1 # Exploring the environmental distribution of the oyster parasite

2 # *Haplosporidium costale*

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17 ## Abstract

18 The protozoan parasite *Haplosporidium costale* is known to occur in the USA where it has been
19 associated with sharp seasonal mortality of the Eastern oyster *Crassostrea virginica* since the 1960's.
20 In 2019, the parasite was detected for the first time in the Pacific oyster *Magallana gigas* in France in
21 the context of light mortality and was subsequently detected in archived material collected since 2008.
22 This detection raised several questions regarding the ability of the parasite to maintain in the
23 ecosystem and the potential involvement of other species in its life cycle. To answer these questions,
24 an integrated sampling approach was deployed seasonally in three oyster farming areas where the
25 parasite was already known to occur. Parasite presence was evaluated after checking the presence of
26 PCR inhibitors and using a previously developed and validated Real Time PCR assay, optimized in this
27 study to detect parasite DNA in various environmental compartments. Parasite DNA was almost only
28 detected in cupped oysters. Considering the high number of oysters found positive with low infection
29 intensity, a complementary experiment was undertaken to better characterize sub-clinical infections
30 in oysters. The presence of the parasite was tested twice a week in water and sediment from aquaria
31 hosting cupped oysters from a known infected site. After one month, oysters were sacrificed and
32 tested regarding the presence of the parasite at the tissular level. Altogether, field and experimental
33 results indicate that the parasite is stably established in oyster, particularly in gills, which may act as a
34 reservoir all along the year. The detection of parasite DNA in nanoplankton and sediment suggests that
35 *H. costale* is released from the oysters outside mortality event. Our results do not support the
36 involvement of other species than cupped oyster in the parasite life cycle except periwinkles, whose
37 role would deserve to be further investigated.

38

39 Keywords: Haplosporida; *Haplosporidium costale*; oyster; *Magallana gigas*, *Crassostrea gigas*;
40 Parasite; eDNA; Parasite distribution

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43 ## Introduction

44 The protozoan parasite *Haplosporidium costale* was detected in France for the first time in 2019 in the
45 context of low mortality of the cupped oysters *Magallana gigas* (Arzul et al. 2022). Following this
46 detection, archived samples collected either in the context of studies or mortality investigations
47 revealed the presence of the parasite in several French oyster production areas at least since 2005
48 (Arzul et al. 2022 ; Cherif-Feildel et al. 2022). *H. costale* belongs to the family Haplosporidiidae, order
49 Haplosporida, phylum Cercozoa (Arzul and Carnegie, 2015). The parasite occurs in eastern oysters
50 *Crassostrea virginica* along the East coast of the United States and Canada and has been more recently
51 reported on the West coast of the USA in *M. gigas* (Burreson and Stokes, 2006). It has been associated
52 with mortality of eastern oysters, *C. virginica* since the 1960's, begetting a pathology known as the
53 Seaside Organisms (SSO) disease (Wood and Andrews 1962).

54 *C. virginica* mortality associated with *H. costale* is usually less than 20% per outbreak, but can reach
55 50% in some years in Virginia, Maryland and Delaware (Andrews and Castagna 1978). Parasite impact
56 on *M. gigas* is less clear. In France, in 2019, the parasite was involved in 7.2 % of cumulative oyster
57 mortality during the nursery period (Arzul et al. 2022) while it was also detected in China, but without
58 mortality observation (Wang et al. 2010). In both oyster species, numerous spores invading the
59 connective tissues in association with an intense hemocyte infiltration were observed in infected
60 moribund oysters.

61 *C. virginica* infection occurs during May-June but remains subclinical until spring of the following year.
62 Infection develops rapidly and sporulation begins typically in late May (Andrews and Castagna 1978).
63 Sporulation coincides with oyster mortality (Couch and Rosenfield 1968, Andrews and Castagna 1978,
64 Andrews 1984). The development of PCR methods allowed detecting the presence of parasite DNA not
65 only between March and May but also in fall (Stokes and Burreson, 2001).

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66 Besides the seasonal features of the infection with *H. costale*, its distribution seems limited to high
67 salinity waters (≥ 25) (Andrews and Castagna 1978). In China its detection in *M. gigas* was observed at
68 salinity between 29 and 32 (Wang et al., 2010). Although no clear relation with temperature is
69 reported, a possible control by temperature over the distribution of haplosporidian species has been
70 suggested (Wang et al., 2010). Finally, even though correlation with oyster age was not found in *C.*
71 *virginica*, *H. costale* prevalence is higher in spat compared to adult *M. gigas* (Lupo et al. 2019 ; Cherif-
72 Feildel et al. 2022).
73
74 If the need of an alternate host has been proposed for the congeneric species *Haplosporidium nelsoni*
75 (Ford et al. 2018), the host range and transmission process of *H. costale* have never yet been formally
76 characterized.
77 The purpose of this study was to determine the environmental distribution of *H. costale* parasite in
78 three French oyster producing areas where the parasite had previously been detected: Bay of
79 Bourgneuf, Bay of Marennes Oléron and Bay of Arcachon (Figure 1) (Arzul et al. 2022). The presence
80 of *H. costale* was seasonally monitored by Real Time PCR (Arzul et al. 2022) over one year in *M. gigas*,
81 sympatric marine invertebrates, plankton and sediment. The PCR assay was optimised in order to test
82 the presence of the parasite in these environmental compartments.
83 Complementary, an experiment was carried out in order to better characterize the sub clinical
84 infections of *H. costale*. For this purpose, oysters collected from Marennes Oléron were maintained
85 for one month in mesocosms. The presence of the parasite in water was checked weekly and the
86 tissular distribution of the parasite in the oysters was investigated by Real Time PCR.
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92 ## Material and methods

93 ### 1- Sampling approach and sample processing to describe environmental distribution of the
94 parasite

95

96 Sampling was carried out every 3 months over one year (2021) in Bourgneuf bay (2 sites); Marennes
97 Oléron bay (1 site) and Arcachon bay (3 sites) (Figure 1 ; Table 1).

98

99

100 Figure 1- Sampling sites investigated in this study

101

102

103 Sites displayed different types of sediment : sand, sandy - mud and mud (Table 1).
104 At each campaign and site the following sampling was carried out:

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105 Thirty adult oysters *M. gigas* were collected in "wild" oyster reefs as well as other invertebrate species
106 occurring ca. 10 to 30 meters around oysters (up to 30 individuals per species). For bivalve species
107 (including oysters), sections of organs including gills, mantle, gonad and digestive gland were prepared
108 from each individual and fixed in Davidson for histology and *in situ* hybridization. In addition, from the
109 same individuals, about 20 mg of gills, digestive gland and mantle were collected and frozen at -20°C
110 or fixed in absolute ethanol for DNA extraction. For the other species, about 20 mg of a pool of all
111 organs were collected for DNA extraction.
112 Six sediment cores were sampled about 5 meters around the oysters using a 20-cm diameter and 5-
113 cm width corer. Within these cores, sub samples were collected at the surface and at 5-cm depth and
114 subsequently frozen –20°C until being processed for DNA extraction (see below). The remaining
115 sediment was sieved on a 400 µm mesh in order to retain benthic fauna. This fauna was sorted by
116 species,genus, family or class(Table S1) and stored in ethanol until being processed for DNA extraction
117 (see below).
118 Meso- and microplankton were collected through two 50-m traits at ebbing tide closed to the oyster
119 reefs using 20 and 200 µm plankton nets. Nanoplankton was obtained from 2 x 5 L of water collected
120 about 75 m from oysters. Once in the laboratory, water was homogenised and 2.5 L were prefiltered
121 at 20 µm and filtered on a 1 µm membrane (250 mL / membrane). Membranes were stored at -20°C
122 until being processed for DNA extraction (see below).
123

Geographic area	Site	GPS coordinates	Type of sediment	Dates of sampling campaigns
Bourgneuf bay	Ecluse	46.968113, -2.043874	Mud	26/01/2021 07/07/2021

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	Passage du Gois	46.922290, -2.105864	Sandy-Mud	20/01/2021 20/04/2021 07/07/2021 03/11/2021
Marennes-Oléron bay	La Floride	45.802636, -1.151221	Sandy-Mud	01/02/2021 03/05/2021 20/07/2021 16/11/2021
Arcachon bay	Comprian	44.6797833, -1.090916	Mud	02/03/2021 27/04/2021 26/07/2021 08/11/2021
	Tès	44.665, -1.138299	Sand	02/03/2021 27/04/2021 26/07/2021 08/11/2021
	Gahignon	44.6806833, -1.160966	Sandy-Mud	02/03/2021

124 *Table 1- Sampling sites and dates*

125

126 ## 2- DNA extraction, PCR inhibitors detection and *Haplosporidium costale* detection by PCR

127

128 - *DNA extraction*

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129 Total DNA was extracted using different protocols depending on the nature of the samples. For oysters
130 and marine invertebrate species in sympatry, total DNA was extracted from approximately 20 mg of
131 tissues using the QIAamp® DNA Mini Kit (Qiagen, Inc) according to Arzul et al. (2022). For Manila clams,
132 DNA was extracted using Wizard ®Genomic DNA Purification Kit (Promega, Inc.).

133 The benthic fauna, meso- and microplankton samples were rinsed between two and three times in
134 PBS. Between 25 and 75 mg of sliced tissues per individual for the benthic fauna and 25 mg of plankton
135 samples were weighted and crushed using a piston pellet prior DNA extraction using the QIAamp® DNA
136 Mini Kit (Qiagen, Inc) according to Manufacturer's protocol except that lysis was extended overnight
137 under stirring.

138 For sediment and nanoplankton samples, DNA extraction was carried out as described by Mérou et al.
139 (2023). For nanoplankton, DNA was extracted from a quarter filtration membrane using the DNeasy®
140 PowerWater® Kit (Qiagen, Inc.) while for sediment, DNA was extracted from 0.25 g using the DNeasy®
141 PowerSoil® Kit (Qiagen, Inc.).
142 Finally, DNA was eluted in 50 µL or 100 µL of buffer AE for environmental samples and invertebrate
143 samples, respectively. DNA concentration was finally measured with NanoDrop 2000 (Thermo
144 Scientific) and samples were stored at 4°C (short term storage) or -20°C (long term storage) until being
145 tested by PCR. Prior PCR analyses, DNA extracted from tissues, meso and micro plankton was adjusted
146 at 5 ng/µL while DNA extracted from nanoplankton was tested without concentration adjustment and
147 DNA extracted from sediment was diluted 10 fold (Mérou et al. 2023).

148

149 - *Detection of PCR inhibitors*

150 The presence of PCR inhibitors was tested using an universal internal control kit (qPCR Internal Positive
151 Control from Eurogentec) on a set of samples of different nature: meso and micro plankton samples
152 (8 samples each representative of the four sampling dates), nanoplankton (20 samples representative
153 of the sampling sites and dates), sediment (18 "surface" and 18 "5-cm deep" samples) and when

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154 possible, 5 to 15 individuals per taxonomic group for marine invertebrates. The analyses were carried
155 out following manufacturer's recommendations. If negative (total inhibition) or if the difference
156 between obtained and control Ct values was above 3 (partial inhibition), DNA suspensions were diluted
157 at 1/10 and 1/100 and tested again.
158

159 • *Detection of* Haplosporidium costale *DNA*

160 Depending on the conclusions of the PCR inhibitor detection tests on a selection of samples (see
161 above), DNA suspensions were eventually diluted prior being tested using the Taqman Real Time PCR
162 assay described in Arzul et al. (2022) targeting a 149 bp fragment of the 18S rRNA gene of
163 *Haplosporidium costale*. Briefly, the PCR mixture included 10 µL TaqMan® Supermix (SsoAdvanced mix
164 from Biorad); 0.3 µL SSO 1358F (Forward Primer) (20 µM) ; 0.3 µL SSO 1507R (Reverse Primer) (20 µM)
165 ; 0.3 µL Probe2 SSO (FAM) (20 µM) ; 4.1 µL bi distilled water and 5 µL extracted DNA. The thermal
166 profile was the following: 95°C for 3 min and 40 (for oysters) and 45 cycles (for other samples than
167 oysters) of amplification at 95°C for 15 sec and 60°C for 1 min.
168 Environmental samples (water, sediment) and plankton were tested in duplicate, bivalves and other
169 invertebrates in simplicate.
170 Positive and negative controls were included in each PCR run. Positive controls consisted of plasmidic
171 DNA including the region targeted by the primers. Negative controls consisted of bi-distilled water
172 used in the extraction and real-time PCR steps.
173 A sample showing a fluorescent signal exceeding the fluorescent background level was considered
174 positive regardless of the threshold cycle (CT) obtained and a sample showing no fluorescent signal
175 above the background level was considered negative.
176

177 ## 3- -Histology and *In situ* hybridization

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178 After 48 h in Davidson's fixative, tissue sections were maintained in 70% ethanol until they were
179 dehydrated and embedded in paraffin for histology according to standard procedures (Howard et al.
180 2004). Two- to three-micrometres thick tissue sections were stained with haematoxylin and eosin.
181 Slide examination was done using a BX50 (Olympus) microscope.
182 *In situ* hybridization protocol was adapted from Stokes and Burreson (2001) and is described in Arzul
183 et al. (2022). Briefly, three-micrometres thick tissue sections on silane-prep™slides (Sigma, France)
184 were dewaxed, rehydrated and treated with proteinase K [100 µg/ml in TE buffer (Tris 50 mM, EDTA
185 10 mM)] at 37°C for 10 min. After dehydration in absolute ethanol, sections were incubated with
186 100 µL of hybridization buffer [50% formamide, 10% dextran sulphate, 4× saline-sodium citrate buffer
187 (0·06 M Na3 citrate, 0·6 M NaCl, pH 7), 250 µg/mL yeast tRNA and 10% Denhardt's solution] containing
188 5 ng/µL of 3'digoxigenin-labelled SSO1318 oligoprobe (Eurogentec). After a denaturation step at 94°C
189 for 5 minutes, hybridization was carried out overnight at 42°C. Sections were then processed for
190 immunological detection using an alkaline phosphatase-conjugated mouse IgG antibody against
191 digoxigenin, stained with NBT/ BCIP and finally observed using a BX 50 microscope (Olympus).
192 Negative controls included samples without digoxigenin-labelled probe in the hybridization mixture or
193 without antibodies during the revelation step. Positive control consisted of *M. gigas* infected with *H.*
194 *costale* characterized in Arzul et al. (2022).
195

196 ## 4- Estimation of the limit of detection in nanoplankton and sediment

197 As the Real-Time PCR limit of detection in oysters was previously determined (Arzul et al. 2022), a
198 similar approach was carried out in sediment and nanoplankton using a synthetic plasmid including
199 691 bp [from position 817 to 1507] of *H. costale* 18S gene in pUC57 (Eurogentec) diluted either in
200 sediment or deposited on a membrane, respectively, and processed for DNA extraction and Real Time
201 PCR for the detection of *H. costale* as described above.

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202 Ten-fold serial dilutions (10^8 -10^0 DNA copies per µl of template) were tested with nine replicates of
203 each dilution level in 2 independent assays (total of 18 replicates per tested dilution). LDpcr was
204 determined by the smallest number of nucleic acid targets given 95% of positive results.
205

206 5- Experimental design used to characterize sub-clinical infections with *Haplosporidium* 207 *costale*

208 Adult oysters were collected in La Floride site from the same population monitored in the field study
209 (same site as in Table 1) on the 3rd of May 2021 and 46 oysters were distributed and maintained for
210 one month in three 50 L aquaria (between 14 and 18 oysters by aquarium). Each aquarium contained
211 about 4500 cm^3 of sediment collected in La Floride and sea water was pumped at 400 meters from La
212 Floride and filtrated through a 5-10 µm membrane. During the experiment, temperature was
213 maintained at 15-16°C, salinity at 32-33 and water was enriched in *Skeletonema costatum* (diatoms).
214 Every 10 days, the water of each aquarium was renewed.
215 Twice a week over one month, three samples of 250 mL of water were collected in each aquarium
216 (before renewal if sampling was concurrent to water renewal), prefiltered at 20 µm and filtered on a
217 1 µm membrane processed as described above for DNA extraction. In addition, once a week, in each
218 aquarium, three samples of 0.25 g of sediment were collected and processed for DNA extraction as
219 described above.
220 Concurrently, when the water was renewed, one sample of water (250 mL) was collected at the
221 entrance of the aquaria and used as controls. Before the beginning of the experiment, water and
222 sediment were also collected and tested as T_0 controls.
223 At the end of the experiment, all the oysters were sacrificed and processed for histology and *in situ*
224 hybridisation as described in section 3. Oysters from two aquaria (n°1 and 3) were processed for the
225 detection of *H. costale* by Real Time PCR as described in section 2 (from a pool of gills, digestive gland
226 and mantle).

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227 In addition, 20 mg of each of the following organs were tested individually for 20 oysters (oysters from
228 aquaria 1 and 2): palps, gills, adductor muscle, digestive gland, gonad, heart, hemolymph. DNA
229 extraction and real time PCR were carried out as described above.

230

231 ## Results

232

233 ### 1- Real Time PCR limits in nanoplankton and sediment

234 PCR results obtained from serial dilutions of plasmids spiked in sediment samples and water filters
235 are presented in Table 2.

	Plasmidic DNA concentration		PCR results		
	Nb of copies / quarter of filter	copies / ml of water	Mean Ct	Nb of positives (out of 18)	% of positive
Nanoplankon	10^8	1.60E+06	19.5	18	100%
	10^7	1.60E+05	21.9	18	100%
	10^6	1.60E+04	26.8	18	100%
	10^5	1.60E+03	31.5	18	100%
	10^4	160	32.3	18	100%
	10^3	16	39.4	18	100%
	100	1.6	39.0	14	78%
	10	0.16	43.4	1	6%
	0	0	N/A	0	0%

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	Nb of copies / tube (250 mg)	copies / mg	Mean Ct	Nb of positives (out of 18)	% of positive
Sediment	10^8	400000	21.8	18	100%
	10^7	40000	24.8	18	100%
	10^6	4000	27.9	18	100%
	10^5	400	31.4	18	100%
	10^4	40	34.9	18	100%
	10^3	4	36.6	7	39%
	100	0.4	N/A	0	0%
	10	0.04	N/A	0	0%
	0	0	N/A	0	0%

236

237 *Table 2- Sensitivity of the Real Time PCR for the detection of* H. costale *DNA from nanoplankton and*
238 *sediment samples. Serial dilutions of plasmids were spiked in 250 mg of sediment and in quarters of*
239 *filters, then samples were processed as field samples. A quarter of filter corresponds to 62.5 mL of*
240 *filtered water. Each dilution was tested in nine replicates in two independent assays.*

241

242 In nanoplankton samples, the lowest dilution producing 100% of positive results was 1000 copies in a
243 quarter of filter, theoretically corresponding to 16 copies / mL of filtered water (when DNA was tested
244 undiluted by PCR). At a theoretical concentration of 1.6 copies / mL, 78% of water samples were found
245 positives.

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246 In sediment samples, the lowest dilution producing 100% of positive results was 10^4 copies in 250 mg,
247 theoretically corresponding to 40 copies / mg of filtered water. At a theoretical concentration of 4
248 copies / mg, 39% of sediment samples were found positives.

249

250 ## 2- Detection of PCR inhibitors

251 Detailed results are available in Table 3. A summary of these results is shown in Figure 2.

252

253 *Figure 2- Percentages of samples showing PCR inhibition using the IPC kit by category of samples-*
254 *Individuals were gathered at the class level– The category "Other" includes anemones, sponges,*
255 *tunicates and flat worms- On the right: the number of samples tested regarding the presence PCR*
256 *inhibition.*

257 Plankton samples displayed the highest detection of PCR inhibitors. Indeed, between 45 and 100% of
258 tested samples presented total or partial inhibition. A 10th and 100th dilution lifted inhibition in nano
259 plankton and micro/meso plankton, respectively.

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260 With the exception of limpets (*Patella* sp.) and top shells (*Gibbula* sp.), all the tested groups within the
261 class Gastropoda had to be diluted to 10 or to 100 for European sting winkles to avoid PCR inhibition.
262 Similarly, within the class Polychaeta, Terebellidae and Sabellidae were also diluted to the 10th to avoid
263 PCR inhibition
264 The only bivalves displaying PCR inhibition in our set of samples were variegated scallops
265 (*Mimachlamys varia*) which were subsequently tested to the 100th.
266 Within the Subphylum Crustacea (Classes Malacostraca and Thecostraca) 20% of isopod samples and
267 25% of amphipod samples showed PCR inhibition which could be lifted after dilution to 10.
268

269 ## 3- Detection of *Haplosporidium costale* in the environment

270

271 Detailed results are available in supplementary material-Supp. 1. Table 3 summarizes these results by
272 nature of samples.

Nature of samples	Number of samples positive/tested for PCR inhibition	DNA dilution allowing to avoid PCR inhibition	Number of samples PCR positive for *H. costale*	Number of samples tested in the whole study	Detection frequency (%) of *H. costale*
Nanoplankton (1-20 µm)	9/20	1/10*	8	105	7.6
Microplankton (20-200 µm)	8/8	1/100	0	41	0
Mesoplankton (>200 µm)	8/8	1/100	0	41	0

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Sediment surface (already diluted 1/10)	0/18		12	126	9.5
Sediment 5cm deep (already diluted 1/10)	0/18		7	126	5.6
Bivalvia (species or genus)	**5/56**		**205**	**1100**	**18.6**
Pacific oyster *(Magallana gigas)*	0/5		203	526	38.6
Mussel (*Mytilus*)	0/5		1	369	0.3
Manila clam *(Ruditapes philippinarum)*	2/15		0	79	0
Common cockle *(Cerastoderma edule)*	2/15		1	64	1.6
Peppery furrow shell *(Scrobicularia plana)*	0/5		0	46	0
Razor clam *(Solen marginatus)*	0/5		0	10	0
Variegated scallop (*Mimachlamys varia)*	1/6	1/100	0	6	0
Gastropoda (species or genus)	**14/42**		**11**	**251**	**4.4**
Common periwinkle (*Littorina littorea*)	6/15	1/10	11	111	9.9
Common periwinkle eggs	0/3		0	7	0
Slipper limpet *(Crepidula fornicata)*	4/5	1/10	0	65	0
Top shell *(Steromphala umbilicalis)*	0/5		0	49	0
European sting winkle (*Ocenebra erinaceus*)	2/5	1/100	0	8	0
Netted dog whelks *(Tritia reticulata)*	2/5	1/10	0	7	0

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Limpets *(Patella)*	0/4		0	4	0
Polychaeta (family)	**6/25**		**2**	**241**	**0.8**
Nephtydae	0/5		2	96	2.1
Nereididae	0/5		0	94	0
Sabellidae	2/5	1/10	0	23	0
Capitellidae	0/5		0	21	0
Terebellidae	4/5	1/10	0	7	0
Malacostraca/Thecostraca**	**4/31**		**1**	**125**	**0.8**
Pea crab (Species *Pinnotheres pisum*)	0/4		0	11	0
Hermit crabs (Family *Diogenidae*)	0/5		1	50	2
Order Amphipoda	3/12	1/10	0	18	0
Order Isopoda	1/5	1/10	0	18	0
Barnacles (Family *Balanidae*)**	0/5		0	14	0
Other (class or species)	**0/18**		**0**	**44**	**0**
Sea sponge (*Hymeniacidon perlevis)*	0/5		0	19	0
Anemone (Class *Anthozoa)*	0/5		0	17	0
Asian tunicate (*Styela clava)*	0/5		0	5	0
Flat worm *(Idiostylochus tortuosus)*	0/3		0	3	0

273 ** for nanoplankton, DNA suspensions were first tested non diluted. No more positive results were*

274 *obtained at 1/10 on tested samples*

275 *** the only Thecostraca specimens were Barnacles (Family Balanidae)*

276 *Table 3- Detection of* Haplosporidium costale *DNA by Real time PCR by category of samples (Individuals*

277 *were gathered at the class level)–The second column indicates the number of samples in which PCR*

278 *inhibitors were detected over the number of samples tested using the IPC kit. The third column specifies*

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279 *the DNA suspension dilution allowing lifting PCR inhibition. The last column shows the detection*
280 *frequency of* H. costale *(%) by category of samples*

281

282 ## 3-1 In oysters *Magallana gigas*

283 Detection frequency of *H. costale* by PCR in cupped oysters is presented in Figure 3 by sampling date
284 and site. Parasite DNA was detected in all the tested sites and at all the sampling dates apart from one
285 sample in Comprian, Arcachon bay carried out in April 2021. However, in this case only five oysters
286 were collected and tested.

287 No particular seasonal pattern could be observed across the different sites. Indeed, in Comprian and
288 Tès (Arcachon bay), detection frequency appeared lower in March-April compared to July-November.
289 However, in La Floride (Marennes Oléron) and Le Gois (Bourgneuf Bay), detection frequency peaked
290 in July and January, respectively.

291 Globally, mean detection frequency was 37%. Whatever the geographic area, maximum detection
292 frequency was between 50 and 70%.

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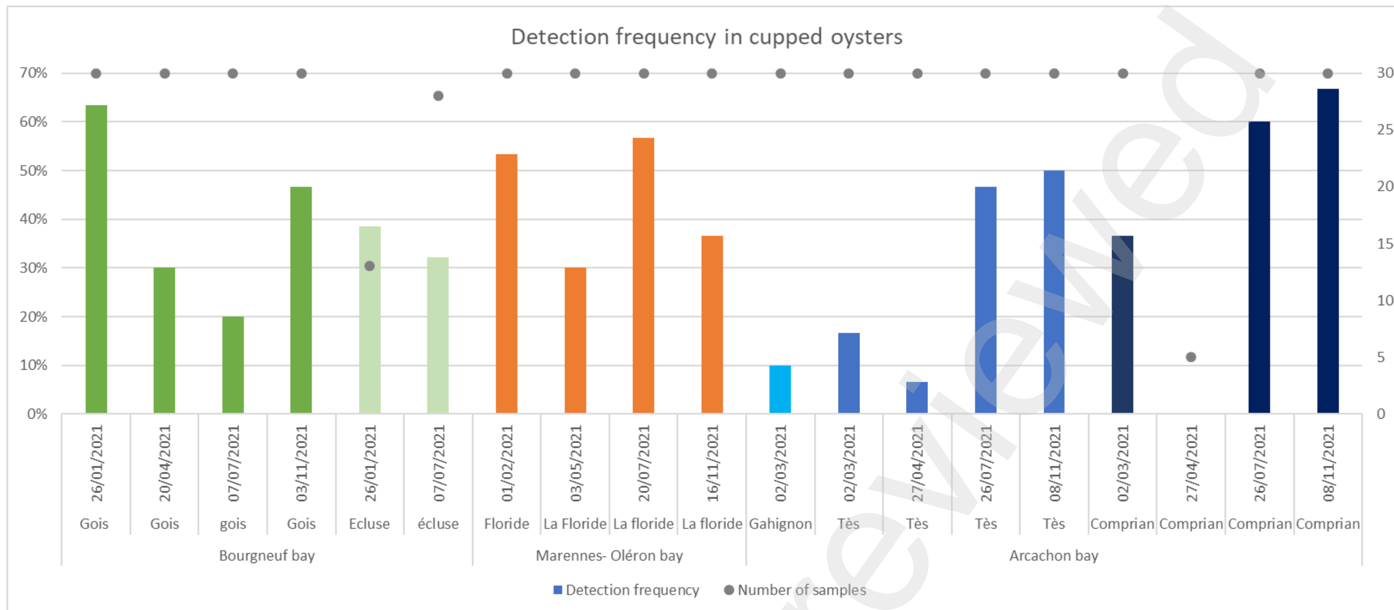
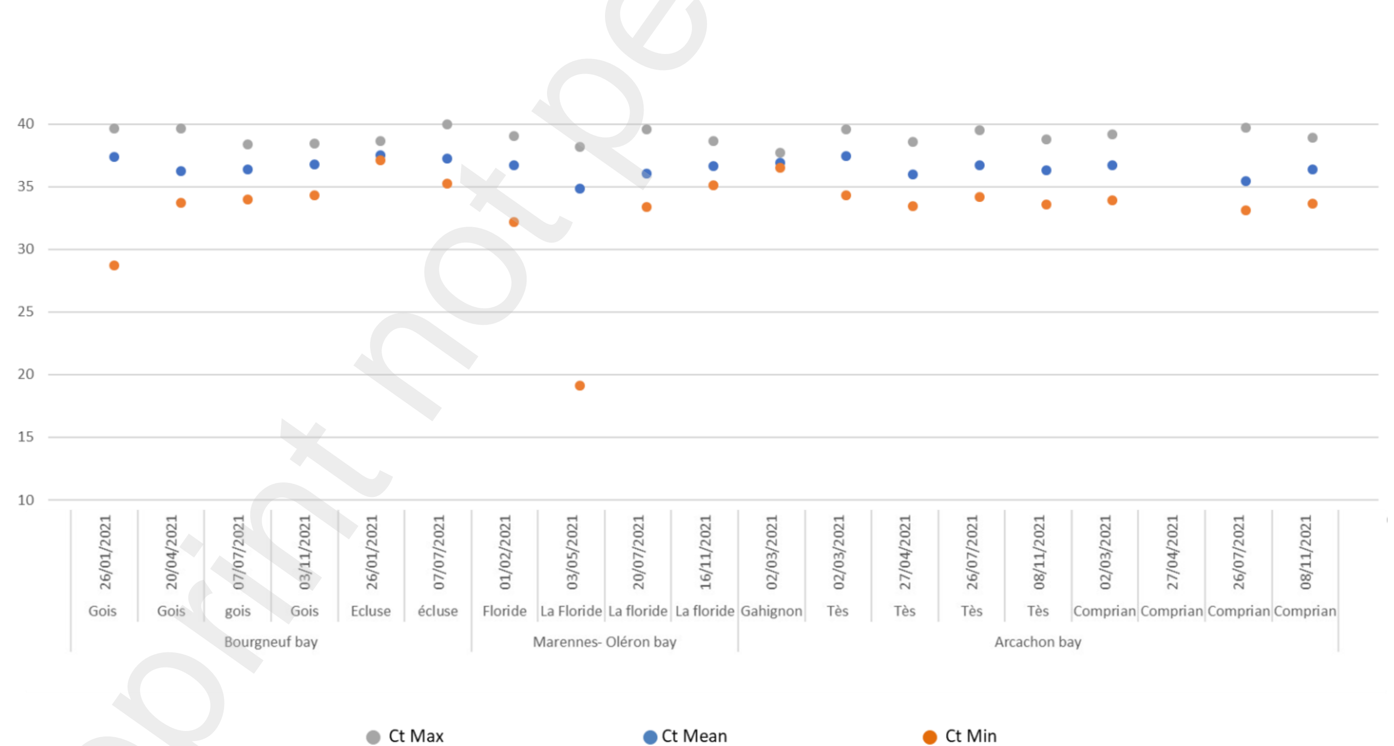


294

295 *Figure 3- PCR detection frequency (%) of* Haplosporidium costale *DNA depending on sampling sites and*
296 *dates. Grey dots show the number of tested samples.*

297



298

299 *Figure 4- Ct values of samples found positive by Real Time PCR for the detection of* Haplosporidium
300 costale. *Blue dots show mean Ct values; grey dots show the maximum Ct value and orange dots show*
301 *the minimum Ct value per sampling site and date.*

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302

303 All the oysters found positive by PCR had Ct values between 30 and 40 except two oysters collected in

304 January in Le Gois (Bourgneuf Bay; Ct = 28.72) and in May in La Floride, (Marennes Oléron Bay; Ct=

305 19.12) (Figure 4).

306 These two oysters were processed for histology and *in situ* hybridization. Spores and multinucleated

307 stages of the parasite were observed in the connective tissues around the digestive gland in the oyster

308 from Marennes Oléron. No parasite was observed in the oyster from Bourgneuf Bay which was

309 confirmed by *in situ* hybridization.

310

311 ## 3-2 In plankton and sediment samples

312 Although PCR inhibitors were detected in nanoplankton samples and 1/10 dilution avoided PCR

313 inhibition (see above), these samples were first tested without dilution. In these conditions, *H. costale*

314 DNA was detected in eight samples. Interestingly, all these samples (7.6% of nanoplankton samples)

315 were from Arcachon bay and 7 out of the 8 samples were collected in March. Ct values ranged between

316 39.82 and 44.54 (Mean Ct 43.02 ±1.86) and were below the detection limit of the method (16 copies

317 mL^{-1} see Result-1). When DNA from the nanoplankton fraction was tested again after a 1/10 dilution,

318 no additional positives were detected.

319 Parasite DNA was not detected in any tested meso and micro plankton samples with or without dilution

320 at 1/100.

321 In sediment, whatever the site, *H. costale* DNA was detected in 12 out of the 126 samples collected at

322 the surface (#9,5%) and in 7 out of the 126 samples collected at 5 cm deep (5.5%). Ct values were lower

323 in 5cm deep samples (mean Ct 37.6 ±3.5) than in surface sediment (Ct mean 39.1 ±3.54) but were all

324 below the detection limit of the method (40 copies mg^{-1}).

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325 Globally, 4.2% of samples from Bay of Bourgneuf and Marennes Oléron were positive while 12% of
326 samples from Arcachon showed amplification. Parasite DNA was detected in more samples collected
327 in July and November than in January-March or April.
328
329
330 3-3 In marine invertebrate other than oysters
331
332 Only two individuals out of 574 bivalves other than cupped oysters tested in this study yielded positive
333 results in PCR: one out of 64 cockles (Ct value = 38.23) and one out of 369 mussels (Ct value = 37.14).
334 The cockle detected positive was collected in Arcachon in November and the mussel in Marennes
335 Oléron in July.
336 Among all the tested invertebrates other than bivalves, *H. costale* DNA was detected in three groups:
337 (i) Malacostraca, hermit crab (1/50, Ct value : 39.35) collected in Arcachon in April; (ii) Polychaeta,
338 *Nephtydae* (2/96 individuals- Ct values : 38.46 and 41) collected in Marennes Oléron in November and
339 in Arcachon in March and (iii) Gastropoda, periwinkles (11/111 individuals- Ct values between 35.99
340 and 40.35). Periwinkles detected positive by PCR were collected mostly in July (6 in Bourgneuf bay and
341 2 in Marennes Oléron) and in a lesser concern in November (2 in Bourgneuf bay and 1 in Marennes-
342 Oléron).
343

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344

345 ## 4- Detection of *Haplosporidium costale* in the experimental mesocosms and different
346 oyster tissues

347

348 ### 4-1. In water, sediment samples and oysters at the individual level

349

350 Controls including water and sediment collected at T0 and water used for renewal were tested
351 negative by real time PCR. Parasite DNA was not detected in any of the 27 tested sediment samples.
352 One sample of >20 µm water and three samples of 1-20 µm water yielded positive but late
353 amplification (Ct >37). These positive signals were only obtained from samples collected in the same
354 aquarium (n°2) (Table 4).

355 The analyses of oysters based on a DNA extraction from a pool of gills, digestive gland and mantle
356 tissues revealed the presence of parasite DNA in 3/14 and 8/18 oysters from the aquaria n°1 and 3,
357 respectively (Table 4).

358

359 ### 4-2. In oysters at the tissular level

360 Oysters from aquaria 1 and 2 (29 in total) were tested at the tissular level regarding the presence of *H.*
361 *costale* DNA.

362 In total, 21 oysters (11/14 in aquarium 1 and 10/15 in aquarium 2) showed at least one tissue positive.
363 Parasite DNA was detected in 21/29 gill samples, 3/29 mantle samples, 1/29 of digestive gland samples
364 and 1/29 hemolymph samples. None of the palp, adductor muscle, gonad and heart samples yielded
365 amplification.

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366 Aquarium 2 showed more positive oysters than aquaria 1 and 3 and was the only aquarium in which
367 some water samples were tested positive.

368 Ct values appeared generally lower in gills (global mean Ct value : 35.2) and mantle (global mean Ct
369 value : 35.49) compared to other positive samples (Ct >37) and compared to the pools of gills/digestive
370 glands and mantle (global mean Ct value : 37.1 Table 4).

	Aquarium 1	Aquarium 2	Aquarium 3
Water 1-20µm	0/27	3/27 (42; 43; 44.4)	0/27
Water >20µm	0/27	1/27 (37.8)	0/27
Sediment	0/9	0/9	0/9
Oysters (pools of gills/digestive gland and mantle)	3/14 (37.38; 38.15; 38.48)		8/18 [35.4; 36.3; 38.1]
Oyster gills	11/14 [34.08; 35.3; 37.86]	10/15 [30.81; 35.04; 37.71]	
Oyster mantle	2/13 (33.13; 36.97)	1/15 (36.37)	
Oyster digestive gland	0/13	1/15 (40.7)	
Oyster Adductor muscle	0/14	0/15	
Oyster gonad	0/14	0/15	
Oyster palp	0/13	0/15	
Oyster heart	0/14	0/13	
Oyster hemolymph	0/14	1/15 (37.37)	

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371 *Table 4- Results of the PCR analyses for the detection of* Haplosporidium costale *DNA for each aquarium*
372 *and type of samples: Number of samples tested positive/Total number of tested samples. Figures in*
373 *brackets () indicate the Ct values of the positive samples. Figures in parentheses [] indicate the*
374 *minimum ; mean and maximum Ct values.*

375

376 Based on PCR results, 20 oysters (11 oysters from aquarium 1 and 9 oysters from aquarium 2) for which
377 gills were positive, were subsequently tested by histology and *in situ* hybridization. Additionally, for
378 four oysters showing Ct values between 30.8 and 34.23, 3 to 5 sections per individual were tested by
379 *in situ* hybridisation. Whatever the Ct value obtained by PCR, no parasite was observed in histology
380 and no positive result was obtained by *in situ* hybridisation.

381

382

383 ## Discussion

384

385 Because of their small size, between 2 µm and 1 mm depending on their stages, their intra-tissular and
386 eventually intracellular location and their low abundance in hosts, micro eukaryotic parasites remain
387 challenging to investigate (Bass et al. 2015). Additionally, the lack of culture and their genetic
388 divergence might increase this complexity.

389 Among micro eukaryotic parasites, haplosporidians are rhizarian parasites of aquatic invertebrates and
390 include causative agents of diseases of commercially important molluscs such as MSX and SSO diseases
391 in oysters. Despite their importance, their diversity and distribution are still poorly known (Hartikainen
392 et al. 2014). In particular, their parasite cycle is not well understood. To our knowledge, although
393 *Haplosporidium costale* has been detected in oysters *Crassostrea virginica* and *Magallana gigas*, its

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394 presence in other bivalve species has never been investigated. Because it has never been possible to
395 reproduce experimentally the disease, the need of an intermediate host has been hypothesized but
396 never demonstrated (Andrews, 1984).

397 "Integrated" field studies testing not only the presence of parasite in host species but also in sympatric
398 species as well as in environmental compartments allow better characterizing the distribution of the
399 parasites at the ecosystem scale (e.g. Mérou et al. 2023). In this context, eDNA based approaches are
400 very powerful to detect the presence of parasites outside their hosts (Bass et al., 2015 ; Bass et al.
401 2023; Ríos-Castro et al., 2021; Rusch et al. 2018). However, molecular tools usually used to detect
402 parasite DNA in host tissues might need to be optimized prior being applied on environmental
403 matrices. Herein, we used a real time PCR previously developed and validated to test the presence of
404 *H. costale* in oysters (Arzul et al. 2022), to investigate the presence of the parasite in other invertebrate
405 species, plankton and sediment. The detection limit of the method was evaluated at 10^3 copies per
406 quarter filter (equivalent to 16 copies. ml^{-1} of water) and 10^4 copies per 250 mg of sediment (equivalent
407 to 40 copies. mg^{-1}). These values appear higher than values estimated using the detection limit of the
408 PCR alone (4.25 copies; μl^{-1} Arzul et al. 2022) and considering the extraction process as 100% efficient.
409 Indeed, in such conditions, the method should allow detecting down to 212 copies per quarter filter
410 and 2120 copies from 25 mg of sediment. These results suggest that part of the plasmids has not been
411 recovered at the end of the DNA extraction process. Likewise, Polinski et al. (2017) developed a
412 method for the detection of the oyster parasite *Mikrocytos mackini*, another Rhizarian, in water and
413 noted that the amount of parasites recovered was consistently less than 28% of initial quantity and
414 attributed this loss to the filtration and extraction steps.

415 An approach similar to the one used in our study was previously developed to detect the flat oyster
416 Rhizarian parasites *Bonamia ostreae* and *Marteilia refringens* in environmental samples and allowed
417 detecting down to 25 parasites per quarter membrane for both parasites and 10 parasites in 25 mg
418 sediment for *M. refringens* (Mérou et al. 2020; 2022). While in these two studies, detection limits were
419 estimated using parasites isolated from infected oysters, in our study we had to use plasmidic DNA,

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420 which could explain the difference observed between detection limit values. Indeed, the genome of *H.*
421 *costale* probably contains several copies of the 18S rRNA genes.

422 Inhibitors may affect the sensitivity of the PCR assay or even lead to false-negative results (Schrader et
423 al. 2012). PCR inhibitors occur in various samples including environmental samples and bivalves
424 (Sanches & Schreier 2020; Hunter et al. 2019; Mancusi et al. 2022). Dilution of extracted DNA allows
425 overcoming PCR inhibition but decrease PCR sensitivity. In our study, we used an exogenous internal
426 positive control to evaluate the presence of PCR inhibitors and adjust DNA concentration. Presence of
427 PCR inhibitors was tested in a set of samples representative of each category of samples collected in
428 the study. Inhibition was observed more particularly in all the fractions of plankton samples,
429 Gastropoda except *Patella* and *Gibbula*, Terebellidae, Sabellidae and the bivalve species *Mimachlamys*
430 *varia*. These results demonstrate the interest to evaluate the presence of inhibitors prior PCR analyses
431 especially when new matrices are to be tested.
432
433 The detection and characterization of *H. costale* in different batches of oysters *M. gigas* in France since
434 2005 (Arzul et al. 2022; Cherif - Salal et al. 2022) raised several questions regarding the ability of the
435 parasite to maintain in the ecosystem and the potential involvement of other species in its life cycle.
436 To answer these questions, an integrated sampling approach was deployed seasonally in three oyster
437 farming areas where the parasite was already known to occur. Parasite presence was evaluated using
438 a previously developed and validated Real Time PCR assay (Arzul et al. 2022), optimized in this study
439 to detect parasite DNA in various environmental compartments.
440
441 Parasite DNA was detected in “wild” oysters from the three oyster farming zones whatever the
442 sampling date. No seasonal pattern could be established based on PCR results. Indeed, detection
443 frequencies ranged between 30 and 67% and peaked in January in Bourgneuf Bay and in July –
444 September in Marennes Oléron and Arcachon. Ct values can be considered as a proxy of the pathogen
445 load or infection level (e.g Walker et al. 2021). In our study, global mean Ct values was 36.54 and Ct

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446 values were always above 30 except for two oysters, one collected in Gois, Bourgneuf bay in January
447 and one collected in La Floride, Marennes Oléron in May. Only this later showed parasite (spores) in
448 histology. Apart from these two oysters, infection intensity appeared very low, between 1 and 10
449 copies/µL (deduced from Arzul et al. 2022). In *Crassostrea virginica*, discrepancy between histology
450 and PCR was previously reported, the use of molecular tools allowing the detection of parasite DNA
451 between spring and fall whereas only multiplication and sporulation stages were observed between
452 March and June when using histology, (Stokes and Burreson, 2001; Andrews et al. 1962; Andrews and
453 Castagna, 1978). In *M. gigas* in France, Real Time PCR allowed detecting parasite DNA in oysters
454 collected between April and November (Arzul et al. 2022; Cherif-Feildel et al. 2022). These two last
455 studies relied on farmed oysters which may have been moved from one site to another. In contrast,
456 our results are based on Real Time PCR analyses of wild oysters, which have never been moved thus
457 reflecting the status of the zone regarding the presence of the parasite. Our results suggest that the
458 parasite maintains in *M. gigas* all along the year at a low intensity level.
459 The low detection frequency and high Ct values observed in other benthic invertebrates do not support
460 their involvement in *H. costale* cycle. The case of periwinkles is different since 9.9% of tested
461 individuals were found positive with Ct values ranging from 35.99 to 40.35. Unfortunately, in this study,
462 fauna associated with oysters was not fixed in histology. It is thus not possible to conclude if the
463 detection of parasite DNA in periwinkles corresponds to a true infection or is the result of their grazing
464 behaviour which might contribute to catch the parasite through nutrition.
465 *HI costale* DNA was not detected in meso- or microplankton samples. However, we cannot exclude
466 that the absence of detection of parasite in these plankton fractions is explained by the presence of
467 PCR inhibitors and the need to dilute DNA suspensions prior PCR analyses.
468 In contrast, parasite DNA was detected in nanoplankton samples. However, the amount of parasite
469 detected in this fraction appeared low. These results suggest that when present in the water, the
470 parasite is rather free than in or attached to phyto- or zooplankton. Most of these detections were

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471 from samples collected in March suggesting that the parasite can occur in the nanoplankton while
472 developing in the oysters.
473 In sediment, parasite DNA was detected in both surface and 5-cm deep samples. Similarly to
474 nanoplankton, the amount of parasites detected was low. Interestingly, more detection was observed
475 in samples collected in July and November than in January-March or April, which supports the
476 hypothesis that the parasite is mostly released from the oysters during mortality, between May and
477 June and should remain detectable the following months depending on the ability of the parasite to
478 survive outside its host. The detection of parasite DNA in 5-cm deep samples might be explained either
479 by the small size of the parasite seeping in sediment particles or by the activity of bioturbating species
480 such as polychaeta or infaunal bivalves.
481
482 Considering the high number of oysters found positive with low infection intensity level, a
483 complementary experiment was undertaken to better characterize these sub-clinical infections.
484
485 Oysters collected in May from La Floride, Marennes Oléron were maintained for one month in
486 mesocosms before being tested regarding the presence of *H. costale* DNA at the individual level from
487 pools of gills, mantle and digestive gland or at the tissular level. All the oysters found positive showed
488 positive results at least in gills. Parasite DNA was also detected, but in a much lesser extent, in mantle,
489 digestive gland and hemolymph. Although both diagnostic approaches (pools of organs and by tissue)
490 were not applied on the same oysters, the detection frequency was globally twice higher from the gills
491 (72% of positive oysters) than from pools (34% of positive oysters). None of the oysters for which gills
492 were found positive by PCR were found hosting the parasite by histology and *in situ* hybridization.
493 These results indicate that the parasite is mostly present in the gills and its abundance is very low. No
494 parasite DNA was detected in sediment samples and only three and one sample of nano and
495 microplankton, respectively were found positive. This low detection of the parasite in the plankton
496 supports the hypothesis that the parasite is stably established in gills rather than just being retained

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497 by gills through filtration activity. Moreover, water introduced in the experimental aquaria was initially
498 tested negative suggesting that the parasite can be released from the oysters in the water outside
499 mortality event and in the absence of sporulation as demonstrated by histology.
500

501 Conclusion

502

503 Altogether, our results show that *Haplosporidium costale* is mostly detected in *Magallana gigas*,
504 particularly in gills, which may act as a reservoir all along the year. *H. costale* was detected in
505 nanoplankton in both field and experimental conditions suggesting that it can be released from the
506 oysters outside mortality event. Once outside the oysters it can spread through current but partly sink
507 on the sediment where it can be detected, especially after the sporulation period. Our results do not
508 support the involvement of other species than cupped oyster in the parasite life cycle except
509 periwinkles. However, the role of this gastropod in the dynamic of the parasite would deserve to be
510 further investigated. Although, our study has also contributed to better characterize sub clinical
511 infections, factors triggering sporulation would definitely need to be clarified in order to understand
512 disease development and finally suggest disease control measures.

513

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CRediT authorship contribution statement

Isabelle Arzul : Data curation, Formal analysis, Methodology, Conceptualization, Writing – original draft, Writing – review & editing ; Cyrielle Lecadet : Data curation, Formal analysis, Investigation, Methodology ; Lydie Canier: Data curation, Formal analysis, Writing – review & editing; Bruno Chollet: Investigation; Delphine Serpin: Investigation; Xavier de Montaudouin: Data curation, Writing – review & editing.

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Table S1 - Detection of *Haplosporidium costale* DNA by Real time PCR (Number of positive/Number of tested samples) by category of samples (Individuals were gathered at the class level) by sampling date and site. The last column indicates information about Ct values obtained for positive samples only.

Zone	**Bourgneuf bay**								**Marennes Oléron bay**				**Arcachon bay**									**Total**	**Info Ct**
Site	Le Gois				Ecluse				La Floride				Comprian				Gahignon	Tès				Nb positive /Nb total (%)	Info Ct (Mean± Standard error [Min - Max]
Sampling Date	26/01/2021	20/04/2021	07/07/2021	03/11/2021	26/01/2021	20/04/2021	07/07/2021	03/11/2021	01/02/2021	03/05/2021	20/07/2021	16/11/2021	02/03/2021	27/04/2021	26/07/2021	08/11/2021	02/03/2021	02/03/2021	27/04/2021	26/07/2021	08/11/2021		
Environmental samples																							
Nanoplankton (1-20 µm)	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	3 /5	0/5	0/5	0/5	3+/5	1/5	0/5	1/5	0/5	8/105 (7,6%)	43,02 ± 1,86 [39,82-44, 54]
Microplankton (20-200 µm)	0/2	0/2	0/2	0/2	0/2	0/1	0/2	0/2	0/2	0/2	0/2	0/2	0/ 2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/41	

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	Mesoplankton (>200 µm)	0/2	0/2	0/2	0/2	0/2	0/1	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/41	
	Sediment (at surface)	0/6	0/6	1/6	0/6	0/6	0/6	0/6	2/6	0/6	0/6	1/6	0/6	0/6	0/6	6/6	1/6	0/6	1/6	0/6	0/6	0/6	12/126 (9,5%)	39,1 ± 3,54 [33,34-43,625]
	Sediment (5cm deep)	0/6	0/6	0/6	1/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	1/6	0/6	0/6	2/6	1/6	0/6	2/6	0/6	0/6	0/6	7/126 (5,5%)	37,6 ± 3,5 [32,425-41, 195]
Bivalvia																								-
	Pacific oyster *(Magallana gigas)*	19/30	9/30	6/30	14/30	5/13		9/28		16/30	9/30	17/30	11/30	11/30	0/5	18/30	20/30	3/30	5/30	2/30	14/30	15/30	203/526 (38,6%)	36,54 ± 0,69 [19,12-39,96]
	Mussel (*Mytilus)*	0/20	0/30	0/30	0/30	0/17	0/9	0/18	0/1	0/13	0/21	1/30	0/30						0/30	0/30	0/30	0/30	1/369	37,14
	Manila clam *(Ruditapes philippinarum)*	0/7		0/3	0/11		0/1			0/9	0/12	0/7	0/6	0/6	0/11	0/2	0/4						0/79	
	Common cockle *(Cerastoderma edule)*	0/17		0/10	0/6					0/23					0/3		1/1					0/4	1/64	38,23
	Peppery furrow shell *(Scrobicularia plana)*					0/30	0/15							0/1									0/46	

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	Razor clam *(Solen marginatus)*																0/10						0/10	
	Variegated scallop (*Mimachlamys varia)*			0/1														0/5					0/6	
Gastropoda (species or genus)																								
	Common periwinkle *(Littorina littorea)*			0/28	0/11	0/3	0/31		1/4	0/6		2/6	1/9	0/1				0/8	0/4				11/111 (10%)	38,33 ± 1,18 [35,99-40,35]
	Common periwinkle eggs																			0/7			0/7	
	Slipper shell *(Crepidula fornicata)*	0/9		0/9	0/6									0/3	0/3			0/10		0/12		0/13	0/65	
	Top shell *(Steromphala umbilicalis)*														0/30					0/19			0/49	
	European sting winkle (*Ocenebra erinaceus*)						0/2			0/1								0/1	0/3		0/1		0/8	
	Netted dog whelks *(Tritia reticulata)*																			0/7			0/7	
	Limpets *(Patella)*					0/1		0/1										0/2					0/4	

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Polychaeta (family)																							
Nephtydae	0/1	0/1	0/1	0/6	0/1			0/2	0/1	0/6	0/11	1/25	1/6	0/3	0/1	0/21	0/1		0/1	0/2	0/6	2/96	38,46 ; 41
Nereididae	0/1	0/3		0/3	0/6	0/23	0/20	0/26	0/1	0/7	0/1							0/1	0/1		0/1	0/94	
Sabellidae	0/2			0/3					0/1					0/3		0/1	0/2	0/11				0/23	
Capitellidae				0/1				0/2			0/5	0/2			0/11							0/21	
Terebellidae												0/7										0/7	
Malacostraca/Thecostraca *																							
Pea crab (Species *Pinnotheres pisum*)							0/1				0/4	0/6										0/11	
Hermit crabs (Family *Diogenidae*)													0/7					0/10	1/30		0/3	1/50	39,35
Order *Amphipoda*	0/1	0/1				0/5			0/1			0/4		0/1	0/2	0/2	0/1					0/18	
Order Isopoda									0/1	0/1		0/16										0/18	

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	*Barnacles (Family Balanidae)**			0/14																			0/14	
Other (class or species)																								
	Sea sponge (*Hymeniacidon perlevis)*																0/10	0/4		0/5			0/19	
	Anemone (*Class Anthozoa)*	-	-	0/8		0/3	0/4		0/2	-													0/17	
	Asian tunicate (*Styela clava)*-																		0/5				0/5	
	Flat worm *(Idiostylochus tortuosus)*																		0/3				0/3	

** the only Thecostraca specimens were Barnacles (Family Balanidae)*

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