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Effects of temperature and salinity on the survival of *Bonamia ostreae*, a parasite infecting flat oysters *Ostrea edulis*

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Abstract:

Bonamiosis due to the intrahaemocytic protistan parasite *Bonamia ostreae* is a European endemic disease affecting the flat oyster *Ostrea edulis*. The parasite has been described in various ecosystems from estuaries to open sea, but no clear correlation has yet been demonstrated between disease development and environmental parameters. In this study, the effect of temperature and salinity on the survival of purified parasites maintained *in vitro* in seawater was investigated by flow cytometry. Purified parasites were incubated in various seawater media (artificial seawater, natural seawater, seabed borewater) at various temperatures (4, 15 and 25°C) and subjected to a range of salinities from 5 to 45 g l^{-1}. Parasites were collected after 12, 24 and 48 h of incubation for flow cytometry analyses including estimation of parasite mortality and parasite viability through detection of non-specific esterase activities. Artificial seawater appeared unsuitable for parasite survival, and results for all media showed a significantly lower survival at 25°C compared to 4°C and 15°C. Moreover, high salinities (≥35 g l^{-1}) favoured parasite survival and detection of esterase activities. Flow cytometry appears to be a suitable technique to investigate survival and activities of unicellular parasites like *B. ostreae* under varied conditions. Although these results contribute to a better understanding of existing interactions between the parasite *B. ostreae* and its environment, validation through epidemiological surveys in the field is also needed.

Keywords: *Bonamia ostreae* · Flow cytometry · *In vitro* assays · Cell viability · Temperature tolerance · Salinity tolerance

50

51 INTRODUCTION

52

53 *Bonamia ostreae* is a protistan parasite belonging to the phylum Haplosporidia (Sprague 1979). It
54 is an intracellular parasite, 2-5 µm in diameter, that infects haemocytes. It can also be observed
55 extracellularly between epithelial or interstitial cells in gills and stomach or in necrotic connective
56 tissue areas. The parasite can be detected in spat (Lynch et al. 2005), however, mortalities mainly
57 affect oysters which are more than 2 year old (Culloty & Mulcahy 1996). At a tissue level, the
58 infection is usually associated with intense haemocyte infiltration of the connective tissue of the
59 gills, mantle and digestive gland. The life cycle is unknown but the disease can be directly
60 transmitted between oysters in a population or experimentally by cohabitation or inoculation
61 (Elston et al. 1986, Hervio et al. 1995) suggesting that intermediate host is not required for parasite
62 cycle accomplishment. Observation of parasites free in gill epithelia potentially associated with
63 gill lesions supports the hypothesis of a parasite release through these organs (Montes et al. 1994).
64 However, the infective form and ways of entry and release remain undetermined. Most of *Bonamia*
65 *ostreae* might be released in the water column after oyster death through tissue lysis.

66 This intrahaemocytic parasite has been described in oysters collected from different ecosystems
67 from estuaries and intertidal zones to deep coastal waters or lagoon and is presently reported in
68 Europe, North America and Morocco. Northern European waters (e.g. Norwegian waters) seem to
69 be free of bonamiosis probably because of the lack of introduction of infected animals. Flat oysters
70 from the Mediterranean Basin are infected by *Bonamia ostreae*, however reported prevalences are
71 low. No clear correlations have been demonstrated between development of the disease and
72 environmental parameters including temperature and salinity. Previous work suggested an impact
73 of temperature on the parasite and / or on the defence capacity of oysters. Although the disease
74 occurs and can be transmitted throughout the year (Tigé & Grizel, 1984), there is a seasonal
75 variation in infection with *Bonamia ostreae*. Prevalence of infection presents peaks in late winter

76 and in autumn (Grizel 1985, Montes 1990, Van Banning 1991, Culloty & Mulcahy 1996, Arzul et
77 al. 2006). A study of bonamiosis prevalence as well as haemocyte activities according to
78 temperature showed that prevalence was higher at low temperature (10°C) compared to higher
79 temperature (20°C) suggesting that low temperatures may affect defence capacities of the oyster
80 and/or the ability of the parasite to infect healthy oysters (Cochennec & Auffret 2002).

81 The lack of suitable tissue culture systems and mollusc cell lines for the culture of the parasite led
82 to the development of a purification protocol (Miahle et al. 1988). The availability of purified
83 *Bonamia ostreae* suspensions allowed experimental infections based on parasite injection (Hervio et
84 al. 1995) and investigations on *in vitro* interactions between parasites and haemocytes (Chagot et al.
85 1992, Mourton et al. 1992). Despite possible survival of purified parasites in filtered sea water (2
86 weeks) as assessed by success of experimental infection (Grizel 1985), purified parasite suspensions
87 have not yet been used to study parasite physiology or its behaviour related to environmental
88 conditions.

89 In aquatic ecology, flow cytometry is classically used to determine abundance, viability and activity
90 of microorganisms including viruses, bacteria, microalgae and planktonic protozoan parasites
91 (Wong & Whiteley 1996, Lindström et al. 2002, Parrow & Burkholder 2002, Binet & Stauber 2006,
92 Hammes et al. 2008). Recent developments aimed at addressing some questions in environmental
93 microbiology including studying microbial physiology under environmentally relevant conditions
94 (Czechowska et al. 2008). Flow cytometry was successfully used to measure cell viability of
95 cultured *Perkinsus marinus*, a parasitic protozoan of the Eastern oyster, *Crassostrea* virginica
96 (Soudant et al. 2005). This tool allows multi parametric analyses on a large number of cells in a
97 very short time and thus presents advantages over microscopic approaches.

98 The objectives of the present study were to test survival of purified *Bonamia ostreae* in different sea
99 water media (artificial, natural and underground salty water) in order to identify the most suitable
100 medium for parasite preservation and to investigate effects of temperature and salinity on the
101 survival of purified parasites by flow cytometry. Purified *B. ostreae* were suspended and maintained

102 *in vitro* in the three different media at three different temperatures and then subjected to a range of
103 salinities in the optimal medium previously defined. Parasite mortality was measured by flow
104 cytometry using propidium iodide staining and parasite viability was estimated by measuring
105 esterase activities using FDA (Fluorescein Diacetate). Esterases are enzymes belonging to the group
106 of hydrolases and are classically measured to estimate global level of viable cell activities
107 (Gagnaire et al. 2006b, Berney et al. 2008, Rault et al. 2008).
108 *In vitro* exposure of purified parasites to ranges of temperature and salinity may improve our
109 knowledge of the disease epidemiology and may provide guidance for oyster farmers for stock
110 management.
111

112 **MATERIAL AND METHODS**

113

114 ***Bonamia ostreae purification***

115 Purification of parasites was performed following the protocol of Miahle et al. (1988) using flat
116 oysters originated from Quiberon Bay (France), an infected area regarding *Bonamia ostreae.*
117 Oysters were maintained 30 days in raceways of 120 litres receiving a constant flow of external
118 seawater at a temperature of 12-15°C and enriched in phytoplankton (*Skeletonema costatum*,
119 *Isochrysis galbana*, *Chaetoceros gracilis* and *Tetraselmis suecica*). Some highly infected flat
120 oysters *Ostrea edulis* were selected by examination of heart tissue imprints under light microscope.
121 Two to three highly infected oysters were used per purification. All organs were homogenized
122 except the adductor muscle. Parasites were concentrated by differential centrifugation on sucrose
123 gradients and then purified by isopycnic centrifugation on a Percoll gradient. Centrifugations were
124 performed at 8°C. Lastly, purified parasites were resuspended in 1 ml of 0.22 µm filtered sea water
125 before being counted using a Malassez-cell haemocytometer. Parasite suspensions were then
126 maintained at 4°C. Salinity of filtered sea water fluctuated between 30 and 34 g l^{-1}.

127 ***Experiment design***

128 Two sets of experiments were performed three times.

129 In a first set of experiments, purified parasites were suspended in three different media and 10 ml of
130 each parasite suspension were distributed in equivalent numbers (5.10^6 cells ml^{-1}) in nine 15 ml-
131 polypropylene tubes per medium. The three media were (1) 0.22 µm filtered prepared artificial sea
132 water with a salinity of 23.4 g l^{-1} and a pH of 6.5 (ASW: 23.4 g NaCl, 1.5 g KCl, 1.2 g $MgSO_4$. 4
133 H_2O, 0.2 g $CaCl_2$. 2 H_2O, H_2O q.s. 1 L) (2) 0.22 µm filtered underground salty water showing a
134 constant salinity of 32 g l^{-1} and a pH of 7.06 (USW, collected at – 110 meters at IFREMER facilities
135 in La Tremblade, France) (3) 0.22 µm filtered natural sea water (NSW) from « La Seudre » estuary,
136 Charente Maritime (France) with a salinity of 30-34 g $l^{-1}$and a pH of 8.06. Parasites maintained in
137 the three different media were subjected to three different temperatures 4°C, 15°C and 25°C (three
138 tubes per condition). The different parasite suspensions were tested by flow cytometry after 12 h, 24
139 h and 48 h of incubation.

140

141 Regarding parasite survival according to previously tested medium and temperature, the second set
142 of experiments, aiming at testing effects of salinity on *Bonamia ostreae* viability, was performed in
143 USW (stable composition in the time compared to natural sea water) and at 15°C (which reflects
144 better natural conditions than 4°C).

145 More precisely, purified parasites were diluted in 0.22 µm filtered USW and distributed in
146 equivalent numbers (5.10^6 cells ml^{-1}) in 15 ml-polypropylene tubes. Distilled water or natural salt
147 from Guérande (Pays de la Loire, France) was added in order to obtain a range of salinities: 5 g l^{-1},
148 15 g l^{-1}, 20 g l^{-1}, 25 g l^{-1}, 30 g l^{-1}, 35 g l^{-1}, 40 g l^{-1}, 45 g l^{-1}. Parasite suspensions (3 tubes per salinity
149 condition) were incubated at 15°C and samples were analysed at 12 h and 48 h by flow cytometry.

150

151 ***Analysis of viability of Bonamia ostreae by flow cytometry***

152 Flow cytometry protocols used in this study were adapted from protocols previously described for

153 *Crassostrea gigas* haemocytes (Gagnaire et al. 2006a). Each measure was carried out three times.
154 For each sample, 5000 events were counted using an EPICS XL 4 (Beckman Coulter). Results were
155 depicted under biparametric representations (density plots) showing parasite cells according to the
156 Forward SCatter (FSC) in abscissa and Side SCatter (SSC) in ordinate and the fluorescence channel
157 corresponding to the marker used. FSC and SSC values, which correspond to diffracted light on the
158 small and right angles, are proportional to cell size and cell complexity, respectively. Recorded
159 fluorescence depended on the monitored parameters: non specific esterase activities were measured
160 using green fluorescence (Fluorescence detector FL1) while cell mortality was measured using red
161 fluorescence (Fluorescence detector FL3).
162 Parasite mortality was estimated after incubating 200 µl of parasite suspensions at 5.10^5 cells ml^{-1} in
163 the dark for 30 min at 4°C with 10 µl of the nucleic acid fluorescent dye propidium iodide (PI, 1.0
164 mg l^{-1}, Interchim). Non specific esterase activities were evaluated by incubating 200 µl of parasite
165 suspensions at 5.10^5 cells ml^{-1} in the dark for 30 min at ambient temperature with 1 µl of the
166 liposoluble substrate fluoresceine diacetate (FDA, 400 µM in DMSO, Molecular Probes,
167 Invitrogen).
168
169 Dead parasites, prepared by boiling cells for 15 min, were used to control efficacy of PI for
170 mortality measurement. The FL3 fluorescence histogram showed 98.1% of PI-stained cells (red
171 fluorescence above 1) considered dead (Fig. 1a). Suspension of live parasites was used to control
172 efficacy of FDA for esterase activities measurement (Fig. 1b). The FL1 fluorescence histogram
173 showed 91% of fluorescent cells after incubation with FDA (green fluorescence above 1)
174 considered alive and presenting esterase activities (Fig. 1b).
175
176 ***Statistical analysis***
177 Data were analyzed statistically using the software Statgraphics® Plus version 5.1. Results were
178 expressed as percentages of positive cells. Mean and standard deviation were calculated for each

179 triplicate. Effect of tested conditions was evaluated performing one-way, two-ways and three-ways
180 ANOVA. Values were converted into r angular arcsinus √(% of positive cells) before analysis to
181 ensure respect of *a priori* assumptions of normality and homogeneity. In the case of rejection of H_0,
182 an *a posteriori* Least Significant Difference Test was used to compare differences between means
183 and to obtain hierarchy between studied factors. Significance was concluded at $p \leq 0.05$.
184
185 **RESULTS**
186
187 ***Size and complexity of parasites***
188 Size and in a lesser concern complexity of parasites varied according to their status: dead or alive.
189 Irrespective of the tested conditions, it was possible to identify two populations of parasite cells
190 (Fig. 2a): a population A consisting in 53 ± 24% of live cells and a population B, smaller in size
191 consisting in a majority of dead cells (mean of 74 ± 23%). Some parasite cells were not included in
192 population A or B and corresponded generally to dead cells showing higher size and higher
193 complexity than cells included in populations A and B. For parasites maintained in NSW at 4°C 12
194 hours after purification, population A and population B included 75.8% and 15.7% of total cells,
195 respectively (Fig. 2a). When only considering non PI stained parasites for the same experimental
196 conditions, population A and population B included 91% and 6.5% of live cells, respectively (Fig.
197 2b). For parasites after boiling, when only considering PI stained cells, population A and population
198 B included 8.6% and 88% of dead cells, respectively (Fig. 2c).
199
200 Population A included more live cells (77.6 ± 6.2%) when mortality rates were below 50%
201 compared to mortality rates above 70% (37.3 ± 24.7%) (Table 1). On the contrary, population B
202 included more dead cells when mortality rates were high (91.7 ± 7.4% for mortality rates above
203 70%) (Table 1).
204

205 ***Impact of medium on cell viability***

206 In the three experiments testing simultaneously the effect of medium, temperature and time of
207 incubation on parasites (three-ways ANOVA), the medium appeared as the most important factor on
208 parasite survival ($p = 0$) and influences more cell mortality and esterase activities than temperature
209 and time of incubation (Table 2).

210 Irrespective of time and temperature of incubation, parasites showed significant better survival in
211 NSW and in USW than in ASW (Fig. 3 and Table 2). However, there was no significant difference
212 between mortality and esterase activity percentages in NSW and USW. Parasite mortality means
213 were 29.1%, 31.4% and 71.1% in NSW, USW and ASW, respectively. The percentage of positive
214 parasites for esterase activities was 44.9 ± 7.8% in NSW, 48.2 ± 6.5% in USW and 30.6 ± 4.1% in
215 ASW, respectively.

216

217 ***Impact of temperature on cell viability***

218 Parasite viability fluctuated according to the tested temperature. Irrespective of medium and time of
219 incubation, mortality appeared significantly higher at 25°C compared to 15°C and 4°C and
220 percentages of cells presenting esterase activities were higher at 4°C compared to 15°C and 25°C
221 (three-ways ANOVA, Table 2).

222 In NSW, irrespective of incubation time, mortality percentages ranged from 11.92 to 25.59% at 4°C,
223 from 16.2 to 31.83% at 15°C and from 39.26 to 75.55 at 25°C (Fig. 4). Cell mortality was thus
224 higher at 25°C compared to 4°C and 15°C especially after 24h and 48h of incubation ($p < 0.0001$)
225 (Fig. 4).

226 In USW, irrespective of time of incubation, the percentage of positive parasites for esterase
227 activities ranged from 52.49 to 73.67% at 4°C, from 31.27 to 43.73% at 15°C and from 21.89 to
228 53.28% at 25°C (Fig. 5).

229

230 ***Impact of incubation time on cell viability***

231 Irrespective of medium and temperature, incubation time did not have significant impact on parasite
232 mortality. However, a difference of percentages of cells presenting esterase activities is noted
233 between 12 and 24 hours of incubation times ($p = 0.003$; three-ways ANOVA, Table 2).
234 In NSW and in USW, parasite survival and parasites presenting esterase activities were higher at
235 4°C and 15°C than at 25°C especially after 48h of incubation (Figs. 4 and 5). In NSW, after 48
236 hours of incubation, parasite mortality mean was 75.55%, 31.83% and 14.73% at 25°C, 15°C and
237 4°C respectively (Fig. 4). At 25°C, mortality was significantly higher after 24 h and 48 h of
238 incubation compared to 12 h ($p < 0.0001$).
239 Similarly, percentage of parasites presenting esterase activities significantly decreased at 25°C after
240 48 h of incubation. In USW at 25°C the percentage of positive cells was 47.23 ± 13.31% after 24 h
241 and 23.54 ± 8.65% after 48 h ($p = 0.0004$) (Fig. 5).
242
243 ***Impact of salinity on cell viability***
244 Incubation length had no significant effect on cell mortality (two-ways ANOVA: $F = 1.81$, $p =$
245 0.186). Therefore, data obtained on independent samples after 12 and 48 hours of incubation were
246 pooled. *A posteriori* tests showed that salinities of 5, 15 and 20 g l^{-1} were associated with highest
247 percentages of mortality whereas salinities of 35, 40 and 45 g l^{-1} allowed better parasite survival
248 (Fig. 6). Higher percentages of positive cells for esterase activities were reported for higher
249 salinities (35 to 45 g l^{-1}) (Fig. 7). Moreover, time of incubation presented a significant impact on
250 parasite esterase activities (two-ways ANOVA, $F = 15.3$, $p = 0$). There was a significant decrease of
251 percentages of positive parasites between 12h and 48h irrespective of tested salinities except at 25 g
252 l^{-1} (Fig. 7).

253 **DISCUSSION**

254

255 Despite 25 years of research on the protozoan *Bonamia ostreae,* its life cycle is poorly known.
256 Whatever is the date at which naïve oysters are placed in an infected area, first known stages of the
257 parasite are observed 3 to 5 months after exposition to the parasite (Tigé & Grizel 1984, Montes
258 1991). Moreover, the infection seems to remain present in areas that have been cleaned and which
259 ceased to produce oysters for several years (Van Banning 1988). Lagtime before infection and
260 persistence of the disease in cleaned areas motivated some authors to investigate potential
261 involvement of macroinvertebrate and zooplankton species in *Bonamia ostreae* life cycle (Lynch et
262 al. 2006). Nevertheless, considering the correlation between density of oysters and prevalence of
263 bonamiosis (Grizel 1985, Hudson & Hill 1991), the parasite mainly depends for its survival and
264 spread on flat oysters *Ostrea edulis* themselves and other aquatic organisms might not be involved
265 as important carriers or transmitters (Van Banning 1988). Transmission of *B. ostreae* between
266 oysters probably occurs through the water column. Water characteristics can have an impact on the
267 survival of the parasites released outside the host and these characteristics can influence the
268 infective capacity of *B. ostreae* as well as the number of oysters newly infected.
269 In that context, the impact of two environmental parameters, the salinity and the temperature, on
270 the parasite viability was investigated at different times of incubation: 12 h, 24 h and 48 h. Trials
271 were stopped at 48 h because some preliminary results were not reproducible beyond this
272 incubation time. In addition, the suitability of three different sea water media for parasite
273 preservation was tested: 0.22 µm filtered natural sea water; 0.22 µm filtered underground salty
274 water (with a constant composition) and 0.22 µm filtered artificial sea water (which is easy to
275 acquire and with a constant composition).

276 Size and complexity of *Bonamia ostreae* were generally homogeneous but depended on the status
277 of the parasite cell i.e. if they were live or dead. Two populations were distinguished: a
278 homogeneous population of small parasites corresponding mainly to dead cells and increasing

279 proportionally to recorded mortality, and another population less homogeneous, larger in size
280 increasing proportionally to survival rates. These results suggest that when dying *B. ostreae*
281 becomes smaller. Such phenomenon is described in apoptotic cells (Cotter et al. 1992, Samali &
282 Cotter 1999, Nasirudeen et al. 2001).

283 Results showed a better survival of purified *Bonamia ostreae* (60 to 80%) in natural filtered sea
284 water and in underground filtered salty water than in artificial filtered sea water (less than 40%)
285 whatever were temperature and time of incubation. This result could be explained by a difference
286 of pH. Indeed, pH of ASW used in this study was more acid (6.5) than NSW (8.06) and USW
287 (7.06). Effect of pH on parasite viability has not been investigated in the present study. Moreover,
288 a difference of salinity was also noticed between tested media: between 30 and 34 g l^{-1} for NSW;
289 32 g l^{-1} for USW and 23.4 g l^{-1} for ASW. Therefore, in addition to be more acid, ASW had a lower
290 salinity than other tested media which could explain the poor conservation of parasites.

291 Although no significant difference of mortality and non specific esterase activities were observed
292 between parasites maintained at 4°C and 15°C in NSW or USW, 25°C did not appear suitable for
293 parasite preservation. In natural conditions, the disease is reported in areas where temperature of
294 sea water rarely reaches 25°C except in Mediterranean Sea where *Bonamia ostreae* is reported but
295 with low prevalence (0.9 ± 1.4%, data from REPAMO, French network for the surveillance of
296 mollusc diseases). In Quiberon bay (Morbihan, France) where prevalence of bonamiosis is
297 estimated at 12.4% ± 6.5 (data from REPAMO), summer water temperatures fluctuated between
298 16.9 and 19.6°C between 1989 and 2003 with a mean estimated at 18.5°C (data from REPHY,
299 French network for the surveillance of phytoplankton and phycotoxins). Some analyses revealed a
300 negative correlation between high summer water temperature and number of oysters detected
301 infected during the following winter (I. Arzul, unpublished data) suggesting that higher
302 temperatures do not favour infection of oysters. A study carried out on *Crassostrea gigas*
303 haemocytes showed that an increase of temperature and a decrease of salinity induced an increase
304 of cell mortality (Gagnaire et al. 2006b) suggesting that these environmental parameters had also

305 an effect on oyster defence capacities. Similarly, previous works demonstrated an impact of
306 temperature on flat oyster *Ostrea edulis* defence mechanisms (Cochennec & Auffret 2002): lower
307 temperatures were associated with increased bonamiosis prevalence. However in the same study,
308 haemocyte activities were tested by flow cytometry and revealed that low temperature (10°C) or a
309 decrease of temperature (from 20°C to 10°C) induced a decrease of enzymatic activities including
310 ROS production involved in defence mechanisms. Several authors described a seasonal variation of
311 infection with *B. ostreae;* prevalence of infection presenting peaks in late winter and in autumn
312 which suggests an involvement of environmental parameters including temperature in the
313 development of the disease (Grizel 1985, Montes 1990, Van Banning 1991, Culloty & Mulcahy
314 1996). Studies carried out on *Bonamia* sp. infecting the Asian oyster *Crassostrea ariakensis* in
315 Atlantic coastal waters of U.S.A showed a strong influence of temperature on seasonal parasite
316 cycling (Carnegie et al. 2008). Interestingly, temperatures around 25°C when oysters were placed
317 in infected area were associated with higher prevalence than temperatures below 20°C.
318 Experimental studies support these results showing that warm temperatures (>20°C) seem to
319 increase *Bonamia* sp. pathogenicity (Audemard et al. 2008a). Epidemiological data available for
320 *Bonamia* (= *Mikrocytos*) *roughleyi* show that the disease expressed under winter oyster mortalities
321 is associated with low temperatures (Wolf 1967). However all these studies consider the parasite
322 inside its host and thus investigate effects of temperature on host-parasite relationships and not
323 directly on parasite survival.

324 Purified *Bonamia ostreae* seems to show a preference for hyper saline media compared to hypo
325 saline media. Three ranges of salinities could be identified from these results: from 5 to 20 g l^{-1},
326 survival and esterase activity measures were very low but a mean of 10% of live cells can still be
327 detected suggesting that the parasite can still be transmitted in these conditions; between 25 and 30
328 g l^{-1} survival was intermediate (estimated at 35% after 12 hours of incubation); between 35 and 45 g
329 l^{-1} survival was higher and estimated at 50% after 12 hours of incubation.

330 As previously mentioned measures of parasite survival in the three tested media (NSW, USW and

331 ASW) supported these results. Indeed, parasite mortality was higher in ASW (salinity of 23.4 g l^{-1})
332 compared to NSW (salinity of 32-34 g l^{-1}) and USW (salinity of 32 g l^{-1}).
333 These results are also concordant with a previous study realised on *Bonamia exiotiosa* in New
334 Zealand in which a salinity of 40 g l^{-1} was associated with highest disease prevalences (Hine 2002).
335 Similarly, infection with *B. roughleyi* seems to be favoured by high salinities (30-35 g l^{-1}) (Farley et
336 al. 1988). Our results are also in concordance with data obtained during a recent study in which
337 salinity below 30 g l^{-1} was associated with lower host mortality and appeared detrimental to
338 *Bonamia* sp. in *Crassostrea ariakensis* (Audemard et al. 2008b).
339 Time of incubation showed an impact on the parasite preservation especially by increasing effect of
340 high temperatures on mortality (higher mortality) and esterase activities (lower percentages of
341 positive cells). Moreover, whatever was the tested salinity, percentages of cells producing esterase
342 activities were lower after 48h compared to 12h. It would be interesting to complete these results by
343 testing a wider range of incubation times in order to evaluate the persistence capacity of *Bonamia*
344 *ostreae* in natural sea water collected from different infected areas. However, mortality and esterase
345 activity measured by flow cytometry are instantaneous and do not allow to follow cumulative
346 mortality.
347 In the present context of global change, data allowing forecasting of disease evolution are
348 requested. Oysters are submitted to environmental changing and parasites as well. Description of
349 the influence of temperature and salinity on *Bonamia ostreae* viability should allow modelling
350 parasite transmission. Moreover these results should contribute to define risky and non risky
351 geographic areas regarding transmission of the disease. These data might also be of interest for
352 oyster farmers. Indeed, by monitoring temperature and salinity parameters, oysters might be moved
353 or sold before suitable conditions for parasites survival are reached.
354

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359

360
361 REFERENCES

362 Arzul I, Miossec L, Blanchet E, Garcia C, François C, Joly J-P (2006) *Bonamia ostreae* and
363 *Ostrea edulis* : a stable host-parasite system in France ? Proceedings of the XIth ISVEE conference,
364 Cairns, Australia

365 Audemard C, Carnegie RB, Bishop M, Peterson CH, Burreson E (2008a) Interacting effects of
366 temperature and salinity on *Bonamia* sp. parasitism in the Asian oyster *Crassostrea ariakensis*. J
367 Invertebr Pathol 98:344-350

368 Audemard C, Carnegie RB, Stokes NA, Bishop M, Peterson CH, Burreson E (2008b) Effects of
369 salinity on *Bonamia* sp. survival in the Asian oyster *Crassostrea ariakensis*. J Shellfish Res 27:535-
370 540

371 Berney M, Vital M, Hülshoff I, Weilenmann HU, Egli T, Hammes F (2008) Rapid, cultivation-
372 independent assessment of microbial viability in drinking water. Water Res 42:4010-4018

373 Binet MT, Stauber JL (2006) Rapid flow cytometric method for the assessment of toxic flagellate
374 cyst viability. Mar Environ Res 62:247-260

375 Carnegie RB, Stokes NA, Audemard C, Bishop M, Wilbur AE, Alphin TD, Posey MH, Peterson
376 CH, Burreson E (2008) Strong seasonality of *Bonamia* sp. infection and induced *Crassostrea*
377 *ariakensis* mortality in Bogue and Masonboro Sounds, North Carolina, USA. J Invertebr Pathol
378 98:335-343

379 Chagot D, Boulo V, Hervio D, Mialhe E, Bachere E, Mourton C, Grizel H (1992) Interactions
380 between *Bonamia ostreae* (Protozoa: Ascetospora) and hemocytes of *Ostrea edulis* and *Crassostrea*
381 *gigas* (Mollusca: Bivalvia): Entry mechanisms. J Invertebr Pathol 59:241-249

382 Cochennec N, Auffret M (2002) European project FAIR-CT98-4120 "Environmental Factors and
383 Shellfish Diseases" 15/05/2002 Final Report.

384 Cotter TG, Lennon SV, Glynn JM, Green DR (1992) Microfilament-disrupting agents prevent
385 formation of apoptotic bodies in tumour cells undergoing apoptosis. Canc Res 25:997-1005

386 Culloty SC, Mulcahy MF (1996) Season-, age-, and sex-related variation in the prevalence of
387 bonamiosis in flat oysters (*Ostrea edulis* L.) on the South coast of Ireland. Aquaculture 144:3–63
388 Czechowska K, Johnson DR, Roelof van der Meer J (2008) Use of flow cytometric methods for
389 single-cell analysis in environmental microbiology. Curr Opin Microbiol 11:205-212
390 Elston RA, Farley CA, Kent ML (1986) Occurrence and significance of bonamiasis in European
391 flat oysters *Ostrea edulis* in North America. Dis Aquat Org 2:49–54
392 Farley CA, Wolf PH, Elston RA (1988) A long-term study of "microcell" disease in oysters with
393 a description of a new genus, *Mikrocytos* (g.n.) and two new species *Mikrocytos mackini* (sp.n.) and
394 *Mikrocytos roughleyi* (sp.n.). Fish Bull 86:581-593
395 Gagnaire B, Thomas-Guyon H, Burgeot T, Renault T (2006a) Pollutant effects on Pacific oyster,
396 *Crassostrea gigas* (Thunberg), hemocytes: screening of 23 molecules using flow cytometry. Cell
397 Biol Toxicol 22:1–14
398 Gagnaire B, Frouin H, Moreau K, Thomas-Guyon H, Renault T (2006b) Effects of temperature
399 and salinity on haemocyte activities of the Pacific oyster, *Crassostrea gigas* (Thunberg). Fish
400 Shellfish Immunol 20:536-547
401 Grizel H (1985) Etudes des récentes épizooties de l'huître plate *Ostrea edulis* L. et de leur impact
402 sur l'ostréiculture bretonne. PhD dissertation, Université des Sciences et Techniques de Languedoc,
403 Montpellier, France
404 Hammes F, Berney M, Wang Y, Vital M, Köster O Egli T (2008) Flow cytometric total bacterial
405 cell counts as a descriptive microbiological parameter for drinking water treatment processes. Water
406 Res 42:269-277
407 Hervio D, Bachere E, Boulo V, Cochennec N, Vuillemin V, Le Coguic Y, Cailletaux G, Mazurie J,
408 Mialhe E (1995) Establishment of an experimental infection protocol for the flat oyster *Ostrea*
409 *edulis* with the intrahaemocytic protozoan parasite *Bonamia ostreae*: application in the selection of
410 parasite-resistant oyster. Aquaculture 132:183–194
411 Hine PM (2002) Severe apicomplexan infection in the oyster *Ostrea chilensis*: a possible

412 predisposing factor in bonamiosis Dis Aquat Org 51:49-60

413 Hudson EB, Hill BJ (1991) Impact and spread of bonamiasis in the UK. Aquaculture 93:279-285

414 Lindström ES, Weisse T, Stadler P (2002) Enumeration of small ciliates in culture by flow

415 cytometry and nucleic acid staining. J Microbiol Meth 49:173-182

416 Lynch SA, Armitage D V, Wylde S, Mulcahy MF, Culloty S C (2005) The susceptibility of young

417 prespawning oysters, *Ostrea edulis*, to *Bonamia ostreae*. J Shellfish Res 24:1019-1025

418 Lynch SA, Armitage DV, Coughlan J, Mulcahy MF, Culloty SC (2006) Investigating the possible

419 role of benthic macroinvertebrates and zooplankton in the life cycle of the haplosporidian *Bonamia*

420 *ostreae*. Exp Parasitol 115:359-368

421 Mialhe E, Bachère E, Chagot D, Grizel H (1988) Isolation and purification of the protozoan

422 *Bonamia ostreae* (Pichot et al. 1980), a parasite affecting the flat oyster *Ostrea edulis* L.

423 Aquaculture 71:293-299

424 Montes J (1990) Development of *Bonamia ostreae* parasitosis of flat oyster, *Ostrea edulis*, from

425 Galicia, northwest Spain. In: Perkins FO, Cheng TC (eds) Pathology in Marine Aquaculture.

426 Academic Press, New York, NY, p 223-227

427 Montes J (1991). Lag time for the infestation of flat oyster (*Ostrea edulis* L.) by *Bonamia ostreae*

428 in estuaries of Galicia (N.W. Spain). Aquaculture 93:235–239

429 Montes J, Anadon R, Azevedo C (1994) A possible life cycle for *Bonamia ostreae* on the basis of

430 electron microscopy studies. J Invertebr Pathol 63 :1–6

431 Mourton C, Boulo V, Chagot D, Hervio D, Bachère E, Mialhe E, Grizel H (1992) Interactions

432 between *Bonamia ostreae* (Protozoa: Ascetospora) and hemocytes of *Ostrea edulis* and *Crassostrea*

433 *gigas* (Mollusca: Bivalvia): *In vitro* system establishment. J Invertebr Pathol 59:235-240

434 Nasirudeen AMA, Tan KSW, Singh M, Yap EH (2001) Programmed cell death in a human

435 intestinal parasite, *Blastocystis hominis.* Parasitology 123:235-246

436 Parrow MW, Burkholder JM (2002) Flow cytometric determination of zoospore DNA content
437 and population DNA distribution in cultured *Pfiesteria* spp. (Pyrrhophyta). J Exp Mar Biol Ecol
438 267:35-51

439 Rault A, Bouix M, Béal C (2008) Dynamic analysis of *Lactobacillus delbrueckii* subsp.
440 *bulgaricus* CFL1 physiological characteristics during fermentation. Appl Microbiol Biotechnol
441 81:559-570

442 Samali A, Cotter TG (1999) Measurement of Cell Death in Culture. *In* : Jenkins N (ed) Animal
443 Cell Biotechnology: Methods and Protocols. Series**:** Methods in Biotechnology. Volume 8. Humana
444 Press Inc, Totowa, NJ, p 155-164

445 Soudant P, Chu FLE, Lund ED (2005) Assessment of the cell viability of cultured *Perkinsus*
446 *marinus* (Perkinsea), a parasitic protozoan of the eastern oyster, *Crassostrea virginica*, using
447 SYBRgreen-Propidium Iodide double staining and flow cytometry. J Euk Microbiol 52:492-499

448 Sprague V (1979) Classification of the Haplosporidia. Mar Fish Rev 41: 40-44

449 Tigé G, Grizel H (1984) Essai de contamination d'*Ostrea edulis* Linne par *Bonamia ostreae*
450 (Pichot et al., 1979) en rivière de Crach (Morbihan). Rev Trav Inst Pêches Marit 46:307–314

451 Van Banning P (1988) Management strategies to control diseases in the Dutch culture of edible
452 oysters. In: Fisher WS (ed), Disease Processes in Marine Bivalve Molluscs. American fisheries
453 Society, Spec Publ 18:243-245

454 Van Banning P (1991) Observations on bonamiasis in the stock of the European flat oyster,
455 *Ostrea edulis*, in the Netherlands, with special reference to the recent developments in Lake
456 Grevelingen. Aquaculture 93:205–211

457 Wolf P (1967) Winter mortality hits state's oyster output. The Fisherman 2:20-22

458 Wong JTY, Whiteley A (1996) An improved method of cell cycle synchronisation for the
459 heterotrophic dinoflagellate *Crypthecodinium cohnii* Biecheler analyzed by flow cytometry. J Exp
460 Mar Biol Ecol 197:91-99

461 **TABLE**

462

463 TABLE 1- Distribution of *Bonamia ostreae* cells in percentages (means (± standard deviation)) in

464 Population A and Population B (as shown on Figures 2 for example) and composition in live and

465 dead cells of these two populations according to the level of mortality rates.

466

Mortality rates	Population A	Population A alive	Population B	Population B dead
< 50%	56,68 (± 18,11)	77,58 (± 6,21)	15,66 (± 5,16)	53,54 (± 22,03)
≥ 50 % and < 70%	50,29 (± 20,78)	56,35 (± 9,16)	22,74 (± 10,65)	74,99 (± 13,79)
≥ 70%	25,64 (± 17,06)	37,33 (± 24,56)	49,51 (±14,44)	91,67 (± 7,43)

467
468

469

470 TABLE 2- ANOVAs comparing the percentages of mortality and the percentages of positive cells

471 for esterase activities according to media, temperatures (= Temp) and times of incubation (= Time).

472 NSW: natural seawater; USW: underground salty water; ASW: artificial seawater. F: Fisher value,

473 p: significance value. Terms significant at $p < 0.05$ are highlighted in bold.

474

		Cell mortality		Esterase activity	
	Ddl	F	p	F	p
Time	2	0.47	0.630	6.35	**0.003**
Medium	2	31.68	**0.000**	8.96	**0.000**
Temp	2	6.81	**0.002**	5.10	**0.009**
LSD tests		Medium ASW > NSW = USW Temp 4 = 15 < 25		Medium ASW < NSW = USW Temp 4 > 15 = 25 Time 12h > 24h = 48h	

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481 **FIGURE LEGENDS**

482

483 Fig. 1a. Red fluorescence histogram of parasites after boiling stained with Propidium Iodide. Gate A
484 corresponds to non stained cells (= live cells) and Gate B corresponds to stained cells (= dead cells).

485

486 Fig. 1b. Green fluorescence histogram of parasites just after purification, in presence of FDA. Gate
487 A corresponds to non fluorescent cells (= non active cells) and Gate B corresponds to fluorescent
488 cells (= active cells).

489

490 Fig. 2a. Cytogram of parasites maintained in natural seawater at 4°C 12 hours after purification.
491 This cytogram shows both Propidium Iodide stained and non-stained cells. Population A: 75.8% of
492 total cells; Population B: 15.7% of total cells.

493

494 Fig 2b. Cytogram of parasites maintained in natural seawater at 4°C 12 hours after purification.
495 This cytogram only shows non Propidium Iodide stained cells. Population A and Population B
496 include 91% and 6.5% of live cells respectively.

497

498 Fig 2c. Cytogram of parasites after boiling. This cytogram only shows Propidium Iodide stained
499 cells. Population A and Population B include 8.6% and 88% of dead cells respectively.

500

501 Fig. 3 - General means and standard errors of cell mortality and esterase activities per medium (time
502 and temperature data pooled); N = 27 replicates. NSW: natural seawater; USW: underground salty
503 water; ASW: artificial seawater.

504

505 Fig. 4 – Parasite mortality (percentages of Propidium Iodide stained cells) in natural sea water
506 according to the temperature and time of incubation (values are mean of three replicates); Bars

507 represent standard errors; *** $p < 0.0001$ compared to data at 25°C and 12 h after incubation and
508 compared to data at 15°C and 4°C
509
510 Fig. 5 - Parasite esterase activities (percentages of positive cells) in underground salty water
511 according to the temperature and time of incubation (Values are mean of three replicates; Bars
512 represent standard errors; *** $p = 0.0004$ compared to data at 25°C 24 h after incubation
513
514 Fig. 6 - Parasite mortality (Percentages of Propidium Iodide stained cells) according to the salinity
515 of underground salty water (time of both data pooled). Values are means ± standard error. N = 6
516 replicates.
517
518 Fig. 7 - Parasite esterase activities (Percentages of positive cells) in underground salty water
519 according to the salinity and time of incubation (Values are mean of three replicates; Bars represent
520 standard errors)； *** $p < 0.001$ compared to data 12 h after incubation
521
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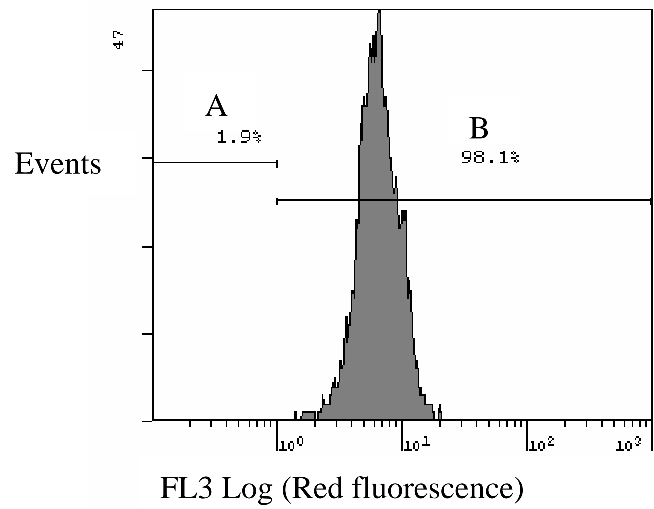

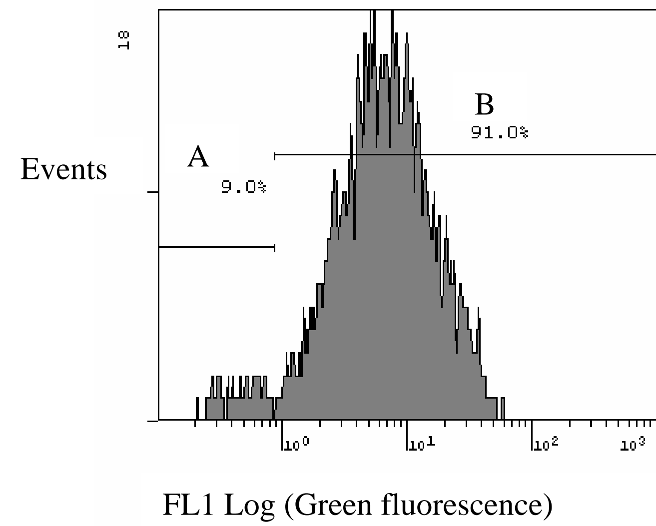

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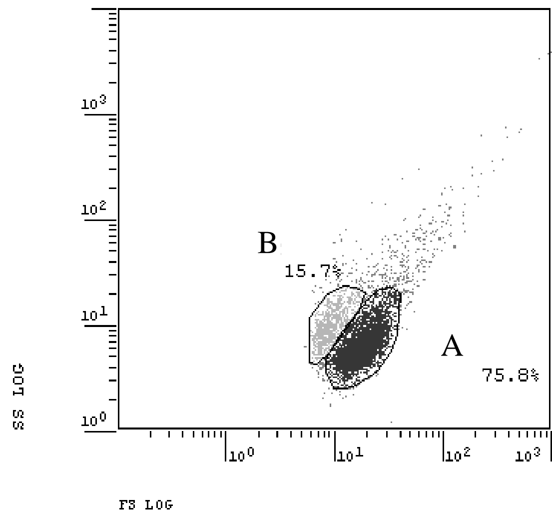

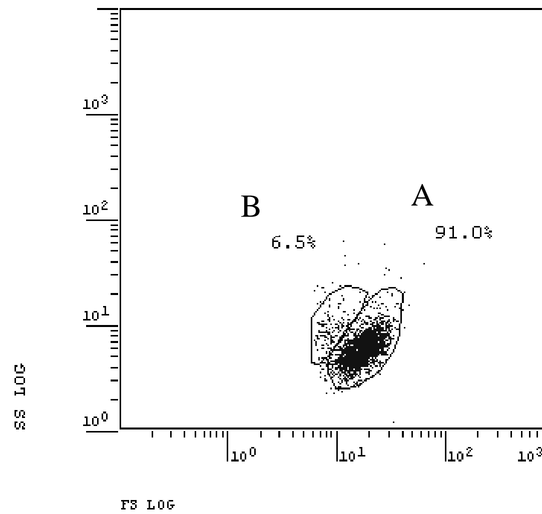

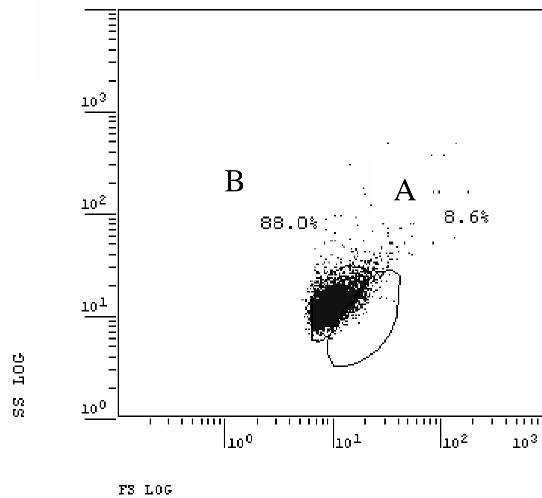

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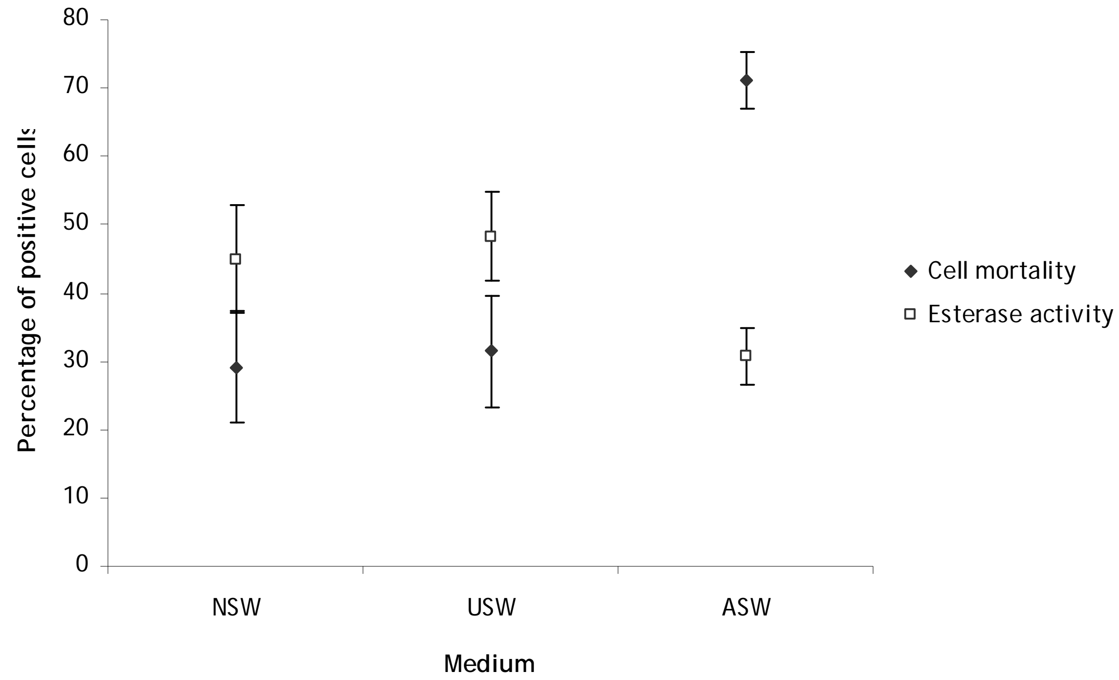

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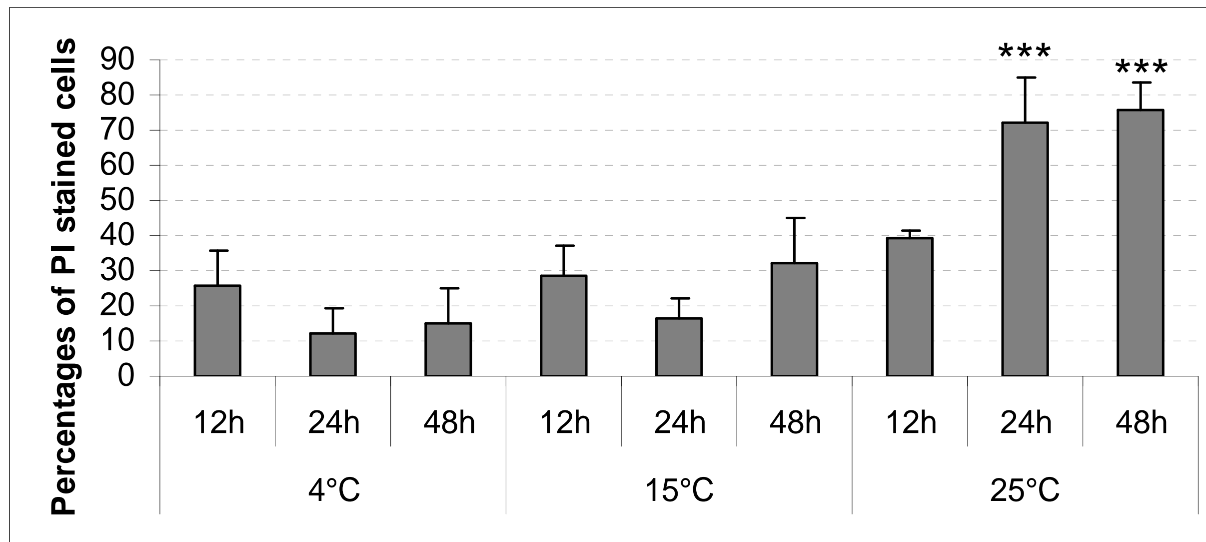

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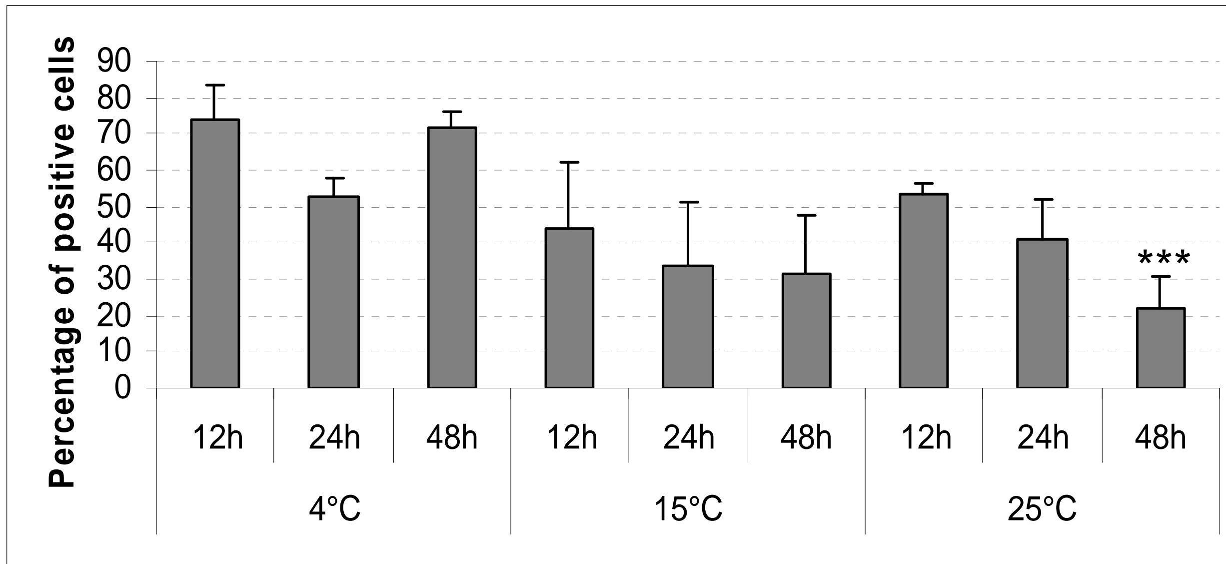

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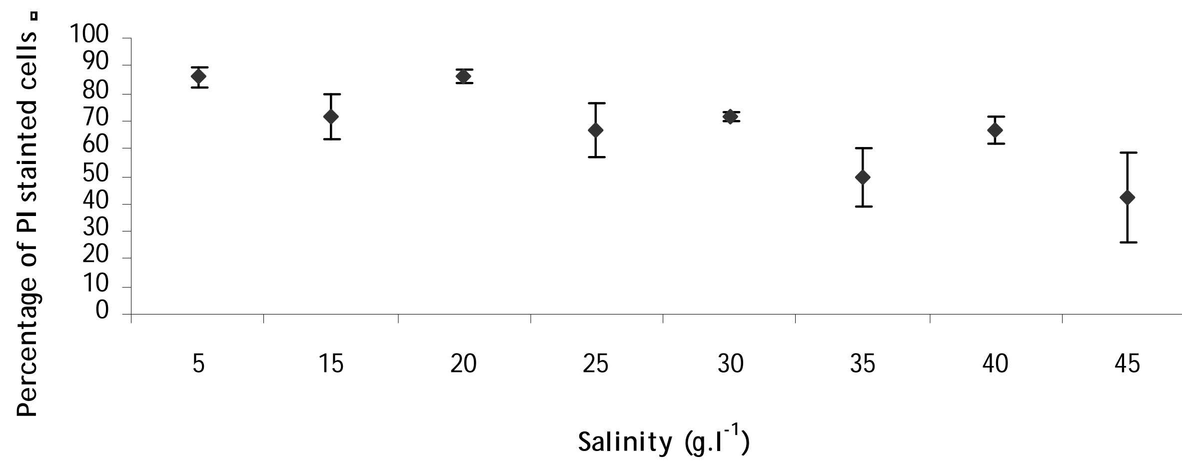

Fig. 6

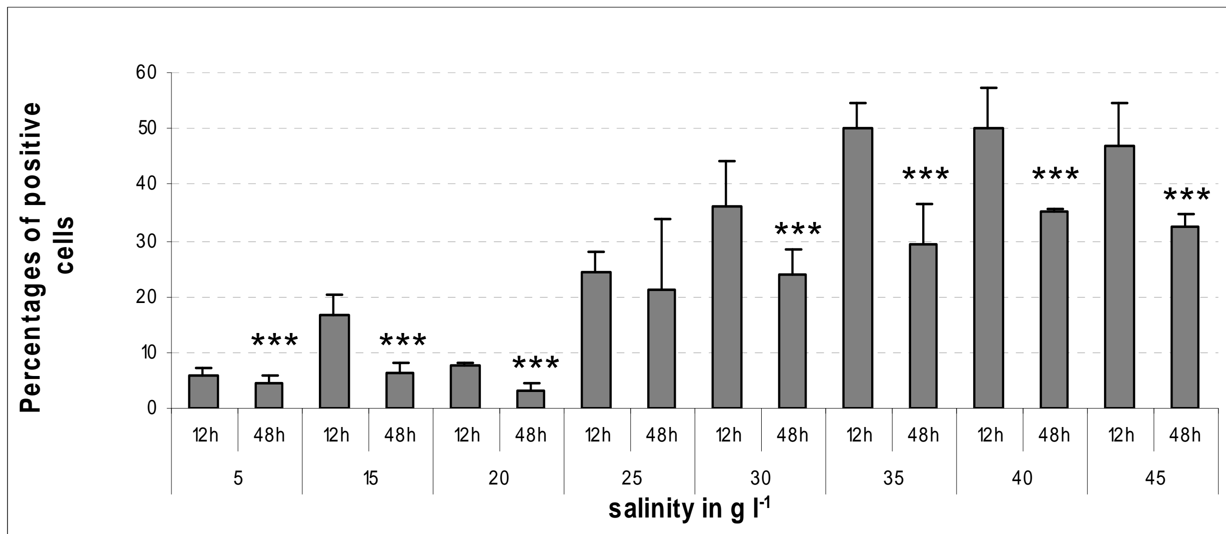

Fig. 7