

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.

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

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INTRODUCTION

Bonamia ostreae is a protistan parasite belonging to the phylum Haplosporidia (Sprague 1979). It is an intracellular parasite, 2 to 5 µm in diameter, that infects haemocytes. It can also be observed extracellularly between epithelial or interstitial cells in gills and stomach or in necrotic connective tissue areas. The parasite can be detected in spat (Lynch et al. 2005); however, mortalities mainly affect oysters that are more than 2 yr old (Culloty & Mulcahy 1996). At the tissue level, the infection is usually associated with intense haemocyte

infiltration of the connective tissue of the gills, mantle and digestive gland. The life cycle is unknown, but the disease can be directly transmitted between oysters in a population or experimentally by cohabitation or inoculation (Elston et al. 1986, Hervio et al. 1995), suggesting that an intermediate host is not required for the parasite to complete its life cycle. Observation of free parasites in gill epithelia potentially associated with gill lesions supports the hypothesis of a parasite release through these organs (Montes et al. 1994). However, the infective form and routes of entry and release remain undetermined. Most *B. ostreae* might

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be released into the water column after oyster death through tissue lysis.

This intrahaemocytic parasite has been described in oysters collected from various ecosystems from estuaries and intertidal zones to deep coastal waters or lagoons and is presently reported in Europe, North America and Morocco. Northern European waters (e.g. Norwegian waters) seem to be free of bonamiosis, probably because of the lack of introduction of infected animals. Flat oysters from the Mediterranean Basin are infected by *Bonamia ostreae*; however, reported prevalences are low (data from REPAMO, the French network for the surveillance of mollusc diseases). No clear correlations have been demonstrated between development of the disease and environmental parameters including temperature and salinity. Previous work suggested an impact of temperature on the parasite and/or on the defence capacity of oysters. Although the disease occurs and can be transmitted throughout the year (Tigé & Grizel 1984), there is a seasonal variation in infection with *B. ostreae*. Prevalence of infection presents peaks in late winter and in autumn (Grizel 1985, Montes 1990, Van Banning 1991, Culloty & Mulcahy 1996, Arzul et al. 2006). A study of bonamiosis prevalence as well as haemocyte activities according to temperature showed that prevalence was higher at low temperature (10°C) compared to higher temperature (20°C), suggesting that low temperatures may affect defence capacities of the oyster and/or the ability of the parasite to infect healthy oysters (Cochennec & Auffret 2002).

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

In aquatic ecology, flow cytometry is classically used to determine abundance, viability and activity of microorganisms including viruses, bacteria, microalgae and planktonic protozoan parasites (Wong & Whiteley 1996, Lindström et al. 2002, Parrow & Burkholder 2002, Binet & Stauber 2006, Hammes et al. 2008). Recent developments have aimed at addressing some questions in environmental microbiology, including studying microbial physiology under environmentally relevant conditions (Czechowska et al. 2008).

Flow cytometry has been successfully used to measure cell viability of cultured *Perkinsus marinus*, a parasitic protozoan of the eastern oyster *Crassostrea virginica* (Soudant et al. 2005). This tool allows multiparametric analyses on a large number of cells in a very short time and thus presents advantages over microscopic approaches.

The objectives of our study were to test survival of purified *Bonamia ostreae* in different sea water media (artificial seawater, natural seawater and seabed borewater) in order to identify the most suitable medium for parasite preservation and to investigate effects of temperature and salinity on the survival of purified parasites by flow cytometry. Purified *B. ostreae* were suspended and maintained *in vitro* in the 3 different media at 3 different temperatures and then subjected to a range of salinities in the optimal medium previously defined. Parasite mortality was measured by flow cytometry using propidium iodide staining, and parasite viability was estimated by measuring esterase activities using fluorescein diacetate (FDA). Esterases are enzymes belonging to the group of hydrolases and are classically measured to estimate global levels of viable cell activities (Gagnaire et al. 2006a, Berney et al. 2008, Rault et al. 2008). *In vitro* exposure of purified parasites to ranges of temperature and salinity may improve our knowledge of the disease epidemiology and may provide guidance for oyster farmers for stock management.

MATERIALS AND METHODS

***Bonamia ostreae* purification.** Purification of parasites was performed following the protocol of Mialhe et al. (1988) using flat oysters that originated from Quiberon Bay (France), an area infected by *B. ostreae*. Oysters were maintained for 30 d in raceways of 120 l receiving a constant flow of external seawater at a temperature of 12 to 15°C and enriched in phytoplankton (*Skeletonema costatum*, *Isochrysis galbana*, *Chaetoceros gracilis* and *Tetraselmis suecica*). Some highly infected *Ostrea edulis* were selected by examination of heart tissue imprints under light microscopy. Two to 3 highly infected oysters were used per purification. All organs were homogenised except the adductor muscle. Parasites were concentrated by differential centrifugation on sucrose gradients and then purified by isopycnic centrifugation on a Percoll gradient. Centrifugations were performed at 8°C. Lastly, purified parasites were resuspended in 1 ml of 0.22 µm filtered seawater before being counted using a Malassez-cell haemocytometer. Parasite suspensions were then maintained at 4°C. Salinity of filtered seawater fluctuated between 30 and 34 g l⁻¹.

Experimental design. Two sets of experiments were performed 3 times. In the first set of experiments, purified parasites were suspended in 3 different media, and 10 ml of each parasite suspension were distributed in equivalent numbers (5×10^6 cells ml^{-1}) in nine 15 ml polypropylene tubes per medium. The 3 media were (1) 0.22 μm filtered prepared artificial seawater (ASW) with a salinity of 23.4 g l^{-1} and a pH of 6.5 (23.4 g NaCl, 1.5 g KCl, 1.2 g $\text{MgSO}_4 \cdot 4\text{H}_2\text{O}$, 0.2 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, H_2O q.s. 1 l) (2) 0.22 μm filtered seabed borewater (SBW) showing a constant salinity of 32 g l^{-1} and a pH of 7.06 (collected at a depth of 110 m at IFREMER facilities in La Tremblade, France) (3) 0.22 μm filtered natural seawater (NSW) from La Seudre estuary, Charente Maritime (France) with a salinity of 30 to 34 g l^{-1} and a pH of 8.06. Parasites maintained in the 3 different media were subjected to 3 different temperatures: 4, 15 and 25°C (3 tubes per condition). The different parasite suspensions were tested by flow cytometry after 12, 24 and 48 h of incubation.

Regarding parasite survival according to previously tested medium and temperature, the second set of experiments, aimed at testing effects of salinity on *Bonamia ostreae* viability, was performed in SBW (stable composition compared to NSW) at 15°C (which better reflects natural conditions than does 4°C). More precisely, purified parasites were diluted in 0.22 μm filtered SBW and distributed in equivalent numbers (5×10^6 cells ml^{-1}) in 15 ml polypropylene tubes. Distilled water or natural salt from Guérande (Pays de la Loire, France) was added to obtain a range of salinities: 5, 15, 20, 25, 30, 35, 40 and 45 g l^{-1} . Parasite suspensions (3 tubes per salinity condition) were incubated at 15°C, and samples were analysed by flow cytometry after 12 and 48 h.

Analysis of viability of *Bonamia ostreae* by flow cytometry. Flow cytometry protocols used in this study

were adapted from protocols previously described for *Crassostrea gigas* haemocytes (Gagnaire et al. 2006b). Each measure was carried out 3 times. For each sample, 5000 events were counted using an EPICS XL 4 (Beckman Coulter). Results were depicted under biparametric representations (density plots) showing parasite cells according to the Forward Scatter (FSc) in abscissa and Side Scatter (SSc) in ordinate and the fluorescence channel corresponding to the marker used. FSc and SSc values, which correspond to diffracted light on the acute and right angles, are proportional to cell size and cell complexity, respectively. Recorded fluorescence depended on the monitored parameters: non-specific esterase activities were measured using green fluorescence (fluorescence detector FL1), while cell mortality was measured using red fluorescence (fluorescence detector FL3).

Parasite mortality was estimated after incubating 200 μl of parasite suspensions at 5×10^5 cells ml^{-1} in the dark for 30 min at 4°C with 10 μl of the nucleic acid fluorescent dye propidium iodide (PI, 1.0 mg l^{-1} , Interchim). Non-specific esterase activities were evaluated by incubating 200 μl of parasite suspensions at 5×10^5 cells ml^{-1} in the dark for 30 min at ambient temperature with 1 μl of the liposoluble substrate fluoresceine diacetate (FDA, 400 μM in DMSO, Molecular Probes, Invitrogen).

Dead parasites, prepared by boiling cells for 15 min, were used to control efficacy of PI for mortality measurement. The FL3 fluorescence histogram showed that 98.1% of PI-stained cells (red fluorescence above 1) were dead (Fig. 1a). Suspension of live parasites was used to control efficacy of FDA for esterase activity measurement (Fig. 1b). The FL1 fluorescence histogram showed that 91% of fluorescent cells after incubation with FDA (green fluorescence above 1) were alive and presented esterase activity (Fig. 1b).

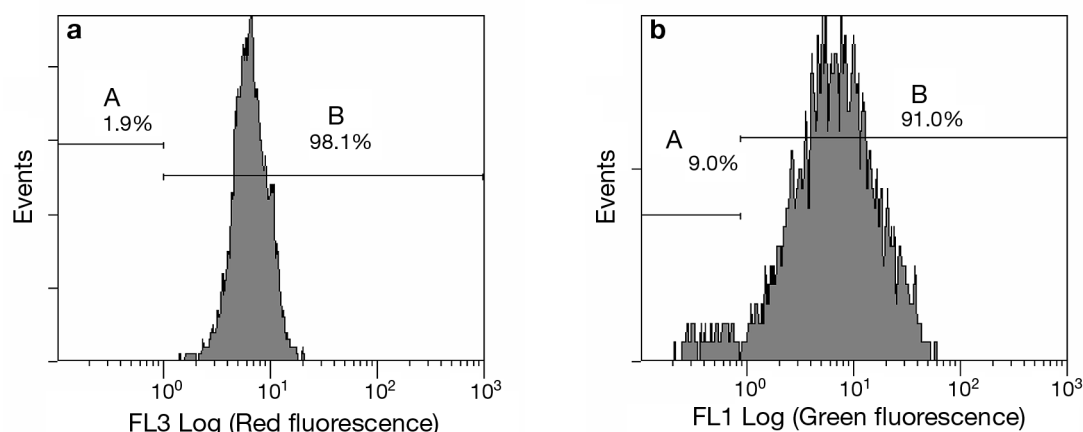


Fig. 1. *Bonamia ostreae*. (a) Red fluorescence of parasites after boiling and staining with propidium iodide. Bracket A corresponds to non-stained cells (= live cells) and Bracket B corresponds to stained cells (= dead cells). (b) Green fluorescence of parasites just after purification, in the presence of fluoresceine diacetate. Bracket A corresponds to non-fluorescent cells (= non-active cells) and Bracket B corresponds to fluorescent cells (= active cells)

Statistical analysis. Data were analysed using the software Statgraphics® Plus version 5.1. Results were expressed as percentages of positive cells. Mean and SD were calculated for each triplicate. The effect of tested conditions was evaluated by 1-way, 2-way and 3-way analyses of variance (ANOVAs). Values were converted into r angular arcsinus $\sqrt{(\% \text{ of positive cells})}$ before analysis to ensure respect of *a priori* assumptions of normality and homogeneity. In the case of rejection of H_0 , an *a posteriori* least significant difference test was used to compare differences between means and to obtain hierarchy between studied factors. Significance was concluded at $p \leq 0.05$.

RESULTS

Size and complexity of parasites

Size and to a lesser degree complexity of parasites varied according to whether they were dead or alive. Irrespective of the tested conditions, it was possible to identify 2 populations of parasite cells (Fig. 2a): population A, comprising $53 \pm 24\%$ of live cells and population B, smaller in size and consisting of a majority of dead cells (mean $74 \pm 23\%$). Some parasite cells were not included in population A or B and corresponded generally to dead cells showing higher size and higher complexity than cells included in populations A and B. For parasites maintained in NSW at 4°C 12 h after purification, populations A and B included 75.8% and 15.7% of total cells, respectively (Fig. 2a). When only considering non-PI-stained parasites under the same experimental conditions, populations A and B included 91% and 6.5% of live cells, respectively (Fig. 2b). After boiling, when only considering PI-stained cells, populations A and B included 8.6% and 88% of dead cells, respectively (Fig. 2c). Population A included more live cells ($77.6 \pm 6.2\%$) when mortality rates were below 50% compared to mortality rates above 70% ($37.3 \pm 24.7\%$; Table 1). In contrast, population B included more dead cells when mortality rates were high ($91.7 \pm 7.4\%$ for mortality rates above 70%; Table 1).

Effect of medium on cell viability

In the 3 experiments simultaneously testing the effect of medium, temperature and incubation time on parasites (3-way ANOVA), medium was the most important factor in parasite survival ($p = 0$) and influenced cell mortality and esterase activity more than did temperature and incubation time (Table 2). Irrespective of incubation time and temperature, parasites

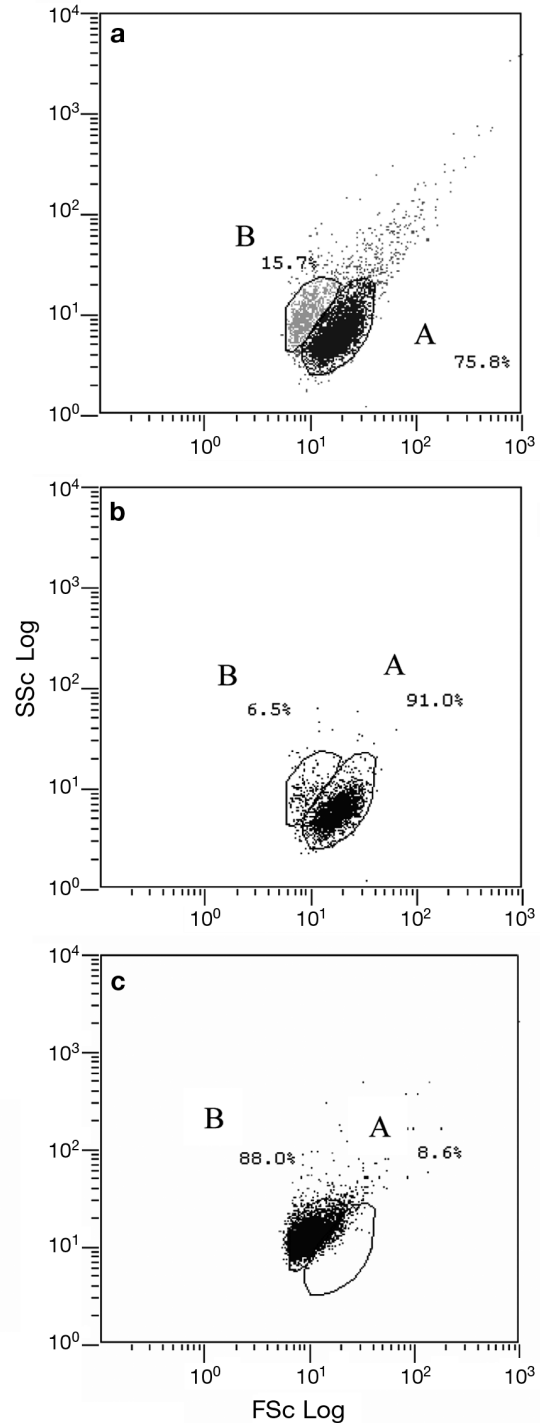


Fig. 2. *Bonamia ostreae*. (a) Parasites maintained in natural seawater at 4°C 12 h after purification. This cytogram shows both propidium iodide (PI)-stained and non-stained cells. Population A: 75.8% of total cells; population B: 15.7% of total cells. (b) Parasites maintained in natural seawater at 4°C 12 h after purification. This cytogram only shows non-PI-stained cells. Populations A and B include 91% and 6.5% of live cells, respectively. (c) Parasites after boiling. This cytogram only shows PI-stained cells. Populations A and B include 8.6% and 88% of dead cells, respectively. FSc/SSc: forward/side scatter, respectively

Table 1. *Bonamia ostreae*. Distribution of cells in percentages (means \pm SD) belonging to populations A and B (as shown in Fig. 2; see text for details) and composition of live and dead cells in these 2 populations according to the parasite mortality rate

Mortality rate	Population A	Population A live	Population B	Population B dead
<50 %	56.68 (\pm 18.11)	77.58 (\pm 6.21)	15.66 (\pm 5.16)	53.54 (\pm 22.03)
\geq 50 % and <70 %	50.29 (\pm 20.78)	56.35 (\pm 9.16)	22.74 (\pm 10.65)	74.99 (\pm 13.79)
\geq 70 %	25.64 (\pm 17.06)	37.33 (\pm 24.56)	49.51 (\pm 14.44)	91.67 (\pm 7.43)

Table 2. ANOVAs comparing the percentages of mortality and the percentages of cells positive for esterase activity according to medium, temperature (Temp) and incubation time (Time). NSW: natural seawater, SBW: seabed borewater, ASW: artificial seawater. Terms significant at $p < 0.05$ are highlighted in **bold** and compared using a least significant difference (LSD) test

	df	Cell mortality		Esterase activity	
		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		Medium	
		ASW > NSW = SBW		ASW < NSW = SBW	
		Temp		Temp	
		4°C = 15°C < 25°C		4°C > 15°C = 25°C	
				Time	
				12 h > 24 h = 48 h	

showed significantly better survival in NSW and SBW than in ASW (Fig. 3, Table 2). However, there was no significant difference between mortality and esterase activity percentages in NSW and SBW. Mean parasite mortality was 29.1 %, 31.4 % and 71.1 % in NSW, SBW and ASW, respectively. The percentage of parasites positive for esterase activity was 44.9 ± 7.8 % in NSW, 48.2 ± 6.5 % in SBW and 30.6 ± 4.1 % in ASW.

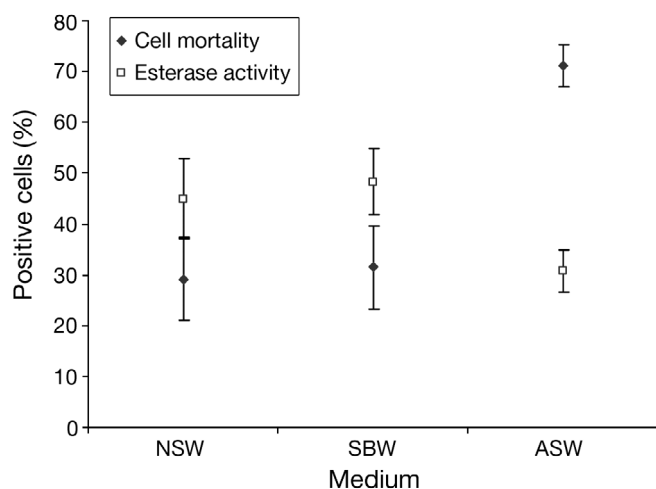


Fig. 3. *Bonamia ostreae*. General means and SE of cell mortality and esterase activity per medium (time and temperature data pooled); $n = 27$ replicates. NSW: natural seawater; SBW: seabed borewater; ASW: artificial seawater

Effect of temperature on cell viability

Parasite viability fluctuated according to the tested temperature. Irrespective of medium and incubation time, mortality appeared significantly higher at 25°C compared to 15°C and 4°C, and percentages of cells presenting esterase activity were higher at 4°C compared to 15°C and 25°C (3-way ANOVA, Table 2). In NSW, irrespective of incubation time, mortality percentages ranged from 11.92 to 25.59% at 4°C, 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 higher at 25°C compared to 4°C and 15°C, especially after 24 and 48 h of incubation ($p < 0.0001$; Fig. 4). In SBW, irrespective of incubation time, the percentage of parasites positive for esterase activity ranged from 52.49 to 73.67% at 4°C, from 31.27 to 43.73% at 15°C and from 21.89 to 53.28% at 25°C (Fig. 5).

Effect of incubation time on cell viability

Irrespective of medium and temperature, incubation time did not have a significant effect on parasite mortality. However, a difference in the percentages of cells presenting esterase activity was noted between 12 and 24 h of incubation ($p = 0.003$; 3-way ANOVA, Table 2). In NSW and SBW, parasite survival and parasites presenting esterase activity were higher at 4°C and 15°C than at 25°C, especially after 48 h of incubation (Figs. 4

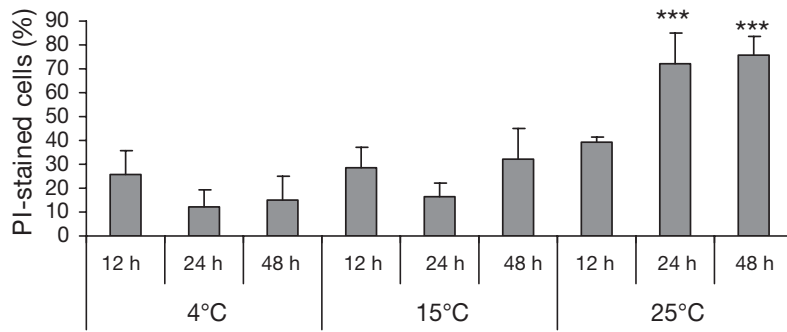


Fig. 4. *Bonamia ostreae*. Mortality (percentages of propidium iodide [PI]-stained cells) in natural seawater according to the temperature and incubation time (values are means + SE of 3 replicates); *** $p < 0.0001$ compared to data at 25°C 12 h after incubation and compared to data at 4°C and 15°C

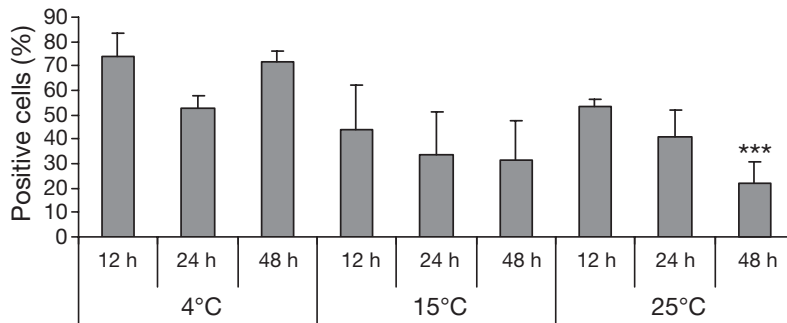


Fig. 5. *Bonamia ostreae*. Esterase activity (percentages of positive cells) in seabed borewater according to the temperature and incubation time (values are means + SE of 3 replicates); *** $p = 0.0004$ compared to data at 25°C 24 h after incubation

& 5). In NSW, after 48 h of incubation, mean parasite mortality was 75.55%, 31.83% and 14.73% at 25°C, 15°C and 4°C, respectively (Fig. 4). At 25°C, mortality was significantly higher after 24 and 48 h of incubation compared to 12 h ($p < 0.0001$). Similarly, the percentage of parasites presenting esterase activity significantly decreased at 25°C after 48 h of incubation. In

SBW at 25°C, the percentage of positive cells was $47.23 \pm 13.31\%$ after 24 h and $23.54 \pm 8.65\%$ after 48 h ($p = 0.0004$; Fig. 5).

Effect of salinity on cell viability

Incubation length had no significant effect on cell mortality (2-way ANOVA: $F_1 = 1.81$, $p = 0.186$). Therefore, data obtained on independent samples after 12 and 48 h of incubation were pooled. A *posteriori* tests showed that salinities of 5, 15 and 20 $g\ l^{-1}$ were associated with the highest mortality, whereas salinities of 35, 40 and 45 $g\ l^{-1}$ allowed better parasite survival (Fig. 6). Higher percentages of cells positive for esterase activity were found at higher salinities (35 to 45 $g\ l^{-1}$; Fig. 7). Moreover, incubation time had a significant effect on parasite esterase activity (2-way ANOVA, $F = 15.3$, $p = 0$). There was a significant decrease in the percentage of positive parasites between 12 and 48 h irrespective of tested salinities except at 25 $g\ l^{-1}$ (Fig. 7).

DISCUSSION

Despite 25 yr of research on the protozoan *Bonamia ostreae*, its life cycle is poorly known. Regardless of the date at which naïve oysters are placed in an infected area, the first known stages of the parasite are observed 3 to 5 mo after exposure to the parasite (Tigé & Grizel 1984, Montes 1991). Moreover, the infection seems to remain present in areas that have been cleaned and that have ceased to produce

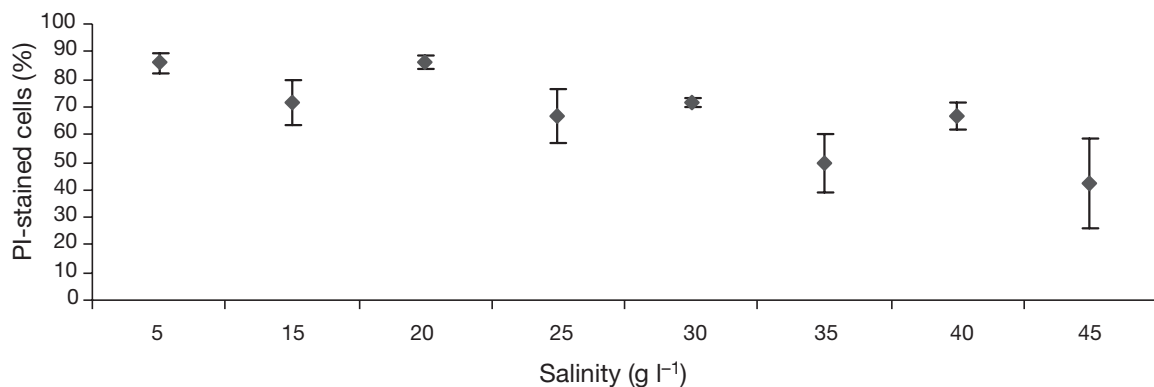


Fig. 6. *Bonamia ostreae*. Mortality (percentages of propidium iodide [PI]-stained cells) according to the salinity of seabed borewater (all time data were pooled). Values are means \pm SE. N = 6 replicates

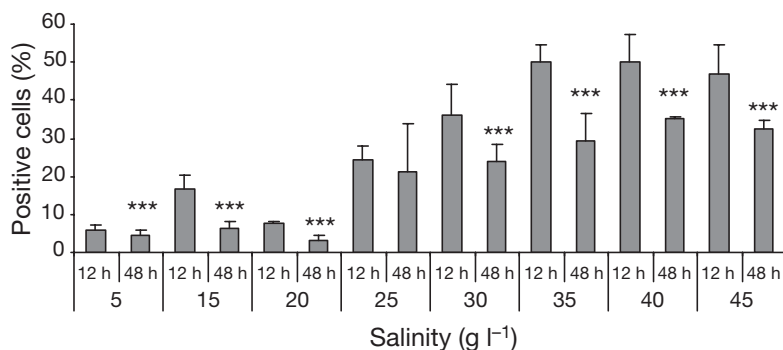


Fig. 7. *Bonamia ostreae*. Esterase activity (percentages of positive cells) in seabed borewater according to the salinity and incubation time (values are means of 3 replicates; bars represent SE); *** $p < 0.001$ compared to data 12 h after incubation

oysters for several years (Van Banning 1988). The lag-time before infection and persistence of the disease in cleaned areas have motivated some authors to investigate potential involvement of macroinvertebrate and zooplankton species in the *B. ostreae* life cycle (Lynch et al. 2007). Nevertheless, considering the correlation between density of oysters and prevalence of bonamiosis (Grizel 1985, Hudson & Hill 1991), the parasite mainly depends on flat oysters *Ostrea edulis* for its survival and spread, and other aquatic organisms might not be involved as important carriers or transmitters (Van Banning 1988). Transmission of *B. ostreae* between oysters probably occurs through the water column. Water characteristics can affect the survival of the parasites released outside the host, and these characteristics can influence the infective capacity of *B. ostreae* as well as the number of oysters newly infected.

In that context, the effects of 2 environmental parameters, salinity and temperature, on parasite viability were investigated at different incubation times: 12, 24 and 48 h. Trials were stopped at 48 h because some preliminary results were not reproducible beyond this incubation time. In addition, the suitability of 3 different seawater media for parasite preservation was tested: 0.22 μm filtered NSW; 0.22 μm filtered SBW (with a constant composition) and 0.22 μm filtered ASW (which is easy to acquire and has a constant composition).

Size and complexity of *Bonamia ostreae* were generally homogeneous but depended on the status of the parasite cells, i.e. whether they were live or dead. Two populations were distinguished: a homogeneous population of small parasites corresponding mainly to dead cells and increasing proportionally to recorded mortality, and another population that was less homogeneous and larger in size increasing proportionally to survival rates. These results suggest that when dying, *B.*

ostreae become smaller. Such a phenomenon has been described in apoptotic cells (Cotter et al. 1992, Samali & Cotter 1999, Nasirudeen et al. 2001).

Results showed a better survival of purified *Bonamia ostreae* (60 to 80%) in filtered NSW and in filtered SBW than in filtered ASW (less than 40%) regardless of temperature and incubation time. This result could be explained by a difference in pH. Indeed, pH of ASW used in this study was more acid (6.5) than NSW (8.06) and SBW (7.06). The effect of pH on parasite viability was not investigated in the present study. Moreover, a difference in salinity was also noticed between tested media: between 30 and 34 g l^{-1} for

NSW, 32 g l^{-1} for SBW and 23.4 g l^{-1} for ASW. Therefore, in addition to being more acidic, ASW had a lower salinity than the other tested media, which could explain the poor survival of parasites.

Although no significant differences in mortality and non-specific esterase activity were observed between parasites maintained at 4°C and 15°C in NSW or SBW, 25°C did not appear suitable for parasite survival. Under natural conditions, the disease is reported in areas where seawater temperature rarely reaches 25°C except in the Mediterranean Sea, where *Bonamia ostreae* has been reported but with low prevalence (0.9 \pm 1.4%; data from REPAMO, the French network for the surveillance of mollusc diseases). In Quiberon Bay (Morbihan, France) where the prevalence of bonamiosis has been estimated at 12.4% \pm 6.5 (data from REPAMO), summer water temperatures fluctuated between 16.9 and 19.6°C between 1989 and 2003, with a mean estimated at 18.5°C (data from REPHY, the French network for the surveillance of phytoplankton and phycotoxins). Some analyses revealed a negative correlation between high summer water temperature and number of infected oysters detected during the following winter (I. Arzul unpubl. data), suggesting that higher temperatures do not favour infection of oysters. A study on *Crassostrea gigas* haemocytes showed that an increase in temperature and a decrease in salinity induced an increase in cell mortality (Gagnaire et al. 2006a), suggesting that these environmental parameters also have an effect on oyster defence capacities. Similarly, previous work has demonstrated an effect of temperature on flat oyster *Ostrea edulis* defence mechanisms (Cochennec & Aufret 2002); lower temperatures were associated with increased prevalence of bonamiosis. However in the same study, haemocyte activities were tested by flow cytometry and revealed that low temperature (10°C) or a decrease in temperature (from 20°C to 10°C) induced

a decrease in enzymatic activity, including production of reactive oxygen species involved in defence mechanisms. Several authors described a seasonal variation of infection with *B. ostreae*, i.e. the prevalence of infection presenting peaks in late winter and in autumn, which suggests an involvement of environmental parameters including temperature in the development of the disease (Grizel 1985, Montes 1990, Van Banning 1991, Culloty & Mulcahy 1996). Studies on *Bonamia* sp. infecting the Asian oyster *C. ariakensis* in Atlantic coastal waters of the US showed a strong influence of temperature on seasonal parasite cycling (Carnegie et al. 2008). Interestingly, when these oysters were placed in infected areas, temperatures around 25°C were associated with higher prevalence than temperatures below 20°C. Experimental studies support these results, showing that warm temperatures (>20°C) seem to increase *Bonamia* sp. pathogenicity (Audemard et al. 2008a). Epidemiological data available for *Bonamia* (= *Mikrocytos*) *roughleyi* show that the disease expressed during winter oyster mortalities is associated with low temperatures (Wolf 1967). However, these studies considered the parasite inside its host and thus investigated the effects of temperature on host–parasite relationships and not directly on parasite survival.

Purified *Bonamia ostreae* seem to show a preference for hypersaline media compared to hyposaline media. Three ranges of salinities could be identified from our results: from 5 to 20 g l⁻¹, survival and esterase activity measures were very low, although a mean of 10% of live cells could still be detected, suggesting that the parasite can still be transmitted under these conditions; between 25 and 30 g l⁻¹, survival was intermediate (estimated at 35% after 12 h of incubation); between 35 and 45 g l⁻¹, survival was higher and estimated at 50% after 12 h of incubation.

As previously mentioned, measures of parasite survival in the 3 tested media (NSW, SBW and ASW) supported these results. Indeed, parasite mortality was higher in ASW (salinity of 23.4 g l⁻¹) compared to NSW (salinity of 32 to 34 g l⁻¹) and SBW (salinity of 32 g l⁻¹).

These results are also concordant with a previous study on *Bonamia exiotosa* in New Zealand, in which a salinity of 40 g l⁻¹ was associated with the highest disease prevalences (Hine 2002). Similarly, infection with *B. roughleyi* seems to be favoured by high salinities (30 to 35 g l⁻¹) (Farley et al. 1988). Our results are also in concordance with data obtained during a recent study in which salinity below 30 g l⁻¹ was associated with lower host mortality and appeared detrimental to *Bonamia* sp. in *Crassostrea ariakensis* (Audemard et al. 2008b).

Incubation time showed an effect on parasite survival, especially by increasing the effect of high tem-

peratures on mortality (higher mortality) and esterase activity (lower percentages of positive cells). Moreover, regardless of the tested salinity, percentages of cells producing esterase activity were lower after 48 h compared to 12 h. It would be interesting to strengthen these results by testing a wider range of incubation times in order to evaluate the persistence capacity of *Bonamia ostreae* in NSW collected from different infected areas. However, mortality and esterase activity measured by flow cytometry are instantaneous and do not allow us to follow cumulative mortality.

In the present context of global climate change, data allowing forecasting of disease evolution are needed. Oysters are subject to environmental change in addition to parasites. Our description of the influence of temperature and salinity on *Bonamia ostreae* viability should allow us to model parasite transmission. Moreover, our results will contribute to defining risky and non-risky geographic areas with regard to disease transmission. These data might also be of interest to oyster farmers. Indeed, by monitoring temperature and salinity parameters, oysters might be moved or sold before suitable conditions for parasite survival are reached.

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LITERATURE CITED

- Arzul I, Miossec L, Blanchet E, Garcia C, François C, Joly JP (2006) *Bonamia ostreae* and *Ostrea edulis*: a stable host–parasite system in France? Proc 11th ISVEE Conference, Cairns
- Audemard C, Carnegie RB, Bishop M, Peterson CH, Burreson E (2008a) Interacting effects of temperature and salinity on *Bonamia* sp. parasitism in the Asian oyster *Crassostrea ariakensis*. J Invertebr Pathol 98:344–350
- Audemard C, Carnegie RB, Stokes NA, Bishop M, Peterson CH, Burreson E (2008b) Effects of salinity on *Bonamia* sp. survival in the Asian oyster *Crassostrea ariakensis*. J Shellfish Res 27:535–540
- Berney M, Vital M, Hülshoff I, Weilenmann HU, Egli T, Hammes F (2008) Rapid, cultivation-independent assessment of microbial viability in drinking water. Water Res 42:4010–4018
- Binet MT, Stauber JL (2006) Rapid flow cytometric method for the assessment of toxic flagellate cyst viability. Mar Environ Res 62:247–260
- Carnegie RB, Stokes NA, Audemard C, Bishop M and others (2008) Strong seasonality of *Bonamia* sp. infection and induced *Crassostrea ariakensis* mortality in Bogue and Masonboro Sounds, North Carolina, USA. J Invertebr Pathol 98:335–343
- Chagot D, Boulo V, Hervio D, Mialhe E, Bachere E, Mourton C, Grizel H (1992) Interactions between *Bonamia ostreae* (Protozoa: Ascetospora) and hemocytes of *Ostrea edulis*

- and *Crassostrea gigas* (Mollusca: Bivalvia): entry mechanisms. *J Invertebr Pathol* 59:241–249
- Cochennec N, Auffret M (2002) European project DISENV FAIR984120 'Environmental Factors and Shellfish Diseases' 15/05/2002 Final Report. Project details available at: <http://cordis.europa.eu/>
- Cotter TG, Lennon SV, Glynn JM, Green DR (1992) Microfilament-disrupting agents prevent formation of apoptotic bodies in tumour cells undergoing apoptosis. *Cancer Res* 25:997–1005
- Culloty SC, Mulcahy MF (1996) Season-, age-, and sex-related variation in the prevalence of bonamiosis in flat oysters (*Ostrea edulis* L.) on the South coast of Ireland. *Aquaculture* 144:53–63
- Czechowska K, Johnson DR, Roelof van der Meer J (2008) Use of flow cytometric methods for single-cell analysis in environmental microbiology. *Curr Opin Microbiol* 11:205–212
- Elston RA, Farley CA, Kent ML (1986) Occurrence and significance of bonamiasis in European flat oysters *Ostrea edulis* in North America. *Dis Aquat Org* 2:49–54
- Farley CA, Wolf PH, Elston RA (1988) A long-term study of 'microcell' disease in oysters with a description of a new genus, *Mikrocytos* (g.n.) and two new species *Mikrocytos mackini* (sp.n.) and *Mikrocytos roughleyi* (sp.n.). *Fish Bull* (Wash DC) 86:581–593
- Gagnaire B, Frouin H, Moreau K, Thomas-Guyon H, Renault T (2006a) Effects of temperature and salinity on haemocyte activities of the Pacific oyster, *Crassostrea gigas* (Thunberg). *Fish Shellfish Immunol* 20:536–547
- Gagnaire B, Thomas-Guyon H, Burgeot T, Renault T (2006b) Pollutant effects on Pacific oyster, *Crassostrea gigas* (Thunberg), hemocytes: screening of 23 molecules using flow cytometry. *Cell Biol Toxicol* 22:1–14
- Grizel H (1985) Etudes des récentes épizooties de l'huître plate *Ostrea edulis* L. et de leur impact sur l'ostréiculture bretonne. PhD dissertation, Université des Sciences et Techniques de Languedoc, Montpellier
- Hammes F, Berney M, Wang Y, Vital M, Köster O, Egli T (2008) Flow cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes. *Water Res* 42:269–277
- Hervio D, Bachere E, Boulo V, Cochennec N and others (1995) Establishment of an experimental infection protocol for the flat oyster *Ostrea edulis* with the intrahaemocytic protozoan parasite *Bonamia ostreae*: application in the selection of parasite-resistant oyster. *Aquaculture* 132:183–194
- Hine PM (2002) Severe apicomplexan infection in the oyster *Ostrea chilensis*: a possible predisposing factor in bonamiosis. *Dis Aquat Org* 51:49–60
- Hudson EB, Hill BJ (1991) Impact and spread of bonamiasis in the UK. *Aquaculture* 93:279–285
- Lindström ES, Weisse T, Stadler P (2002) Enumeration of small ciliates in culture by flow cytometry and nucleic acid staining. *J Microbiol Methods* 49:173–182
- Lynch SA, Armitage DV, Wylde S, Mulcahy MF, Culloty SC (2005) The susceptibility of young prespawning oysters, *Ostrea edulis*, to *Bonamia ostreae*. *J Shellfish Res* 24:1019–1025
- Lynch SA, Armitage DV, Coughlan J, Mulcahy MF, Culloty SC (2007) Investigating the possible role of benthic macroinvertebrates and zooplankton in the life cycle of the haplosporidian *Bonamia ostreae*. *Exp Parasitol* 115:359–368
- Mialhe E, Bachère E, Chagot D, Grizel H (1988) Isolation and purification of the protozoan *Bonamia ostreae* (Pichot et al. 1980), a parasite affecting the flat oyster *Ostrea edulis* L. *Aquaculture* 71:293–299
- Montes J (1990) Development of *Bonamia ostreae* parasitosis of flat oyster, *Ostrea edulis*, from Galicia, northwest Spain. In: Perkins FO, Cheng TC (eds) *Pathology in marine aquaculture*. Academic Press, New York, p 223–227
- Montes J (1991) Lag time for the infestation of flat oyster (*Ostrea edulis* L.) by *Bonamia ostreae* in estuaries of Galicia (N.W. Spain). *Aquaculture* 93:235–239
- Montes J, Anadon R, Azevedo C (1994) A possible life cycle for *Bonamia ostreae* on the basis of electron microscopy studies. *J Invertebr Pathol* 63:1–6
- Mourton C, Boulo V, Chagot D, Hervio D, Bachère E, Mialhe E, Grizel H (1992) Interactions between *Bonamia ostreae* (Protozoa: Ascetospora) and hemocytes of *Ostrea edulis* and *Crassostrea gigas* (Mollusca: Bivalvia): *in vitro* system establishment. *J Invertebr Pathol* 59:235–240
- Nasirudeen AMA, Tan KSW, Singh M, Yap EH (2001) Programmed cell death in a human intestinal parasite, *Blastocystis hominis*. *Parasitology* 123:235–246
- Parrow MW, Burkholder JM (2002) Flow cytometric determination of zoospore DNA content and population DNA distribution in cultured *Pfiesteria* spp. (Pyrrhophyta). *J Exp Mar Biol Ecol* 267:35–51
- Rault A, Bouix M, Béal C (2008) Dynamic analysis of *Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1 physiological characteristics during fermentation. *Appl Microbiol Biotechnol* 81:559–570
- Samali A, Cotter TG (1999) Measurement of cell death in culture. In: Jenkins N (ed) *Animal cell biotechnology: methods and protocols*. Series: methods in biotechnology, Vol 8. Humana Press, Totowa, NJ, p 155–164
- Soudant P, Chu FLE, Lund ED (2005) Assessment of the cell viability of cultured *Perkinsus marinus* (Perkinsea), a parasitic protozoan of the eastern oyster, *Crassostrea virginica*, using SYBRgreen-Propidium Iodide double staining and flow cytometry. *J Eukaryot Microbiol* 52:492–499
- Sprague V (1979) Classification of the Haplosporidia. *Mar Fish Rev* 41:40–44
- Tigé G, Grizel H (1984) Essai de contamination d'*Ostrea edulis* Linne par *Bonamia ostreae* (Pichot et al., 1979) en rivière de Crach (Morbihan). *Rev Trav Inst Pêch Marit* 46:307–314
- Van Banning P (1988) Management strategies to control diseases in the Dutch culture of edible oysters. In: Fisher WS (ed) *Disease processes in marine bivalve molluscs*. *Am Fish Soc Spec Publ* 18:243–245
- Van Banning P (1991) Observations on bonamiasis in the stock of the European flat oyster, *Ostrea edulis*, in the Netherlands, with special reference to the recent developments in Lake Grevelingen. *Aquaculture* 93:205–211
- Wolf P (1967) Winter mortality hits state's oyster output. *Fisherman* 2:20–22
- Wong JTY, Whiteley A (1996) An improved method of cell cycle synchronisation for the heterotrophic dinoflagellate *Cryptocodinium cohnii* Biecheler analyzed by flow cytometry. *J Exp Mar Biol Ecol* 197:91–99