary methods, easier to implement in the laboratory and for stakeholders, to provide tools to quantify viable, infective parasites in the environment.

In vitro cell culture assays are alternative approaches to bioassays. They have been widely applied associated with qPCR or RT-qPCR methods to detect infectious viruses (36, 37), and *Cryptosporidium parvum* (38-43) and *T. gondii* oocysts (44, 45) following sporozoite excystation. In these latter studies, excystation relied on a mechanical treatment to obtain free sporocysts, followed by their incubation with bile salts to release the sporozoites. However, this method is long and non-reproducible and results in a poor sporozoite yield, mainly because of a parasite loss due to mechanical treatment and bile salts exposure. The objective of this study was therefor to propose a rapid, accessible and sensitive method to detect infectious *T. gondii* oocysts, for water quality monitoring based on the use of bivalves as indicators of water contamination. An approach based on the infection of cell cultures using free sporocysts, combined with qPCR, was first developed in simple matrix (water) and characterized in terms of limit of detection and correlation with mouse bioassays. Then, the developed method was adapted to the detection of oocysts by using the continental zebra mussel (*Dreissena polymorpha*) and the coastal blue mussel (*Mytilus edulis*) as reliable indicators of water quality assessment in the freshwater-seawater continuum.

Results

Selection of the protocol for the release of sporocysts from *T. gondii* oocysts

As sporozoite excystations protocols usually lead to low sporozoite yield, we chose to test different protocols to break the oocyst wall while keeping the sporocysts intact for their

cell culture. For this, viable oocysts were exposed to different mechanical disruption protocols to optimize the release of intact sporocysts. The assessed parameters were: types (ceramic, glass, or a mix (Lysing Matrix E from MP Biomedicals)) and sizes (0.4 to 2 mm) of beads, suspension solution (0.05% SDS, IMDM growth media), time of agitation, and instrument for agitation (vortex, TissueLyser). However, considering the high variability observed using vortex (data not shown), the TissueLyser was selected for further optimization (Fig. S1,).

Overall, irrespective of the time of TissueLyser agitation and of the suspension solution (0.05% SDS and IMDM growth media), less than 40% of sporocysts were released with ceramic and glass beads. A higher percentage was obtained following agitation with small glass beads (425-600 µm) for 30 s in 0.05% SDS and with larger glass beads (2 mm) for 3 min in 0.05% SDS. However, in both conditions, the inter-assay variability was high (11%). A mechanical disruption with TissueLyser associated with Lysing Matrix E tube led to the release of more than 25% of sporocysts irrespective of the agitation time. The highest percentage rate was obtained after 30 s in IMDM growth media with 84% ± 14% of released sporocysts. Hence, a 30 s agitation of oocysts for 30 s in the Lysing Matrix E tube in IMDM growth media was condition retained for further sporocyst-CC-qPCR experiments.

CC-qPCR based on the infection of cells challenged with *T. gondii* sporocysts

The optimal contact time required for the sporocysts to naturally excyst and release sporozoites and for the sporozoites to penetrate the cells was determined *in vitro*. To that aim, Vero cells and sporocysts were left in contact for 2 hours or 1 day (contact time before washing (D0, Fig. 1A and B). Irrespective of the contact time, *T. gondii* DNA was systematically detected by qPCR in total DNA extracted from the cell pellet. These results suggest that the two tested contact times were sufficient to allow some sporozoites to penetrate into the cells and/or that some sporocysts remained stuck to the surface of Vero

169 cells, even after washing. Then, the ability of the sporozoites to differentiate into replicative
170 tachyzoites in Vero cells was assessed by qPCR following two to six days (D2 to D6) of
171 culture. When cell culture was stopped at D2 or D3 following a contact time of 2 h (Fig. 1A),
172 Cq values did not vary significantly (p value > 0.05), suggesting that no or few tachyzoites
173 was multiplied within the cells. But after six days of cell culture, Cq significantly decreased
174 compared to D0 (CqD0 – CqD6 = 7.6, p value < 0.05) demonstrating the presence of
175 replicative tachyzoites (Fig. 1A).
176 When the contact between cells and sporocysts was lengthened to one day, qPCR signal
177 decreased from 25.51± 0.70 at D0 to 23.03 ± 0.95 95 at D2, and 20.40 ± 1.17 at D3 of culture.
178 Although increasing the time of culture resulted in a larger decrease of Cq values (CqD0 –
179 CqD6 = 6.2; p-value < 0.05),), we selected the protocol allowing the detection of infectious
180 oocysts within four days (one day contact time + three days cell culture) as an optimum. This
181 assay was able to specifically detect infective oocysts as demonstrated by the absence of
182 qPCR signal reduction following contact of cells with heat inactivated oocysts (Fig. 1B).
183 In order to assess the effect of the TissueLyser method on the infective potential of
184 sporocysts, free sporocysts were stored for one and five months in PBS at 4°C (Fig. 2). The
185 cell culture was stopped at D6 to be in the most favorable condition to observe parasite
186 infectivity. Irrespective of storage time, Cq decrease was similar between D0 and D6 (CqD0 –
187 CqD6 = 6, Fig. 2). Thus, mechanical agitation by TissueLyser did not alter the infectivity of
188 sporocysts stored up to five months, demonstrating the robustness of the sporocyst stage
189 compared to sporozoites which are very fragile.
190
191 **Limit of detection of the *T. gondii* sporocyst-based CC-qPCR assay in simple matrix.**
192 The protocol allowing the detection of infective oocysts within the shortest time, i.e. four days
193 (one day contact time + three days cell culture), was selected for further characterization. A

serial dilution ranging from 10^1 to 10^4 oocysts was used to prepare sporocysts for Vero cells infection using the protocol selected above. Ten infective oocysts were consistently detected (3/3 positive replicates at Day 3, Table 1) indicating that the limit of detection of the method was less than 10 oocysts. The linear correlation between the initial number of infectious parasites and the Cq values from 10 000 to 100 ($R^2 > 0.99$) suggested that this model could be quantitative in this range and that the limit of quantification was between 10 and 100 oocysts.

Assessing infectivity of heat-treated *T. gondii* oocysts by CC-qPCR and bioassay.

To be reliable, the measure of infectivity by sporocyst-based CC-qPCR needs to correlate with bioassay which is the reference method to detect infective *T. gondii* oocysts. To address this question, oocysts were inactivated by heating for 5 min at 99°C, 2 min at 80°C, or 2 min at 60°C. The two latter treatments were selected because they were shown to lead to absence of correlation between animal bioassays and viability method based on reverse transcriptase-qPCR (47). However, the developed sporocyst-based CC-qPCR showed a valid correlation with mouse bioassays irrespective of the applied heat treatment (Table S1). Indeed, untreated oocysts were infectious using both methods while no tachyzoites were detected by sporocyst-based CC-qPCR and mice remained seronegative for *T. gondii*. Hence, the proposed sporocyst-based CC-qPCR assay showed a good agreement with bioassay to determine the infectivity of oocysts exposed to heat treatments and is able to specifically detect infective oocysts.

Potential of the CC-qPCR assay to determine the infectivity of *T. gondii* oocysts in spiked mussels (*M. edulis* and *D. polymorpha*).

To determine the infectivity of oocysts in mussel matrices, some adjustments of the sporocyst-based CC-qPCR assay were required (see details in material and methods). First,

the oocysts recovered from *M. edulis* and *D. polymorpha* were purified using a Percoll gradient (30%) combined with a sodium hypochlorite treatment, prior to sporocyst release. Second, in order to avoid cell culture contamination due to mussel tissues, cell infection was performed in 24-well plates to dilute mussel tissue homogenates, and the contact time between sporocysts and cells was reduced to three hours instead of one day.

The kinetics of Vero cell infection with sporocysts isolated from *M. edulis* and *D. polymorpha* homogenates are presented in Figure 3. For *D. polymorpha*, a significant decrease of Cq was observed from 62 h (p-value <0.05) and was maximal after six days (CqD0-CqD6 = 8.52). For *M. edulis*, the decrease was remarkable after three days of culture (D3) and was maximal after six days (ΔCq = 9.3). In order to detect, infectious oocysts in mussel matrix as quickly as possible, a culture time of D3 was retained for both mussel matrices. Then, the limit of detection of the sporocyst-based assays to detect infective parasite in both mussel species was determined (Table II). To that aim, mussel tissues were spiked with a number of oocysts ranging from 10 to 10^4, and then processed as described in material methods to recover oocysts and to release sporocysts before infecting cell cultures. For both mussels, as low as 10 infective oocysts initially spiked to tissues led to parasite multiplication and cell infection within three days (Cq = 24.1 for *M. edulis* and Cq = 19.44 for *D. polymorpha*, Table II). These results indicated that the limit of detection of this sporocyst-based CC-qPCR was below 10 oocysts. However, the Cq values obtained at D3 were not correlated to the initial amount of oocysts spiked on tissues ($r^2 \leq 0.81$, Table II) suggesting this *in vitro* model could not be quantitative.

Discussion

The waterborne transmission route of *T. gondii* to humans via the dissemination of oocysts through surface and drinking waters and its epidemiological impact are now more significant

than previously believed (4, 7, 48, 49). Up to 2010, *T. gondii* was the etiologic agent in seven reported waterborne outbreaks in Brazil, Panama, Canada, French Guiana, and India (7, 9). The parasite has been detected in surface water bodies used for recreation, commercially harvested shellfish generally consumed raw or undercooked (see review 40, 50) and for drinking water that could infect humans. Moreover, contrary to running water, using filter-feeding attached organisms makes the contamination measurement representative of the sample or exposure site. Measurements in biological matrices (mussels) could i) limit the variability (temporal integration) of measurements compared to those taken in water, and ii) provide more reliable data on the degree of contamination of water bodies, thus facilitating their comparison. The continuous improvement of methods to detect *T. gondii* oocysts could help to determine the prevalence of the parasite in different environmental samples and to study a large spatial scale (freshwater-seawater continuum), marine and seawater invertebrates could be tested at the same time.

In order to assess the human health risk linked to *T. gondii* oocysts, information is required on parasite viability. Viability assays postulate that only viable oocysts can lead to host infection. However, viable parasites are not all necessarily infectious and consequently such assays can lead to an overestimation of the risk. This is particularly the case of non-sporulated *T. gondii* oocysts that are non-infectious while being viable. The current method used to assess oocyst infectivity is bioassay, which is considered the gold standard. However, as well as being expensive, there are ethical concerns related to the use of animal models. Cell culture assays have been described as promising alternative methods in terms of cost and response delay. However, although cell culture combined with qPCR has been largely described for *C. parvum* oocysts (38, 39, 42, 43, 51), only a few studies have described cell culture from *T. gondii* oocysts. They are based on sporozoite-host cell co-culture and the subsequent detection of proliferating parasites by qPCR (44, 45). Moreover, no study has reported CC-qPCR assays

with the objective of detecting small amounts of *T. gondii* oocysts in conditions closed to the natural contamination of environmental samples (31). Here we describe a cell culture assay based on *T. gondii* sporocysts, which is suitable for environmental applications in terms of performance and implementation.

Several protocols have been described for the excystation of *T. gondii* oocysts. The effectiveness of the sporozoite releasing methods represents a critical step. Given the robust nature of the oocyst wall (52), protocols usually lie on its mechanical breaking to allow release of the sporocysts. Then, sporozoite excystation is achieved by incubating sporocysts with biliary salt suspension. We tested different protocols to break the oocyst wall while keeping the sporocyst wall intact (44, 45, 53). Here, TissueLyser combined to Lysis Matrix E gave the best results.

To obtain the best conditions opening the sporocyst wall, several studies used bile salt or sodium taurocholate treatment associated or not with Na_2CO_3 and medium saturated with CO_2 (54, 55). However, we observed significant sporozoite loss, huge mortality and no reproducible protocols because of sporozoite fragility (data not shown). This has already been described for *Isospora suis* (56) but not corroborated with the release of 80.6% sporozoites (45). As no concluding results were obtained for the sporozoite release step, we tested the ability of sporocysts to naturally excyst in cell culture at 37°C. Only mechanical grinding with TissueLyser (30 s) associated with Lysing Matrix E tube in IMDM growth media achieved the release of 84 ± 14% of sporocysts. However, this percentage of released sporocysts is probably underestimated because 10%-15% of oocysts never sporulate (32). This method can be easily implemented in laboratories and can be applied to oocysts isolated from environmental or food samples as mussels.

We checked that the TissueLyser did not alter the infectivity of the sporocysts in cell culture in water after prolonged storage (up to 5 months) in water and in mussel matrix. In order to

294 determine the optimal conditions to measure the infectivity, two parameters were studied: (i)
295 the contact time between the sporocysts and cells that is required for the release of sporozoites
296 and cell infection; (ii) the time of cell culture that is required to measure parasite
297 multiplication by qPCR.
298 In water, infective oocysts could be detected following one day of contact between sporocysts
299 and cells, and two days of cell culture, demonstrating that sporocysts were able to excyst, to
300 release sporozoites that could eventually invade cells and differentiate into replicative
301 tachyzoites in three days. However, the optimal sporocyst-based CC-qPCR condition was
302 established at three days of cell culture following one day of contact, allowing a large and
303 significant parasite multiplication in the shortest time (i.e. four days). Our results also
304 demonstrated that mechanical grinding with TissueLyser combined to Lysis Matrix E did not
305 damage fresh sporocysts, as well as stored sporocysts (up to five months at 4°C) that were
306 shown to still be able to induce cell infection. Using this four days sporocyst-based CC-qPCR
307 assay, the time-to-response was divided by 2.5, relative to the 10 days usually described for
308 cell-culture based on sporozoites infection (44, 45). Obviously, this sporocyst-based CC-
309 qPCR assay also represented a significant improvement compared to animal models (three
310 weeks). Reducing the time contact between the sporocysts and the cells to two hours led to an
311 increase of the time-to-response, with the requirement of six days of cell culture to
312 significantly detect the parasite multiplication. The limit of detection of sporocyst-based CC-
313 qPCR assay was less than 10 oocysts in water that makes it suitable for environmental
314 applications where the oocyst level of contamination can be low. Moreover, a linear
315 correlation between the initial number of infective oocysts (10^1 to 10^4) and the Cq values
316 obtained after 3 days of cell culture was observed suggesting that this approach could be used
317 to assess treatment efficacy in water.

318 To obtain a reliable assessment of the exposure of humans to infective oocysts, the proposed
319 approach needs to correlate with infectivity measure using the reference method, i.e. animal
320 models. Heat treatments that showned correlation between bioassays and viability method
321 based on reverse transcriptase(RT)-qPCR at 5 min 99°C, but not at 2 min 80°C or 2 min 60°C
322 (47), were applied to oocysts. Irrespective of the treatment, no parasite multiplication could be
323 detected using the sporocyst-based CC-qPCR assay, indicating that this approach was able to
324 specifically detect infective oocysts. Moreover, results from the sporocyst-based CC-qPCR
325 assay were in agreement with bioassay, indicating that this approach is as reliable as animal
326 models, to assess the exposure of humans or mammals to infective oocysts.
327 The sporocyst-based CC-qPCR developed in water matrix was adapted to mussel matrices (*M.*
328 *edulis* and *D. polymorpha*). Percoll gradient purification has been yet used on clams but also
329 on other types of matrix such as meat, cheese, eggs, fish (59). We tested a 30% Percoll
330 gradient purification step for eliminating most of the mussel matrix contaminants and sodium
331 hypochlorite for removing bacteria and we observed the infectivity of *T. gondii* oocysts in
332 mussel matrices. So percoll did not alter *T. gondii* oocyst infectivity as shown for *C. parvum*
333 oocysts (60) and seems therefore compatible with sporocyst-CC-qPCR on a mollusk matrix.
334 In order to avoid fungi growth during cell culture, a 24-well plate format assay was used to
335 dilute the mussel matrices still present after Percoll separation and sodium hypochlorite
336 treatment, and the contact time between sporocysts and cells had to be reduced to 3 hours (vs.
337 1 day for oocysts in water). Longer culture times enhanced detection of infectious parasites in
338 mussel matrices, i.e. 62 and 72 hours of cell culture for respectively *D. polymorpha* and *M.*
339 *edulis*. These results are consistent with previous observations for *C. parvum* oocysts, that
340 highlighted that increasing the culture time to 72h led to higher levels of infection (62). With
341 the objective of using the sporocyst-based CC-qPCR assay in monitoring application, the
342 assay allowing the significant detection of infective oocysts at high level within three days

(three hours contact between sporocysts and cells + three days of cell culture) was selected. This method was able to detect less than 10 infective *T. gondii* oocysts in both *M. edulis* and *D. polymorpha* with a result in only 3 days. In comparison, *in vivo*, the limit of detection on the *Mytella guyanensis* mussel was between 10 and 100 *T. gondii* oocysts with a result obtained after eight weeks (12). Nevertheless, the sporocyst-based CC-qPCR appeared to be not quantitative in mussels indicating that it could not be used to study the efficiency of industrial processes in these matrices. But, considering that a single oocyst is sufficient to cause infection/disease (in some hosts), rather than quantification, a low level of detection is crucial in the context of risk assessment. Thus, the sporocyst-based CC-qPCR method represents a promising alternative to bioassays to detect infective *T. gondii* oocysts in *M. edulis* and *D. polymorpha* in environmental monitoring.

To conclude, we have developed a laboratory-accessible method based on Vero cell infection using sporocysts cell culture combines with qPCR detection, allowing the specific characterization of less than 10 infective oocysts in water (four days) and in mussels (three days). This new approach, which correlates with bioassays, is relevant to assess the exposure to infective *T. gondii* oocysts in water, by direct analyses of water samples, but also mussels as biosentinels for a more representative characterization of the risk. Overall, this tool appears to be of benefit to the food/water industries to help them to assess the health risk associated to the presence of infective oocysts.

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362 **Materials and methods**

363

364 ***T. gondii* oocysts**

365 Oocysts of type III strain VEG were produced as described previously (46) containing 87.2%
366 of sporulated oocysts, provided in H_2SO_4 aqueous solution (2%) and stored at 4°C until use.
367 Before experiments, oocysts were washed three times in sterile distilled water (dH_2O) to
368 remove sulfuric acid. The concentrations of parasite suspensions used for experiments were
369 calibrated by counting oocysts in sodium dodecyl sulfate (SDS) aqueous solution (0.5%) on
370 Kova Slide (Kova® Slide 10) using a phase contrast microscope (Axioskop 40, Zeiss).
371 Oocysts used throughout this study were aged less than 14 months and their viability was
372 controlled before use by RT-qPCR targeting the *sporo-SAG* gene as previously described
373 (31). Oocysts were inactivated by heating in dry heating block as followed: 5 min at +99°C, 2
374 min at +80°C and 2 min at +60°C. To minimize experimental variability, each treatment
375 condition was tested the same day for CC-qPCR and mouse bioassay. Three aliquots were
376 dedicated to bioassays and six to CC-qPCR analyses.

377

378 **Release of sporocysts**

379 To optimize the release of sporocysts, 10^4 oocysts in IMDM growth media (see below) or
380 0.05% SDS (qsp 350 µL) were subjected to mechanical agitation using vortex (33 rpm) and
381 TissueLyser (QIAGEN) at 33 Hz. Different sizes and types of beads were tested (2 mm or
382 425-600 µm in glass, 1.2 mm in ceramic or using a Lysing Matrix E tube from MP
383 Biomedicals that contains 1.4 ceramic spheres, 0.1 mm silica spheres, and one 4 mm glass
384 bead), as well as different beating times (0 sec, 30 sec, 1 min, 2 min, 3 min or 5 min). The
385 respective number of free sporocysts and oocysts was counted in 18 Kova slide cell count. To
386 calculate the percentage of released sporocysts (= number of sporocysts observed / number of

theoretical sporocysts), we considered that 10^4 oocysts corresponded to 2.10^4 sporocysts assuming that 100% oocysts were sporulated and contained each two sporocysts. Each experiment was performed in single or duplicate.

Sporocyst-based cell culture infection assay for *T. gondii* oocysts in simple matrix

Vero cell line (ATCC, CCL-81) was used to support the development of *T. gondii* parasites from sporocysts. Cells were maintained at 37°C in IMDM growth media containing glutaMAX^{TM} (ThermoFisher, USA) supplemented with 5% (v/v) of heat-inactivated fetal bovine serum (Eurobio, France), 100 µg/mL of streptomycin (Gibco®) and 100 µg/mL of penicillin (Gibco®). Cells were grown to 80% confluence on 75 cm² culture flask (VWR, Canada) in 5% CO_2 atmosphere at 37°C. Once confluent, cells were washed with pH 7.4 PBS and trypsinized to remove the cell monolayer from the flask. About 2.10^5 cells were seeded into each well of 96-well culture plates and grown in medium during 24 h as indicated above. The sporocysts obtained from 10^4 oocysts in cell culture medium were deposited on the confluent monolayer allowing the sporozoites to be spontaneously released and to invade cells for 2 h or 1 day. After the contact time, cells were washed to remove the parasites that had not penetrated the cells and considered as “none infective” (D0). Then, cells were cultivated for 2 (D2), 3 (D3) or 6 days (D6) at 37°C allowing conversion from sporozoites to tachyzoites and their detection by qPCR. Control culture was incubated alone without sporocysts. A serial dilution of oocysts in cell culture medium, ranging from 10 to 10^4, was used to estimate the limit of detection of the sporocyst-based CC-qPCR assay in simple matrix. For each dilution, three independent experiments were performed. The limit of detection was defined as the lowest quantity of oocysts that led to qPCR signal in all the replicates.

Sporocyst-based cell culture infection assay for *T. gondii* oocysts recovered from mussels

412 The seawater blue mussels (4–5 cm shell length), *Mytilus edulis*, were collected on the
413 intertidal rocky shore of Yport (Seine-Maritime, France). The freshwater Zebra mussels,
414 *Dreissena polymorpha*, were collected at about 5 m depth in November at the Lac-du-Der-
415 Chantecoq (Marne, France). Whole mussels were frozen before being sliced with a scalpel.
416 Then, tissues from one mussel were introduced in stomacher bags (Bagpage R400,
417 Interscience, Saint-Nom-la-Bretèche, France) and then spiked with 10^4 oocysts for infection
418 kinetics characterization or with different amounts of oocysts (ranging from 10 to 5.10^4) to
419 estimate the limit of detection of the sporocyst-based CC-qPCR assay. Each spiking was
420 performed in triplicates. A negative control was performed without oocysts. Each stomacher
421 bag was incubated during 2 h at room temperature and then overnight at 4°C. Mussel tissue
422 digestion was performed using 1X Trypsin (Thermo Fisher Scientific, Villebon-sur-Yvette,
423 France) for 1h30 at 37°C at 90 rpm agitation. Filtrates were collected and 25 mL of NaCl
424 0.9% were added to wash the stomacher bag. Samples were washed twice at 2 500g +10°C
425 during 10 min. The pellets were suspended with 1.5 mL of Percoll 30% (density 1.04). After 5
426 min at 12 000 g, 1 mL of supernatant was discarded and 1 mL of NaCl 0.9% was added to
427 break the Percoll gradient. Samples were then exposed to household bleach containing 1.6%
428 sodium hypochlorite during 10 min at 4°C to prevent bacterial contamination. The oocysts
429 were then washed twice at 5 000 g during 5 min in IMDM growth media to remove bleach
430 before sporocyst release using Lysing Matrix E tube and agitation for 30 s with TissueLyzer.
431 The pellet was resuspended in culture medium and inoculated into wells of a 24-well culture
432 plate, containing each, a monolayer of 1.10^6 Vero cells established as indicated above. After
433 three hours of contact between sporocysts and cells, cells were washed (D0) and then cultured
434 for 62 h, three (D3), and six days (D6). A serial dilution of oocysts ranging from 10 to 5.10^4
435 was used to spike mussels to estimate the limit of detection of CC-qPCR infection assay in

436 mussels which was defined as the lowest quantity of oocysts that led to qPCR signal in all the
437 replicates.
438
439 **DNA extraction and real time qPCR**
440 Using the QIAamp DNA Mini kit (QIAGEN), the supernatant was discarded from each well
441 and cells were suspended in 180 µL of the ATL buffer and 20 µL of proteinase K. The
442 suspension was transferred into a 1.5 mL Eppendorf tube and the sample was processed
443 further for DNA extraction by following the manufacture-recommended procedures.
444 The 529 bp-repeat element was targeted using previously described primers and probe (62).
445 qPCR was performed using a SimpliAmp™ Thermal Cycler (ThermoFisher, Scientific Inc,
446 Villebon-sur-Yvette, France) in a final volume of 25 µL containing 12.5 µL of iQ™ Supermix
447 (Bio Rad, Marnes la Coquette, France), 1 µL of BSA 10 mg/mL (SIGMA, France), 1 µL of
448 each primer 20 µM (forward, 5'-AGAGACACCGGAATGCGATCT-3'; reverse, 5'-
449 CCCTCTTCTCCACTCTTCAATTCT-3'), 0.5 µL of probe 10 µM (5' FAM-
450 ACGCTTTCCTCGTGGTGATGGCG-3' BHQ1), 5 µL of DNA template and 4 µL of
451 DNAse-RNAse free water. Each DNA extract was tested in duplicate. The cycling parameters
452 included a denaturation step at +95°C for 3 min followed by 40 cycles at +95°C for 15 s and
453 +65°C for 1 min. A decrease of Cq (quantification cycle) values between D0 and the end of
454 culture (water matrix: D2, D3, and D6; mussel matrices: 62 h, D2, D3 or D6) highlighted the
455 infectivity of the parasites and a signal difference (Cq_{D0} - $Cq_{end\ culture}$) was calculated.
456
457 **Mouse bioassay for infectivity**
458 As described in a previous study (11), 10^3 *T. gondii* oocysts were inoculated in outbreed
459 female Swiss Webster mice (Charles River Laboratory, Neuilly-sur-Seine, France) weighing
460 20-30 g. Each mouse was intraperitoneally inoculated as this procedure allows control of the

dose of inoculation. Mice were housed in cages providing granules and water *ad libitium* and tested for *T. gondii* seroconversion with the modified agglutination test (MAT) four weeks post-inoculation (11).

Statistical analysis

The Cq values obtained in qPCR were compared using the non-parametric Kruskal Wallis test. If the null hypothesis H_0 (the tested conditions have no effect on the measured value) was rejected, then the post hoc Wilcoxon-Mann-Whitney test was applied for two independent samples. All statistical tests were performed using StatXact7. Statistical difference was considered as p value < 0.05.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

488 REFERENCES

489 1. AFSSA, 2005. Toxoplasmose : état des connaissances et évaluation du risque lié à
490 l'alimentation – Rapport du groupe de travail "*Toxoplasma gondii*".

491 2. Belluco S, Mancin M, Conficoni D, Simonato G, Pietrobelli M, Ricci A. 2016.
492 Investigating the determinants of Toxoplasma gondii prevalence in meat: a systematic
493 review and meta-regression. PLoS One 11: e0153856.
494 https://doi.org/10.1371/journal.pone.0153856

495 3. Condoleo R, Rinaldi L, Sette S, Mezher Z. 2018. Risk Assessment of human
496 toxoplasmosis associated with the consumption of pork meat in Italy. Risk Analysis
497 38 :1202-1222. https://doi.org/10.1111/risa.12934

498 4. Jones JL, Dubey JP. 2010. Waterborne toxoplasmosis-recent developments. Exp
499 Parasitol 124:10–25. https://doi.org/10.1016/j.exppara.2009.03.013

500 5. Dumètre A, Dardé ML, 2003. How to detect *Toxoplasma gondii* oocysts in
501 environmental samples? FEMS Microbiol Rev 27 :651–661.
502 https://doi.org/10.1016/S0168-6445(03)00071-8

503 6. Lindsay DS, Holliman D, Flick GJ, Goodwin DG, Mitchell SM, Dubey JP. 2008.
504 Effects of high pressure processing on *Toxoplasma gondii* oocysts on raspberries. J
505 Parasitol 94:757–758. https://doi.org/10.1645/GE-1471.1

506 7. Baldursson S, Karanis P. 2011. Waterborne transmission of protozoan parasites:
507 Review of worldwide outbreaks _ An update 2004–2010. Water Research 45:6603–
508 6614. https://doi.org/10.1016/j.watres.2011.10.013

509 8. Meireles LR, Ekman CC, Andrade HF, Luna EJ. 2015. Human toxoplasmosis
510 outbreaks and the agent infecting form finding from a systematic review. Rev Inst
511 Med Trop Sao Paulo. 57:369-376. https://doi.org/10.1590/S0036-46652015000500001

9. Karanis P, Kourenti C, Smith H. 2007. Waterborne transmission of protozoan parasites: a worldwide review of outbreaks and lessons learnt. J Water Health 5:1-38.

10. Plutzer J, Karanis P. 2016. Neglected waterborne parasitic protozoa and their detection in water. Water Res 101:318-332. https://doi.org/10.1016/j.watres.2016.05.085

11. Villena I, Aubert D, Gomis P, Ferté H, Inglard JC, Denis-Bisiaux H, Dondon JM, Pisano E, Ortis N, Pinon JM. 2004. Evaluation of a strategy for *Toxoplasma gondii* oocyst detection in water. Appl Environ Microbiol 70:4035–4039. https://doi.org/10.1128/AEM.70.7.4035-4039.2004

12. Esmerini PO, Gennari SM, Pena HF. 2010. Analysis of marine bivalve shellfish from the fish market in Santos city, São Paulo state, Brazil, for *Toxoplasma gondii*. Vet Parasitol 170:8-13. https://doi.org/10.1016/j.vetpar.2010.01.036

13. Lass A, Pietkiewicz H, Szostakowska B, Myjak P. 2012 ; The first detection of *Toxoplasma gondii* DNA in environmental fruits and vegetables samples. Eur J Clin Microbiol Infect Dis. 6:1101-8. doi: 10.1007/s10096-011-1414-8.

14. Daniels ME, Hogan J, Smith WA, Oates SC, Miler MA, hardin D, Shapiro K, Huertos ML, Conrad PA, Dominik C, Watson FGR. 2014. Estimating environmental conditions affecting protozoal pathogen removal in surface water wetland systems using a multi-scale, model-based approach. Sci Total Environ 493: 1036-1046. https://doi.org/10.1016/j.scitotenv.2014.06.053

15. Roslev P, Bukh AS, Iversen L, Sonderbo H, Iversen N. 2010. Application of mussels as biosamples for characterization of faecal pollution in coastal recreational waters. Water Sci Technol 62 :586-593. https://doi.org/10.2166/wst.2010.910

16. Grodzki M, Schaeffer J, Piquet J-C, Le Saux J-C, Chevé J, Ollivier J, Le Pendu J, Le Guyader FS. 2014. Bioaccumulation efficiency, tissue distribution, and environmental

536 occurrence of hepatitis e virus in bivalve shellfish from France. Appl Environ
537 Microbiol 80:4269–4276. https://doi.org/10.1128/AEM.00978-14
538 17. Ayres PA, Burton HW, Cullum ML. 1978. Sewage pollution and shellfish. In
539 Techniques for the study of mixed populations. Society for applied bacteriology
540 technical series Number 11 ed. Lovelock DM and Davies R. pp 51-62. London
541 Academic Press.
542 18. Graczyk TK, Conn DB, Marcogliese DJ, Graczyk H, De Lafontaine Y. 2003.
543 Accumulation of human parasites by zebra mussels (*Dreissena polymorpha*) and
544 Asian freshwater clams (*Corbicula fluminea*). Parasitol Res 89:107-112.
545 https://doi.org/10.1007/s00436-002-0729-x
546 19. Robertson, L.J., Gjerde, B., 2008. Development and use of a pepsin digestion method
547 for analysis of shellfish for *Cryptosporidium* oocysts and *Giardia* cysts. J Food Protect
548 71:959-966.
549 20. Palos Ladeiro M, Aubert D, Villena I, Geffard A, Bigot A. 2014. Bioaccumulation of
550 human waterborn protozoa by zebra mussel (*Dreisseina polymorpha*): interest for
551 water biomonitoring. Water Res 48:148-155.
552 https://doi.org/10.1016/j.watres.2013.09.017
553 21. Aksoy U, Marangi M, Papini R, Ozkoc S, Bayram Delibas S, Giangaspero A. 2014.
554 Detection of *Toxoplasma gondii* and *Cyclospora cayetanensis* in *Mytilus*
555 *galloprovincialis* from Izmir Province coast (Turkey) by real time PCR/ high-
556 resolution melting analysis (HRM). Food Microbiol 2 :128-135.
557 https://doi.org/10.1016/j.fm.2014.05.012
558 22. Shapiro K, Van Wormer E, Aguilar B, Conrad PA. 2015. Surveillance for *Toxoplasma*
559 *gondii* in California mussels (*Mytilus californianus*) reveals transmission of atypical

genotypes from land to sea. Environ Microbiol 17:4177-4188. https://doi.org/10.1111/1462-2920.12685

23. Staggs SE, Keely SP, Ware MW, Schable N, See MJ, Gregorio D, Zou X, Su C, Dubey JP, Villegas E. 2015. The development and implementation of a method using blue mussels (*Mytilus sp*.) as biosentinels of *Cryptosporidium spp*. and *Toxoplasma gondii* contamination in marine aquatic environments. Parasitol Res 114:4655-4667. https://doi.org/10.1007/s00436-015-4711-9

24. Kerambrun E, Palos Ladeiro M, Bigot-Clivot A, Dedeourge-Geffard O, Dupuis E, Villena I, Aubert D, Geffard A. 2015. Zebra mussel as a new tool to show evidence of freshwater contamination by waterborne *Toxoplasma gondii*. J Appl Microbiol 120:498-508. https://doi.org/10.1111/jam.12999

25. Lindsay DS, Phelps KK, Smith SA, Flick G, Sumner SS, Dubey JP. 2001. Removal of *Toxoplasma gondii* oocysts from sea water by eastern oysters (*Crassostrea virginica*). J Eukaryot Microbiol Supp:197S-198S.

26. Arkush KD, Miller MA, Leutenegger CM, Gardner IA, Packham AE, Heckeroth AR, Tenter AM, Barr BC, Conrad PA, 2003. Molecular and bioassay-based detection of *Toxoplasma gondii* oocyst uptake by mussels (*Mytilus galloprovincialis*). Int J Parasitol 33:1087-1097. https://doi.org/10.1016/S0020-7519(03)00181-4

27. Lindsay DS, Dubey JP. 2009. Long-term survival of *Toxoplasma gondii* sporulated oocysts in seawater. J Parasitol 95:1019-1020. https://doi.org/10.1645/GE-1919.1

28. Giangaspero, A., R. Cirillo, V. Lacasella, A. Lonigro, M. Marangi, P. Cavallo, F. Berrilli, D. Di Cave, and O. Brandonisio. 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

584 29. Tedde T, Marangi M, Papini R, Salza S, Normanno G, Virgilio S, Giangaspero A.
585 2019. *Toxoplasma gondii* and Other Zoonotic Protozoans in Mediterranean Mussel
586 (*Mytilus galloprovincialis*) and Blue Mussel (*Mytilus edulis*): A Food Safety Concern?
587 J Food Prot 27:535-542. https://doi.org/10.4315/0362-028X.JFP-18-157
588 30. Garcés G, Effenberger M, Najdrowski M, Wackwitz C, Gronauer A, Wilderer PA,
589 Lebuhn M. 2006. Quantification of *Cryptosporidium parvum* in anaerobic digesters
590 treating manure by (reverse-transcription) quantitative real-time PCR, infectivity and
591 excystation tests. Water Sci Technol 53:195-202
592 31. Rousseau A, La Carbona S, Dumètre A, Robertson LJ, Gargala G, Escotte-Binet S,
593 Favennec L, Villena I, Gérard C, Aubert D. 2018. Assessing viability and infectivity
594 of foodborne and waterborne stages (cysts/oocysts) of *Giardia duodenalis*,
595 *Cryptosporidium* spp., and *Toxoplasma gondii*: a review of methods. Parasite 25:14.
596 https://doi.org/10.1051/parasite/2018009.
597 32. Rousseau A, Villena I, Dumètre A, Escotte-Binet S, Favennec L, Dubey JP, Aubert D,
598 La Carbona S. 2019. Evaluation of propidium monoazide-based qPCR to detect viable
599 oocysts of *Toxoplasma gondii*. Parasitol Res 118:999-1010.
600 https://doi.org/10.1007/s00436-019-06220-1
601 33. Smith JJ, Gunasekera TS, Barardi CRM, Veal D, Vesey G. 2004. Determination of
602 *Cryptosporidium parvum* oocyst viability by fluorescence in situ hybridization using a
603 ribosomal RNA-directed probe. J Appl Microbiol 96:409–417.
604 https://doi.org/10.1046/j.1365-2672.2004.02150.x
605 34. Habtewold T, Groom Z, Duchateau L, Christophides GK. 2015. Detection of viable
606 Plasmodium ookinetes in the midguts of *Anopheles coluzzi* using PMA-qrtPCR.
607 Parasites & Vectors 8:455. https://doi.org/10.1186/s13071-015-1087-8

608 35. Kniel KE, Lindsay DS, Sumner SS, Hackney CR, Pierson MD, Dubey JP. 2002.
609 Examination of attachment and survival of *Toxoplasma gondii* oocysts on raspberries
610 and blueberries. J Parasitol 88:790-793. https://doi.org/10.1645/0022-
611 3395(2002)088[0790:EOAASO]2.0.CO;2
612 36. Grabow WO, Taylor MB, de Villiers JC. 2001. New methods for the detection of
613 viruses: call for review of drinking water quality guidelines. Water Sci Technol 43:1-
614 8.
615 37. Lee HK, Jeong YS. 2004. Comparison of total culturable virus assay and multiplex
616 integrated cell culture-PCR for reliability of waterborne virus detection. Appl Environ
617 Microbiol 70: 3632-3636. https://doi.org/10.1128/AEM.70.6.3632-3636.2004
618 38. Rochelle PA, Marshall MM, Mead JR, Johnson AM, Korish DG, Rosen JS, De Leon
619 R. 2002. Comparison of *in vitro* cell culture and a mouse assay for measuring
620 infectivity of *Cryptosporidium parvum*. Appl EnvironMicrobiol 68 :3809–3817.
621 https://doi.org/10.1128/AEM.68.8.3809-3817.2002
622 39. Jenkins M, Trout J, Higgins J, Dorsch M, Veal D, Fayer R. 2003. Comparison of tests
623 for viable and infectious *Cryptosporidium parvum* oocysts. Parasitol Res 89:1–5.
624 https://doi.org/10.1007/s00436-002-0720-6
625 40. Palos Ladeiro M, Bigot A, Aubert D, Hohweyer J, Favennec F, Villena I, Geffard A.
626 2013. Protozoa interaction with aquatic invertebrate: interest for watercourses
627 biomonitoring. Environ Sci Pollut Res 20:778–789. https://doi.org/10.1007/s11356-
628 012-1189-1
629 41. Parr JB, Sevilleja JE, Samie A, Alcantara C, Stroup SE, Kohli A, Fayer R, Lima AA,
630 Houpt ER, Guerrant RL. 2007. Detection and quantification of *Cryptosporidium* in
631 HCT-8 cells and human fecal specimens using real-time polymerase chain reaction.
632 Am J Trop Med Hyg 76:938-942.

42. Shahiduzzaman M, Dyachenko V, Keidel J, Schmäschke R, Daugschies A. 2010. Combination of cell culture and quantitative PCR (cc-qPCR) to assess disinfectants efficacy on *Cryptosporidium* oocysts under standardized conditions. Vet Parasitol 167:43–49. https://doi.org/10.1016/j.vetpar.2009.09.042

43. Johnson AM, Di Giovanni GD, Rochelle PA. 2012. Comparison of assays for sensitive and reproducible detection of cell culture-infectious *Cryptosporidium parvum* and *Cryptosporidium hominis* in drinking water. Appl Environ Microbiol 78:156–162. https://doi.org/10.1128/AEM.06444-11

44. Ware MW, Augustine SAJ, Erisman DO, See MJ, Wymer L, Hayes SL, Dubey, JP, Villegas EN. 2010. Determining UV inactivation of *Toxoplasma gondii* oocysts by using cell culture and a mouse bioassay. Appl EnvironMicrobiol 76:5140-5147. https://doi.org/10.1128/AEM.00153-10

45. Villegas EN, Augustine SAJ, Villegas LF, Ware MW, See MJ, Lindquist HDA, Schaefer III FW, Dubey JP. 2010. Using quantitative reverse transcriptase PCR and cell culture plaque assays to determine resistance of *Toxoplasma gondii* oocysts to chemical sanitizers. J Microbiol Methods 81:219–225. https://doi.org/10.1016/j.mimet.2010.03.023

46. Dubey JP, Lunney JK, Shen SK, Kwok OCH, Ashford DA. 1996. Infectivity of low numbers of *Toxoplasma gondii* oocysts to pigs. J Parasitol 82: 428-443.

47. Travaillé E, La Carbona S, Gargala G, Aubert D, Guyot K, Dumètre A, Villena I, Houssin M. 2016. Development of a qRT-PCR method to assess the viability of *Giardia intestinalis* cysts, *Cryptosporidium spp*. and *Toxoplasma gondii* oocysts. Food Control 59:359–365. https://doi.org/10.1016/j.foodcont.2015.06.007

48. Karanis K, Aldeyarbi HM, Mirhashemi EM, Khalil KM. 2013. The impact of the waterborne transmission of *Toxoplasma gondii* and analysis efforts for water

658 detection: an overview and updat. Environ Sci Pollut Res 20 :86–99.
659 https://doi.org/10.1007/s11356-012-1177-5
660 49. Efstratiou A, Ongerth JE, Karanis P. 2017. Waterborne transmission of protozoan
661 parasites: Review of worldwide outbreaks _ An update 2011-2016. Water Res 144:14–
662 22. https://doi.org/10.1016/j.watres.2017.01.036
663 50. Hohweyer J, Dumètre A, Aubert D, Azas N, Villena I. 2013. Tools and methods for
664 detecting and characterizing *Giardia*, *Cryptosporidium*, and *Toxoplasma* parasites in
665 marine mollusks. J Food Protect 9: 1488-1657.
666 https://doi.org/10.1016/j.fm.2016.01.002
667 51. Najdrowski M, Joachim A, Daugschies A. 2007. An improved in vitro infection model
668 for viability testing of *Cryptosporidium parvum* oocysts. Vet Parasitol 150 :150-154.
669 https://doi.org/10.1016/j.vetpar.2007.09.005
670 52. Dumètre A, Dubey JP, Ferguson DJ, Bongrand P, Azas N, Puech PH, 2013.
671 Mechanics of the *Toxoplasma gondii* oocyst wall. Proc Natl Acad Sci U S A.
672 110(28):11535-11540. doi: 10.1073/pnas.1308425110
673 53. Everson WV, Ware MW, Dubey JP, Lindquist HD. 2002. Isolation of purified oocyst
674 walls and sporocysts from *Toxoplasma gondii*. J Eukaryot Microbiol 49:344-349.
675 54. Kasper LH, Bradley MS, Pfefferkorn ER. 1984. Identification of stage-specific sporozoite
676 antigens of *Toxoplasma gondii* by monoclonal antibodies. J Immunol 132:443-449.
677 55. Freyre A, Falcon J. 2004. Massive excystation of *Toxoplasma gondii* sporozoites. Exp
678 Parasitol 107:72-77. https://doi.org/10.1016/j.exppara.2004.03.014
679 56. Lindsay DS, Current WL, Ernst JV. 1983. Excystation of *Isospora suis* Biester, 1934
680 of swine. Fûr Prasasitenkd 69:27-34.
681 57. Johnson LL. 1992. SCID mouse models of acute and relapsing chronic *Toxoplasma*
682 *gondii* infections. Infect Immun 60:3719–3724.

683 58. Wainwright KE, Lagunas-Solar M, Miller MA, Barr BC, Melli AC, Packham AE,
684 Zeng N, Truong T, Conrad PA. 2010. Radiofrequency-induced thermal inactivation of
685 *Toxoplasma gondii* oocysts in water. Zoonoses Public Health, 57:74-81.
686 https://doi.org/10.1111/j.1863-2378.2009.01280.x

687 59. Fukushima H, Katsube K, Hata Y, Kishi R, Fujiwara S. 2007. Rapid separation and
688 concentration of food-borne pathogens in food samples prior to quantification by
689 viable-cell counting and real-time PCR. Appl Environ Microbiol 73:92-100.
690 https://doi.org/10.1128/AEM.01772-06

691 60. Suresh P, Rehg JE. 1996. Comparative evaluation of several techniques for
692 purification of *Cryptosporidium parvum* oocysts from rat feces. J Clin Microbiol
693 34:38-40.

694 61. Di Giovanni GD, Lechevallier MW. 2005. Quantitative-PCR assessment of
695 *Cryptosporidium parvum* cell culture infection. Appl Environ Microbiol 71 :1495–
696 1500. https://doi.org/10.1128/AEM.71.3.1495-1500.2005.

697 62. Lélu M, Villena I, Dardé ML, Aubert D, Geers R, Dupuis E, Marnef F, Poulle ML,
698 Gotteland C, Dumètre A, Gilot- Fromont E. 2012. Quantitative estimation of the
699 viability of *Toxoplasma gondii* oocysts in soil. Appl Environ Microbiol 78:5127-5132.
700 https://doi.org/10.1128/AEM.00246-12

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Figure legends

Fig. 1: Kinetics of cell infection by *T. gondii* sporocysts in simple matrix assessed by sporocyst-CC-qPCR assay. Following 2 h (A) or 1 day (B) of contact between the sporocysts and Vero cells (contact time: D0), the cells were cultivated for 2 (D2), 3 (D3) or 6 days (D6). Sporocysts were obtained after TissueLyser agitation (30 s, 33Hz) from viable and heat inactivated oocysts (5 min at 99°C). The y axis indicates mean qPCR signal (Cq) and standart deviation (2<n<5 independent experiments). Negative control corresponds to Vero cells without sporocysts. Asterisks show significant differences (p-value < 0.05).

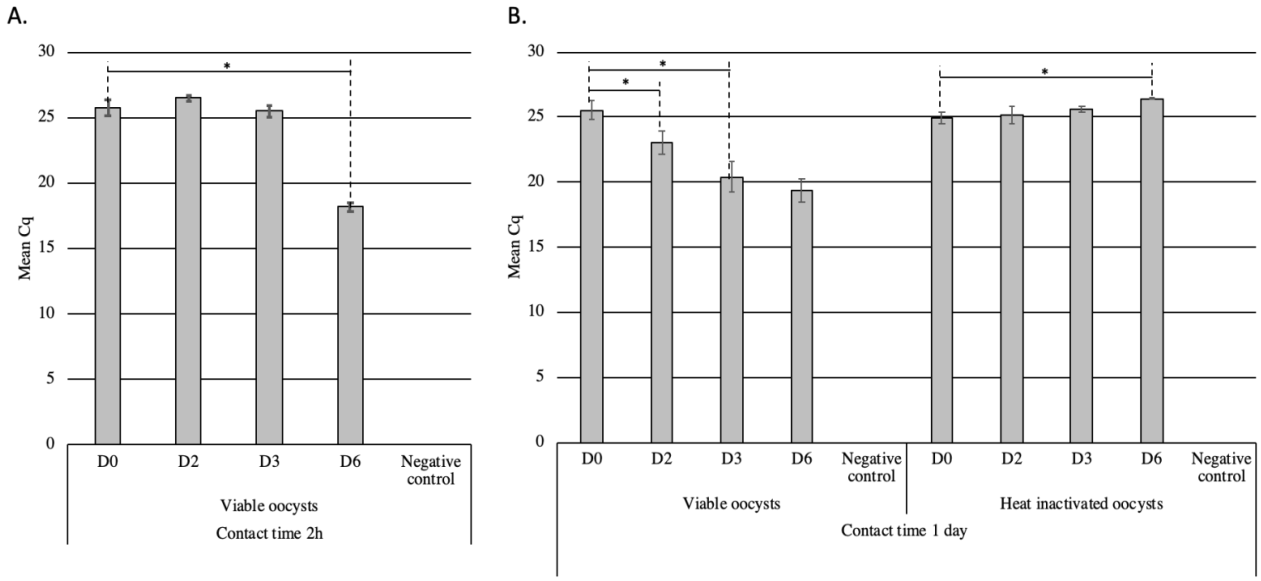
Fig. 2: Infectivity of *T. gondii* sporocysts after storage at 4°C assessed by sporocyst-CC-qPCR. After 1 day of contact between the sporocysts and Vero cells (contact time: D0), the cells were cultivated for 6 days (D6). Sporocysts were obtained after TissueLyser agitation (30 s, 33Hz) from viable oocysts and stored 0, 1 or 5 months at 4°C**.** The y axis indicates mean qPCR signal (Cq) (2 < n < 9 independent experiments). Asterisks show significant differences (p-value < 0.05).

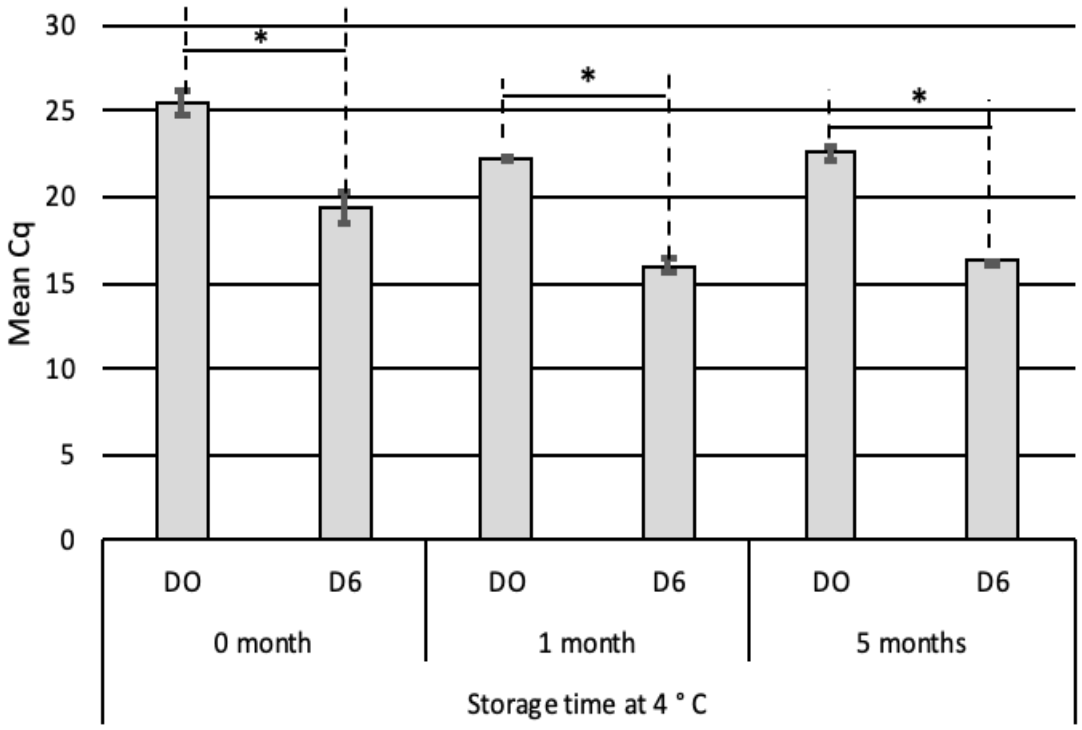
Fig. 3: Kinetics of cell infection by *T. gondii* sporocysts isolated from *D. polymorpha and M. edulis* assessed by sporocyst-CC-qPCR. Sporocysts were released from oocysts isolated from mussels and following 3 h of contact between the sporocysts and Vero cells (D0), the cells were cultivated for 62 h, 3 (D3) or 6 days (D6). The y axis indicates mean qPCR signal (Cq) (n=3 independent experiments). Asterisks show significant differences in Cq (p-value <0.05).

Table captions

Table I. Sensitivity of the *T. gondii* sporocyst-CC-qPCR assay to detect infective parasites in simple matrix. A serial dilution ranging from10 to 10 000 *T. gondii* oocysts was used to obtain sporocysts resuspended in culture medium and then deposited on Vero cells (80% confluence) incubated at 37°C. qPCR specific to *T. gondii* was performed from the cell pellet DNA extract after a contact time between sporozoites and cells of 1 day (D0) and after culture of the cells for 3 days (D3) (n=3 independent experiments). Asterisks show significant differences in Cq values between CqD0 and CqD3 (p-value <0.05). Positive well: positive signal by qPCR in well culture.

Table II. Sensitivity of the *T. gondii* sporocyst-CC-qPCR assay to detect infective parasites in *D. polymorpha* and *M. edulis.* Oocysts (10 to 10^4) were spiked to tissues from one mussel. Then the tissues were processed to recover oocysts and to release sporocysts before infecting cells (see material and methods section). qPCR was performed at D0 (i.e., after 3 hours of contact time between sporocysts and cells) and at D3 (i.e., after 3 days of cell culture) (n=3 independent experiments). SD: standard deviation.





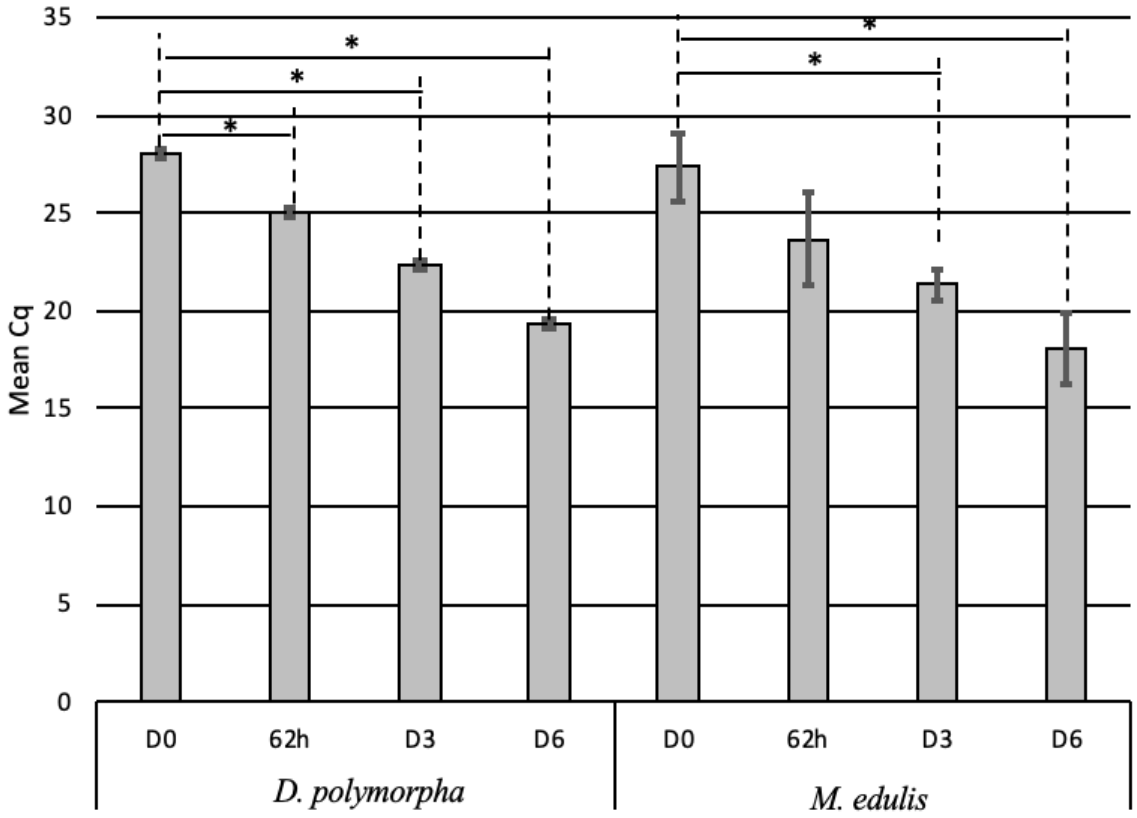


Table I

	D0			D3		
Oocysts quantity	Mean Cq	± SD	Positive experiment	Mean Cq	± SD	Positive experiment
10 000	25.36	± 0.59	3/3	20.40*	± 1.17	3/3
1 000	29.37	± 0.27	3/3	24.06*	± 0.09	3/3
100	32.71	± 0.41	3/3	27.38*	± 0.75	3/3
10	33.33	± 1.08	3/3	28.01*	± 2.07	3/3

TABLE II

	Oocysts quantity	*D. polymorpha*		*M. edulis*	
		Mean Cq	± SD	Mean Cq	± SD
D0	10 000	25.73	± 0.42	27.34	± 1.73
D3	50 000	20.25	± 0.36	18.13	± 0.21
	10 000	21.83	± 0.54	18.82	± 0.41
	5 000	22.43	± 0.55	19	± 0.34
	1 000	23.85	± 0.13	19.49	± 0.64
	500	23.77	± 0.44	20.15	± 0.16
	100	24.42	± 0.17	19.94	± 0.69
	50	25.08	± 1.23	19.72	± 0.14
	10	24.1	± 0.20	19.44	± 0.36
Equation		y = -1.14x + 26.47		y = -0.39x + 20.47	
r^2		0.81		0.57	