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

Isabelle Arzul1,*, Béatrice Gagnaire2, Céline Bond1, Bruno Chollet1, Benjamin Morga1, Sylvie Ferrand1, Maeva Robert1, Tristan Renault1

1 Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER), Laboratoire de Génétique et Pathologie (LGP), avenue de Mus de Loup, 17390 La Tremblade, France
2 Laboratory of Radioecology and Ecotoxicology, DEI/SECRE/LRE, Institute of Radioprotection and Nuclear Safety (IRSN), Cadarache, Building 186, BP 3, 13115 St-Paul-lez-Durance Cedex, France

* Corresponding author : I. Arzul, email address : iarzul@ifremer.fr

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
INTRODUCTION

*Bonamia ostreae* is a protistan parasite belonging to the phylum Haplosporidia (Sprague 1979). It is an intracellular parasite, 2-5 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 which are more than 2 year old (Culloty & Mulcahy 1996). At a 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 intermediate host is not required for parasite cycle accomplishment. Observation of parasites free 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 ways of entry and release remain undetermined. Most of *Bonamia ostreae* might be released in the water column after oyster death through tissue lysis.

This intrahaemocytic parasite has been described in oysters collected from different ecosystems from estuaries and intertidal zones to deep coastal waters or lagoon 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. 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 *Bonamia 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 (Miahle 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 weeks) as assessed by 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 aimed at addressing some questions in environmental microbiology including studying microbial physiology under environmentally relevant conditions (Czechowska et al. 2008). Flow cytometry was 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 multi parametric analyses on a large number of cells in a very short time and thus presents advantages over microscopic approaches.

The objectives of the present study were to test survival of purified Bonamia ostreae in different sea water media (artificial, natural and underground salty water) 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 three different media at three 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 FDA (Fluorescein Diacetate). Esterases are enzymes belonging to the group of hydrolases and are classically measured to estimate global level of viable cell activities (Gagnaire et al. 2006b, 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.

MATERIAL AND METHODS

Bonamia ostreae purification

Purification of parasites was performed following the protocol of Miahle et al. (1988) using flat oysters originated from Quiberon Bay (France), an infected area regarding Bonamia ostreae. Oysters were maintained 30 days in raceways of 120 litres receiving a constant flow of external seawater at a temperature of 12-15°C and enriched in phytoplankton (Skeletonema costatum, Isochrysis galbana, Chaetoceros gracilis and Tetraselmis suecica). Some highly infected flat oysters Ostrea edulis were selected by examination of heart tissue imprints under light microscope. Two to three highly infected oysters were used per purification. All organs were homogenized 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 sea water before being counted using a Malassez-cell haemocytometer. Parasite suspensions were then maintained at 4°C. Salinity of filtered sea water fluctuated between 30 and 34 g l⁻¹.
Experiment design

Two sets of experiments were performed three times.

In a first set of experiments, purified parasites were suspended in three different media and 10 ml of each parasite suspension were distributed in equivalent numbers \((5 \times 10^6 \text{ cells ml}^{-1})\) in nine 15 ml-polypropylene tubes per medium. The three media were:

1. 0.22 µm filtered prepared artificial sea 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 H\(_2\)O, 0.2 g CaCl\(_2\) . 2 H\(_2\)O, H\(_2\)O q.s. 1 L) (2) 0.22 µm filtered underground salty water showing a constant salinity of 32 g l\(^{-1}\) and a pH of 7.06 (USW, collected at – 110 meters at IFREMER facilities in La Tremblade, France) (3) 0.22 µm filtered natural sea water (NSW) from « La Seudre » estuary, Charente Maritime (France) with a salinity of 30-34 g l\(^{-1}\) and a pH of 8.06. Parasites maintained in the three different media were subjected to three different temperatures 4°C, 15°C and 25°C (three tubes per condition). The different parasite suspensions were tested by flow cytometry after 12 h, 24 h and 48 h of incubation.

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

More precisely, purified parasites were diluted in 0.22 µm filtered USW and distributed in equivalent numbers \((5 \times 10^6 \text{ cells ml}^{-1})\) in 15 ml-polypropylene tubes. Distilled water or natural salt from Guérande (Pays de la Loire, France) was added in order to obtain a range of salinities: 5 g l\(^{-1}\), 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 condition) were incubated at 15°C and samples were analysed at 12 h and 48 h by flow cytometry.

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. 2006a). Each measure was carried out three 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 small 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 98.1% of PI-stained cells (red fluorescence above 1) considered dead (Fig. 1a). Suspension of live parasites was used to control efficacy of FDA for esterase activities measurement (Fig. 1b). The FL1 fluorescence histogram showed 91% of fluorescent cells after incubation with FDA (green fluorescence above 1) considered alive and presenting esterase activities (Fig. 1b).

Statistical analysis

Data were analyzed statistically using the software Statgraphics® Plus version 5.1. Results were expressed as percentages of positive cells. Mean and standard deviation were calculated for each
triplicate. Effect of tested conditions was evaluated performing one-way, two-ways and three-ways ANOVA. Values were converted into r angular arcsinus √(% of positive cells) before analysis to ensure respect of a priori assumptions of normality and homogeneity. In the case of rejection of H₀, 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 ≤ 0.05.

RESULTS

Size and complexity of parasites

Size and in a lesser concern complexity of parasites varied according to their status: dead or alive. Irrespective of the tested conditions, it was possible to identify two populations of parasite cells (Fig. 2a): a population A consisting in 53 ± 24% of live cells and a population B, smaller in size consisting in a majority of dead cells (mean of 74 ± 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 hours after purification, population A and population B included 75.8% and 15.7% of total cells, respectively (Fig. 2a). When only considering non PI stained parasites for the same experimental conditions, population A and population B included 91% and 6.5% of live cells, respectively (Fig. 2b). For parasites after boiling, when only considering PI stained cells, population A and population B included 8.6% and 88% of dead cells, respectively (Fig. 2c).

Population A included more live cells (77.6 ± 6.2%) when mortality rates were below 50% compared to mortality rates above 70% (37.3 ± 24.7%) (Table 1). On the contrary, population B included more dead cells when mortality rates were high (91.7 ± 7.4% for mortality rates above 70%) (Table 1).
Impact of medium on cell viability

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

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

Impact of temperature on cell viability

Parasite viability fluctuated according to the tested temperature. Irrespective of medium and time of incubation, mortality appeared significantly higher at 25°C compared to 15°C and 4°C and percentages of cells presenting esterase activities were higher at 4°C compared to 15°C and 25°C (three-ways 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 24h and 48h of incubation ($p < 0.0001$) (Fig. 4).

In USW, irrespective of time of incubation, the percentage of positive parasites for esterase 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 53.28% at 25°C (Fig. 5).

Impact of incubation time on cell viability
Irrespective of medium and temperature, incubation time did not have significant impact on parasite mortality. However, a difference of percentages of cells presenting esterase activities is noted between 12 and 24 hours of incubation times (p = 0.003; three-ways ANOVA, Table 2).

In NSW and in USW, parasite survival and parasites presenting esterase activities were higher at 4°C and 15°C than at 25°C especially after 48h of incubation (Figs. 4 and 5). In NSW, after 48 hours of incubation, parasite mortality mean 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 h and 48 h of incubation compared to 12 h (p < 0.0001).

Similarly, percentage of parasites presenting esterase activities significantly decreased at 25°C after 48 h of incubation. In USW at 25°C the percentage of positive cells was 47.23 ± 13.31% after 24 h and 23.54 ± 8.65% after 48 h (p = 0.0004) (Fig. 5).

**Impact of salinity on cell viability**

Incubation length had no significant effect on cell mortality (two-ways ANOVA: F = 1.81, p = 0.186). Therefore, data obtained on independent samples after 12 and 48 hours of incubation were pooled. *A posteriori* tests showed that salinities of 5, 15 and 20 g l⁻¹ were associated with highest percentages of mortality whereas salinities of 35, 40 and 45 g l⁻¹ allowed better parasite survival (Fig. 6). Higher percentages of positive cells for esterase activities were reported for higher salinities (35 to 45 g l⁻¹) (Fig. 7). Moreover, time of incubation presented a significant impact on parasite esterase activities (two-ways ANOVA, F = 15.3, p = 0). There was a significant decrease of percentages of positive parasites between 12h and 48h irrespective of tested salinities except at 25 g l⁻¹ (Fig. 7).
Despite 25 years of research on the protozoan *Bonamia ostreae*, its life cycle is poorly known. Whatever is the date at which naïve oysters are placed in an infected area, first known stages of the parasite are observed 3 to 5 months after exposition to the parasite (Tigé & Grizel 1984, Montes 1991). Moreover, the infection seems to remain present in areas that have been cleaned and which ceased to produce oysters for several years (Van Banning 1988). Lagtime before infection and persistence of the disease in cleaned areas motivated some authors to investigate potential involvement of macroinvertebrate and zooplankton species in *Bonamia ostreae* life cycle (Lynch et al. 2006). Nevertheless, considering the correlation between density of oysters and prevalence of bonamiosis (Grizel 1985, Hudson & Hill 1991), the parasite mainly depends for its survival and spread on flat oysters *Ostrea edulis* themselves 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 have an impact on 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 impact of two environmental parameters, the salinity and the temperature, on the parasite viability was investigated at different times of incubation: 12 h, 24 h 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 three different sea water media for parasite preservation was tested: 0.22 µm filtered natural sea water; 0.22 µm filtered underground salty water (with a constant composition) and 0.22 µm filtered artificial sea water (which is easy to acquire and with a constant composition).

Size and complexity of *Bonamia ostreae* were generally homogeneous but depended on the status of the parasite cell i.e. if 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 less homogeneous, larger in size increasing proportionally to survival rates. These results suggest that when dying *B. ostreae* becomes smaller. Such phenomenon is 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 natural filtered sea water and in underground filtered salty water than in artificial filtered sea water (less than 40%) whatever were temperature and time of incubation. This result could be explained by a difference of pH. Indeed, pH of ASW used in this study was more acid (6.5) than NSW (8.06) and USW (7.06). Effect of pH on parasite viability has not been investigated in the present study. Moreover, a difference of salinity was also noticed between tested media: between 30 and 34 g l⁻¹ for NSW; 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 salinity than other tested media which could explain the poor conservation of parasites.

Although no significant difference of mortality and non specific esterase activities were observed between parasites maintained at 4°C and 15°C in NSW or USW, 25°C did not appear suitable for parasite preservation. In natural conditions, the disease is reported in areas where temperature of sea water rarely reaches 25°C except in Mediterranean Sea where *Bonamia ostreae* is reported but with low prevalence (0.9 ± 1.4%, data from REPAMO, French network for the surveillance of mollusc diseases). In Quiberon bay (Morbihan, France) where prevalence of bonamiosis is estimated at 12.4% ± 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, French network for the surveillance of phytoplankton and phycotoxins). Some analyses revealed a negative correlation between high summer water temperature and number of oysters detected infected during the following winter (I. Arzul, unpublished data) suggesting that higher temperatures do not favour infection of oysters. A study carried out on *Crassostrea gigas* haemocytes showed that an increase of temperature and a decrease of salinity induced an increase of cell mortality (Gagnaire et al. 2006b) suggesting that these environmental parameters had also
an effect on oyster defence capacities. Similarly, previous works demonstrated an impact of
temperature on flat oyster *Ostrea edulis* defence mechanisms (Cochennec & Auffret 2002): lower
temperatures were associated with increased bonamiosis prevalence. However in the same study,
haemocyte activities were tested by flow cytometry and revealed that low temperature (10°C) or a
decrease of temperature (from 20°C to 10°C) induced a decrease of enzymatic activities including
ROS production involved in defence mechanisms. Several authors described a seasonal variation of
infection with *B. ostreae*; 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 carried out on *Bonamia* sp. infecting the Asian oyster *Crassostrea ariakensis* in
Atlantic coastal waters of U.S.A showed a strong influence of temperature on seasonal parasite
cycling (Carnegie et al. 2008). Interestingly, temperatures around 25°C when oysters were placed
in infected area 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 under winter oyster mortalities
is associated with low temperatures (Wolf 1967). However all these studies consider the parasite
inside its host and thus investigate effects of temperature on host-parasite relationships and not
directly on parasite survival.

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

As previously mentioned measures of parasite survival in the three tested media (NSW, USW and
ASW) supported these results. Indeed, parasite mortality was higher in ASW (salinity of 23.4 g l\(^{-1}\)) compared to NSW (salinity of 32-34 g l\(^{-1}\)) and USW (salinity of 32 g l\(^{-1}\)).

These results are also concordant with a previous study realised on *Bonamia exiotiosa* in New Zealand in which a salinity of 40 g l\(^{-1}\) was associated with highest disease prevalences (Hine 2002). Similarly, infection with *B. roughleyi* seems to be favoured by high salinities (30-35 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).

Time of incubation showed an impact on the parasite preservation especially by increasing effect of high temperatures on mortality (higher mortality) and esterase activities (lower percentages of positive cells). Moreover, whatever was the tested salinity, percentages of cells producing esterase activities were lower after 48h compared to 12h. It would be interesting to complete these results by testing a wider range of incubation times in order to evaluate the persistence capacity of *Bonamia ostreae* in natural sea water collected from different infected areas. However, mortality and esterase activity measured by flow cytometry are instantaneous and do not allow to follow cumulative mortality.

In the present context of global change, data allowing forecasting of disease evolution are requested. Oysters are submitted to environmental changing and parasites as well. Description of the influence of temperature and salinity on *Bonamia ostreae* viability should allow modelling parasite transmission. Moreover these results should contribute to define risky and non risky geographic areas regarding transmission of the disease. These data might also be of interest for oyster farmers. Indeed, by monitoring temperature and salinity parameters, oysters might be moved or sold before suitable conditions for parasites survival are reached.

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**TABLE 1** - Distribution of *Bonamia ostreae* cells in percentages (means (± standard deviation)) in Population A and Population B (as shown on Figures 2 for example) and composition in live and dead cells of these two populations according to the level of mortality rates.

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<td>&lt; 50%</td>
<td>56.68 (± 18.11)</td>
<td>77.58 (± 6.21)</td>
<td>15.66 (± 5.16)</td>
<td>53.54 (± 22.03)</td>
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<tr>
<td>≥ 50 % and &lt; 70%</td>
<td>50.29 (± 20.78)</td>
<td>56.35 (± 9.16)</td>
<td>22.74 (± 10.65)</td>
<td>74.99 (± 13.79)</td>
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<tr>
<td>≥ 70%</td>
<td>25.64 (± 17.06)</td>
<td>37.33 (± 24.56)</td>
<td>49.51 (± 14.44)</td>
<td>91.67 (± 7.43)</td>
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TABLE 2- ANOVAs comparing the percentages of mortality and the percentages of positive cells for esterase activities according to media, temperatures (= Temp) and times of incubation (= Time).

NSW: natural seawater; USW: underground salty water; ASW: artificial seawater. F: Fisher value, p: significance value. Terms significant at p < 0.05 are highlighted in bold.

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<td>Ddl F</td>
<td>p</td>
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<td>Time</td>
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LSD tests

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<td>ASW &lt; NSW = USW</td>
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<table>
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<td>4 = 15 &lt; 25</td>
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<table>
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<th>Time</th>
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<td>12h &gt; 24h = 48h</td>
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FIGURE LEGENDS

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

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

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

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

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

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

Fig. 4 – Parasite mortality (percentages of Propidium Iodide stained cells) in natural sea water according to the temperature and time of incubation (values are mean of three replicates); Bars
Fig. 5 - Parasite esterase activities (percentages of positive cells) in underground salty water according to the temperature and time of incubation (Values are mean of three replicates; Bars represent standard errors; *** p < 0.0001 compared to data at 25°C and 12 h after incubation and compared to data at 15°C and 4°C

Fig. 6 - Parasite mortality (Percentages of Propidium Iodide stained cells) according to the salinity of underground salty water (time of both data pooled). Values are means ± standard error. N = 6 replicates.

Fig. 7 - Parasite esterase activities (Percentages of positive cells) in underground salty water according to the salinity and time of incubation (Values are mean of three replicates; Bars represent standard errors); *** p < 0.001 compared to data 12 h after incubation
Fig 1.
Fig 2.
Fig. 3
Fig. 4
Fig. 5
Fig. 6

Percentage of PI stained cells vs. Salinity (g.l$^{-1}$)
Fig. 7