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

Angélique Rousseau, Sandie Escotte-Binet, Stéphanie La Carbona, Aurélien Dumètre, Sophie Chagneau, Loïc Favennec, Sophie Kubina, Jitender Dubey, Didier Majou, Aurélie Bigot-Clivot, et al.

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1 **Assessing *Toxoplasma gondii* oocyst infectivity using a sporocyst-based cell-culture assay**  
2 **combined with qPCR for environmental applications.**

3

4 **Running title:**

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

6

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39 **ABSTRACT**

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

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

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

65

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

68

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

94 parameters, which are particularly important in the present context of global climate change.  
95 To circumvent the main drawbacks of water analyses, new alternative approaches to water  
96 analyses have recently emerged in water quality surveys using host-associated  
97 microorganisms as natural biosamplers (15). Special attention has been paid to bivalves  
98 because their intense filtering activity could lead to high accumulation of pathogens (15,16).  
99 Hence, studying bivalves can highlight pathogen contamination while water analysis results  
100 are negative (17). Laboratory studies have shown that marine and freshwater bivalves can  
101 concentrate waterborne protozoan parasites (18-20). Consistent with this, some studies have  
102 reported the detection of *T. gondii* oocysts in different marine (12, 21-23) or continental (24)  
103 bivalves, allowing the study of a large spatial scale (freshwater-seawater continuum).  
104 Experiments have demonstrated that *T. gondii* oocysts can sporulate in seawater, be  
105 concentrated by mussels and remain infectious for laboratory mice (25-27). Usually, DNA-  
106 based methods are applied to detect protozoa in mollusks (23, 28, 29). However, DNA can  
107 persist for a long time in dead cells (30), thus preventing a distinction between viable and  
108 dead parasites. As only viable parasites are potentially infectious and can lead to illness,  
109 viability is a major feature for assessing the health risk. Methods to measure the viability and  
110 infectivity of protozoa including *T. gondii* have been recently reviewed (31). Among  
111 molecular techniques, Propidium MonoAzide based-PCR assays appeared not relevant to  
112 measure the viability of *T. gondii* oocysts (32) and RNA based-methods overestimated the  
113 exposure of humans to viable oocysts because of persistence of RNA in dead parasites (33,  
114 34). Considering that all viable parasites are not necessarily infectious, i.e. able to replicate  
115 within host cells, the methods allowing the characterization of infectivity remained the most  
116 reliable ones.  
117 Many authors have used animal models, the gold standard, to evaluate the infectivity of *T.*  
118 *gondii* oocysts spiked on raspberries or blueberries (6, 35) or in naturally contaminated

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

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

## 139 **Results**

140

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

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

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

150 Overall, irrespective of the time of TissueLyser agitation and of the suspension  
151 solution (0.05% SDS and IMDM growth media), less than 40% of sporocysts were released  
152 with ceramic and glass beads. A higher percentage was obtained following agitation with  
153 small glass beads (425-600  $\mu\text{m}$ ) for 30 s in 0.05% SDS and with larger glass beads (2 mm) for  
154 3 min in 0.05% SDS. However, in both conditions, the inter-assay variability was high (11%).  
155 A mechanical disruption with TissueLyser associated with Lysing Matrix E tube led to the  
156 release of more than 25% of sporocysts irrespective of the agitation time. The highest  
157 percentage rate was obtained after 30 s in IMDM growth media with  $84\% \pm 14\%$  of released  
158 sporocysts. Hence, a 30 s agitation of oocysts for 30 s in the Lysing Matrix E tube in IMDM  
159 growth media was condition retained for further sporocyst-CC-qPCR experiments.

160

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

162 The optimal contact time required for the sporocysts to naturally excyst and release  
163 sporozoites and for the sporozoites to penetrate the cells was determined *in vitro*. To that aim,  
164 Vero cells and sporocysts were left in contact for 2 hours or 1 day (contact time before  
165 washing (D0, Fig. 1A and B). Irrespective of the contact time, *T. gondii* DNA was  
166 systematically detected by qPCR in total DNA extracted from the cell pellet. These results  
167 suggest that the two tested contact times were sufficient to allow some sporozoites to  
168 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  $25.51 \pm 0.70$  at D0 to  $23.03 \pm 0.95$  at D2, and  $20.40 \pm 1.17$  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  $4^{\circ}\text{C}$  (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

194 serial dilution ranging from  $10^1$  to  $10^4$  oocysts was used to prepare sporocysts for Vero cells  
195 infection using the protocol selected above. Ten infective oocysts were consistently detected  
196 (3/3 positive replicates at Day 3, Table 1) indicating that the limit of detection of the method  
197 was less than 10 oocysts. The linear correlation between the initial number of infectious  
198 parasites and the Cq values from 10 000 to 100 ( $R^2 > 0.99$ ) suggested that this model could be  
199 quantitative in this range and that the limit of quantification was between 10 and 100 oocysts.

200

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

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

214

#### 215 **Potential of the CC-qPCR assay to determine the infectivity of *T. gondii* oocysts in 216 spiked mussels (*M. edulis* and *D. polymorpha*).**

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

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

224 The kinetics of Vero cell infection with sporocysts isolated from *M. edulis* and *D. polymorpha*  
225 homogenates are presented in Figure 3. For *D. polymorpha*, a significant decrease of Cq was  
226 observed from 62 h (p-value <0.05) and was maximal after six days (CqD0-CqD6 = 8.52).  
227 For *M. edulis*, the decrease was remarkable after three days of culture (D3) and was maximal  
228 after six days ( $\Delta Cq = 9.3$ ). In order to detect, infectious oocysts in mussel matrix as quickly as  
229 possible, a culture time of D3 was retained for both mussel matrices. Then, the limit of  
230 detection of the sporocyst-based assays to detect infective parasite in both mussel species was  
231 determined (Table II). To that aim, mussel tissues were spiked with a number of oocysts  
232 ranging from 10 to  $10^4$ , and then processed as described in material methods to recover  
233 oocysts and to release sporocysts before infecting cell cultures. For both mussels, as low as 10  
234 infective oocysts initially spiked to tissues led to parasite multiplication and cell infection  
235 within three days (Cq = 24.1 for *M. edulis* and Cq = 19.44 for *D. polymorpha*, Table II).  
236 These results indicated that the limit of detection of this sporocyst-based CC-qPCR was below  
237 10 oocysts. However, the Cq values obtained at D3 were not correlated to the initial amount  
238 of oocysts spiked on tissues ( $r^2 \leq 0.81$ , Table II) suggesting this *in vitro* model could not be  
239 quantitative.

240

## 241 Discussion

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

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

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

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

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

280 To obtain the best conditions opening the sporocyst wall, several studies used bile salt or  
281 sodium taurocholate treatment associated or not with Na<sub>2</sub>CO<sub>3</sub> and medium saturated with CO<sub>2</sub>  
282 (54, 55). However, we observed significant sporozoite loss, huge mortality and no  
283 reproducible protocols because of sporozoite fragility (data not shown). This has already been  
284 described for *Isospora suis* (56) but not corroborated with the release of 80.6% sporozoites  
285 (45). As no concluding results were obtained for the sporozoite release step, we tested the  
286 ability of sporocysts to naturally excyst in cell culture at 37°C. Only mechanical grinding with  
287 TissueLyser (30 s) associated with Lysing Matrix E tube in IMDM growth media achieved  
288 the release of 84 ± 14% of sporocysts. However, this percentage of released sporocysts is  
289 probably underestimated because 10%-15% of oocysts never sporulate (32). This method can  
290 be easily implemented in laboratories and can be applied to oocysts isolated from  
291 environmental or food samples as mussels.

292 We checked that the TissueLyser did not alter the infectivity of the sporocysts in cell culture  
293 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 C<sub>q</sub> 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 showed 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

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

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

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<sub>2</sub>SO<sub>4</sub> aqueous solution (2%) and stored at 4°C until use.  
367 Before experiments, oocysts were washed three times in sterile distilled water (dH<sub>2</sub>O) 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<sup>4</sup> 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

387 theoretical sporocysts), we considered that  $10^4$  oocysts corresponded to  $2 \cdot 10^4$  sporocysts  
388 assuming that 100% oocysts were sporulated and contained each two sporocysts. Each  
389 experiment was performed in single or duplicate.

390

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

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

410

#### 411 **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  $\mu$ L of the ATL buffer and 20  $\mu$ L 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  $\mu$ L containing 12.5  $\mu$ L of iQ™ Supermix

447 (Bio Rad, Marnes la Coquette, France), 1  $\mu$ L of BSA 10 mg/mL (SIGMA, France), 1  $\mu$ L of

448 each primer 20  $\mu$ M (forward, 5'-AGAGACACCGGAATGCGATCT-3'; reverse, 5'-

449 CCCTCTTCTCCACTCTTCAATTCT-3'), 0.5  $\mu$ L of probe 10  $\mu$ M (5' FAM-

450 ACGCTTTCCTCGTGGTGATGGCG-3' BHQ1), 5  $\mu$ L of DNA template and 4  $\mu$ L 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 C<sub>q</sub> (quantification cycle) values between D<sub>0</sub> 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 (C<sub>qD0</sub> - C<sub>qend culture</sub>) was calculated.

456

#### 457 **Mouse bioassay for infectivity**

458 As described in a previous study (11), 10<sup>3</sup> *T. gondii* oocysts were inoculated in outbred

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

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

464

#### 465 **Statistical analysis**

466 The C<sub>q</sub> values obtained in qPCR were compared using the non-parametric Kruskal Wallis  
467 test. If the null hypothesis H<sub>0</sub> (the tested conditions have no effect on the measured value) was  
468 rejected, then the post hoc Wilcoxon-Mann-Whitney test was applied for two independent  
469 samples. All statistical tests were performed using StatXact7. Statistical difference was  
470 considered as p value < 0.05.

471

472

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485

486 **CONFLICT OF INTEREST**

487 The authors declare no conflict of interest.

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702

**Figure legends**

703

**Fig. 1: Kinetics of cell infection by *T. gondii* sporocysts in simple matrix assessed by**

704

**sporocyst-CC-qPCR assay.** Following 2 h (A) or 1 day (B) of contact between the

705

sporocysts and Vero cells (contact time: D0), the cells were cultivated for 2 (D2), 3 (D3) or 6

706

days (D6). Sporocysts were obtained after TissueLyser agitation (30 s, 33Hz) from viable and

707

heat inactivated oocysts (5 min at 99°C). The y axis indicates mean qPCR signal (Cq) and

708

standart deviation ( $2 < n < 5$  independent experiments). Negative control corresponds to Vero

709

cells without sporocysts. Asterisks show significant differences (p-value &lt; 0.05).

710

711

**Fig. 2: Infectivity of *T. gondii* sporocysts after storage at 4°C assessed by sporocyst-CC-**

712

**qPCR.** After 1 day of contact between the sporocysts and Vero cells (contact time: D0), the

713

cells were cultivated for 6 days (D6). Sporocysts were obtained after TissueLyser agitation

714

(30 s, 33Hz) from viable oocysts and stored 0, 1 or 5 months at 4°C. The y axis indicates

715

mean qPCR signal (Cq) ( $2 < n < 9$  independent experiments). Asterisks show significant

716

differences (p-value &lt; 0.05).

717

718

**Fig. 3: Kinetics of cell infection by *T. gondii* sporocysts isolated from *D. polymorpha* and**

719

***M. edulis* assessed by sporocyst-CC-qPCR.** Sporocysts were released from oocysts isolated

720

from mussels and following 3 h of contact between the sporocysts and Vero cells (D0), the

721

cells were cultivated for 62 h, 3 (D3) or 6 days (D6). The y axis indicates mean qPCR signal

722

(Cq) (n=3 independent experiments). Asterisks show significant differences in Cq (p-value

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&lt;0.05).

724

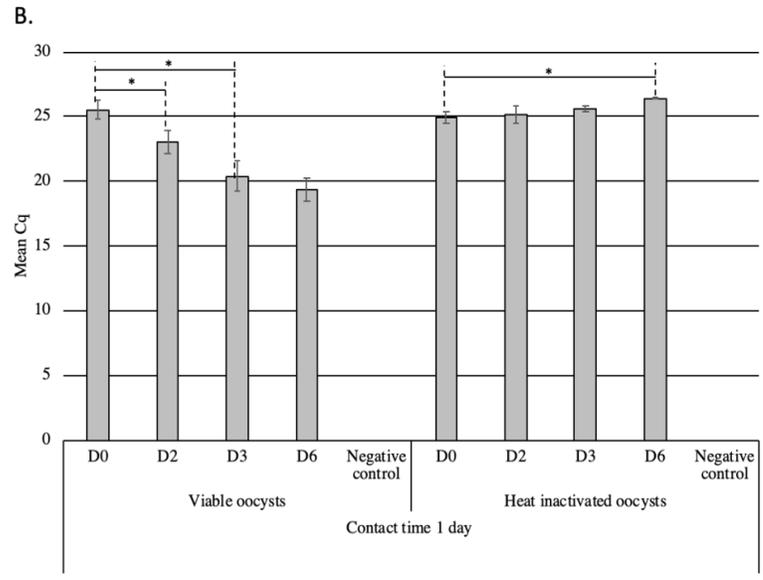
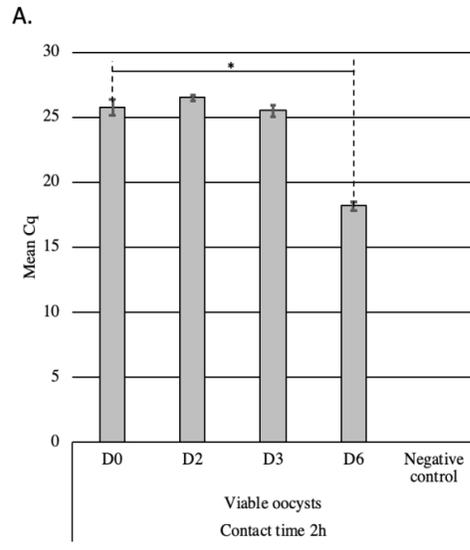
725 **Table captions**

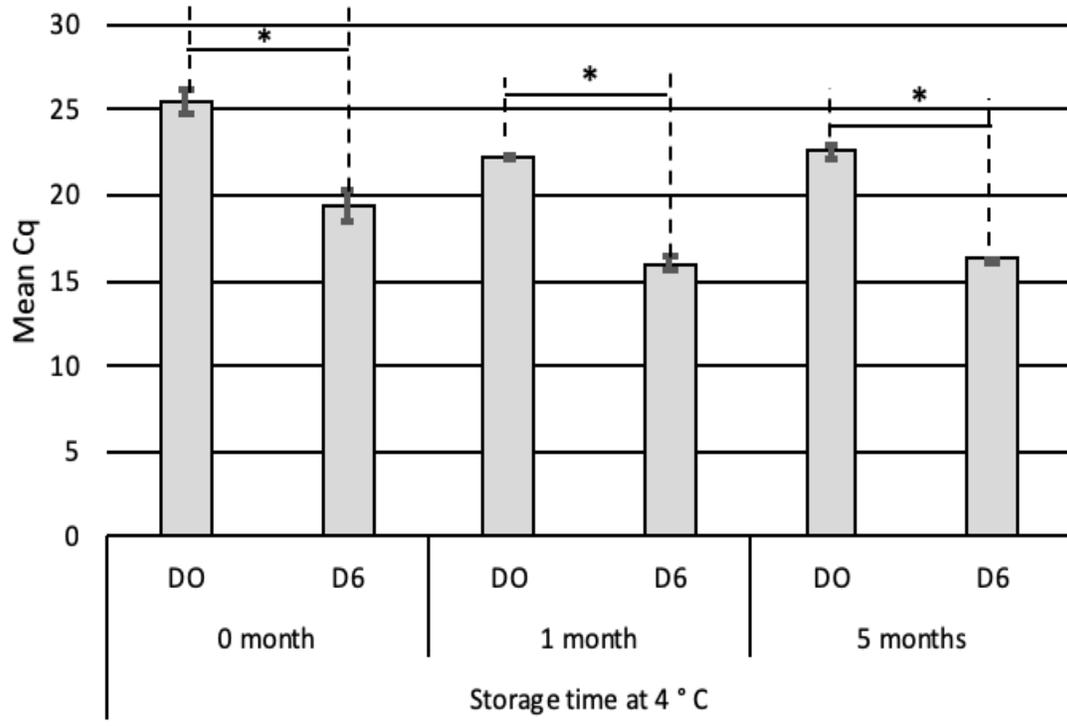
726

727 **Table I. Sensitivity of the *T. gondii* sporocyst-CC-qPCR assay to detect infective**  
728 **parasites in simple matrix.** A serial dilution ranging from 10 to 10 000 *T. gondii* oocysts was  
729 used to obtain sporocysts resuspended in culture medium and then deposited on Vero cells  
730 (80% confluence) incubated at 37°C. qPCR specific to *T. gondii* was performed from the cell  
731 pellet DNA extract after a contact time between sporozoites and cells of 1 day (D0) and after  
732 culture of the cells for 3 days (D3) (n=3 independent experiments). Asterisks show significant  
733 differences in C<sub>q</sub> values between C<sub>q</sub>D0 and C<sub>q</sub>D3 (p-value <0.05). Positive well: positive  
734 signal by qPCR in well culture.

735

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





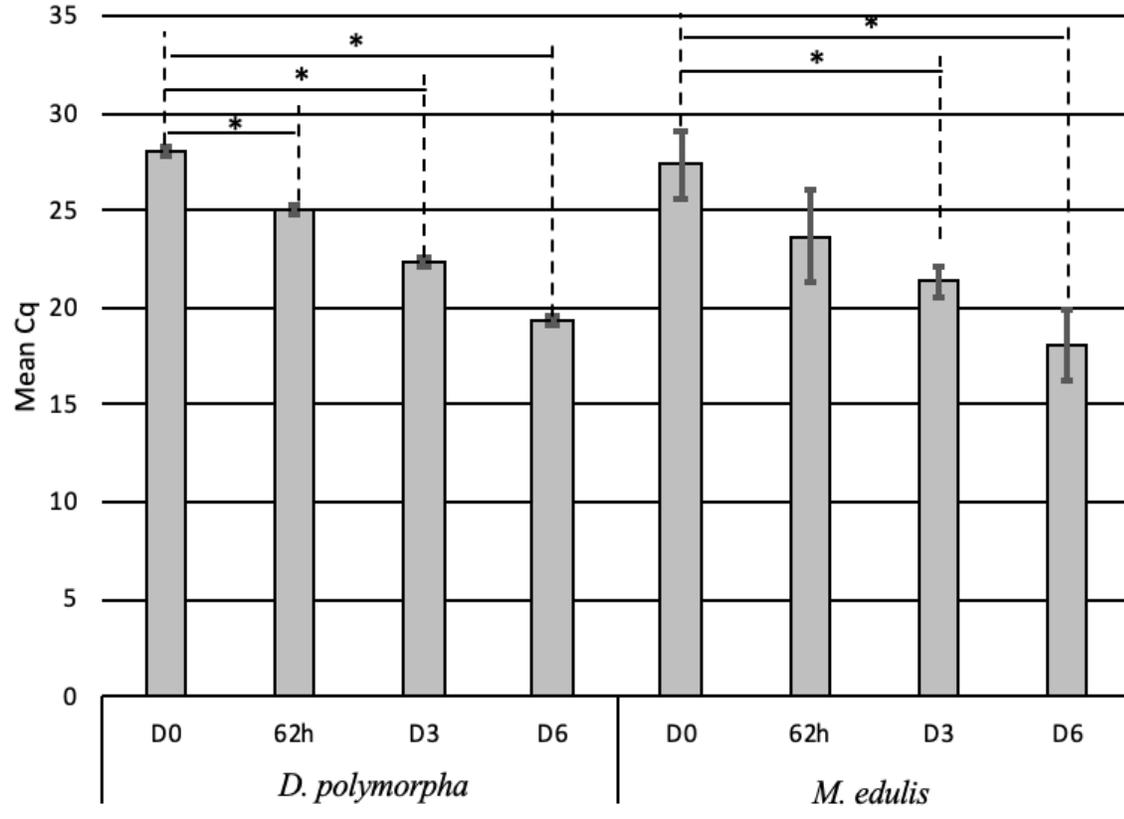


Table I

Oocysts quantity	D0			D3		
	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	<i>D. polymorpha</i>		<i>M. edulis</i>	
		Mean Cq	± SD	Mean Cq	± SD
<b>D0</b>	10 000	25.73	± 0.42	27.34	± 1.73
<b>D3</b>	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
<b>Equation</b>		$y = -1.14x + 26.47$		$y = -0.39x + 20.47$	
<b>r<sup>2</sup></b>		0.81		0.57	