

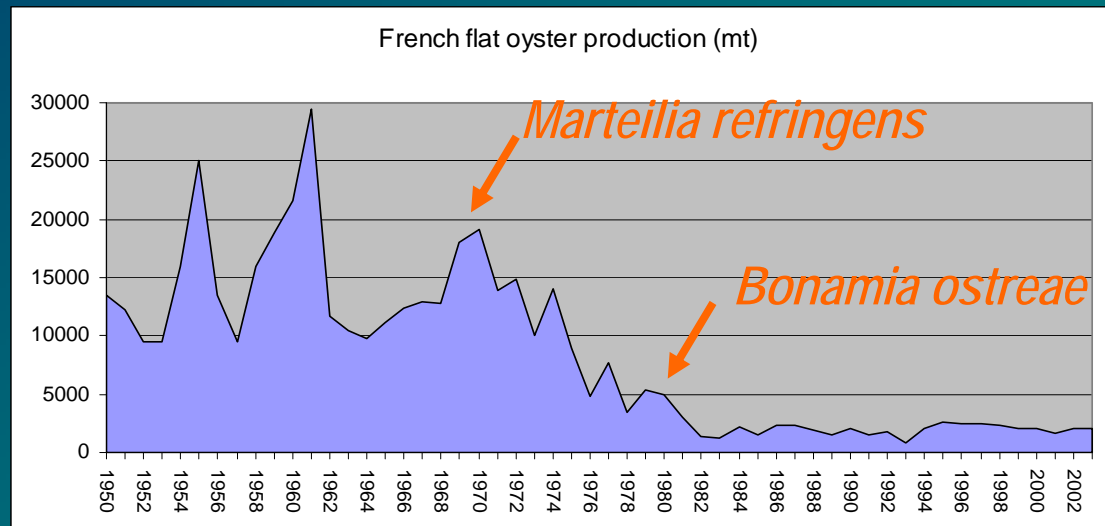
Effects of temperature and salinity  
on the survival of *Bonamia ostreae*,  
parasite infecting flat oyster *Ostrea*  
*edulis*, in sea water

Isabelle Arzul, Céline Bond, Béatrice Gagnaire, Benjamin Morga,  
Bruno Chollet, Sylvie Ferrand, Maeva Robert and Tristan Renault\*

IFREMER- Laboratoire de Génétique et Pathologie - 17390 La Tremblade, France  
iarzul@ifremer.fr

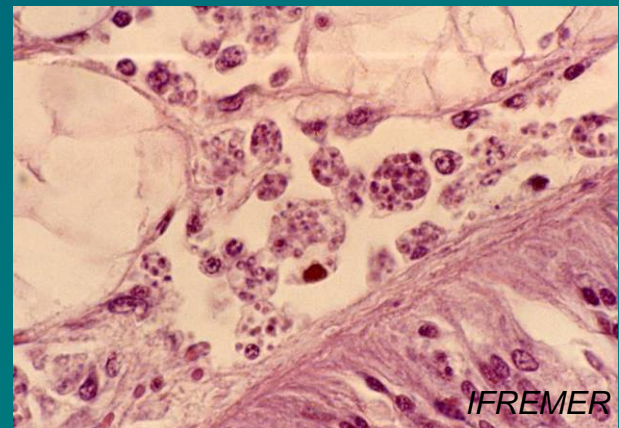
# Introduction

- The flat oyster *Ostrea edulis* was the flagship of the Breton oyster production until two diseases due to the protozoans *Bonamia ostreae* and *Marteilia refringens* spread in the 1970's.
- These diseases drastically reduced the flat oyster production from nearly 20,000 t per year in 1970 to less than 2,000 t nowadays.



# Introduction

- The protozoan *Bonamia ostreae* was first reported in June 1979, in oyster farms of Tudy Island, Brittany, in association with abnormal mass **mortalities** (80-90%) (Pichot *et al.*, 1979).
- During the following months, the same parasite was detected in all the Brittany farming centres and then has **rapidly spread** to most European oyster stocks (both reared and wild).
- The introduction is believed to have occurred with transfers of flat oysters, *Ostrea edulis* moved **from California** to France and Spain.



# Introduction



- This intrahaemocytic parasite has been described in **different ecosystems** from estuaries to open sea and is presently reported in Europe, North America and Morocco.
- The life cycle is unknown but the disease can be **transmitted directly** by cohabitation.
- No clear correlations could be demonstrated between development of the disease and environmental parameters including **temperature and salinity**
- However, previous works suggested an **impact of the temperature** on the parasite and / or on the defence capacity of oysters.

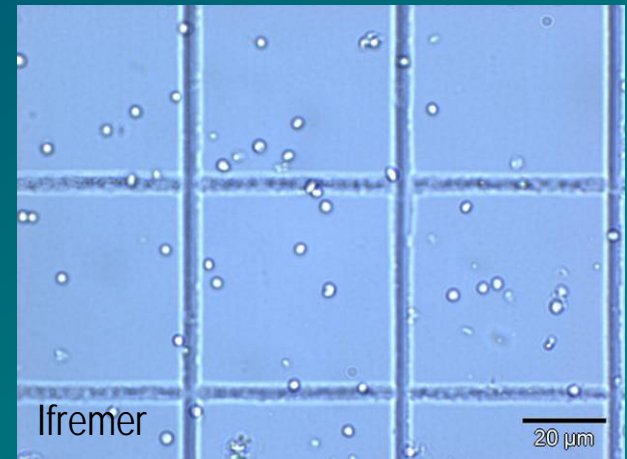
# Aim of the study

- Testing survival of purified *Bonamia ostreae* in **different sea water media** (artificial, natural and underground salty sea water)
  - Investigating the impact of **temperature and salinity** on the survival of purified parasites
- ⇒ by measuring parasite mortality and esterase activities by **flow cytometry**

# Material and method

## Purification of parasite (Miahle et al. 1988)

1. Selection of **highly infected** flat oysters by heart imprints
2. Homogenization of all organs except the adductor muscle
3. Parasites concentration by differential centrifugation on **sucrose gradients** and separation by centrifugation on a **Percoll gradient**
4. Purified parasites are then suspended in filtered sea water (0.22  $\mu\text{m}$ ) and counted using a Malassez-cell hemocyter.

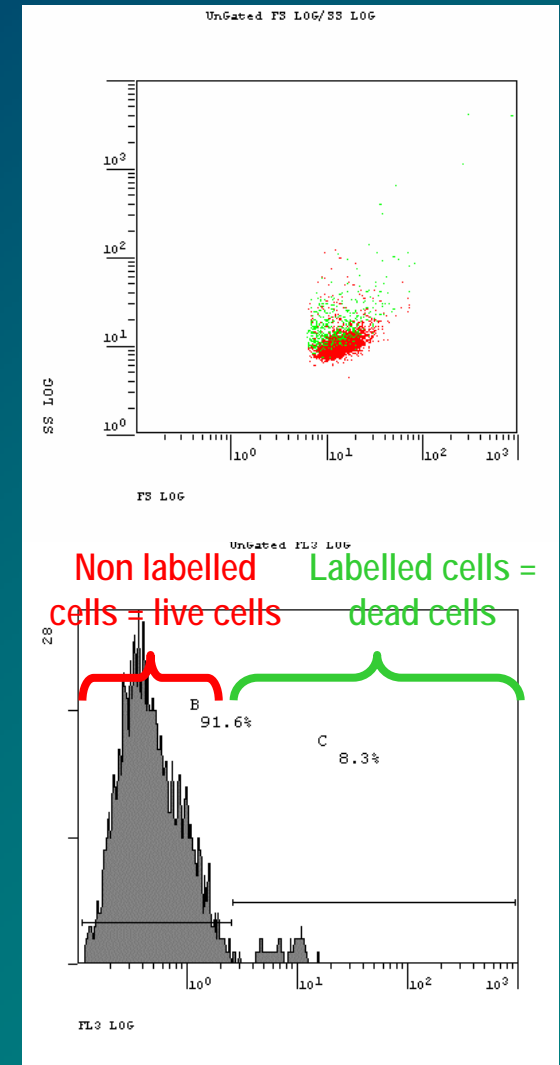


*Bonamia ostreae* after purification

# Material and method

## Flow cytometry analyses

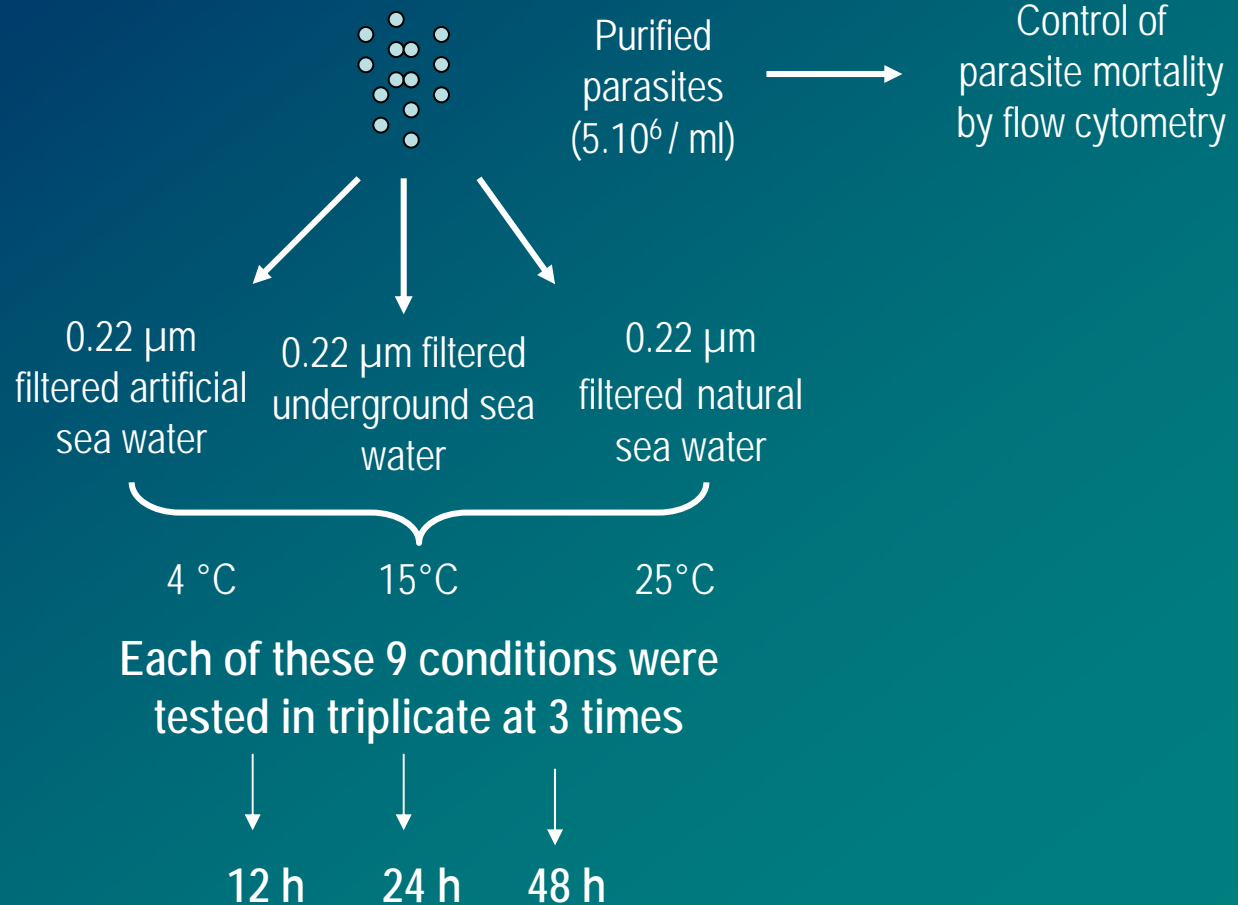
- For each sample, 5000 events were counted using an EPICS XL 4 (Beckman Coulter)
- Results were depicted as cell cytograms indicating cell size (FSC value) and cell (complexity) (SSC value) and the fluorescence channel corresponding to the marker used (green for non specific esterase activities and red for cell mortality)
- Mortality and esterase activities were quantified using 200  $\mu\text{L}$  of parasite suspension containing  $5 \times 10^5$  cells/ml.
- For mortality measure, parasites were incubated in the dark for 30 min at  $4^\circ\text{C}$  with 10  $\mu\text{L}$  of propidium iodide (1.0 mg/L)
- For measure of esterase activities, parasites were incubated in the dark for 30 min at ambient temperature with 1  $\mu\text{L}$  of F.D.A. (400  $\mu\text{M}$ )



IP labelled *Bonamia ostreae* 12 hours after purification.  
Discriminant= 5. Number of events = 5000 (B. Morga)

# Material and method

## Experiment 1 (realised three times)





# Material and method

## Experiment 2 (realised three times)

Purified parasites in 0.22  
 $\mu\text{m}$  filtered underground  
salty water ( $5 \cdot 10^6 / \text{ml}$ )  
maintained at  $15^\circ\text{C}$



Control of  
parasite mortality  
by flow cytometry



5 g/L 15 g/L 20 g/L 25 g/L 30 g/L 35 g/L 40 g/L 45 g/L

Each of these 8 salinity conditions  
were tested in triplicate at 4 times



12 h



24 h



48 h



1 w

# Material and method

## Statistical analyses

Results were expressed as percentages of positive cells

Mean and standard deviation were calculated for each triplicate

Values were converted into  $r$  angular arcsinus before analysis to ensure respect of a priori assumptions of normality and homogeneity

Effect of tested conditions was evaluated performing an ANOVA (Statgraphics<sup>®</sup> Plus version 5.1)

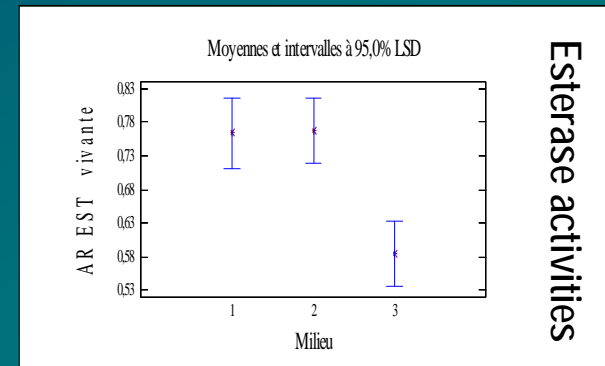
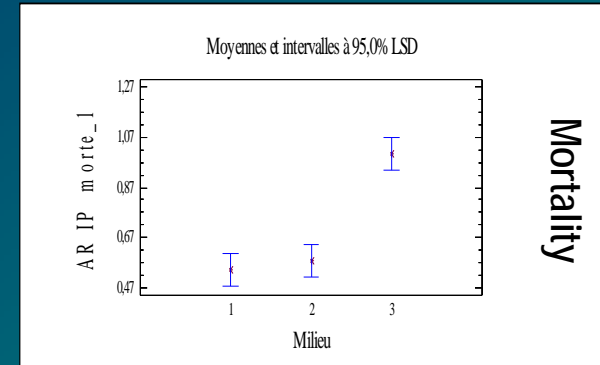
# Results (experiment 1)

Impact of the medium on parasite survival and activities

The medium appears as the **most important factor** on parasite survival ( $p < 0.5\%$ )

Whatever are temperatures and times post purification, parasite shows higher mortality and lower esterase activities in **artificial sea water**

No significant difference of survival and esterase activities was observed between underground salty water and natural sea water

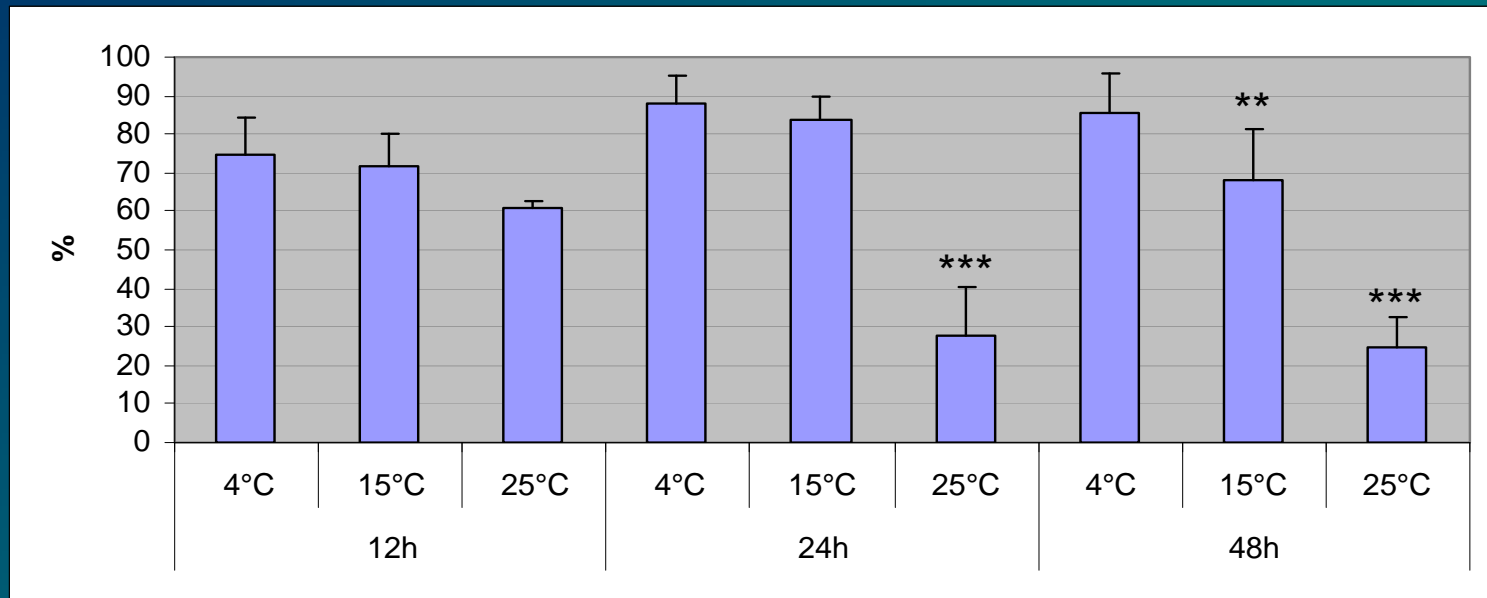


Mortality and esterase activities of parasites according to the medium (1= NSW; 2= USW; 3=ASW)

# Results (experiment 1)

## Impact of temperature on parasite survival and activities

In natural sea water and underground salty water, parasite mortality appeared higher at 25°C than at 4°C and 15°C especially 24 hours after purification.



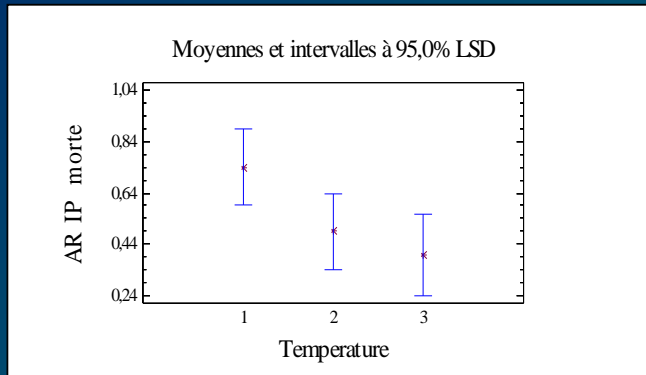
Parasite survival in natural sea water (deducted from IP labelling) according to the temperature and time of incubation (Values are mean of three replicates; Bars represent standard deviation; \*\* p=0.005; \*\*\* p<0.0001)

# Results (experiment 1)

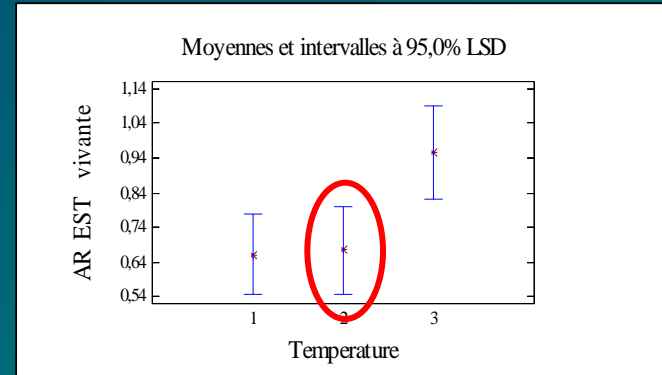
## Impact of temperature on parasite survival and activities

Measure of non specific esterase activities gave similar results to IP labelling at 4°C and 25°C. However temperature of 15°C does not seem suitable for parasite esterase activities in NSW.

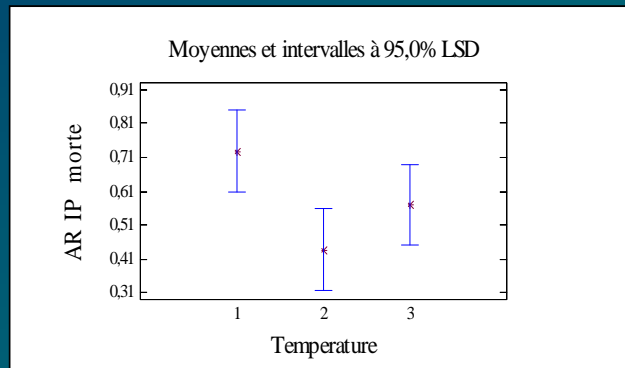
Mortality



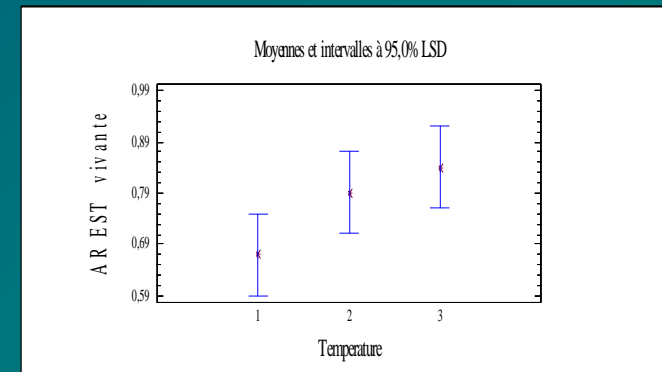
NSW



Esterase activities



USW

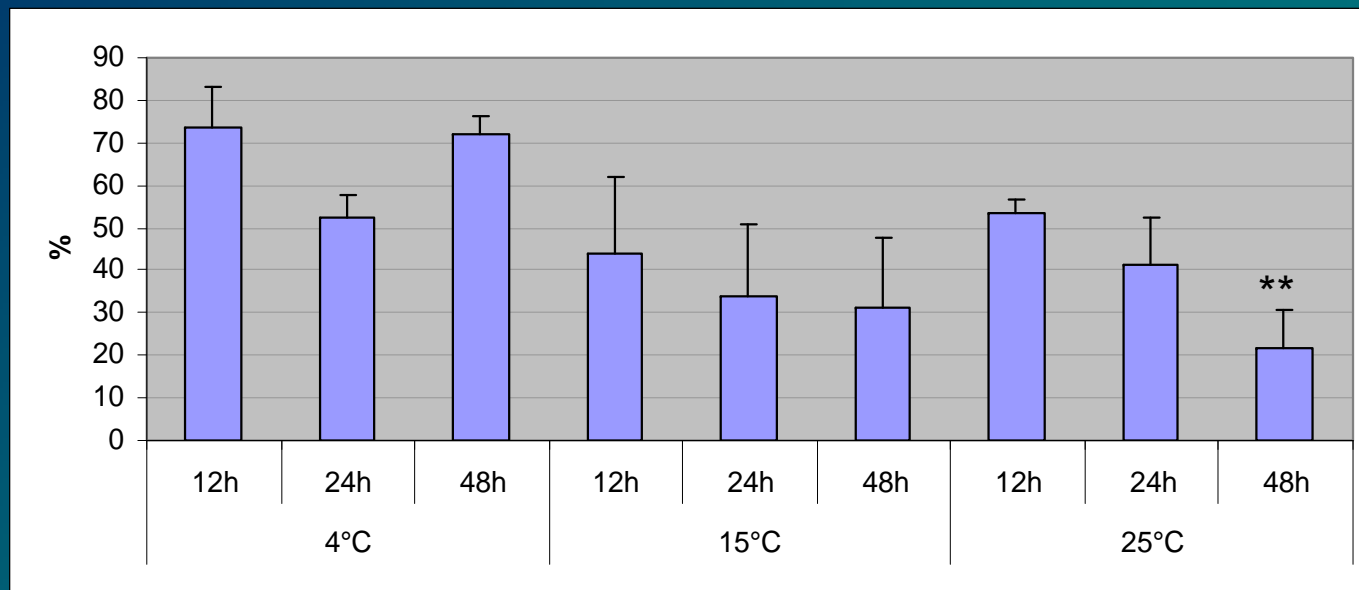


Mortality and esterase activities of parasites according to the temperature of incubation  
(1= 25°C; 2= 15°C; 3=4°C)

# Results (experiment 1)

## Impact of time on parasite survival and activities

Parasite is still alive 48 hours after purification. Time does not have significant impact neither on parasite mortality nor on esterase activities at 4°C and 15°C.



Parasite esterase activities in underground salty water according to the temperature and time of incubation (Values are mean of three replicates; Bars represent standard deviation; \*\*  $p=0.0004$  )

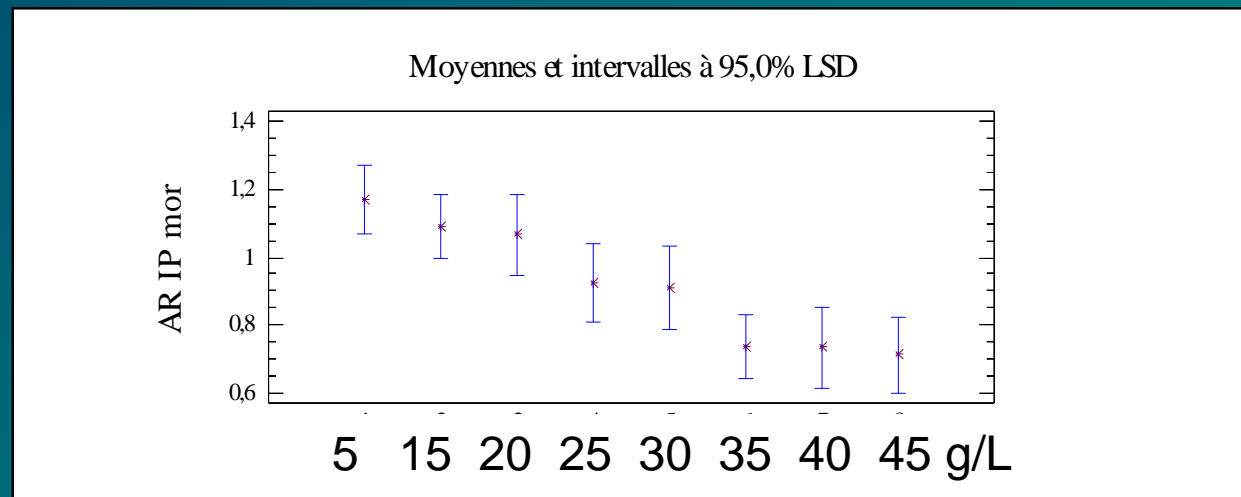
# Results (experiment 2)

Impact of salinity on survival and activities of parasites maintained in underground salty water at 15°C

Why these conditions? Underground salty water is stable in time and seems to offer good conditions for parasite survival and activities at 4 and 15°C. Temperature of 15°C has been chosen because it is closer to natural conditions than 4°C.

Multiple range test shows that salinities of 5, 15 and 20 g/L induce a higher percentage of parasite mortality and salinities of 35, 40 and 45 g/L induce a better parasite survival.

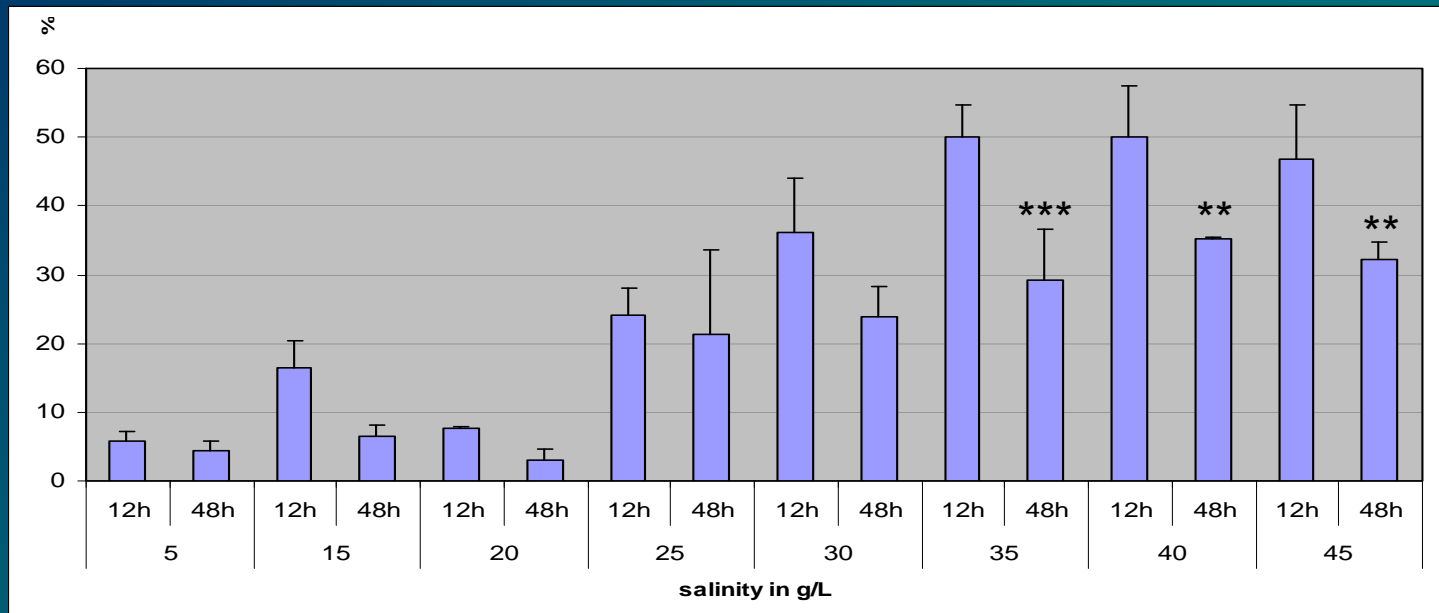
Parasite mortality according to the salinity of underground salty water



# Results (experiment 2)

Impact of salinity and time on survival and activities of parasite maintained in underground salty water at 15°C

Measure of non specific esterase activities gave similar results to IP labelling : percentage of positive parasites for this parameter is higher for higher salinities. Moreover, it is possible to see a significant impact of time of incubation for salinities higher than 35 g/L.



Parasite esterase activities in underground salty water according to the salinity and time of incubation (Values are mean of three replicates; Bars represent standard deviation; \*\*\* p=0.002 ; \*\* p= 0.03)



# Conclusion-Discussion

- Purified *Bonamia ostreae* showed a better survival (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).
- No significant difference of mortality and esterase activities could be observed between parasites maintained at 4°C and 15°C in NSW or USW. However, 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 .
- Previous works demonstrated an impact of temperature on oyster defence mechanisms (Cochennec & Auffret, not published): lower temperatures increased bonamiosis prevalence. Several authors have studied the cycle of the disease which seems to differ according to each country depending on environmental parameters including temperature (Montes & Melendez, 1987; Culloty et al. 1996). However all these studies consider parasite inside its host and thus investigate effect of temperature on host-parasite relationships and not directly on parasite survival.

# Conclusion-Discussion

- Purified *Bonamia ostreae* showed a better survival and higher esterase activities in hyper saline ( $\geq 35$  g/L) than in hypo saline media ( $\leq 20$  g/L). This result fits in with a previous study realised on *B. exitiosa* in New Zealand in which a salinity of 40g/L was associated with highest disease prevalences (Hine et al. 2002). Our results are also supported by a recent study in which salinity below 30 parts per thousand appeared detrimental to *Bonamia* sp. in *Crassostrea ariakensis* (Audemard et al. 2005).
- Time of incubation did not appear to have a clear impact on parasite survival and activities in our study. It was possible to detect up to 58% of live cells after 1 week of incubation in underground salty water maintain at 4°C. However, mortality and esterase activity measures by flow cytometry are instantaneous and probably do not reflect true percentages when cells are too damaged.

# Perspectives

- Flow cytometry appears as an interesting tool to study survival of pathogens like *Bonamia ostreae* in different conditions and could also be used to test efficacy of some media to preserve parasites after purification.
- Impact of temperature on *Bonamia ostreae* mortality and activity needs to be completed notably by including a wider range of tested temperatures
- These results should be completed by observations under an epifluorescent microscope especially after one week of incubation in order to determine true percentages of live cells
- Such results may be interesting for oyster farmers for stock management. Indeed, by monitoring parameters like temperature and salinity, oysters may be moved or sold when suitable conditions for parasite survival are reached
- However, further studies are still needed to improve our knowledge concerning life cycle of *Bonamia ostreae*

Thanks for your  
attention

