# Chronic oyster mortality in summer in the Bay of Marennes-Oléron; field and laboratory experiments

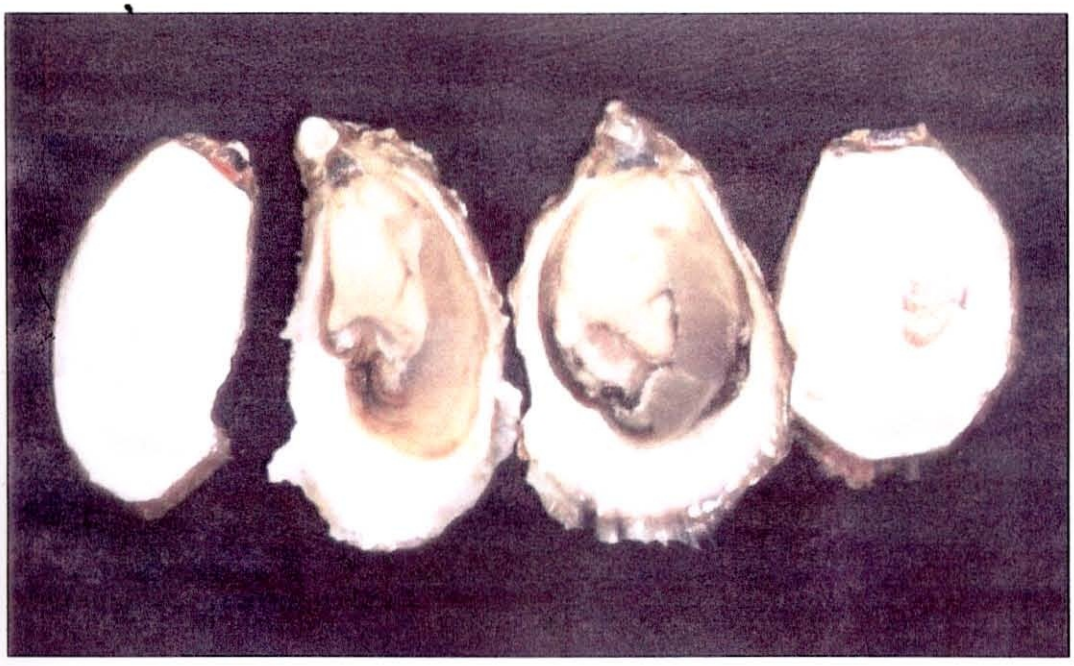

**Photo of *C. gigas* by A. Bakker**

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*June 2000*
*Laboratoire Conchylicole du Poitou-Charentes, Ronce les Bains in France*

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## Summary

Oyster culture is of great economic value for France. A large part of oyster production is situated in the Southwest of France, in the Bay of Marennes-Oléron. The last 20 years a phenomenon called summer mortality of the oyster species *Crassostrea gigas* occurs in the bay. A high percentage of the oysters die during the spring and summer when maturation takes place. Pollution, predation and illness seem not the primal cause of summer mortality in the Bay of Marennes-Oléron. Neither can over-stocking problems explain the occurring mortalities.

Two different culture types are used in the bay. The first is seeding the oysters on the sediment (on bottom culture), the second is placing the oysters in bags on tables made of iron bars (on table culture). I compared the mortality rate for the different culture types in 1998 and 1999 for 4 sites at the banks of Ronce-Perquis. Furthermore, I studied temperature, pH, dissolved oxygen and salinity in the field. Tests in the laboratory were developed to test the influence of different abiotic conditions (temperature, pH and dissolved oxygen) on mortality rate in oysters. Biochemical analyses were done on oysters at the start of the experiment and on the oysters that died during the experiment.

In 1998 I did not find a significant difference in accumulated mortality rate, averaged over four sites, between the two culture types. In 1999 accumulated mortality rate, averaged over four sites, is significantly different for the two culture types. The significant difference per period averaged over sites occurs in the periods from half May till half July. This is also the period in which maturation of the oyster takes place. I did not find a trend in temperature difference between the two culture types in 1998 and 1999. Dissolved oxygen and salinity data from site 3 in 1999 did not show a difference in trend between the height of the two culture types during April till June. However pH recordings from site 3 in 1999, showed that pH was always higher at table culture height than at ground culture height in April till June. The laboratory results showed that placing mature oysters (that are rich in carbohydrates) every day in a dry oven at 35° C for three hours followed by three hours in a tank with seawater of pH of 9.5-10 (value also found in the field) causes a significant mortality rate. The group of oysters that had only the temperature 'stress' did not show a significant mortality rate. Neither did the group of oysters that was subjected to the temperature 'stress' followed by one hour in a tank with seawater with 4-5 mg/l of dissolved oxygen (value also found in the field) show a significant effect on mortality rate. I did not find a significant difference in mortality rate of the same groups during a period of starvation after the 'stress' experiments. Oysters from the bay that have a lower carbohydrates percentage might however show significant mortality rate.

The biochemistry of the oysters that died in this stress experiment differ significantly in lipids and glycogen but not in dry weight and carbohydrates from the oysters at $t_0$. Both lipid concentration and glycogen concentration is lower in the oysters that died showing an influence of stress on the energy resource of glycogen that is also used when the oyster closes its shell to withstand stress. Overall pH stress can be of great importance for mortality rate difference between the two types of *Crassostrea gigas* culture in the Bay of Marennes-Oléron. A combination of different abiotic stress can effect glycogen content (used for anaerobic respiration during low tide) in mature oysters and therefore play an important part in summer mortality.

# Introduction

## *Oyster culture in the Bay of Marennes-Oléron*

The Bay of Marennes-Oléron in the Southwest of France (fig. 1) has been an important site for oyster culture since the end of the 19th century (Goulletquer and Heral, 1997). The species of oyster that is cultivated here nowadays is from Japanese origin; *Crassostrea gigas*, also named the Pacific cupped oyster. Besides this species, there is a much smaller cultivation of the flat oyster *Ostrea edulis* in the Bay of Marennes-Oléron. At the moment the production of the Pacific cupped oyster is between 30.000 and 40.000 tons per year with a stock of 110.000 tons (Soletchnik *et al.*, 1999).
The Pacific cupped oyster was introduced in France in 1972 when gill and viral diseases ran their course at the European coasts and destroyed the entire Portuguese oyster (*Crassostrea angulata*) culture of France within a few years (Comps, 1988 and Goulletquer & Heral, 1997). Some years later *C. gigas* dominated the oyster culture in many oyster cultures sites around the world.
Oyster culture is still increasing in the Bay of Marennes-Oléron and some problems have arisen. One of them is the occurrence of summer mortality. A high percentage of the oysters, that are cultivated on the banks of the bay within the intertidal area, die during the spring and summer. The same trend is also found in other oyster culture grounds in France and in other parts of the world like Japan, north America (West coast), Alaska and Morocco (Beattie *et al.*, 1988, Imaï *et al.*, 1965, Maurer & Comps, 1986, Meyers *et al.*, 1990, Perdue *et al.*, 1981, and Shafee, 1986). The intensity of mortality varies in time and place. In May 1988 and June 1993 estimated mortality reached a height of respectively 41 % and 30 % at the banks of Ronce-Perquis in the bay of Marennes-Oléron (Bodoy *et al.*, 1990 and Héral, 1993).

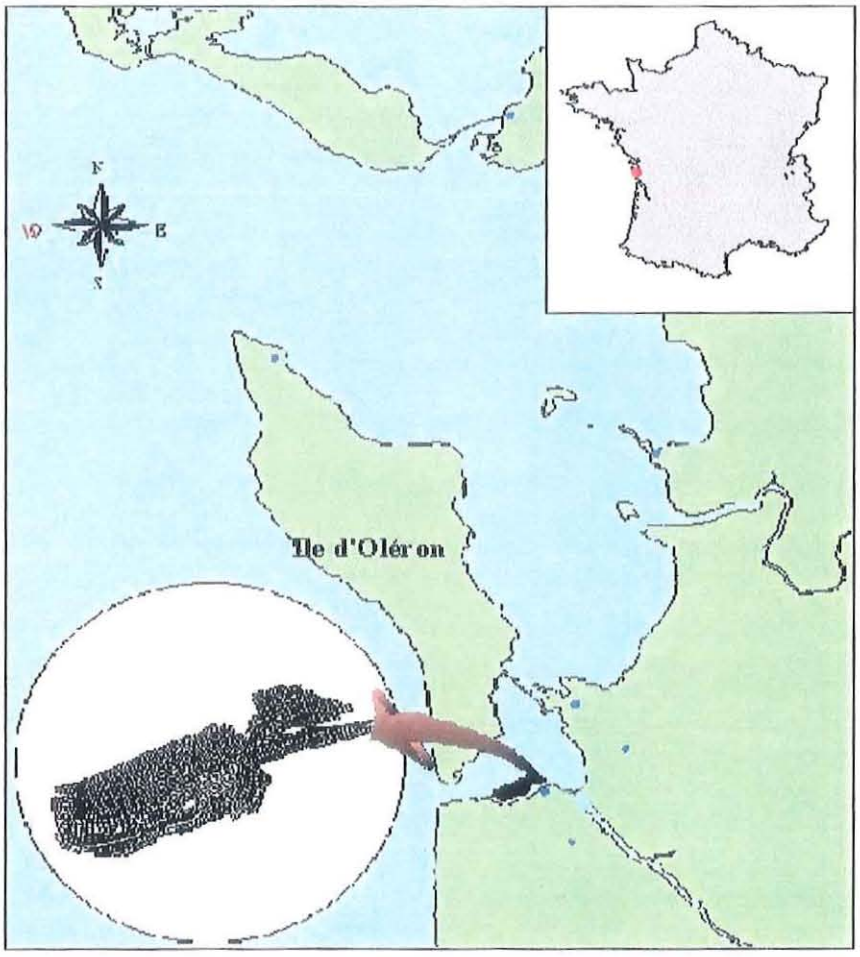



***Fig. 1. The bay of Marennes-Oléron with the oyster banks of Ronce-Perquis in the Southwest of France.***

## Causes for mortality in oysters

Causes of death in oysters can be divided into natural death, predation and sickness caused by pollution or bacteria and viruses. Natural death can be death due to old age (up to 10 years) or caused by environmental factors. These environmental factors causing death can be covering by sediment, exposure to great heat or cold, damage of the shell and long exposure to air or fresh water.
Predators and pests like the boring sponge, the boring sea worm, certain burrowing shrimps, the parasitic copepod, certain crabs, oyster drill and certain starfish can also cause mortality in oysters (Quayle, 1969). There are also some diseases that can cause high mortality in oyster culture like the foot disease and the Denman disease (Quayle, 1969). Both these diseases leave scars on the shell and are easily recognised. Some virus infections of oysters are more difficult to detect. Herpes like viruses have especially been causing high mortalities in juvenile oysters at different hatcheries (Comps, 1988 and Renault *et al.*, 1995).
The knowledge on virology of oysters is increasing but still limited compared to virology of vertebrates and insects.

Since 1988 IFREMER (Institut Français de Recherche pour l'Exploitation de la Mer) has monitored the occurrence of summer mortality of the Pacific cupped oyster in the bay of Marennes-Oléron and is conducting research to find the cause or combination of causes for this phenomenon. Possible causes like pollution of the seawater, toxic products produced by organisms like phytoplankton, predation and illness of oysters are monitored. Also the food resources for the oysters and general and local abiotic environmental conditions like temperature in the bay are being investigated.
Pollution, predation and illness seem not the primal cause of summer mortality in the Bay of Marennes-Oléron (Soletchnik *et al.*, 1999). Neither can over-stocking problems explain the occurring mortalities (Héral, 1991). Soletchnik *et al.* (1997) and Perdue *et al.* (1981) found that mortality is correlated with temperature rising in spring causing algae bloom and the maturation of the oyster. Both the temperature rise and the rise in food supply (the algae bloom) stimulate a fast growing rate, rapid metabolism and sexual maturation in oysters. During sexual maturation the glycogen of the oysters is converted into lipids for the production of eggs or sperm in the gonads (Deslous-Paoli & Héral, 1988 and Gabbott, 1983). Therefore glycogen content can drop to only a few percent of the total body weight. Furthermore Soletchnik *et al.* (1997) have shown that filtration rate in highly mature oysters is lower than in oysters in other stages and found that mature 1.5 year old oysters had a negative energy budget reaching – 90 J/h due to a reduced absorption efficiency.
The combination of a fast growing rate, rapid metabolism and low glycogen content can make oysters more susceptible for infections or the effects of abiotic stress. The oyster is dependent of glycogen for anaerobic respiration like during low tide (de Zwaan, 1983). Oxygen depletion of the surrounding seawater of the oyster during its immersion or other unfavourable conditions like elevated pH and high salinity of the surrounding seawater could reduce oyster activity (opening to feed and "breath") and thus weight heavily on the glycogen content of the oyster.
We must also take into account that due to elevated temperatures of the seawater during summer bacterial growth will be stimulated and therefore infections will occur more frequently.

## Difference in mortality rate for two types of culture

In the Bay of Marennes-Oléron two ways of culturing oysters are used. The first is the traditional way; the oysters are spread out on the sediment at low tide and left to grow till they are harvested with a rake. The second acquires some material like iron tables that are placed on the sediment and on which bags with oysters are placed but can easily be harvested by removing the bags.
Although summer mortality seems to effect the two practised types of oyster culture, on bottom and on table (fig 1a and 1b), it appears to be higher for on bottom culture of oysters than for on table culture of oysters (Soletchnik *et al.*, 1999).
Possible causes for the difference in mortality rate between these two types of culture can be a difference in environmental stress caused by the temperature, pH, dissolved oxygen and salinity of the direct environment. Some studies describe the effects of abiotic factors on filtration or respiration in oysters (Bernard, 1983 and Bougrier *et al.*, 1995). There have also been some research in a controlled environment (laboratory) on the effects of temperature and oxygen stress on mortality in juvenile

oysters (Glémarec, 1996 and Glémarec *et al.*, 1997). However, there have not been studies in a controlled environment (laboratory) on the effects of abiotic factors on mortality in mature oysters. Neither have I found records of experiments regarding elevating or lowering the pH to study its effects on the mortality of oysters. Furthermore, in most studies the biochemistry (energy resources like carbohydrates and glycogen) of the oysters that were used in the experiment are not described. This gives difficulties for interpreting what the results will mean for oyster culture in the field.

In this research I like to describe the difference in mortality rate between on bottom and on 50-cm high table culture. Furthermore, I like to describe the differences of some environmental conditions of on bottom and on 50-cm high table culture. I therefore analyse data on mortality, temperature, pH, oxygen and salinity that have been collected in 1998 and 1999 by the Laboratoire Conchylicole Poitou-Charentes (LCPC) IFREMER.
Secondly, I will try to find the influences of temperature stress separate and in combination with pH or oxygen stress by a controlled experiment in the laboratory. By simulating the field conditions (found by the data analyses) of temperature, pH and oxygen in this laboratory experiment I hope to find answers regarding mortality causes for the two types of oyster culture and the differences and similarities between them.

**Goal: Studying differences in on bottom and on table culture of Pacific cupped oysters by analysing field data collected on mortality, temperature, pH, dissolved oxygen and salinity of both cultures in 1998 and 1999. Based on the trends that I find in abiotic factors in the field, I will replicate certain abiotic conditions in a controlled laboratory experiment to study their possible effects on mortality.**

1a. What are the trends in mortality at 4 culture sites in 1998 and 1999?
1b. What are the differences and/or similarities in mortality rate for the two types of culture (on the ground and on tables) in 1998 and 1999?
1c. What are the differences in mortality rate for two types of on bottom culture; 1) in bag on table at sediment level and 2) plain on the sediment in 1999?

2. What are the trends and the differences in temperature for the two types of culture (on the ground and on tables) in 1998 and 1999?

3a. What are the trends in pH, dissolved $O_2$ and salinity recording at 10 cm height in 1998?
3b. What are the trends and the differences in pH, dissolved $O_2$ and salinity recording at 10 cm high and at 50 cm high from the sediment in 1999?

4a. What are the effects of a certain temperature, pH and oxygen 'stress' on oyster mortality in a laboratory experiment?
4b. Is mortality related with lipid, carbohydrate or glycogen content of the oyster?

# Material & methods

## *Field experiments*

In '98 and '99 at four sites named: 1) Le Sec, 2) Le Lézard, 3) Le Nanti et 4) Le Profond in the oyster banks of Ronce-Perquis within the intertidal area, temperature and Pacific cupped oyster mortality are monitored (fig. 2). The sites differ in sediment composition, height and time of immersion (table 1). The nursing grounds or cultivation place of the oyster to grow it to a certain size, differs per year as does the age of the oysters that are used for the field experiments (see table 2).
At each site three batches of oysters where placed in bags on culture tables. Furthermore, at each site oysters are placed plain on the sediment in parks (see protocol 1a). About once a month the dead oysters are counted and removed from the bags and the parks (but not removed from the parks in 1998, see protocol 1b). Mortality data from the first sampling date are not taken into account because of the high mortality in the first weeks caused by the handling and the change of environment.

| site | height (m) | emersion % |
|---|---|---|
| 1 | 3.4 | 50 |
| 2 | 2.8 | 38 |
| 3 | 2.5 | 33 |
| 4 | 1.5 | 20 |

***Table 1. Height in metres and emersion % per day of the four different sites measured at sediment level.***

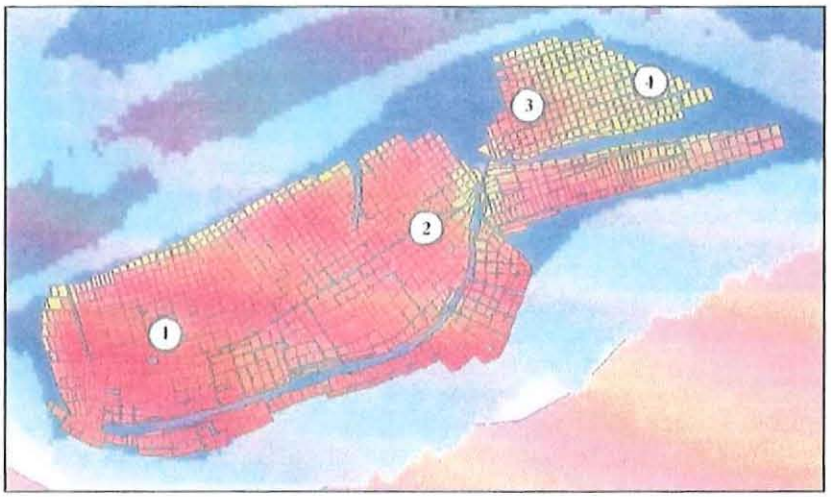



***Fig. 2 (by IFREMER LCPC). The four sites were the field experiments take place at the oyster culture banks of Ronce-Perquis.***

***Photo 1 (by V. Lebourgeois). Some large size ground parks in the middle with some small ground parks on the right and some oyster tables on the left.***

At each site in 1998 and 1999 and for both types of culture (on bottom and on table) temperature is registered by a temperature probe (see protocol 2a). It is placed in one of the bags on the tables and next to the oysters on the ground in one park.
In 1998 a multiparameter probe (Solomat) is placed at about 10 cm height from the sediment to measure certain abiotic parameters like temperature (air and seawater), salinity, pH and dissolved $O_2$ at site 3 (Le Nanti) (see protocol 2b).
In 1999 two multiparameter probes are placed to measure also temperature, salinity, pH and dissolved $O_2$ at site 3 (Le Nanti). One is measuring at 10 cm above the sediment and the other is measuring at the level of the 50-cm high culture tables.
In 1999 also at site two (Le Lézard) and three (Le Nanti) mortality rate is measured for two different types of on bottom culture. Three batches of 200, 3 year old oysters were each placed in bags on 0 cm high culture tables (at bottom level) and three batches of oysters were placed plain on the sediment.

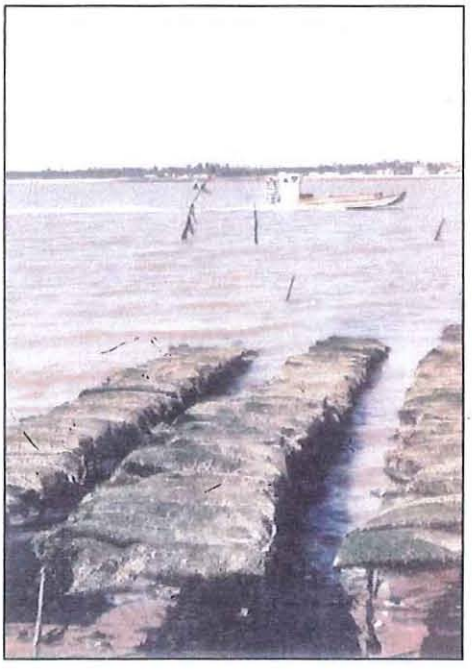


***Photo 2 and 3 (by A. Bakker). On table culture of oyster and the bags containing the oysters.***

| Years | 1998 | 1999 |
|---|---|---|
| **Sites** | 1,2,3,4 | 1,2,3,4 |
| **Start date (placing the oysters on the site)** | 23-4-98 | 1-4-99 |
| **End date** | 7-9-98 | 30-8-99 |
| **Oyster age** | 2 years | 3 years |
| **Origin of oysters** | Bonne Anse | Seudre estuary |
| **Nursing grounds** | Charet | Ronce |
| **Type of culture** | on bottom and on table | on bottom and on table |
| **Height of table** | 50 cm | 50 cm |
| **Number of bags per site times number of oysters per bag** | 3 x 200 | 3 x 200 |
| **Total number of oysters on table per site** | 600 | 600 |
| **Size of on bottom culture park** | 10 $m^2$ or 50 $m^2$ | 0.5 $m^2$ |
| **Number of parks per site times density** | 1 x 400/$m^2$ | 3 x 400/$m^2$ |
| **Number of sampling per park per date** | 6 or 10 times from 0,5 $m^2$ | 1 time |
| **Temperature measurements and height of the probe** | at all sites, at 0 cm and at 50 cm height from the sediment | at all sites, at 0 cm and at 50 cm height from the sediment |
| **Type of probe** | Tinytalk Pt100 Datalogger | StowAway Optic |
| **Extra experiment** | * | yes |
| **Type of culture** | * | plain on bottom and in bag on bottom |
| **Site** | * | 2 and 3 |
| **Number of parks per site times number of oysters per park** | * | 3 x 200 |
| **Number of bags per site times number of oysters per bag** | * | 3 x 200 |
| **Number of sampling per park per date** | * | 1 time |
| **Extra abiotic data recording** | water quality monitor (Solomat) recording | water quality monitor (Solomat) recording |
| **Duration** | 30-4-98 till 8-9-98 (132 days) | 6-4-99 till 17-6-99 (72 days) |
| **Site** | 3 | 3 |
| **Height of water quality monitor (Solomat) from sediment** | 10 cm | 10 cm and 50 cm |

***Table 2. Description of the different experiments and the methods used in 1998 and 1999. * means does not apply for this.***

## *Laboratory experiment*

By simulating in a laboratory experiment some differences of on bottom and on table culture in the field, we can test hypotheses of the effects of certain parameters found in the field that could be the cause of the difference in mortality rate between these culture types. These can be parameters such as high temperatures during a certain time (air exposure), low oxygen when the seawater rises during the night and high pH of the surrounding seawater. What I want to find is the influence of these parameters on mortality separated from the other complicated interactions with the sediment.
In this laboratory experiment I therefore test a temperature 'stress' influence on mortality in mature oysters separate and in combination with pH and $O_2$ 'stress'.

The experiment is applied on a batch of oysters during 50 days and is divided in two parts; the stress experiment and the starvation experiment.

## History of oyster batch

A batch of Pacific cupped oysters, a crossbreeding of 36 females with 10 males from GrainOcean, that have a homogenous history and are highly mature, is bought. The spawning of the crossbreeding took place on 12-2-1999. Fixation of the larvae took place on 12-3-1999. They were taken to the nursery on 6-4-1999. They were left on ropes in the bay shore at 3-5-1999. At 29-6-1999 they were placed in an oyster pond (shallow earth basins near the sea were oysters are left to fatten) till they arrived in the LCPC on the 26-4-2000.

Oysters are first placed in a tank with underground seawater (see protocol 3) of 17° C, pH of 8, salinity of 32‰ and saturated with oxygen, for a period of three days. Unfortunately, during the following four days the oysters could not stay in the underground seawater tank and are therefore placed in air in a cold room at 11° C. Following this, the oysters are left at 22° C for 30 min to check mortality and then they are put back in the underground seawater of 17° C at the 2nd of May. No mortality is recorded and neither the next two days. On the 4th of May the oysters are taken out of the underground seawater tank and left in the air at 22° C for 4 hours during preparation of material. After this they are placed back in the underground seawater tank. The next day every oyster is marked with a number on a plastic label glued onto the oyster with two-component glue to be individually recognised. During this numbering, the oysters are all 2.5 hours out of the seawater and at room temperature (22° C). After the numbering, they are placed in raceways in which the underground seawater slowly rises to let the oysters acclimatise. Nutrition (see protocol 3) is administered from this moment during three and a half days to let the oysters recuperate from the stress but not renewal their reserves before the start of the experiment.

## Biochemistry

The individual total weight of all oysters is measured three days before the start of the experiment to describe the batch of oysters that I used for the experiment.
To describe the batch of oysters that is used in the experiment, we take measurements of 30 oysters randomly chosen from the total batch at $t_0$ (the day of the start of the experiment). Total weight, shell weight, wet meat weight and dry meat weight are measured and biochemical analyses (carbohydrates, glycogen and lipid content) are done for 30 oysters of the total batch. Sex and maturity stage is noted for 20 oysters of these 30 oysters. We measure also carbohydrates, glycogen and lipids of oysters that die during the stress experiment and compare them with each other to see if we can find a trend in the energy resources. Then we compare them with the individual difference in the oysters at $t_0$. For more details see protocols 4, 5 and 6.

## General conditions during the stress experiment

From day one the oysters are placed in seawater that is pumped from 100 metres deep (underground seawater) and which is filtered (see protocol 3). Its temperature is controlled and constant (17-18° C) as well as its pH (8.2). It is saturated in oxygen content (100% or 7.4 mg) and has a salinity of 32 ‰. There is a constant addition of a food mix in the raceways, which are each equipped with a flow-through system (see protocol 3). The waterflow is 200 ml seawater per 6-7 seconds since the 5th of May. The nutrition (see protocol 3) is about 55 ml nutrition per minute for the three and a half days, starting the 5th till the morning of the 9th of May before the experiment and about 200 ml nutrition per minute during the stress experiment.
The temperature of the seawater in the raceways is always between 17.0° C and 18.5° C and in the plastic tanks during the stress experiment between 17° C at the beginning rising till sometimes 20° C at the end. The pH of the underground seawater is between 8.0 and 8.3.
The ten oysters of one repeat are placed in plastic racks. The three repeats of each different stress-group are divided over the three raceways.

## The stress experiment (table 3)

One batch of 3 x 10 oysters will be subjected every day during the week to temperature 'stress'. One batch of 3 x 10 oysters will be subjected every day during the week to temperature 'stress' and pH 'stress'. One batch of 3 x 10 oysters will be subjected every day to temperature 'stress' and oxygen 'stress'. The rest of the time the oysters will be in the raceways with optimal conditions and nutrition. 3 x 10 oysters will be the control group and will not be subjected to any of the above mentioned 'stress'. When an oyster is dying, the date will be noted with its number. The oyster number is later also noted on the minigrip pocket for freeze drying to be able to find back the date of dying when the biochemical analyses have to be done. The dying oysters are removed to be frozen and then freeze dried. For more details see protocol 3.

## Temperature 'stress'

All oysters are placed out of the seawater including the control group during the temperature 'stress' period. This is because when temperature stress is applied to the assigned 9 x 10 oysters, they will be without food and so the control group has to be without food for the same period of time to avoid differences in biometry caused by feeding time per day. Besides, it resembles more the field situation where oysters are always several hours a day out of the seawater. The control group is placed at a room temperature that is always between 18° C and 24° C. The oysters that are put under temperature 'stress' are placed in a dry oven. To make sure all oysters are tightly closed before they are put in the oven, they are rinsed a few seconds with fresh water. The duration of temperature 'stress' will be 3 hours and the temperature in the dry oven will be between 34.6-36.8° C except for the first 45 minutes when the temperature of the oven rises from 32° C to the desired temperature. This is because at the beginning, after opening the doors to place the oysters inside, the oven needs a bit of time to warm up. The temperature in the field can reach 35° C as well.

## pH 'stress'

By adding a basic solution like NaOH (sodium hydroxide) to the seawater of a plastic tank, the pH is elevated. The oysters are left in the tank and food is manually applied. The pH is elevated to a pH of 9.5-10 for a period of 3 hours in the tank without flow-through system for the pH 'stress' group of 3 x 10 oysters. In the field the pH near the sediment is often round 9.3 and stays constant for a much longer period than 3 hours. It will be impossible to induce the pH 'stress' for such a long period in this experiment therefore I use a higher pH during a shorter period.

The reaction that takes place when sodium hydroxide is added to the seawater is;

$$NaOH_{(s)} \rightarrow Na^{+}_{(l)} + OH^{-}_{(l)}$$

## Oxygen 'stress'

By adding $Na_2SO_3$ (sodium sulphite) to the seawater of the plastic tank, the oxygen content in the seawater is lowered. The oysters are left in the tank and food is manually applied. The oxygen concentration is lowered to 4-5 mg per litre, which is also found in the field near the sediment. The oxygen 'stress' is applied for about an hour, as is the case in the field, in the tank without flow-through system for the oxygen 'stress' group of 3 x 10 oysters.

The reaction that takes place when sodium sulphite is added to the seawater is;

$$2\ Na_2SO_{3(s)} \rightarrow 4\ Na^{+}_{(l)} + 2\ SO^{2-}_{3(l)}$$
$$2\ SO^{2-}_{3(l)} + O_{2(g)} \rightarrow 2\ SO^{-}_{4(l)}$$

## Combinations

Stress is induced at different times for the cases of combining different stress factors on a batch. Temperature 'stress' is always induced first in the morning and pH or oxygen 'stress' in the afternoon. This will resemble the situation in the field where pH 'stress' and oxygen 'stress' occur in the seawater

and not simultaneously with temperature 'stress'. During the pH and oxygen 'stress' the other oysters are also placed in a tank without flow-through system to make sure that there is no difference in food supply. In the tanks, oxygen is monitored and added if needed.

## Food and cleaning race-ways

The low food supply during the three days before the experiment to let the oysters acclimatise is constant and consists of three phytoplankton species. During the experiment the normal food supply is also constant and consists of the same three phytoplankton species before and during the entire stress experiment (see protocol 3). The flow of the nutrients is regulated by a tab. During the tests in the tanks 200 ml of nutrition is added per 10 litres of seawater per hour from the beginning of the experiment.
The race-ways with oysters are cleaned at least every two days. They are rinsed with fresh water when the oysters are in the plastic tanks for the experiments.

## The starvation experiment

In the morning of the 17th day, which is the end of the stress experiment, the oysters are taken out of the race-ways and rinsed with fresh water. Then they are left for half an hour at room temperature to dry. Then they are put in the oven at 25° C with two glass beakers of fresh water to have saturated air humidity. The oysters are left for a total of 29.5 hours in the dry oven. However, in the morning the temperature in the dry oven is rising up to 28.4° C. The heater of the dry oven is turned off and the temperature lowers in a few hours till 22° C. Because mortality rate seems to go to fast, the raceways with underground seawater from the stress experiment are prepared again, this time without any nutrition. I place all the oysters in the seawater again. Every morning, mortality will be checked and noted until the morning of the 28$^{th}$ day when the experiment ends and the remaining oysters are placed in the freezer.

| **Day** | **Start of experiment** | **Stress applied in the morning** | **Period between morning and afternoon** | **Stress applied in the afternoon** | **During the night** |
|---|---|---|---|---|---|
| 1-4 | all oysters are rinsed, all oysters are left in air during 30 min. | temperature stress during 3 hours | oysters are left in air during 30 min. | pH stress during 3 hours/oxygen stress during 1 hour | * |
| 5 & 6 | * | * | * | * | * |
| 7-11 | all oysters are rinsed, all oysters are left in air during 30 min. | temperature stress during 3 hours | oysters are left in air during 30 min. | pH stress during 3 hours/oxygen stress during 1 hour | * |
| 12 & 13 | * | * | * | * | * |
| 14-16 | all oysters are rinsed, all oysters are left in air during 30 min. | temperature stress during 3 hours | oysters are left in air during 30 min. | pH stress during 3 hours/oxygen stress during 1 hour | * |
| 17 | all oysters are rinsed, all oysters are left in air during 30 min. | all oysters in the dry oven at 25°C | all oysters in the dry oven at 25°C | all oysters in the dry oven at 25°C | all oysters in the dry oven at 25°C |
| 18 | all oysters in the dry oven at 25°C | all oysters in the dry oven at 25°C | all oysters in the dry oven at 25°C | all oysters in the seawater without nutrition | all oysters in the seawater without nutrition |
| 19-28 | all oysters in the seawater without nutrition | all oysters in the seawater without nutrition | all oysters in the seawater without nutrition | all oysters in the seawater without nutrition | all oysters in the seawater without nutrition |

***Table 3. Summary description of the actions per day during the stress experiment and the starvation experiment.* means the oysters are in the seawater with nutrition.***

### *Statistics*

For the data collected on temperature and seawater quality (oxygen, pH and salinity) we use descriptive statistics (Zolman, 1993) with Microsoft Excel 97. For the data on mortality in field and for the data of the laboratory experiment we use one-way analysis of variance (ANOVA) and the Kruskal-Wallis test of rank in StatGraphics Plus 4.0.

# Results

## *Mortality rate in the field*

### Mortality rate of on table and on bottom culture

In 1998 mortality of oysters is checked for six periods for the two culture types, on bottom and on table culture. In 1999 mortality of oysters is checked for five periods for the two culture types, on bottom and on table culture. The first period of both years is not taken into account because of the influence of handling the oysters and the time of adaptation of the oysters to their new environment. The other periods are numbered 1 till 5 for 1998 and 1 till 4 for 1999 (table 4).

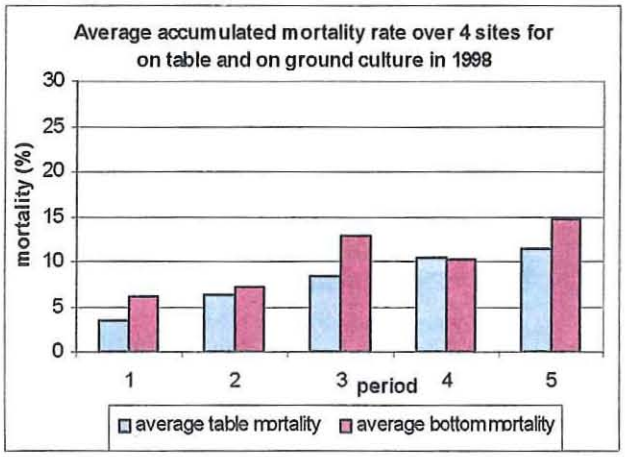
| period | year 1998 | year 1999 |
|---|---|---|
| *1* | 25-5 till 10-6 | 19-4 till 17-5 |
| *2* | 10-6 till 26-6 | 17-5 till 14-6 |
| *3* | 26-6 till 10-7 | 14-6 till 12-7 |
| *4* | 10-7 till 23-7 | 12-7 till 30-8 |
| *5* | 23-7 till 7-9 | |

***Table 4. The periods in which mortality at all sites is checked in 1998 and 1999.***

The mortality rate differs per period and per site and also in the different years (Annex 1 and 2). The highest mortality rate for 1998 occurs at site 4 in period 3 for on bottom culture and is 10.5 % (Annex 2a). However, movement of the seawater displaced the oysters and a storm in July made some dead oysters (shells) disappear from the on bottom culture area. I therefore find a negative mortality rate (accumulated mortality rate period x minus the accumulated mortality rate in period x-1) in the period 2 and 4 at certain sites and a low mortality rate in period 5 (Annex 1b). The highest mortality rate in 1999 occurred at site 1 in period 1 for on bottom culture and is 12.2 % (Annex 2b). However, I do not have data of on bottom mortality rate at site 4 for 1999 because more than half of the oysters were removed by some outsiders.

The accumulated mortality rate for on table culture ranges from 9.0-13.0 % in 1998 and from 12.0-23.8 % in 1999 (Annex 4a and 4b). The accumulated mortality rate for on bottom culture ranges from 7.2-20.9 % in 1998 and from 22.8-29.4 % in 1999 (Annex 4a and 4b).
The accumulated mortality rate at the end of the 5 periods in 1998 is highest at site 1 for on bottom culture and is 20.9 % (Annex 3a,b and 4a). The accumulated mortality rate at the end of the 4 periods in 1999 is highest at site 3 for on bottom culture and is 29.4 % (Annex 3c,d and 4b). However, I do not have data of on bottom mortality rate at site 4 for 1999.

When comparing in 1998 the accumulated mortality rate for the two different culture types (on bottom and on table culture) averaged over the four sites, I find that the accumulated mortality rate in period 5 is 14.7 % for on bottom culture and 11.5 % for on table culture, (fig. 3a).
When comparing in 1999 the accumulated mortality rate for the two different culture types (on bottom and on table culture) averaged over the four sites, I find that the accumulated mortality rate in period 4 is 25.3 % for on bottom culture and 17.8 % for on table culture, (fig. 3b).



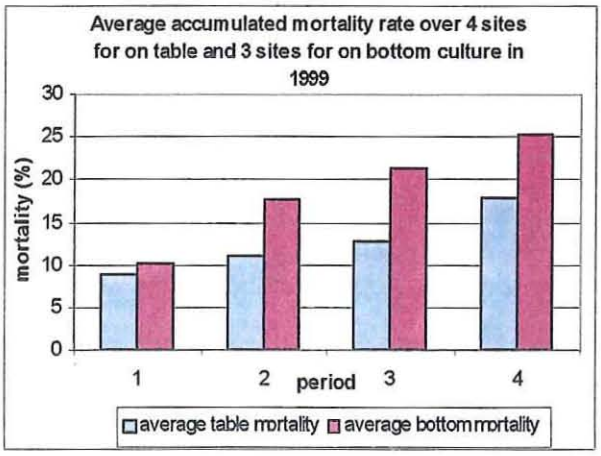




***Fig. 3a and 3b. Accumulated mortality rate for different periods averaged over the sites for the years of 1998 and 1999. In 1998 the average for on bottom culture and on table culture is taken over 4 sites. In 1999 the average is taken over 4 sites for on table culture and over 3 sites for on bottom culture.***

I can not compare the mortality rate of the different culture types in 1998 for significant difference for the five periods separately. This is because I can only calculate the average mortality rate for the ground culture with the data on accumulated mortality rate that I have (dead oysters are not removed after counting).
When comparing the mortality rate of the different culture types in 1999 (averaged over sites 1, 2 and 3), there is a significant difference between them for the periods 2 and 3, but not for the periods 1 and 4 (table 5).
There is a significant difference in 1999 in accumulated mortality rate (averaged over sites 1, 2 and 3) for the different culture types over the four periods (table 6). However, there is no significant difference in 1998 in accumulated mortality rate (averaged over four sites) for the different culture types over the four periods (table 6).
Therefore, the sites are analysed separately for the difference in accumulated mortality rate in 1998 between the different culture types for the four periods (table 7). Only site one shows a significant difference in the accumulated mortality rate for the two culture types.

| **comparing of on bottom and on table culture** | **mortality rate (%) 1999** |
|---|---|
| *period 1* | p = 0.4013 (n = 18) |
| *period 2* | p = 0.0003 (n = 18) |
| *period 3* | p = 0.0091 (n = 18) |
| *period 4* | p = 0.3311 (n = 18) |

***Table 5. Significance of difference (using a Kruskal-Wallis test of rank) in mortality rate between on bottom and on table culture over four sites. The mortality rate is compared per period. n is the number of observations.***

| **comparing on bottom with on table culture** | **acc. mortality rate (%) over all sites** | **n** |
|---|---|---|
| *1998* | p = 0.194 | 40 |
| *1999* | p = 0.011 | 24 |

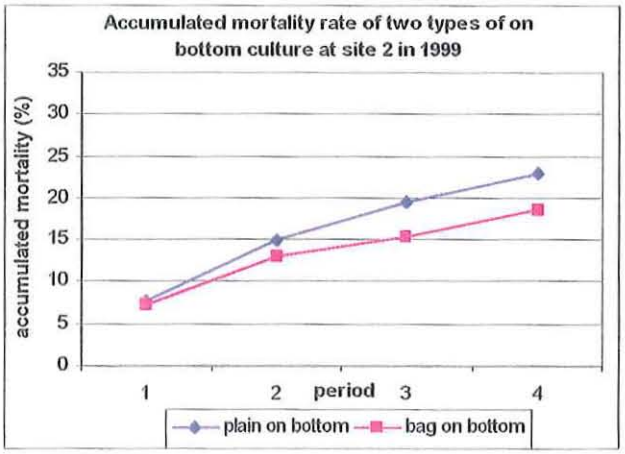
***Table 6. Significance of difference (using a Kruskal-Wallis test of rank) in accumulated mortality rate, in the last period for each year, between on bottom and on table culture. n is the number of observations.***

| comparing on bottom with on table culture | acc. mortality rate (%) in 1998 |
|---|---|
| *site 1* | p = 0.016 (n = 10) |
| *site 2* | p = 0.754 (n = 10) |
| *site 3* | p = 0.347 (n = 10) |
| *site 4* | p = 0.465 (n = 10) |

***Table 7. Significance of difference (using a Kruskal-Wallis test of rank) in accumulated mortality rate in period 5 in 1998 between on bottom and on table culture for the four different sites. n is the number of observations.***

## Mortality rate of two types of on bottom culture

In 1999 an extra experiment is conducted to measure mortality rate of two types of on bottom culture; 1) in bag on table at sediment level and 2) plain on the sediment for 4 periods (see table 4).
The accumulated mortality rate in period 4 is 22.9 % for plain on bottom culture and 18.7 % for in bag on bottom culture, when comparing the accumulated mortality rate for the two types of ground culture at site 2 (fig. 4a and Annex 5). The accumulated mortality rate in period 4 is 30.9 % for plain on bottom culture and 25.3 % for in bag on bottom culture, when comparing the accumulated mortality rate for the two types of ground culture at site 3 (fig. 4a and Annex 5).
Although the accumulated mortality rate is higher in all periods for plain on bottom culture compared to bag on bottom culture at both sites (fig. 4), there is no significant difference in accumulated mortality rate, over the two sites, between bag and plain culture on bottom (table 8). Comparing the mortality rate between bag and plain culture on bottom, over the two sites, separately for the different periods does not give a significant difference either in any of the periods (table 8).



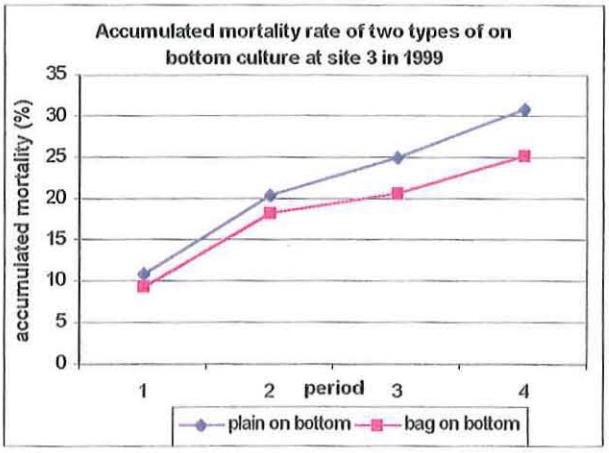




***Fig. 4a and 4b. Accumulated mortality rate of plain on bottom and in bag on bottom culture for two sites in 1999.***

| comparing plain on bottom with in bag on bottom culture | acc. mortality rate (%) over the two sites | mortality rate (%) over the two sites in 1999 |
|---|---|---|
| *period 1* | p = 0.401 (n = 16) | p = 0.631 (n = 12) |
| *period 2* | | p = 0.423 (n = 12) |
| *period 3* | | p = 0.109 (n = 12) |
| *period 4* | | p = 0.423 (n = 12) |

***Table 8. Significance of difference in mortality rate and accumulated mortality rate (using a Kruskal-Wallis test of rank) of plain on bottom and in bag on bottom culture over two sites in 1999. The mortality rate is compared per period. n is the number of observations.***

### *Temperature trends at the sites*

The temperature range in the spring and summer is different in time for the different sites in 1998 (Annex 7). The maximum temperature that was recorded was 42.4° C at site 2 in May 1998 for on table culture (Annex 7b and table 9a). When comparing the two different culture types I find that for site 1, 2 and 4 the maximum recorded temperature is higher at on table than at on bottom culture (table 9a).

| temperature 1998 | max ground | max table | min ground | min table | mode ground | mode table |
|---|---|---|---|---|---|---|
| *site 1* | 33.9 | 35.8 | 7.2 | 7.0 | 19.4 | 20.1 |
| *site 2* | 34.2 | 42.4 | 8.1 | 7.6 | 20.1 | 20.6 |
| *site 3* | 26.1* | 37.8 | 8.0* | 7.7 | 13.5 | 20.6 |
| *site 4* | 27.4 | 38.5 | 9.5 | 8.8 | 20.6 | 20.6 |

***Table 9a. Temperature data recorded on the four sites in 1998 from the months April till September. Minimum and maximum temperatures recorded at on bottom and on table culture. Mode is the most common temperature that has been recorded. For on bottom culture at site 3 temperature was only recorded in April and May and is therefor noted with *.***

The amplitude of the temperature in the spring and summer is difficult to compare in time for the different sites in 1999 due to gaps in the data caused by problems with recording (Annex 8). The maximum temperature that is recorded was 38.6° C at site 2 in May 1999 for on bottom culture (Annex 8b and table 9b).
There are no temperature data for on table culture at site 4 due to a failure of the recording material.

| temperature 1999 | max ground | max table | min ground | min table | mode ground | mode table |
|---|---|---|---|---|---|---|
| *site 1* | 30.0* | 31.9* | 5.0* | 4.7* | 15.8 | 13.1 |
| *site 2* | 38.6 | 37.3 | 5.0 | 5.7 | 20.8 | 20.7 |
| *site 3* | 30.9** | 31.2** | 5.4** | 6.1** | 13.3 | 18.0 |
| *site 4* | 34.9*** | x | 7.2*** | x | 18.6 | x |

***Table 9b. Temperature data recorded by ... on the four sites in 1999 from the months April till August. Minimum and maximum temperatures recorded at on bottom and on table culture. Mode is the most common temperature that has been recorded. For site 1 temperature was only recorded in April and May and is therefor noted with *. For site 3 temperature data are recorded with the water quality monitor (Solomat) for the months April till June and therefore noted with **. For site 4 temperature was recorded from April till July and is therefore noted with ***.***
***x temperature was not recorded.***

Due to the tide, the tables emerge before the on bottom culture and also stay longer in the air. Based on the water quality data of 1999 at the site 3, I found that this difference in emersion time between the two types of culture can reach up to 60 minutes (table culture in the air when on bottom culture is still in the water) when the tide goes down, plus 90 minutes (table culture in the air when the on bottom culture is already in the water) when the tide goes up. This gives a total difference of 2.5 hours. Sometimes the table culture is emerged during up to 220 minutes due to the tide but the on bottom culture does not emerge at all during this period called neap-tide.

I compared the times that temperature is ≥ 2.0° C higher on one culture type compared to the other culture type at that same moment (Annex 6). In 1998 at site 1, 2 and 3 more often a higher temperature is recorded at on table culture than on bottom culture (table 10a). Only at site 4 it is the opposite.

| 1998 | temp table ≥ 2.0 °C higher than temp ground | temp ground ≥ 2.0°C higher than temp table | total measures |
|---|---|---|---|
| *site 1* | 5.0 % | 3.5 % | 9755 |
| *site 2* | 4.9 % | 1.2 % | 8610 |
| *site 3* | 8.7 % | 1.2 % | 1353 |
| *site 4* | 1.4 % | 2.0 % | 12564 |

***Table 10a. Percentage of times that temperature data collected at table culture was 2.0° C or more higher than the temperature data collected at that moment at ground culture and number of times than temperature data collected at ground culture was 2.0° C or more higher than the temperature data collected at that moment at table culture.***
***Total measures means the number of times that temperature was measured for both cultures at the same moment.***

In 1999 however, at site 1 and 2 and 3 more often a higher temperature is recorded at on bottom culture than on table culture (table 10b). Temperature data of site 4 are missing for on table culture due to a failure of the recording material.

| 1999 | temp table ≥ 2.0 °C higher than temp ground | temp ground ≥ 2.0°C higher than temp table | total measures |
|---|---|---|---|
| *site 1* | 1.7 % | 6.9 % | 5364 |
| *site 2* | 2.0 % | 9.9 % | 12554 |
| *site 3* | 1.0 % | 4.3 % | 8378 |
| *site 4* | * | * | * |

***Table10b. Percentage of times that temperature data collected at table culture was 2.0° C or more higher than the temperature data collected at that moment at ground culture and number of times than temperature data collected at ground culture was 2.0° C or more higher than the temperature data collected at that moment at table culture. Total measures means the number of times that temperature was measured for both cultures at the same moment.***

## Water quality data 1998 and 1999

For the seawater quality (Solomat) data collected at site 3 in 1998 I find that in April the salinity of the water is rising (Annex 9). Overall, oxygen is declining in time after the end of June and reaches a value around 1 mg/l in September (Annex 9). The trend in pH does not change in time and the value stays around 9. In 1998 the pH range is 2.9, the range of oxygen is 11.7 mg and the range of salinity is 14.8 ‰ (table 11).

| site 3 1998 | mean | max | min | mode |
|---|---|---|---|---|
| *oxygen (mg/l) 10 cm* | 6.9 | 12.3 | 0.6 | 9.1 |
| *pH 10 cm* | 8.7 | 10.7 | 7.8 | 8.8 |
| *salinity (‰) 10 cm* | 32.8 | 39.8 | 25.0 | 32.9 |

***Table 11. Oxygen, pH and salinity recording by the water quality monitor (Solomat) at 10 cm height at site 3 in 1998 from the 30th of April till the 8th of September. Mode is the most common value that has been recorded.***

In 1999 data on seawater quality (temperature, salinity, pH, and dissolved oxygen) have been collected at sediment level (10 cm height from the sediment) and at 50 cm from the sediment (table culture height) from the 6th of April till the 17th of June at site 3.

### pH in 1999

Overall the pH of the seawater near the sediment is higher than the pH at 50 cm height from the sediment (Annex 10a). In April the average value of the pH at 10 cm and at 50 cm differs little. In May, the average pH at 50 cm is a bit lower than in April but the average pH at 10 cm is higher than in April and has larger amplitudes than before. At the end of May the average pH at 50 cm has gone up a bit again and the average pH at 10 cm has gone down a bit causing less difference between them (Annex 10a). There is no trend in pH difference between night periods and day periods. At 10 cm height the pH reached a value of 10.4 while at 50 cm height the maximum recorded pH is 8.3 (table 12). The mode for pH at 10 cm and 50 cm height do not differ much (table 12).

### Oxygen in 1999

The dissolved oxygen in the seawater is usually low during the first hour of seawater rising compared to the period when the multiparameter probes and thus the oyster cultures at site 3 are totally immersed. Dissolved oxygen reaches lower values at the 10 cm height than at 50 cm height from the sediment (Annex 10b). There are however a lot of gaps in the data. There is a trend of low oxygen during the night (period of darkness) compared to the day. The mean values are around 9 mg dissolved oxygen/litre in the seawater and they do not differ much between the two heights (table 12). However, the mode for oxygen is 8.5 mg/l at 10 cm and 9.6 mg/l at 50 cm and thus does differ (table 12).

### Salinity in 1999

I find not much difference in salinity trend for the two recording heights (Annex 10c). There is no difference in the means of salinity recorded at 10 cm height and at 50 cm height neither do the recorded maximum and minimum value of salinity differ much (table 12). The mode for the salinity is 31.4 at 10 cm and 30.7 at 50 cm (table 12).

| site 3 1999 | mean | max | min | mode |
|---|---|---|---|---|
| *oxygen (mg/l) 10 cm* | 9.1 | 12.1 | 5.2 | 8.5 |
| *oxygen (mg/l) 50 cm* | 8.9 | 10.7 | 5.9 | 9.6 |
| *pH 10 cm* | 8.6 | 10.4 | 7.9 | 8.2 |
| *pH 50 cm* | 8.0 | 8.3 | 7.6 | 8.0 |
| *salinity (‰) 10 cm* | 31.3 | 34.5 | 27.9 | 31.4 |
| *salinity (‰) 50 cm* | 31.3 | 34.2 | 28.0 | 30.7 |

***Table 12. Oxygen, pH and salinity recording by the multiparameter probe (Solomat) at 10 cm and 50 cm height at site 3 in 1999 from the 6th of April till the 17th of June. Mode is the most common value that has been recorded.***

### Laboratory experiment

The analysis of 30 oysters at the day of the start of the stress experiment represents the situation at $t_0$ (Annex 11).
We find that 14 of the 20 oysters checked for gender are females, 5 of the 20 are males and there is one hermaphrodite. This gives an estimation of 70% females, 20 % males and 10% hermaphrodites for the total batch. There are 16 out of 19 oysters in maturity state 3 and 3 out of 19 in state 2 (the oyster that was hermaphrodite is not taken into account) (see protocol 4). This gives an estimation of 80% of oysters that are in maturity state 3 and 11% that are in state 2 for the total batch. In table 13a biochemistry and biometry of the oysters at $t_0$ are described. Carbohydrates consists of glycogen and other carbohydrates.

| $t_0$ group | mean | max | min |
|---|---|---|---|
| *lipids (%)* | 16.45 | 22.99 | 10.71 |
| *carbohydrates (%)* | 12.17 | 18.79 | 5.38 |
| *glycogen (%)* | 10.24 | 17.29 | 3.74 |
| *total weight (g)* | 91.2 | 141.5 | 62.8 |
| *shell weight (g)* | 54.6 | 89.7 | 33.2 |
| *wet meat weight (g)* | 12.08 | 22.70 | 9.05 |
| *dry meat weight (g)* | 2.52 | 5.56 | 1.76 |

***Table 13a. Biometry and biochemical analysis results (measured in percentage per dry weight) of the oyster group of 30 individuals at $t_0$***

| stress group | mean | max | min |
|---|---|---|---|
| *lipids (%)* | 12.84 | 17.55 | 10.14 |
| *carbohydrates (%)* | 10.27 | 15.59 | 3.73 |
| *glycogen (%)* | 2.59 | 7.17 | 0.47 |
| *total weight at $t_0$ (g)* | 90.3 | 110.1 | 70.3 |
| *shell weight (g)* | 52.5 | 62.5 | 44.4 |
| *dry meat weight (g)* | 2.49 | 3.53 | 1.70 |

***Table 13b. Biometry and biochemical analysis results (measured in percentage per dry weight) of the oysters that died during the stress experiment.***

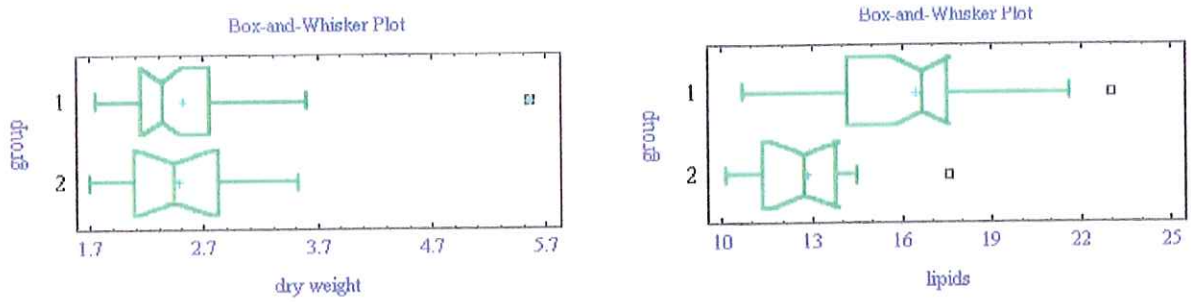



***Fig. 5a and 5b. Dry weight and lipid concentrations per dry weight, in percentages. Group 1 is the group of oysters analysed at $t_0$ and group 2 are the oysters that died during the stress experiment. Means confidence level intervals of accumulated mortality rate of the stress experiment. The data are divided into quartiles of equal frequency around the mean. The small squares show the out lying values.***

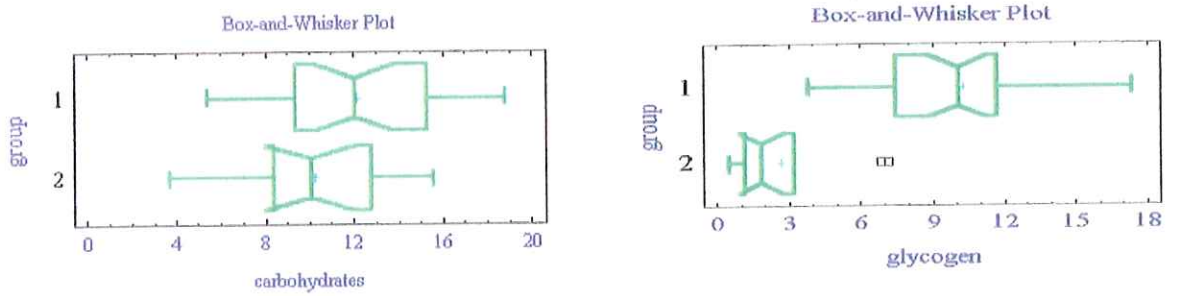



***Fig. 5c and 5d. Carbohydrates and glycogen concentrations per dry weight, in percentages. Group 1 is the group of oysters analysed at $t_0$ and group 2 are the oysters that died during the stress experiment. Means confidence level intervals of accumulated mortality rate of the stress experiment. The data are divided into quartiles of equal frequency around the mean. The small squares show the out lying values.***

## Stress experiment

During the stress experiment (day 1-16) a total of 12 oysters died. Oysters died during the days 1-11 but no dead are found in the days 12 till the morning of day 17. 8 oysters from the pH stress group died, 3 oysters from the oxygen group died, 1 oyster from the temperature group died and no oysters from the control group died (Annex 13).
There is a significant difference in accumulated mortality rate during the stress experiment between the control group and the pH group and between the temperature group and the pH group. However, no significance difference exists between the control group and the temperature group or oxygen group (table 14 and fig.6).
When comparing the means of biochemistry (measured in percentage per dry weight) of the oysters at $t_0$ and the oysters that died during the stress experiment I find that biochemistry has changed but not the dry weight nor the shell weight (table 13a and 13b and Annex 12). There is a significant difference in lipid and glycogen concentration but not in dry weight and carbohydrates between the oysters at $t_0$ and the ones that died during the stress experiment (table 15 and fig. 5).

| **acc. mortality rate** | **temperature** | **pH** | **oxygen** |
|---|---|---|---|
| *control* | p = 0.317 (n = 6) | p = 0.034 (n = 6) | p =0.121 (n = 6) |
| *temperature* | * | p = 0.043 (n = 6) | p = 0.346 (n = 6) |
| *pH* | * | * | p = 0.072 (n = 6) |
| *oxygen* | * | * | * |

***Table 14. Significance of difference in accumulated mortality rate (using a Kruskal-Wallis test of rank) between the four groups. n is the number of observations.***

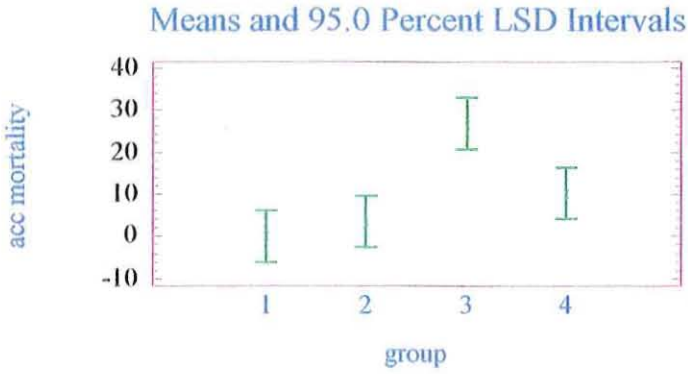



***Fig. 6. Accumulated (acc) mortality in percentage. Group 1 is the control group, 2 is the temperature group, 3 is the pH group and 4 is the oxygen group. Means and 95.0 % confidence level intervals of accumulated mortality rate of the stress experiment.***

| comparing group $t_0$ with group dead from stress | group $t_0$ = 30 individuals group stress = 12 individuals |
|---|---|
| *dry weight (g)* | p = 0.8893 (n = 42) |
| *lipids (%)* | p = 0.0003 (n = 42) |
| *carbohydrates (%)* | p = 0.1814 (n = 42) |
| *glycogen (%)* | p = 0.0000 (n = 42) |

***Table 15. Significance of difference in dry weight and biochemistry (using a Kruskal-Wallis test of rank) between the oysters that died during the stress experiment and the group at $t_0$. The mortality rate is compared per period. n is the number of observations.***

## Starvation experiment

In the starvation experiment the mortality rate of the oysters is irregular and not continuous. During some days there is no mortality (fig. 6). At the start of the experiment when the oysters were kept in the dry-oven for 29.5 hours, the pH group responded with a high mortality rate compared to the other groups (fig. 7). We use angular transformation to use the normal distribution, by taking the arc sine square root of the percentage of survival (Snedecor & Cochran, 1989). This is done to take into account the number of oysters at the start of the starvation experiment. When we compared the four groups for difference among them at day 20, we found no significance for among group difference (table 16). We then compared the difference between the pH group and the three other groups assembled together and found a significant difference. The same analysis is done at the end of the experiment and no significant difference is found between the groups neither when comparing only the pH group with the control group.
The $LT_{50}$ (time for 50 % mortality to occur) do differs among the groups and is shortest for the pH group then the oxygen group, the temperature group and is longest for the control group (table 17).

| comparing | F-ratio $t_{20}$ | p value $t_{20}$ | F-ratio $t_{50}$ | p value $t_{50}$ |
|---|---|---|---|---|
| *between groups* | 2.89 | 0.1023 (n = 12) | 0.24 | 0.8654 (n = 12) |
| *between pH group and the other groups* | 10.35 | 0.0092 (n = 12) | * | * |
| *between pH group and control group* | * | * | 0.50 | 0.5196 (n = 6) |

***Table 16. The significance of difference for comparing between groups at day 20 ($t_{20}$) or day 50 ($t_{50}$) and the start of the starvation experiment. * means not tested.***

| group | $LT_{50}$ (days) | number of individuals at the start |
|---|---|---|
| *control* | 50 | 30 |
| *temperature* | 45 | 29 |
| *pH* | 31 | 22 |
| *oxygen* | 40-41 | 27 |

***Table 17. The $LT_{50}$ for the different groups and the number of individuals at the start of the starvation experiment for each group.***

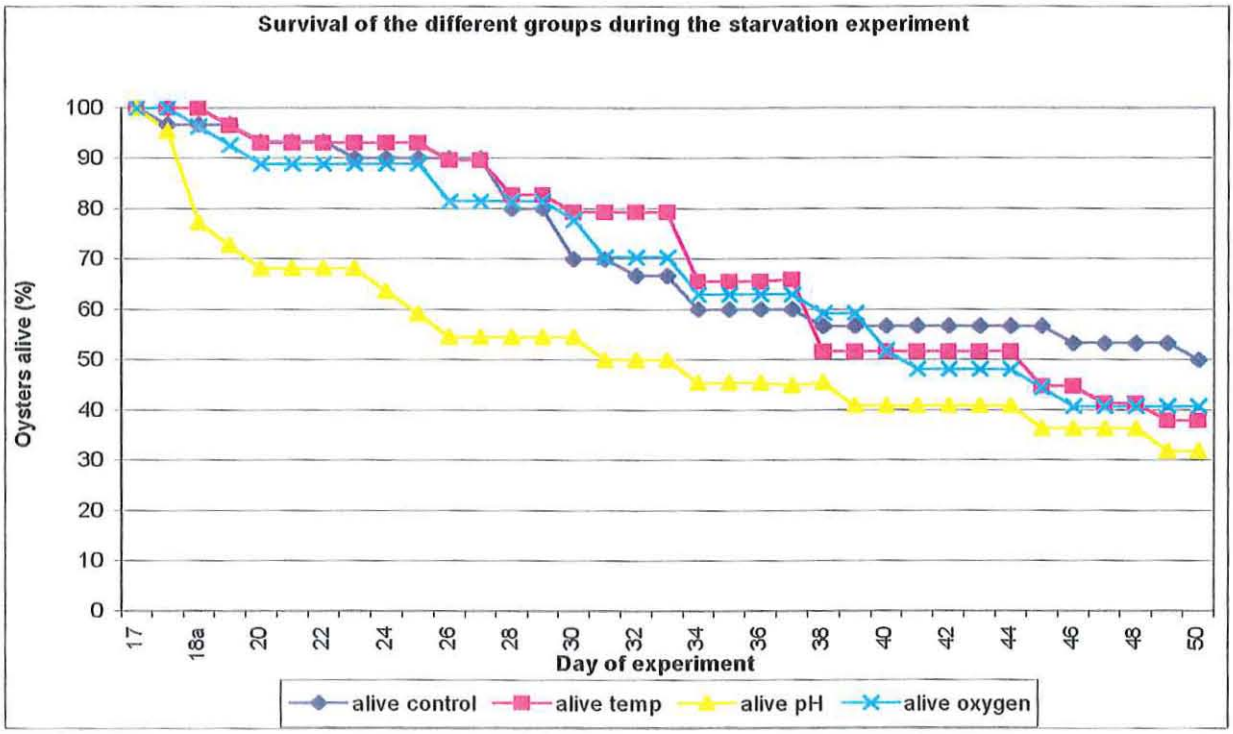



*Fig 7. Survival in percentage of the oysters from the different groups during the starvation experiment.*

# Discussion

## *Mortality trends in two type of cultures*

I find that accumulated mortality rate averaged over 4 sites is significantly different for the two culture types in 1999 but not in 1998. It is possible that due to the problems with the protocol used in 1998 the significance in difference is effected. The accumulated mortality rate in 1999 over the four sites is considerably high; 25.3 % or about one fourth of the total on bottom culture died in the period between half April and the end of August 1999. This is however lower than mortality rate observed at on bottom culture during mass mortality years like in 1988 and 1993 when at certain sites a higher mortality rate was reached even within a shorter period (Bodoy *et al.*, 1990 and Héral, 1993). The accumulated mortality rate in 1998 is lower than in 1999 but the periods when mortality rate is measured differ. 17.8 % of the total on table culture, averaged over four sites, died in 1998 in the period between half April and the end of August. The on table culture accumulated mortality rate over a period from end May till the beginning of October and averaged over four sites is 11.5 %.
Accumulated mortality rate, averaged over four sites, for on bottom culture in 1998 is less than two third than that in 1999 averaged over three sites. Mortality rate in small parks is different than in large ones due to loss of oysters or moving of oysters due to storms etc. in the large parks in 1998. The storm in July 1998 and the negative or low mortality rate in period 4 and 5 might explain some of the difference in average accumulated mortality rate for on bottom culture between 1998 and 1999.
According to Perdue *et al.* (1981) and Soletchnik *et al.* (1997) summer mortality is related to fast growth and maturation under high nutrition conditions. Le Moine *et al.* (2000) monitored nutrition and growth in 1998. They found low carbohydrate reserves in the oysters in the bay in the spring of 1998 and also maturation and spawning effort were therefore lower than in 1997 and 1999. The results of lower mortality rate in 1998 than in 1999 are conform with the theory that mortality is related to high gonad development.
In both years the highest accumulated mortality rate occurs for on bottom culture but it differs for the sites.
When I compare the mortality rate for the different periods I found that significance in difference between the different culture types occurs only in the periods half May till half June and half June till half July. The oysters are maturing during these periods and the peak of spawning occurs at the end of July (Goulletquer *et al.*, 1998).

There is no significant difference in mortality rate between culturing oysters plain on the sediment or in a bag (normally only used for on table culture) when tested at two sites in 1999. This supports that the significant difference in accumulated mortality rate between on bottom and on table culture is not caused by the use of a bag for the oysters at on table culture.

## *Temperature trends and water quality data 1998 and 1999*

I do not find a reason to suggest that temperature is in generally higher at on bottom culture than on table culture based on the different results of the analysed temperature data in 1998 and 1999.
Maximum temperatures of both years are reached at half of May. The temperature reaches values of 42.4° C in 1998 and 37.6° C in 1999 (when fewer data are recorded). These maximums are higher than the temperature of 35° C of the dry oven that I used in the laboratory experiment.
The pH of the seawater can rise due to the reduction of $H_2S$ in the sediment caused by certain bacteria (pers. comm. Kantin). There is a clear trend in pH difference between the two recording heights. Overall the pH near the sediment is higher than at 50 cm from the sediment. The maximum pH recorded in 1998 is 10.7 at 10 cm height and is 10.4 in 1999 also at 10 cm height from the sediment at site 3. pH difference between the two heights (cultures) is largest in the month May at site 3 in 1999. A significance difference in mortality rate in 1999 occurs in the same month. It occurs also in the month of June but then the pH difference between the two heights is smaller again.
Day-night differences in oxygen are caused by uptake of oxygen by diatoms on the surface of the sediment from the seawater. The minimum value reached in 1998 is in the month of September when dissolved oxygen is less than 1 mg/l. Due to the rising of the temperature less oxygen dissolves in the

seawater. In 1999 the value of dissolved oxygen reaches values around 5 mg/l in half of June. In the laboratory experiment I lowered the dissolved oxygen to a value of 4-5 mg for the oxygen group compared to the normal 7-7.5 mg of dissolved oxygen in the seawater in the tanks of the other groups. I do not find a trend in difference in salinity between the two different heights were the multiparameter probe was placed. Furthermore the mean value of salinity is the same for both heights. Based on this I do not think that salinity has an effect on the difference in mortality rate between on bottom and on table culture in the bay. When I compare the salinity values found in 1998 and 1999 with values tested by Bernard (1983) I find that the optimum value for somatic growth in *C. gigas* lies at 24 ‰ and the range is 16-31 ‰. The mean and maximum of the recorded values in the Bay of Marennes-Oléron at site 3 were sometimes higher and salinity might play some role in summer mortality.

### *Laboratory experiment*

During the experiment all oysters that died, died in the first eleven days. The six following days no mortality occurred. Bivalves like oysters can adapt their metabolic rates and processes to cope with periods of stress (Bernard, 1983 and Eertman & de Zwaan). This might also be the case in this experiment where the oysters are subjected to stress at always the same time and for the same duration and after the stress they are placed over night in seawater with optimal conditions.
There is a significant mortality rate difference when comparing the pH group (combination of temperature 'stress' in the morning and pH 'stress' in the afternoon) with the control group. However, no significant effect on mortality of the treatment of the other groups (temperature and oxygen) has been detected. I elevated the pH value in the tank till a value of 9.5-10 compared to the normal pH of 8.2 in other tanks. This simulates the difference in pH in the field in the month of May between the two heights were the two types of cultures are situated. I therefore think that pH plays an important part in the difference in mortality rate between the two culture types. In the field the pH reached values over 10 in May at sediment height in both 1998 and 1999. Therefore the effects on mortality rate in mature oysters can be even stronger than what I found in the stress experiment.

I did not find a significant effect of the temperature 'stress' in the dry oven at 35° C for three hours on mortality. Furthermore there is a significant difference in mortality rate between the temperature group and the pH group. This means that not the temperature 'stress' applied to the pH group is responsible for the mortality rate difference between this group and the control group. I also did not detect a significant difference in mortality rate between oxygen group (combination of temperature 'stress' and oxygen 'stress') and control in the stress experiment. So in this case, the lowering of the dissolved oxygen in the seawater to a value of 4-5 mg/l for one hour in combination with temperature 'stress' in the morning does not effect mortality. Lower values in oxygen, than what I created in the stress experiment, occur normally in July (data 98) and might give effect. It is also important to take into account that in the field the oysters are twice a day exposed to air due to the low tide instead of in our experiment one time a day (a total of 4 hours: just before, after and during the temperature 'stress'). Therefore the oysters in the field probably have a greater need of oxygen for energy taking processes like filtration during the period that they immersed.
I expected that during the starvation experiment the oysters that had a history of stress due to the applications during the stress experiment, would give mortality rates conform to the intensity of the stress experienced by the oyster. The $LT_{50}$ of the pH group (combination of temperature and pH 'stress') was shorter than for the other groups supporting the results of the stress experiment. I also found a that the stress in air had more effect on pH group compared to the other groups. However, I did not find a significant difference at the end of the experiment between the groups that had been subjected to different stress. There might be an effect of the initial number of oysters at the beginning of the starvation experiment.

### *Biochemistry*

The dry weight is not significantly different between the group of oysters that died during the stress experiment and the group at $t_0$. Deslous-Paoli & Héral (1988) show that after spawning or after a period of about month of poor trophic conditions, dry weight and carbohydrate can diminish

significantly. This means that in my experiment the oysters that died did not spawn neither did they experience starvation effects due to low nutrition.
I also compared biochemistry with one-way ANOVA with dry weight as a covariable. However this did not change significance and not significance that I found in the differences for biochemistry. I found that carbohydrates are not significantly different between the group of oysters that died during the stress experiment and the group at $t_0$. Glycogen concentration is significant lower in the group of oysters that died during the stress experiment than the group at $t_0$. This drop in glycogen content might be the effect of the use of glycogen during closure of the oyster as a reaction to withstand the stress. I noticed that for the pH group the oysters were more often closed during the pH 'stress' when I compared it with the percentage of closed oysters of the temperature and control group.
Lipid concentration is significant lower in the group of oysters that died during the stress experiment than the group at $t_0$. An explanation could be that the oysters reconvert lipids into glycogen due to the need of glycogen to withstand the periods of stress. The re-absorption of gonad tissue containing high concentrations of lipids and conversion into carbohydrates is also seen in oysters after spawning (pers. comm. Goulletquer).

### *Comparing with oysters in the field*

I used mature oysters that come from the oyster pond and thus differ in energy resources from oysters in the field when they reach the same maturity. It is therefore hard to predict what would exactly be the effects of this experiment on the mortality rate of these oysters. However, the glycogen content is twice as high in the oysters that I used for the laboratory experiment than for the oysters in the field at the banks of Ronce-Perquis (Deslous-Paoli & Héral, 1988). They found concentrations of carbohydrates (calculated per dry meat weight) between 0 and 4 % in oysters in the bay, of which about half is glycogen. The oysters used for my laboratory experiment had carbohydrates at $t_0$ that consisted in generally of more than 75% of glycogen. Because the oysters that we used have a higher carbohydrate and glycogen content than the oysters we find in the field at the same stage of maturity, it would be logical that oysters from the field would be more sensitive in the case of withstanding stress by closure of the shells during the stress applications.
That I did not find significant effects of the temperature 'stress' and the combination of temperature and oxygen 'stress' on mortality rate in the oysters that I used does not mean that they can not play a role in the mortality of the oysters from the field.

## Suggestions for further research

This research was a first approach to measure effects of combinations of temperature, pH and oxygen 'stress' on *C. gigas* mortality. More tests or tests of a longer duration with multiple stress factors like pH, temperature and oxygen with different intensity and duration should be conducted to find out more on the effects of abiotic stress on mortality of oysters. It might also be a good idea to do the tests with a homogenous batch of oysters from the field and using normal seawater and plankton from the bay to create more natural conditions. The importance is always to compare different tests and to attach not too much value to one experiment.

In my experiment I used only combinations of certain pH and oxygen conditions with temperature 'stress' in a row. However in the field pH and oxygen stress can occur simultaneously and therefore have an extra effect on the oysters and should thus be tested.
Other means to measure stress effects on oysters is to measure filtration, oxygen consumption and growth of oysters during tests with pH and oxygen stress. Measuring biochemistry for the oysters that are subjected to different stress could show the influence of the stress on energy resources in the oyster.

Due to the connection between the stress experiment and the starvation experiment in this research and the maximum number of test individuals that I could use for conducting the experiment, I could not sample biochemistry of the control group at the end of the stress experiment. It would be wise to take this into account for further experiments as well as using a larger number of test individuals at the beginning of laboratory experiment (stress with starvation experiment) to get more accurate biochemistry differences and $LT_{50}$ for the different groups. Besides placing oysters in seawater without any nutrition it is also possible to distinguish in 'history of stress' by placing them in a cold room at 11° C and monitoring mortality.

## Acknowledgements

I would like to thank Jean-Luc Seugnet and Roger Kantin for their company, interesting conversations and driving skills, making me arrive safely at work and at the end of the day safely home. I thank Nicole Faury and Philippe Geairon for helping me with the biochemical analyses. Further, I like to thank Philippe Goulletquer and Patrick Soletchnik who supervised my internship. Patrick has also helped me a lot with the statistics and listened often patiently to my never-ending questions.
The rest of the Mickeys have been there to help me (each within its own speciality) or answers all the questions that I had.
There have been a lot of persons who checked the oyster mortality in my raceways during the weekends and I like to thank them hereby as well. Fred and Stephan 'Macgyver' helped me with the installations for the tanks and raceways and Christophe prepared the nutrition for the oysters. Without them all the oysters would have been long dead before the experiment even got well started.
I would like to thank Aurore, Greg and the students at IFREMER for making my stay in France very pleasant during the weekends and evenings. They also have enlarged my personal dictionary of the France language although not always with very common or decent expressions.
My friends in Holland, my parents and Cédric have been always supporting me with e-mails, phone calls and visits.

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# Protocols

## *1a. Culture of oysters in the different field experiments*

The oysters that are used for the field experiments are first calibrated and homogeneously distributed over all batches of each 200 oysters. 200 Oysters are put into bags to be brought to the field. The bags are made of plastic with mazes of 14 mm, are 100 cm long, 50 cm wide in 1998 and 75 cm by 75 cm in 1999 (see photo 3). They are closed with rubbers and hooks on both sides. For the on table culture, the bags are placed flat and adjacent to each other on tables made of iron bars. The tables stand 50 cm high of the sediment, are about 50 cm wide and differ in length (photo 2). In periods of algae bloom the bags are turned and shaken to remove the thick algae layer on the bag.
The oysters for the on bottom culture are taken from the bag and spread out on a parcel of the sediment which is closed off by a low plastic fence (photo 1). Before seeding the oysters on the parcel, the sediment is checked for remaining oysters and shells of an old culture. These are removed. The size of the parcel is made equal to the size of the bags on the tables. However, in 1998 a big parcel is used of respectively 10 $m^2$ and 50 $m^2$ with a density of about 200 oysters per half a square meter.
When sampling mortality of on bottom culture in 1998, six measurements are taken per sampling date.
For the experiment in 1999 with oysters for different types of bottom culture, a table is placed at 0 cm height from the sediment. On this table the bags with oysters are placed.

## *1b. Measuring mortality rate*

For the oysters placed in bags: The bags are opened about once a month in the field and spread in a plastic basket. The number of dead oysters (empty or open shells) are counted and noted and the ones alive are put back in the bags and placed back on the table.
For the oysters placed plain on the bottom: In 1998 either 6 or 10 times, dead and alive oysters are counted on squares of 0.5 $m^2$ at a random place in the large bottom park. The shells/ dead oysters are not removed. In 1999 the dead and alive oysters are taken out and counted for the three small 0.5 $m^2$ bottom parks. The shells/dead oysters are removed and the alive oysters are put back in their park.

## 2a. Temperature measuring

In one of the oyster bags or between the oysters for the on bottom culture, a temperature probe is placed. In 1998 this temperature probe is a Tinytag Pt 100 Datalogger (ADRM, Bordeaux) which can measure from – 40° C up to 75° C and in 1999 it is a StowAway Optic (ADRM, Bordeaux) which can measure from –36° C up to 75° C. They are programmed with the reading kit of the probe and an analogue/digital RS 232 converter, using Logbook (Microsoft), to start at a certain time and to take the temperature every 15 minutes. After a period of maximum 82 days, it is replaced by an empty one. The data from the probe are extracted with the reading kit of the probe, into Logbook (Microsoft). The data during changing and just after putting it in place are removed.
Once every year the probes are tested (calibrated) by programming them to start at the same time with measuring every 5 minutes and then putting each of them simultaneous through the same temperature conditions. Testing them to varying conditions can be done by putting them a few hours in running water and after this lying them some centimetres apart on a concrete plateau (which has no shadow) during a night and a morning. In the end we place them another hour in running water. In this way the working of the probes during the differences in immersion and bare (dry) periods and large temperature leaps that the oyster go through, can be tested. The time of handling and replacing of the probes is noted and this will be taken into account when the comparing of the probes.
Another test is done by placing the probes in a dry oven at 49° C and 57° C, at room temperature, in the refrigerator and the freezer to see if their measurements differed more than 0.5° C from the real temperature. To test how they work with 100% humidity at high and low temperatures they could be placed in an erlemeyer with water that has been in the oven or fridge for some time. At the end of the test, the water in the erlemeyer can be measured by a normal thermometer.

## 2b. Measurements with the multiparameter probe (Solomat)

A water quality monitor, type WP 803 with the 803 PS multiparameter probe (Solomat, Neotronics), is placed in the field at 10 cm height at site 3 in 1998. Two water quality monitors are placed at site 3, one at 10 cm height from the sediment and one at 50 cm height from the sediment, in 1999.
The probe, which is placed in the water, is connected to the water quality monitor and a car battery. The battery and the water quality monitor are placed in a large PCV tube and closed off with a tightly fitting PVC plate with O-ring and four skews (to keep the seawater out). The whole construction is then attached to a plate of concrete, to keep the material from moving.
In 1998 temperature, pH, oxygen and salinity is measured every 15 minutes and in 1999 they are measured every 10 minutes.
In both years the multiparameter probe is cleaned from algae every week especially at the membrane and the galvametric probe were the temperature, pH, oxygen and salinity are registered. At least once a month it is taken to the laboratory to clean it thoroughly, to extract the data with analogue/digital converter RS 232 in CS6.bat and to reprogram it.
Data from the first recording time till the first emersion as well as data recorded during cleaning are not taken into account because of the differences caused by handling the equipment.

## 3. *Stress experiment*

1. Turn on the dry oven for 35° C at 9.00 h
2. Lift the oysters carefully out of the water else the leaking of the water out of the oysters will create differences between individuals.
3. Note the dead oysters (gaping oysters that do not close their cells on touch) take them out and place them in the freezer.
4. At 9.30 h, the 120 oysters in 12 plastic trays with each replication of each group representative in a different raceway (3 in total) have to be rinsed with fresh water before taken to the dry oven.
5. Put them with their tray in the dry oven at 10.00. The fresh air is installed at 6.
6. The control group 3 x 10 will be left on a table at room temperature in the place where the oven is.
7. Clean the pH tanks with soap and sponge.
8. Fill the 6 small and the 3 large tanks with seawater, the small ones with 10 litre and the big with 30 litre (about one litre for every oyster).
9. Prepare the oxygen (3) tanks by adding 2-3 ml $Na_2SO_3$ (1 M) to the water (it takes 45 min for the oxygen to lower to a steady value).
10. Fill a bucket with nutrition.
11. Take the oysters out of the dry oven at 13.00 (after 3 hours) and leave them at room temperature in the place where the oven is for half an hour to cool.
12. Prepare the pH (3) tanks by adding about 10 ml NaOH (4 M) to the water.
13. At 13.30 the pH and oxygen stress experiments start with the two batches of 3 x 10 oysters each.
14. The other 6 x 10 oysters are placed back in the two big tanks, 30 in each.
15. Place oysters gently with flat side up in the pH and oxygen tanks.
16. Add every hour 200 ml nutrition mix per 10 oysters.
17. Install the oxygen system for the pH tanks and the two big tanks.
18. Check oxygen and pH every half an hour. For elevating the pH add 1 ml NaOH to 200 ml of the water of the tank and mix it. Then pour it back in the tank and mix it with the rest of the water in the tank.
19. Cleaning the tanks in the mean time if necessary.
20. At 14.30 the 30 oxygen stress oysters and the tanks need to be rinsed with seawater and the tanks with the oysters will be refilled with seawater and nutrition.
21. Install the oxygen system for the oxygen tanks.
22. At 16.30 the oysters of the pH experiment will be rinsed with seawater.
23. All the oysters are now placed back in the raceways.
24. The tanks are washed with fresh water and the pH tanks are cleaned with soap to remove the precipitation.

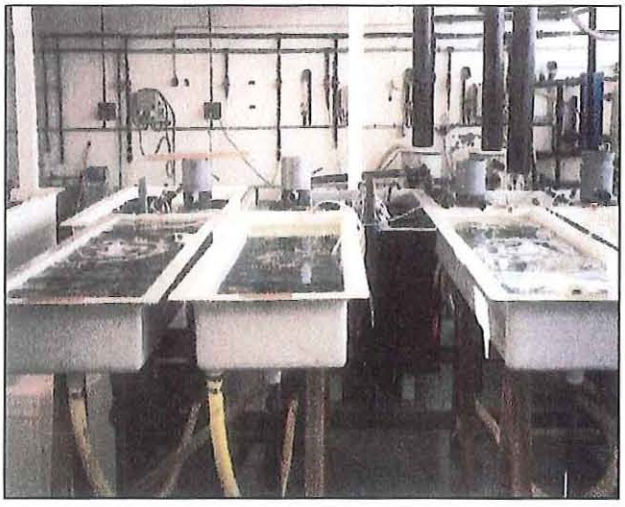
| **Group** | **Numbers** | **Colour label** |
|---|---|---|
| control | 1-30 | black |
| temperature | 31-1 | red |
| temperature & pH | 61-90 | yellow |
| temperature & oxygen | 91-120 | green |

| **Number of oysters (at the start)** | **Litres of water (during the entire experiment)** | **Nutrition (during the entire experiment)** |
|---|---|---|
| 10 | 10 | 200 ml/10 litres of water/hour |
| 30 | 30 | 200 ml/10 litres of water/hour |

**Material**

- Cold room of 11°C

- Tank for min 160 (150+10 extra) oysters, with a flow-through system with forage seawater of 17 ° C, a normal pH (around 8) and oxygen content (around 100%) to have starvation conditions till maximum three days before the start of the experiment
- Three raceways (each 120 litres) with a flow-through system with forage seawater of a regulated temperature around 17 ° C, a constant pH around 8, saturated oxygen content (accomplished by the air diffusers) and a constant food supply
- Dry oven with space for 9 x 10 oysters
- Three plastic 'stress' tanks (closed system) for 3 x 10 oysters for oxygen stress. Oxygen meter and Na2SO3 (1 M) solution to lower the oxygen
- Three plastic 'stress' tanks (closed system) for 3 x 10 oysters for pH stress. pH meter and NaOH (4 M) solution to rise the pH
- Two big plastic tanks (closed system), for the controlled group (30) plus the temperature 'stress' group (30) placed mixed in the two tanks: total 60 oysters
- Removable systems to add oxygen in any of the plastic tanks by pumping air with an air pressure machine through air diffusers into the water
- Glass beaker of 200 ml to add nutrition to the plastic tanks
- Two plastic beaker of 5 litres to fill the plastic tanks with seawater
- Nutrition: the nutrition used for the experiment exist of three phytoplankton species.
  A mix was made with the following concentrations
  Tetraselmis suecica: 800 000 cells/ml
  Isochrisis galbana: 6500 000 cells/ml
  Chaetoceros calcitrans: 4500 000 cells/ml
  with 300 l of this mix we make the nutrition, by adjusting the mix with forage seawater till a final volume of 2000 l.
- Underground seawater: pumped from 100 metres deep, is filtered and treated for iron precipitation. Its temperature is controlled and constant (17-18° C) as well as its pH (8.2). It is saturated in oxygen content (100% or 7.4 mg) and has a salinity of 32 ‰ (see table on the next page).



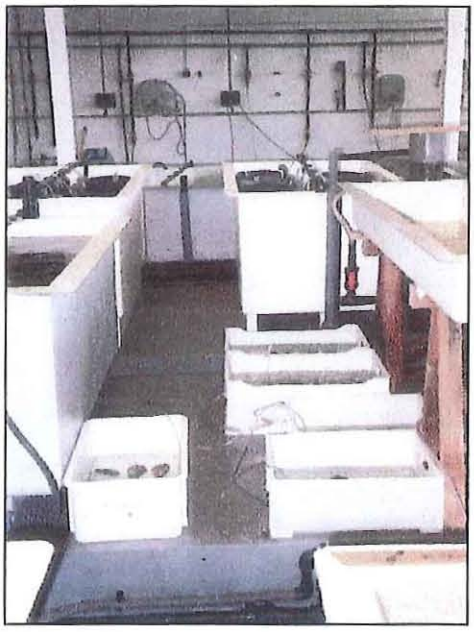


***Photo 1 and 2. The three raceways on photo 1 and the installation of the tanks for applying stress on photo 2.***

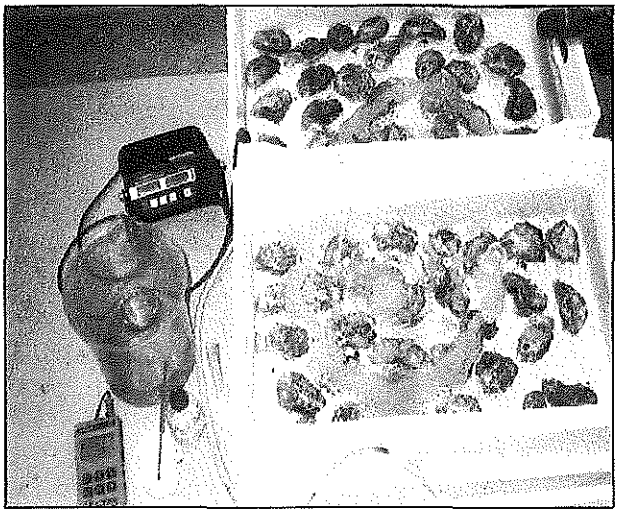


***Photo 3 and 4. Photo 3 shows the no stress tanks with the control group and the temperature group with the adding of extra oxygen in the water. The oxygen-meter and the pH-meter as well as the nutrition are also on the photo. Photo 4 shows the tanks with the oxygen group during the stress application.***

| **Substances in underground seawater at the end of filtration and manipulation** | |
|---|---|
| $CO_2$ (mmol/l) | 0.11 |
| $HCO_3$ (mmol/l) | 7.19 |
| $CO_3$ (mmol/l) | 0.36 |
| Fe (mg/l) | 0.06 |
| Mn (mg/l) | 0.41 |
| N-$NH_4$ (µmol/l) | 29.32 |
| N-$NO_3$ (µmol/l) | 286.9 |
| P-$PO_4$(µmol/l) | 8.6 |
| N-$NO_2$ (µmol/l) | 46.84 |
| SI-$SIO_4$ (µmol/l) | 294.45 |

## 4. *Biometry and sex determination of oysters*

1. Washing the oysters with fresh water and brushing them clean.
2. Letting them dry for 15 minutes.
3. Weighting them.
4. Opening the oysters, pouring off the oyster juice and placing the fresh meat on special absorbent tissues that takes up the oyster juice.
5. Numbering and weighing the minigrip bags.
6. Leaving the fresh meat to leak for 30 minutes.
7. At this moment we can determine the sex and the maturation stage by putting a bit of gonad tissue on a microscope glass and look at it with a microscope when it is still fresh and has not dried yet.
8. Maturation from 0 to 3: 0 is no gonad activity
   1 is the gonads are developing but no distinctions between cells
   2 is ovules and sperm clearly present but not a lot of sperm activity nor are the ovules all in the same stage and without much tissue between them
   3 is ovules all ripe and pear shaped lying close together or high sperm activity
9. Putting the fresh meat in the minigrip bags and weighing them (wet weight).
10. Putting the fresh meat in the freezer at about –18 °C for a day or longer.
11. The shells are left in the dry oven at 57° C for a day.
12. The shells are then weighted.
13. After being frozen the minigrips containing the samples are taken out of the freezer and put in the freeze dryer at about -52° C with the bags open, for four days.
14. The samples are weighted for the dry weight.
15. The samples are then stored for analyses in a drykeeper.

**Material**

- Plastic Minigrip bags
- Kimtex Lite absorbent tissues
- Microscope
- freeze dryer Christ beta 1-8 (Fisher Bioblock Scientific)
- dry keeper (Sanplatec Corp.)

## 5. Analysis of quantity of lipids

*Extraction and purification according to Bligh and Dyer (1959)*
*Dosage according to Marsh and Weinstein (1966)*

1. The samples that have been dried are weighted and then grinned in a centrifugal ball mill.
2. Rinse 1 glass tube of 10 x 1.2 cm and 1 glass tube of 11 x 1.5 cm per sample that you want to analyse, with chloroform in the fume hood.
3. Let the tubes dry and the chloroform evaporate.
4. Weight an amount of between 8 and 11 mg of the grinned oyster in the 10 x 1.2 cm glass tube, note the weight and close it with the cap.
5. Add 1 ml of chloroform to each tube (do this in the fume hood).
6. Add 2 ml of methanol to each tube.
7. Homogenise with the vortex.
8. Centrifuge the samples at 2000 rpm for 10 minutes.
9. Pour off the supernatant (containing the lipids) in the glass tube of 11 x 1.5 cm.
10. Repeat steps 5 to 9 to extract again the lipids from the grinned oyster.
11. Add 4 ml MilliQ water to each glass tube containing the supernatant.
12. Shake the tubes to homogenise
13. Centrifuge at 2000 rpm for 10 minutes.
14. Remove the upper phase above the white band by sucking carefully with a fast pump machine.
15. Leave the rest with the white band in a dry oven between 36 and 40°C for about two days.

For the reference we make a solution of about 1500 $\mu$g/ml glycerol tripalmitate in chloroform. Rinse 11 glass tubes of 11 x 1.5 cm with chloroform and let it dry/evaporate.
We weight about 1500 $\mu$g glycerol tripalmitate and note the exact weight. Then we solve this in 10 ml chloroform. Shake the solution to solve the glycerol tripalmitate. Put in the 11 glass tubes each 0, 100, 200, 300, till 1000 ml of the glycerol tripalmitate solution to get concentrations of 0, 150, 300, 450 etc. till 1500 µg.
Leave the tubes also in the dry oven between 36 and 40°C for about two days.

16. Add 10 ml $H_2SO_4$ to the pellet in each tube.
17. Place the tube 20 minutes at 200°C.
18. Let the tubes cool by placing them in cold water for some minutes.
19. Check the plates of the iEMs Reader/Dispenser photospectrometer using the Biolise program 1.65 for Windows.
20. Pipette three times 200 µl for each sample in the plate.
21. Read the samples with an iEMs Reader/Dispenser photospectrometer at 360 nm using the Biolise program 1.65 for Windows.
22. The results are expressed in equivalents of tripalmitate.

**Material**

- centrifugal ball mill (Frisch)
- chloroform absolute
- MilliQ water
- methanol absolute
- fast pump machine (home made)
- glycerol tripalmitate (Merck)
- $H_2SO_4$ 96%
- dry heater (Liebish)
- iEMs Reader/dispenser MF 1401 (Labsystems)

## 6. Analyses of quantity of carbohydrates and glycogen

*According to Dubois et al. (1956)*

1. The samples that have been dried are weighted and then grinned in a centrifugal ball mill.
2. Weight an amount of between 8 and 11 mg of the grinned oyster in the 10 x 1.2 cm glass tube, note the weight and close it with the cap.
3. Add 3 ml TCA to each tube.
4. Place a spatula in the tube and place the tube then on the vortex to homogenise the solution.
5. Rinse the spatula with a bit of TCA between each tube.
6. Let the TCA extract the carbohydrates for 1 hour at 4 °C in the fridge.
7. Centrifuge at 2000 rpm for 10 minutes.
8. Pipette 0.5 ml of the supernatant (upper phase) in a polypropylene tube of 10 ml 9.3 x 2 cm for the carbohydrates analyses.
9. Pipette 0.5 ml of the supernatant (upper phase) in a polypropylene tube of 10 ml 9.3 x 1.5 cm for the glycogen analyses.

For the reference we use a standard solution of 1000 µg/µl glucose. We make (10 ml 9.3 x 2 cm) tubes with a concentration of glucose from 0 till 500 µg in a total volume of 2 ml. Put in the tubes each 0, 0.1, 0.2 etc. till 1 ml of the glucose solution and add 2, 1.9, 1.8 etc. till 0 ml of TCA. We mix the solutions with the vortex and take 0.5 ml of each. Then we follow the steps below as for the carbohydrates.

*Carbohydrates*

1. Add 1 ml of 5 % phenol to the tube, in the fume hood.
2. Wait for 40 minutes.
3. Add 5 ml of $H_2SO_4$ to the tube, in the fume hood.
4. Wait for 10 minutes.
5. Mix carefully on the vortex.
6. Check the plates of the iEMs Reader/Dispenser photospectrometer using the Biolise program 1.65 for Windows.
7. Pipette three times 200 µl for each sample in the plate.
8. Read the samples with an iEMs Reader/Dispenser photospectrometer at 490 nm using the Biolise program 1.65 for Windows.
9. The results are expressed in equivalents of glucose.

*Glycogen*

1. Add 4 ml ethanol absolute to precipitate the glycogen.
2. Mix with the vortex.
3. Centrifuge during ten minutes at 2000 rpm.
4. Throw the supernatant away.
5. Boil MilliQ water in a glass beaker with an electric heater.
6. Add 0.5 ml boiling MilliQ water to each tube.
10. Add 1 ml of 5 % phenol to the tube, in the fume hood.
11. Wait for 40 minutes.
12. Add 5 ml of H2SO4 to the tube, in the fume hood.
13. Wait for 10 minutes.
14. Mix carefully on the vortex.
15. Check the plates of the iEMs Reader/Dispenser photospectrometer using the Biolise program 1.65 for Windows.
16. Pipette three times 200 µl for each sample in the plate.
17. Read the samples with an iEMs Reader/Dispenser photospectrometer at 490 nm using the Biolise program 1.65 for Windows.
18. The results are expressed in equivalents of glucose.

**Material**

- centrifugal ball mill (Frisch)
- TCA is 15 % Trichloracetic Acid: solution of 99 % pure Trichloracetic Acid pellets in MQ
- standard solution 1000 $\mu$g/µl glucose (Sigma Diagnostics)
- ethanol absolute
- MilliQ water
- 5 % phenol: diluted with MQ from a 100 % phenol solution
- $H_2SO_4$ 96 %
- iEMs Reader/dispenser MF 1401 (Labsystems)

## 7. *Biochemistry analysis*

The photospectrometer results are saved as a text file and then converted to Excel. We use three macro's (biolyse 1, 2 and 3) in Excel (that have been already created before). The first macro gives the average of the three repeats that have been done for each sample. If one of the repeats is responsible for a high change in the average of the three, it is deleted.
Now we correct the concentrations of the standard for the lipids with the exact amount that we used to make the standard solutions. We make a graph of the OD of the range of the standard solutions on the x-axe and its solutions with different concentrations of lipids/glucose on the y-axe (see fig 1a and 1b).

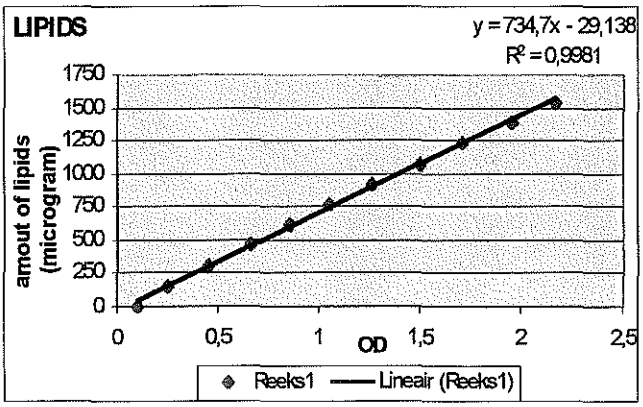



*Fig1a.*

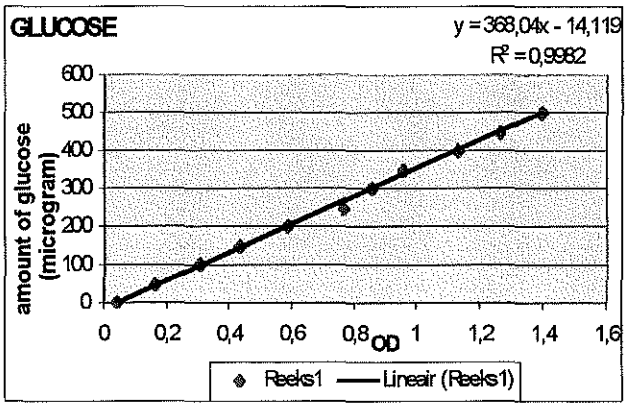
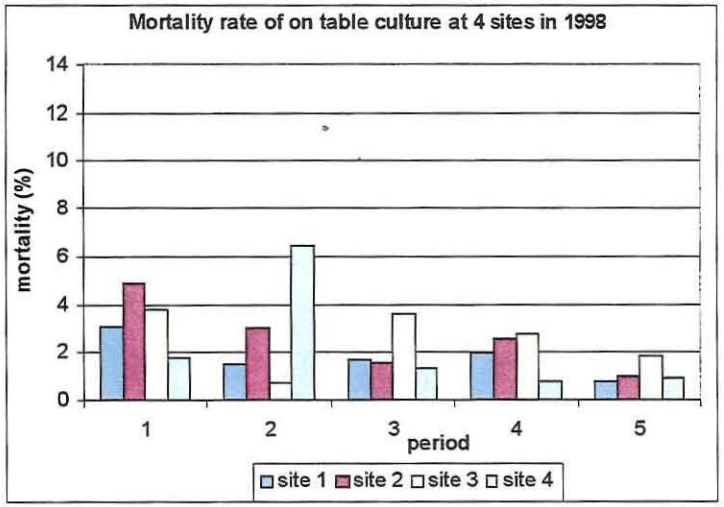


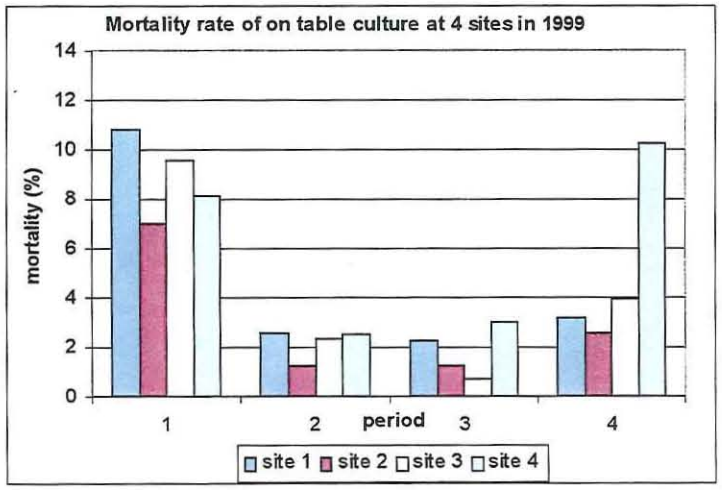

*Fig 1b.*

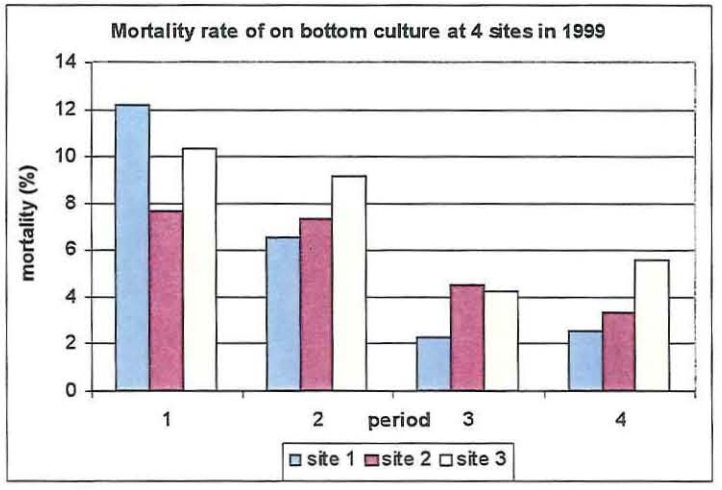
We draw a line through the points of the graph, which gives an equation (see fig 1a and 1b). By filling in the OD values of the samples for x in the equation we find the concentration of lipids/carbohydrates and glycogen. Then we have to make a correction for the milligram weight of powder that we used of each sample to calculate the percentage of lipids/carbohydrates and glycogen in each sample, which is expressed in equivalents of tripalmitate/glucose.
Because we added 3 ml of TCA at the beginning (see protocol 6) but used only 0.5 ml of the solution for the rest of the analyse (dilution by six) and we also diluted the standard solution by two, we therefore still have to multiply the percentage carbohydrates and glycogen by three.

# Annex 1a, 1b, 1c and 1 d.







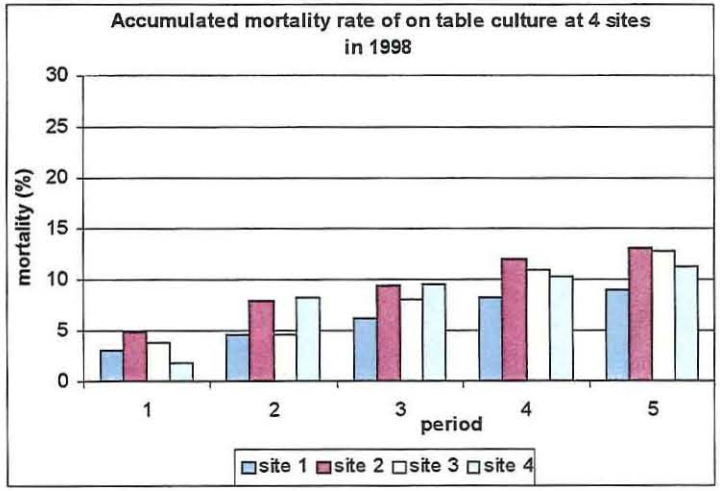



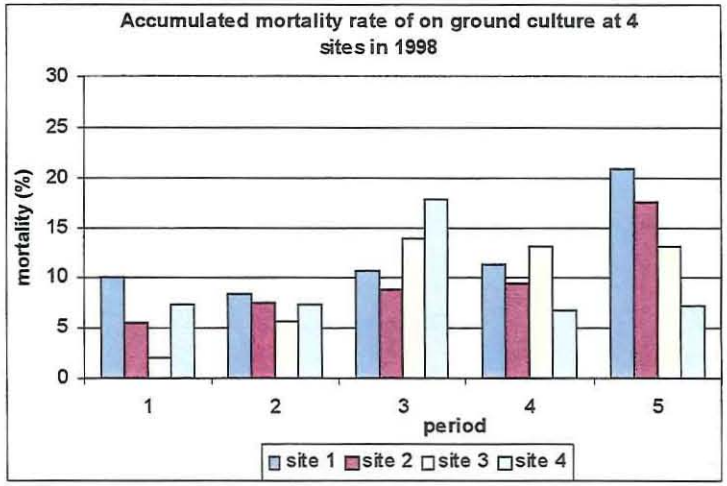
## Annex 2a and 2b.

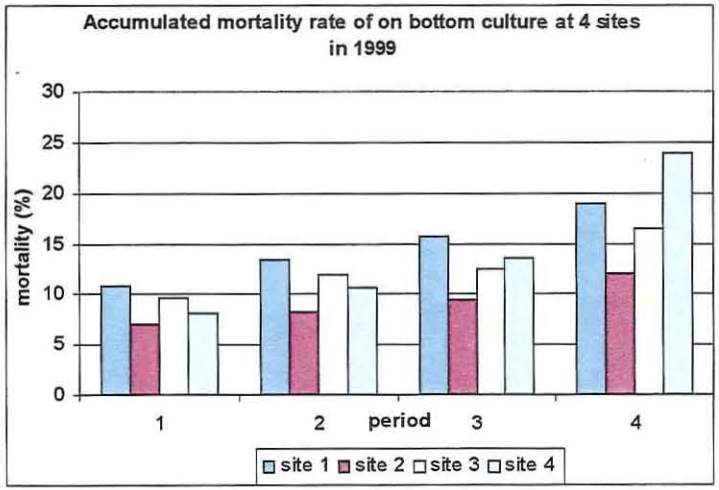
| 1998 period | site 1 table | site 2 table | site 3 table | site 4 table | average table mortality | site 1 bottom | site 2 bottom | site 3 bottom | site 4 bottom | average bottom mortality |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 3,1 | 4,9 | 3,8 | 1,8 | 2,4 | 10,0 | 5,5 | 1,9 | 7,3 | 6,2 |
| 2 | 1,5 | 3,0 | 0,7 | 6,4 | 2,0 | -1,7 | 2,0 | 3,8 | 0,0 | 1,0 |
| 3 | 1,7 | 1,6 | 3,6 | 1,3 | 1,7 | 2,3 | 1,3 | 8,2 | 10,5 | 5,6 |
| 4 | 1,9 | 2,5 | 2,8 | 0,8 | 1,4 | 0,6 | 0,5 | -0,7 | -11,1 | -2,7 |
| 5 | 0,8 | 1,0 | 1,8 | 0,9 | 1,8 | 9,6 | 8,2 | 0,0 | 0,5 | 4,6 |
| total | 9,0 | 13,0 | 12,7 | 11,2 | 11,5 | 20,9 | 17,6 | 13,1 | 7,2 | 14,7 |

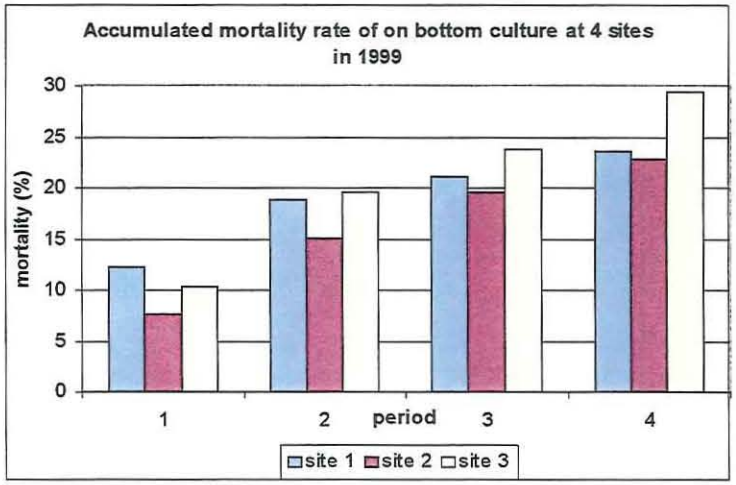
| 1999 period | site 1 table | site 2 table | site 3 table | site 4 table | average table mortality | site 1 bottom | site 2 bottom | site 3 bottom | site 4 bottom | average bottom mortality |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 10,8 | 7,0 | 9,6 | 8,1 | 8,9 | 12,2 | 7,7 | 10,3 | | 10,1 |
| 2 | 2,6 | 1,2 | 2,3 | 2,5 | 2,2 | 6,6 | 7,3 | 9,2 | | 7,7 |
| 3 | 2,3 | 1,3 | 0,7 | 3,0 | 1,8 | 2,3 | 4,5 | 4,3 | | 3,7 |
| 4 | 3,2 | 2,6 | 4,0 | 10,3 | 5,0 | 2,6 | 3,3 | 5,6 | | 3,8 |
| total | 18,9 | 12,0 | 16,5 | 23,8 | 17,8 | 23,6 | 22,8 | 29,4 | | 25,3 |

## Annex 3a, 3b, 3c and 3d.



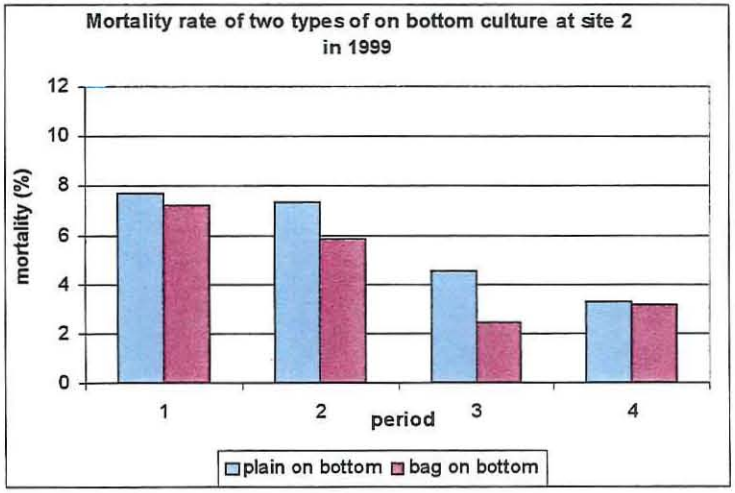



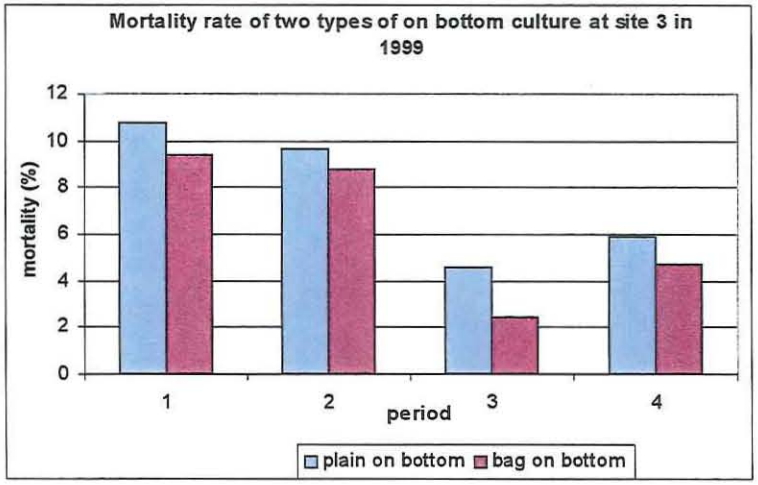
## Annex 4a and 4b.

| 1998 period | site 1 table | site 2 table | site 3 table | site 4 table | average acc. table mortality | site 1 bottom | site 2 bottom | site 3 bottom | site 4 bottom | average acc. bottom mortality |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 3,1 | 4,9 | 3,8 | 1,8 | 3,4 | 10,0 | 5,5 | 1,9 | 7,3 | 6,2 |
| 2 | 4,6 | 7,9 | 4,5 | 8,2 | 6,3 | 8,3 | 7,5 | 5,7 | 7,3 | 7,2 |
| 3 | 6,3 | 9,4 | 8,1 | 9,5 | 8,3 | 10,6 | 8,9 | 13,8 | 17,8 | 12,8 |
| 4 | 8,2 | 12,0 | 10,8 | 10,3 | 10,3 | 11,3 | 9,4 | 13,1 | 6,7 | 10,1 |
| 5 | 9,0 | 13,0 | 12,7 | 11,2 | 11,5 | 20,9 | 17,6 | 13,1 | 7,2 | 14,7 |

| 1999 period | site 1 table | site 2 table | site 3 table | site 4 table | average acc. table mortality | site 1 bottom | site 2 bottom | site 3 bottom | site 4 bottom | average acc. bottom mortality |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 10,8 | 7,0 | 9,6 | 8,1 | 8,9 | 12,2 | 7,7 | 10,3 | | 10,1 |
| 2 | 13,4 | 8,2 | 11,9 | 10,6 | 11,0 | 18,8 | 15,0 | 19,5 | | 17,8 |
| 3 | 15,7 | 9,5 | 12,5 | 13,6 | 12,8 | 21,0 | 19,5 | 23,8 | | 21,5 |
| 4 | 18,9 | 12,0 | 16,5 | 23,8 | 17,8 | 23,6 | 22,8 | 29,4 | | 25,3 |

## Annex 5a, 5b, 5c and 5d.




| period | mortality (%) site 2 plain on bottom | mortality (%) site 2 bag on bottom | mortality (%) site 3 plain on bottom | mortality (%) site 3 bag on bottom |
|---|---|---|---|---|
| 1 | 7,7 | 7,2 | 10,8 | 9,4 |
| 2 | 7,3 | 5,9 | 9,7 | 8,8 |
| 3 | 4,5 | 2,4 | 4,5 | 2,4 |
| 4 | 3,3 | 3,2 | 5,9 | 4,7 |

| period | acc. mortality (%) site 2 plain on bottom | acc. mortality (%) site 2 bag on bottom | acc. mortality (%) site 3 plain on bottom | acc. mortality (%) site 3 bag on bottom |
|---|---|---|---|---|
| 1 | 7,7 | 7,2 | 10,8 | 9,4 |
| 2 | 15,1 | 13,1 | 20,4 | 18,2 |
| 3 | 19,6 | 15,5 | 25,0 | 20,6 |
| 4 | 22,9 | 18,7 | 30,9 | 25,3 |

## Annex 6a and 6b.

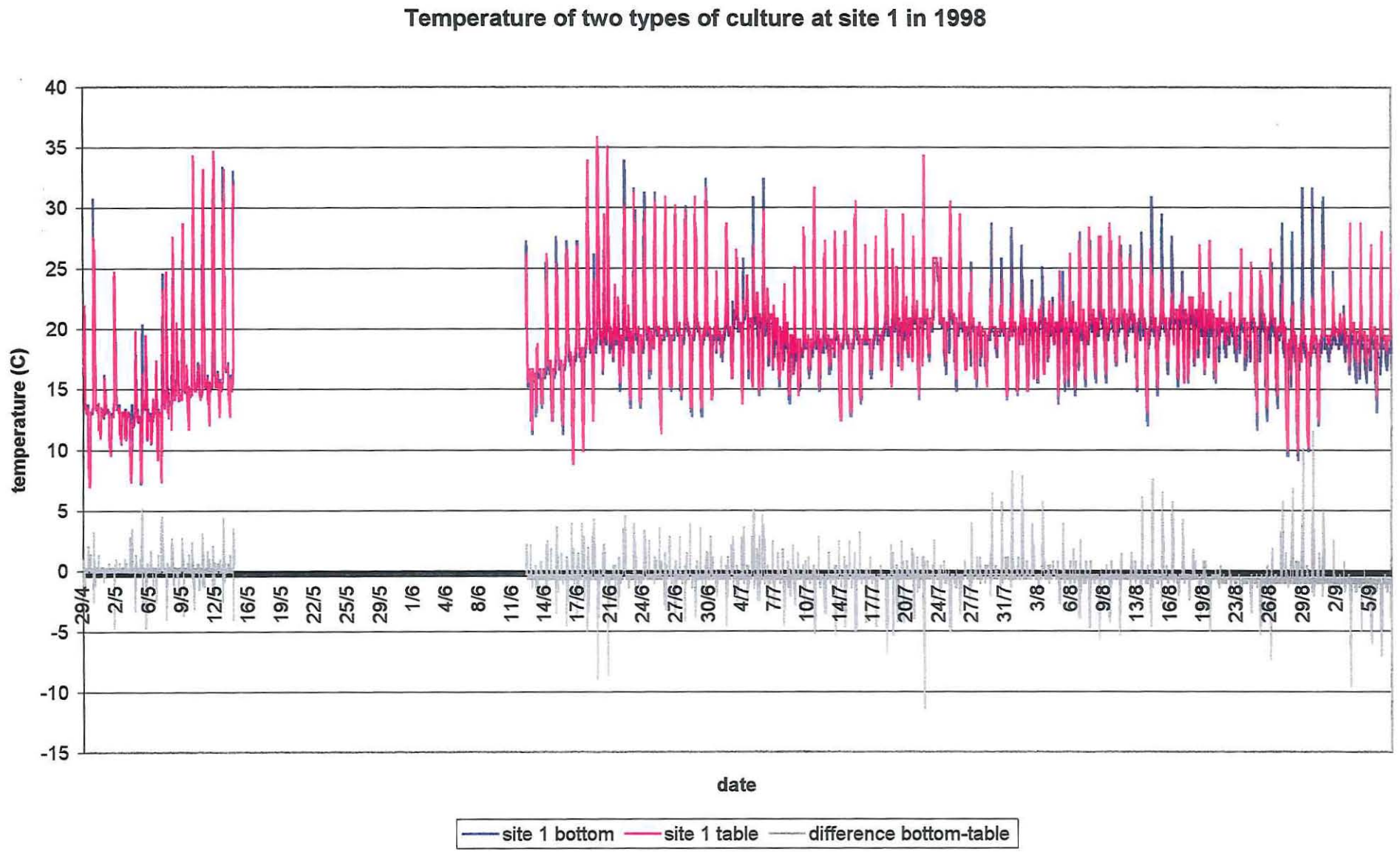
| 1998 Site | Temp table ≥ 2.0° C higher temp than bottom | Temp bottom ≥ 2.0° C higher temp than table | Total measures |
|---|---|---|---|
| 1 | 487 | 343 | 9755 |
| 2 | 420 | 101 | 8610 |
| 3 | 118 | 16 | 1353 |
| 4 | 170 | 249 | 12564 |

*Table with number of times that temperature data collected at table culture was 2.0° C or more higher than the temperature data collected at that moment at bottom culture and number of times than temperature data collected at bottom culture was 2.0° C or more higher than the temperature data collected at that moment at table culture.*
*Total measures means number of times that temperature was measured for both cultures at the same moment.*

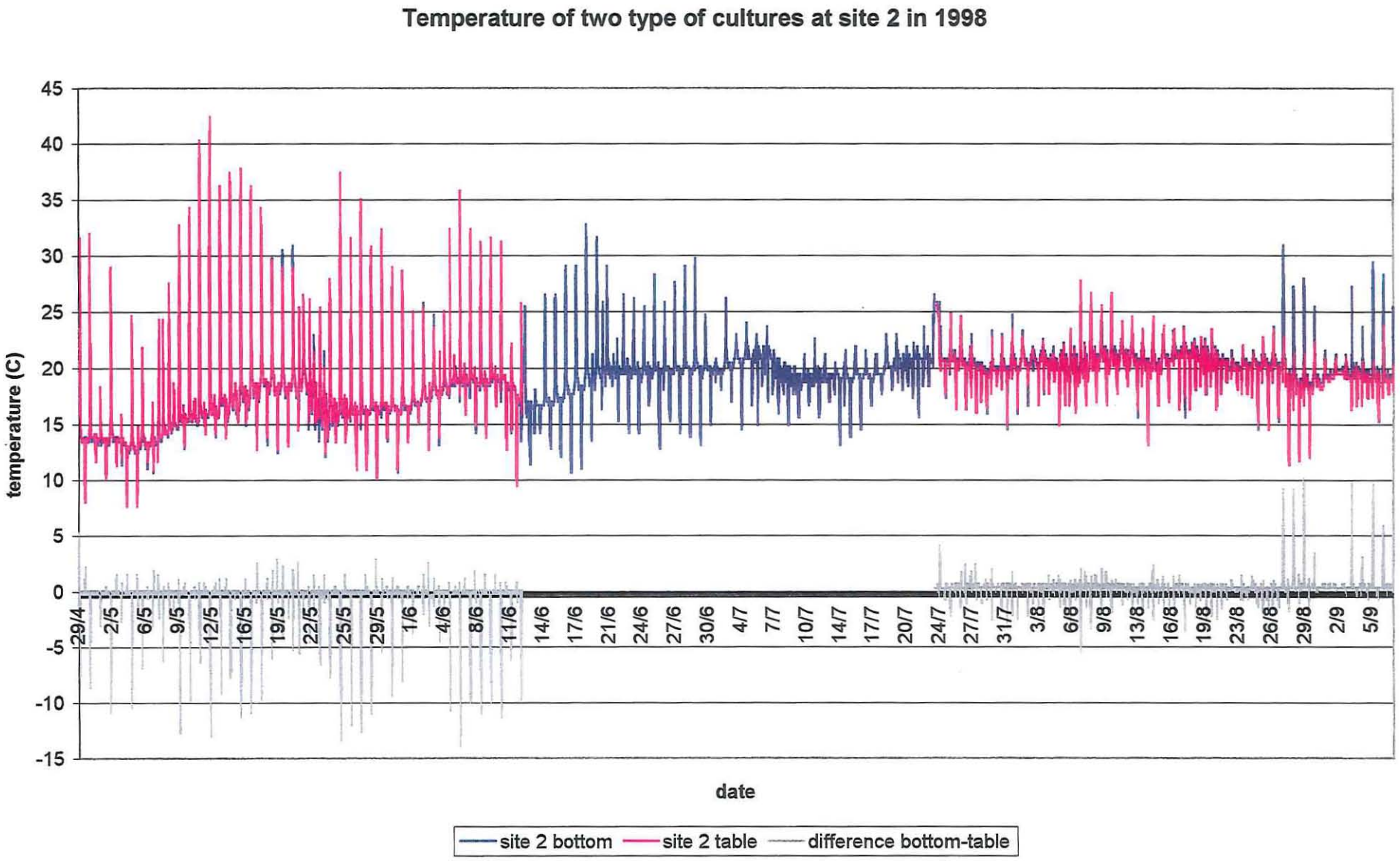
| 1999 Site | Temp table ≥ 2.0° C higher temp than bottom | Temp bottom ≥ 2.0° C higher temp than table | Total measures |
|---|---|---|---|
| 1 | 93 | 369 | 5364 |
| 2 | 247 | 1242 | 12554 |
| 3 | 87 | 362 | 8378 |
| 4 | * | * | * |

*Table with number of times that temperature data collected at table culture was 2.0° C or more higher than the temperature data collected at that moment at bottom culture and number of times than temperature data collected at bottom culture was 2.0° C or more higher than the temperature data collected at that moment at table culture.*
*Total measures means number of times that temperature was measures for both cultures at the same moment.*

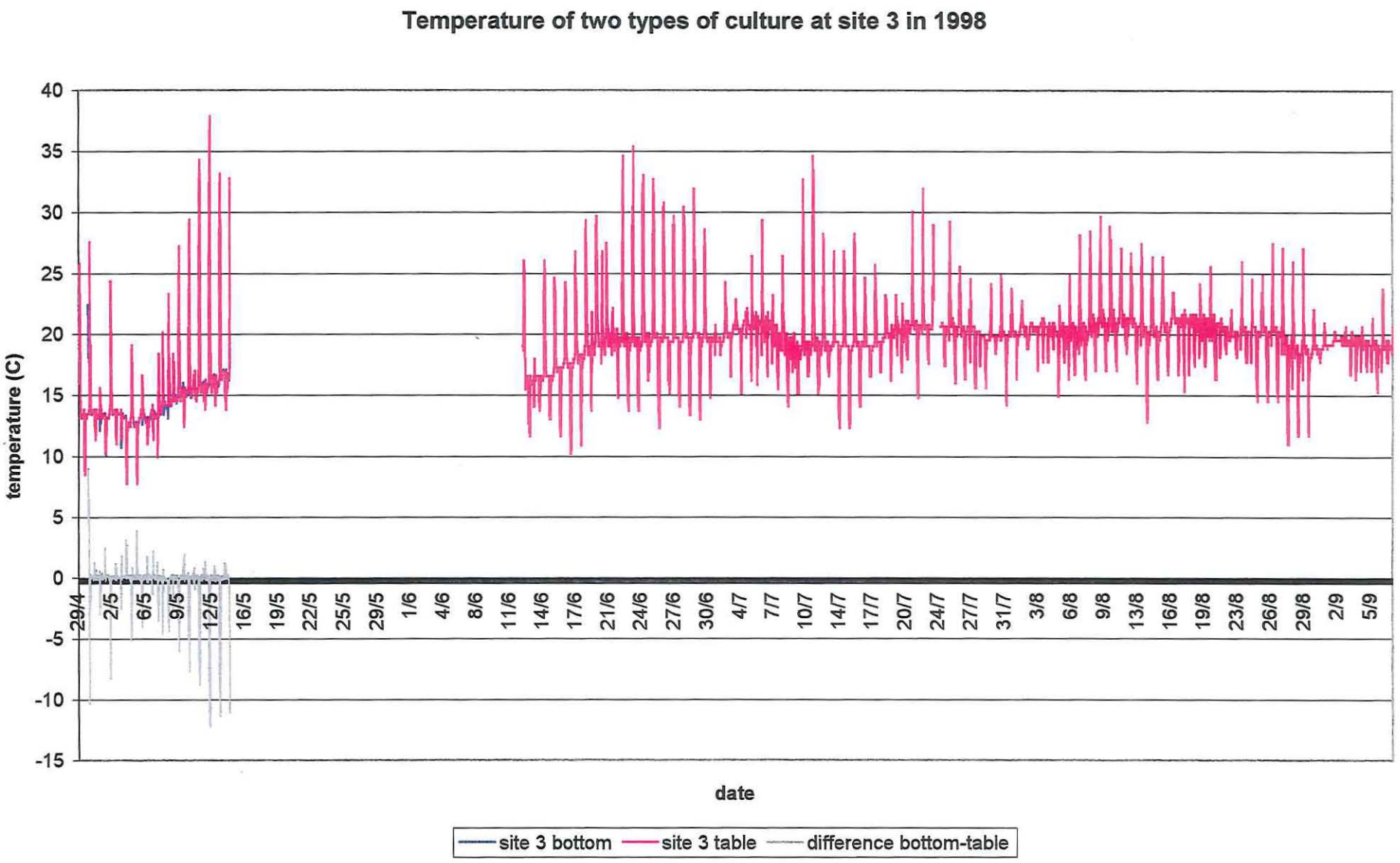
## Annex 7a.


Temperature of two types of culture at site 1 in 1998
temperature (C)
40
35
30
25
20
15
10
5
0
-5
-10
-15
29/4
2/5
6/5
9/5
12/5
16/5
19/5
22/5
25/5
29/5
1/6
4/6
8/6
11/6
14/6
17/6
21/6
24/6
27/6
30/6
4/7
7/7
10/7
14/7
17/7
20/7
24/7
27/7
31/7
3/8
6/8
9/8
13/8
16/8
19/8
23/8
26/8
29/8
2/9
5/9
date
site 1 bottom
site 1 table
difference bottom-table

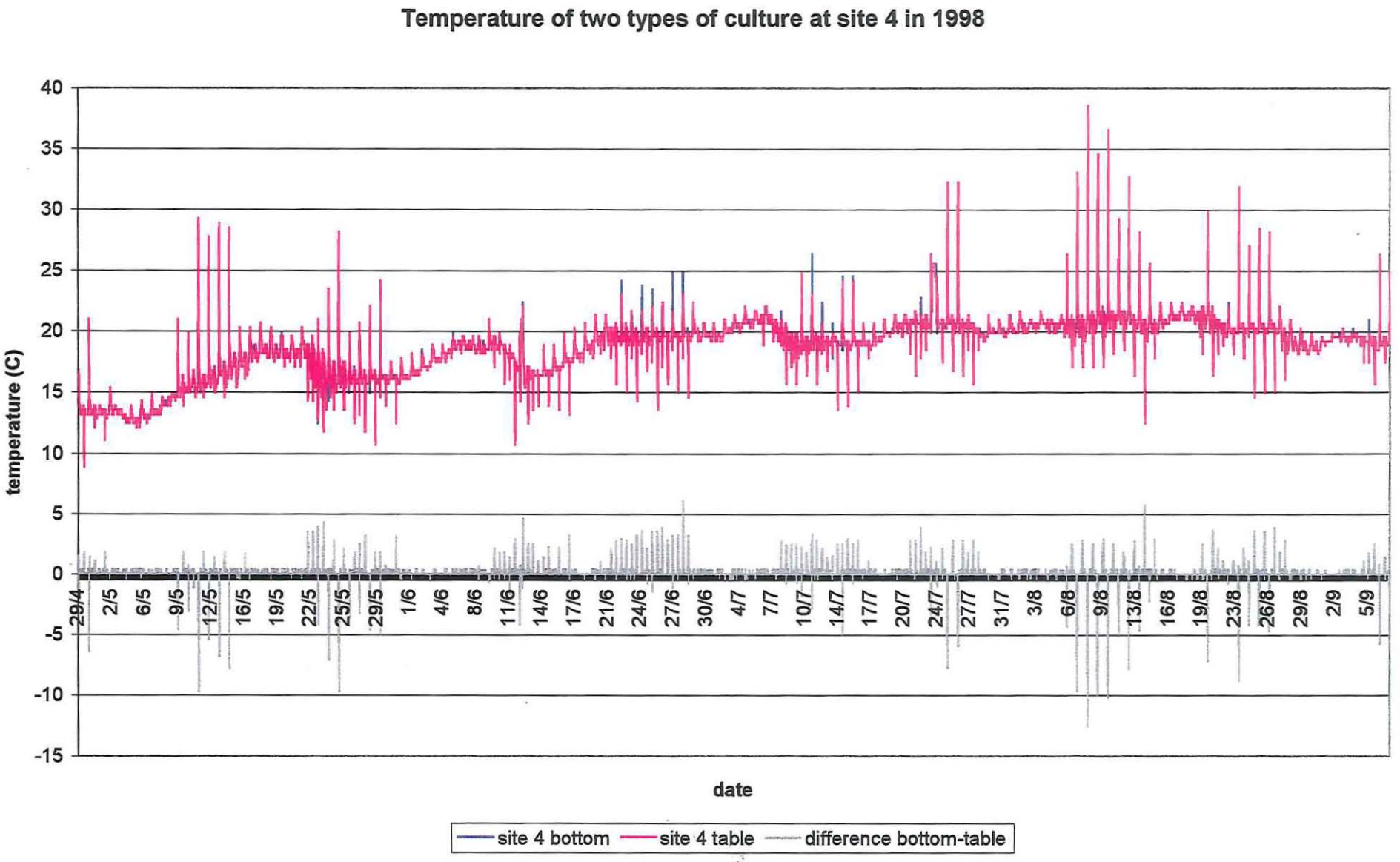
**Annex 7b.**


Temperature of two type of cultures at site 2 in 1998
temperature (C)
45
40
35
30
25
20
15
10
5
0
-5
-10
-15
29/4
2/5
6/5
9/5
12/5
16/5
19/5
22/5
25/5
29/5
1/6
4/6
8/6
11/6
14/6
17/6
21/6
24/6
27/6
30/6
4/7
7/7
10/7
14/7
17/7
20/7
24/7
27/7
31/7
3/8
6/8
9/8
13/8
16/8
19/8
23/8
26/8
29/8
2/9
5/9
date
site 2 bottom
site 2 table
difference bottom-table

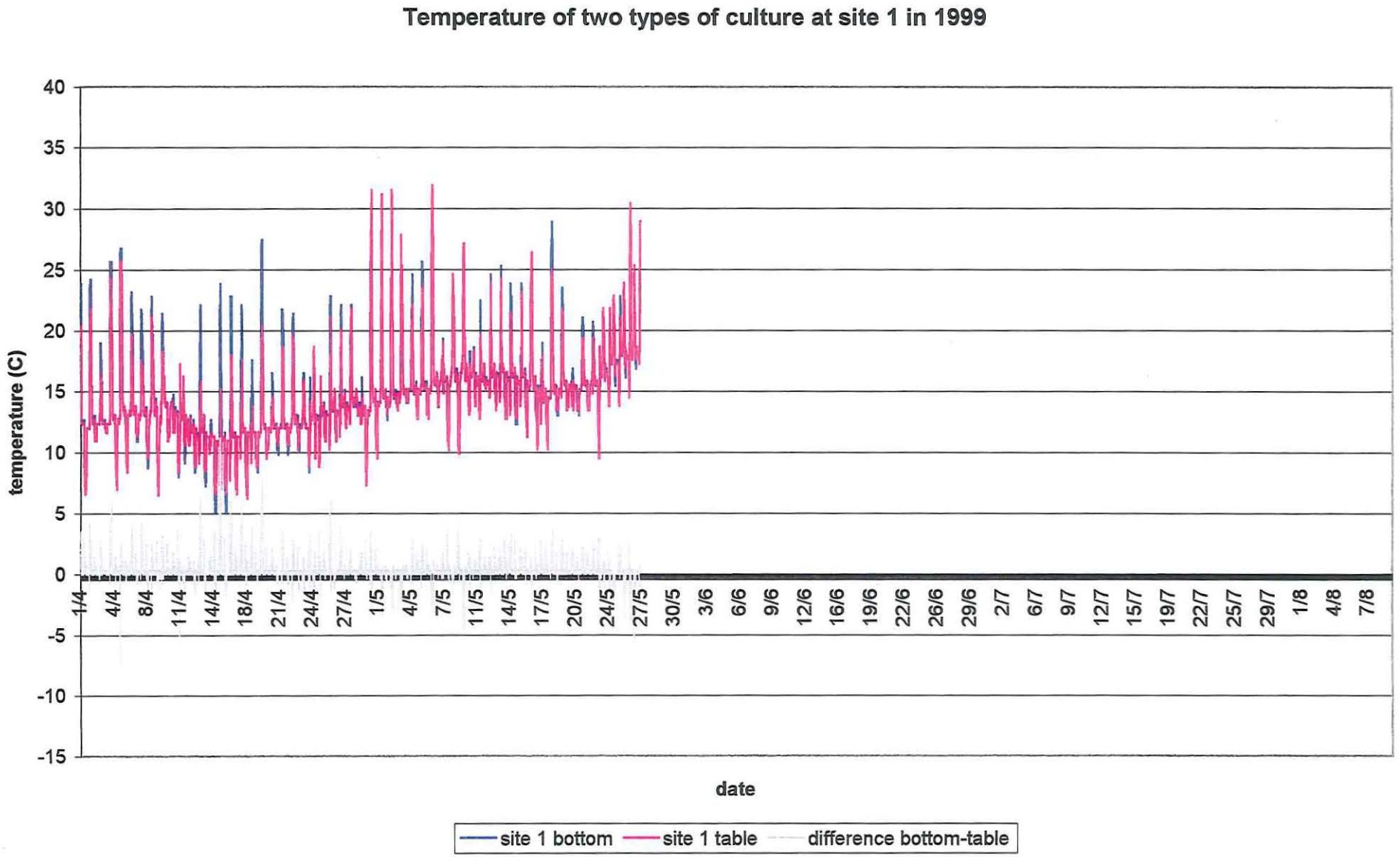
## Annex 7c.


Temperature of two types of culture at site 3 in 1998
temperature (C)
40
35
30
25
20
15
10
5
0
-5
-10
-15
29/4
2/5
6/5
9/5
12/5
16/5
19/5
22/5
25/5
29/5
1/6
4/6
8/6
11/6
14/6
17/6
21/6
24/6
27/6
30/6
4/7
7/7
10/7
14/7
17/7
20/7
24/7
27/7
31/7
3/8
6/8
9/8
13/8
16/8
19/8
23/8
26/8
29/8
2/9
5/9
date
site 3 bottom
site 3 table
difference bottom-table

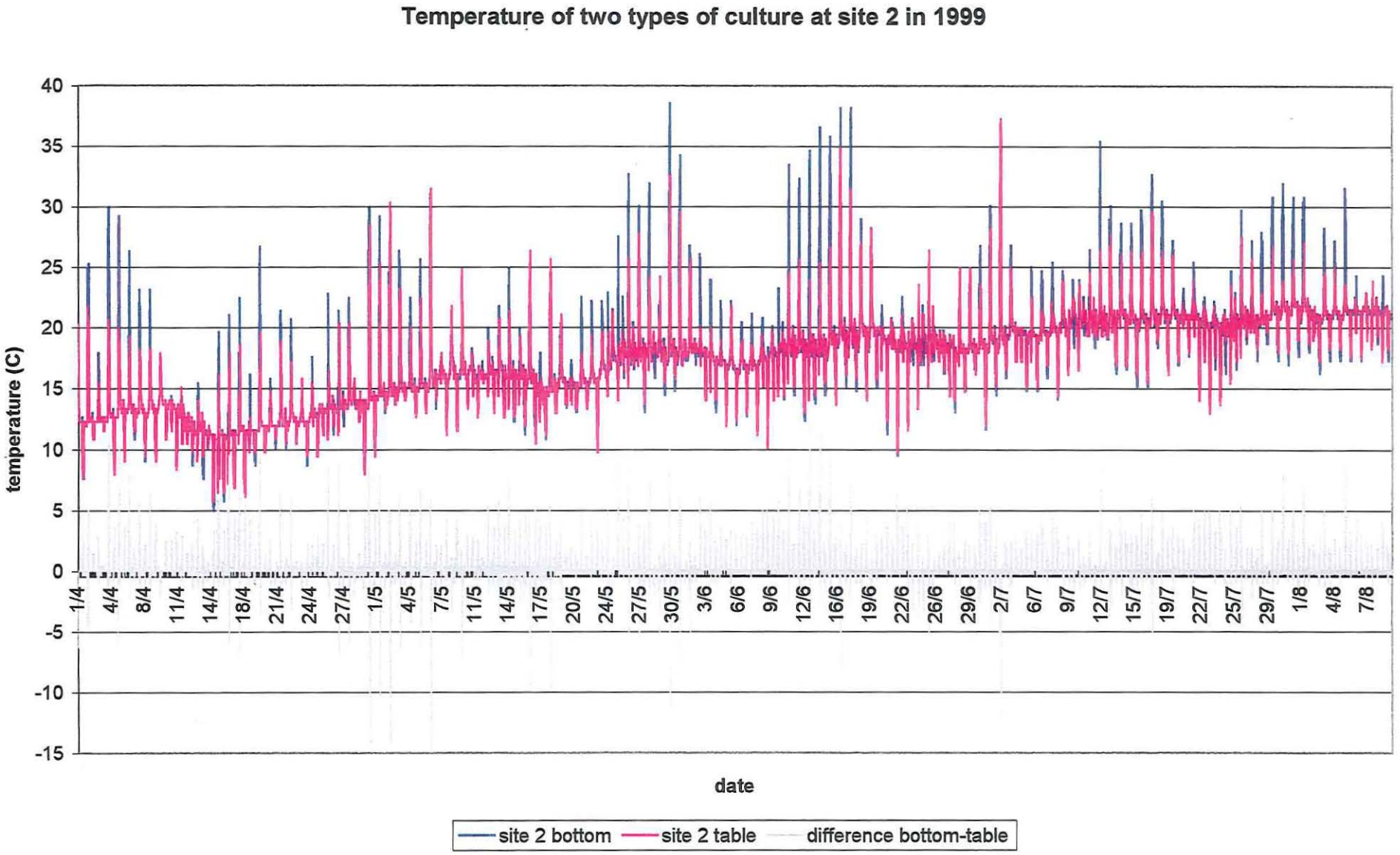
**Annex 7d.**


Temperature of two types of culture at site 4 in 1998
temperature (C)
40
35
30
25
20
15
10
5
0
-5
-10
-15
29/4
2/5
6/5
9/5
12/5
16/5
19/5
22/5
25/5
29/5
1/6
4/6
8/6
11/6
14/6
17/6
21/6
24/6
27/6
30/6
4/7
7/7
10/7
14/7
17/7
20/7
24/7
27/7
31/7
3/8
6/8
9/8
13/8
16/8
19/8
23/8
26/8
29/8
2/9
5/9
date
site 4 bottom
site 4 table
difference bottom-table

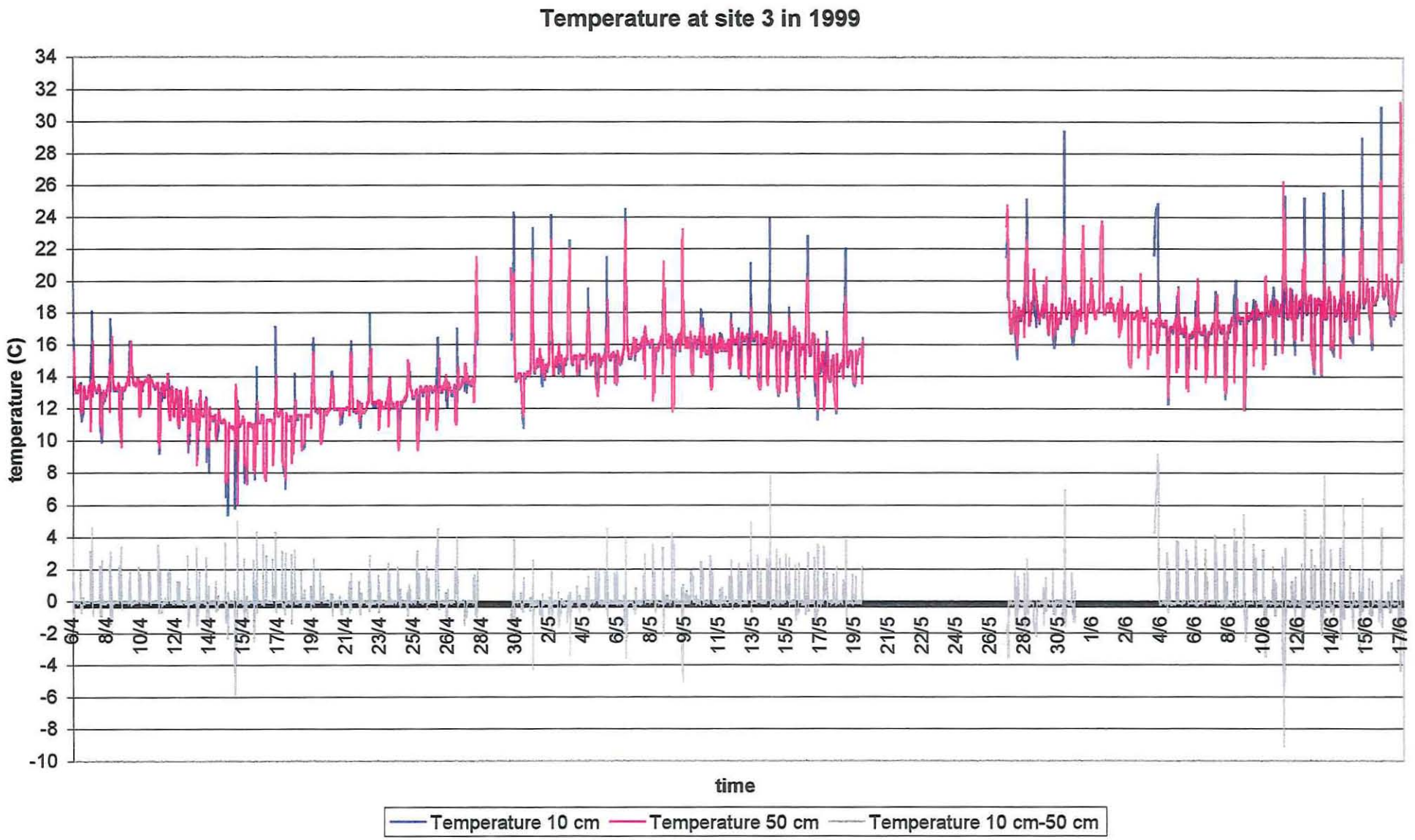
## Annex 8a.


Temperature of two types of culture at site 1 in 1999
temperature (C)
40
35
30
25
20
15
10
5
0
-5
-10
-15
1/4
4/4
8/4
11/4
14/4
18/4
21/4
24/4
27/4
1/5
4/5
7/5
11/5
14/5
17/5
20/5
24/5
27/5
30/5
3/6
6/6
9/6
12/6
16/6
19/6
22/6
26/6
29/6
2/7
6/7
9/7
12/7
15/7
19/7
22/7
25/7
29/7
1/8
4/8
7/8
date
site 1 bottom
site 1 table
difference bottom-table

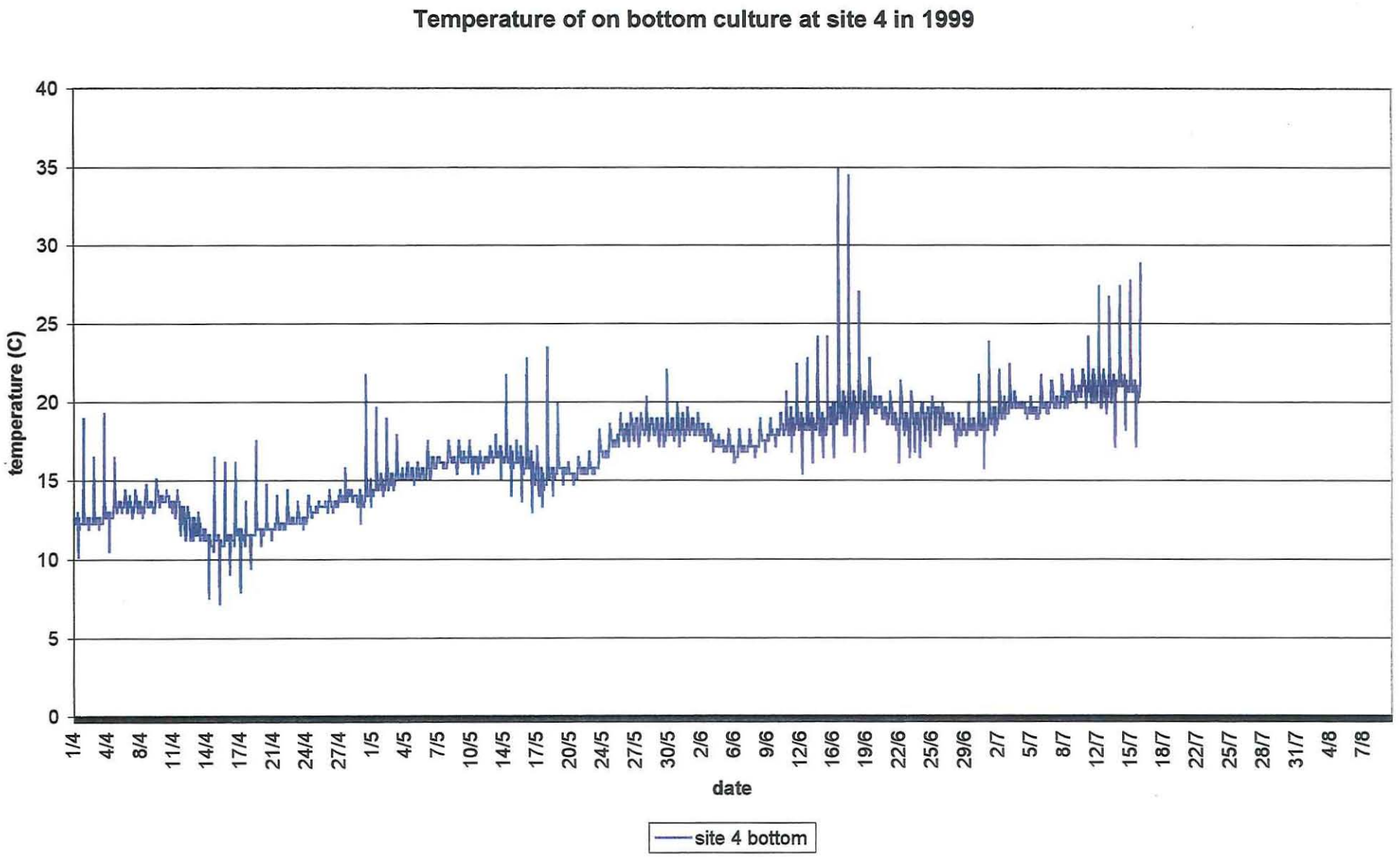
**Annex 8b.**


Temperature of two types of culture at site 2 in 1999
temperature (C)
40
35
30
25
20
15
10
5
0
-5
-10
-15
1/4
4/4
8/4
11/4
14/4
18/4
21/4
24/4
27/4
1/5
4/5
7/5
11/5
14/5
17/5
20/5
24/5
27/5
30/5
3/6
6/6
9/6
12/6
16/6
19/6
22/6
26/6
29/6
2/7
6/7
9/7
12/7
15/7
19/7
22/7
25/7
29/7
1/8
4/8
7/8
date
site 2 bottom
site 2 table
difference bottom-table

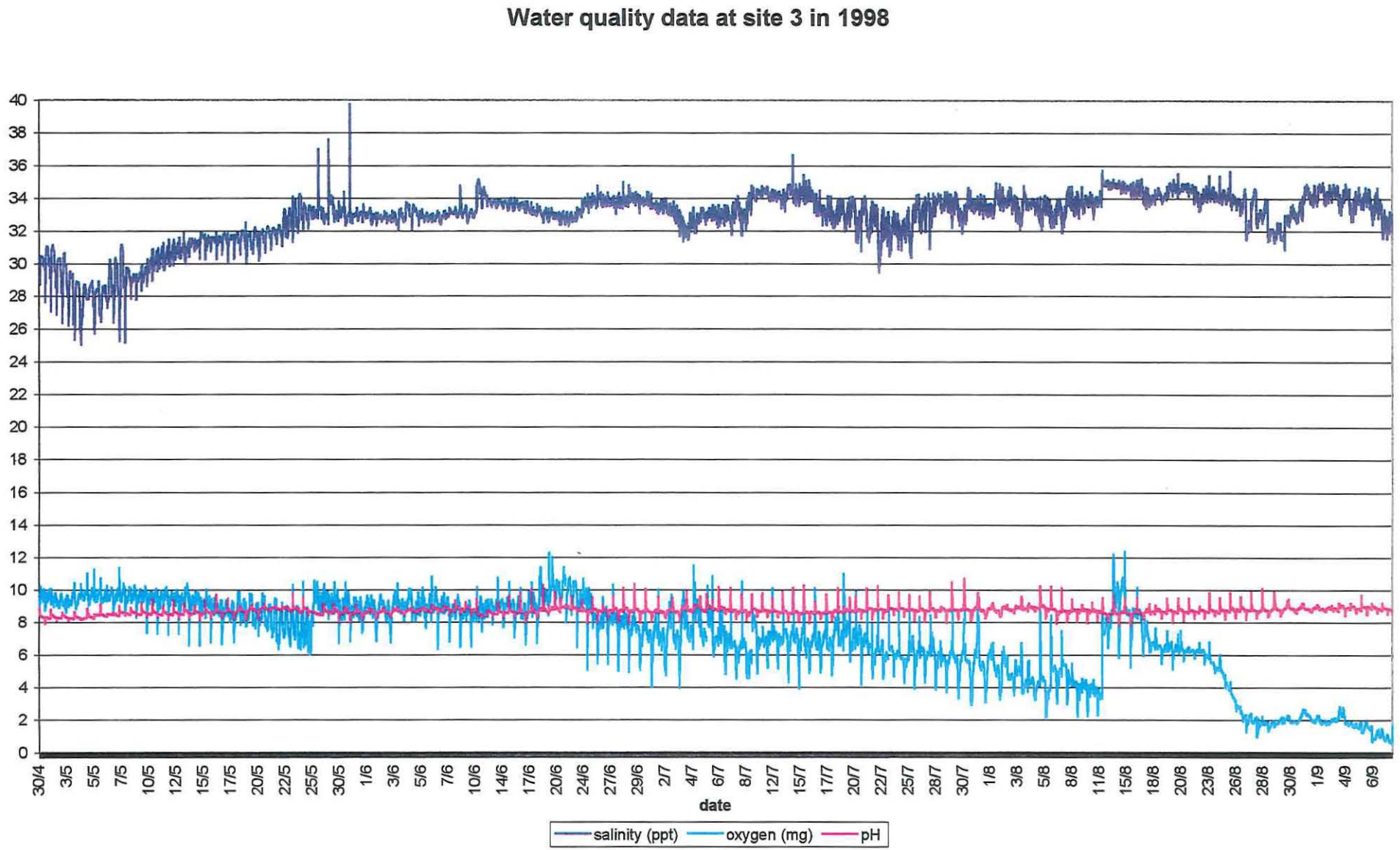
## Annex 8c.


Temperature at site 3 in 1999
temperature (C)
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
-2
-4
-6
-8
-10
6/4
8/4
10/4
12/4
14/4
15/4
17/4
19/4
21/4
23/4
25/4
26/4
28/4
30/4
2/5
4/5
6/5
8/5
9/5
11/5
13/5
15/5
17/5
19/5
21/5
22/5
24/5
26/5
28/5
30/5
1/6
2/6
4/6
6/6
8/6
10/6
12/6
14/6
15/6
17/6
time
Temperature 10 cm
Temperature 50 cm
Temperature 10 cm-50 cm

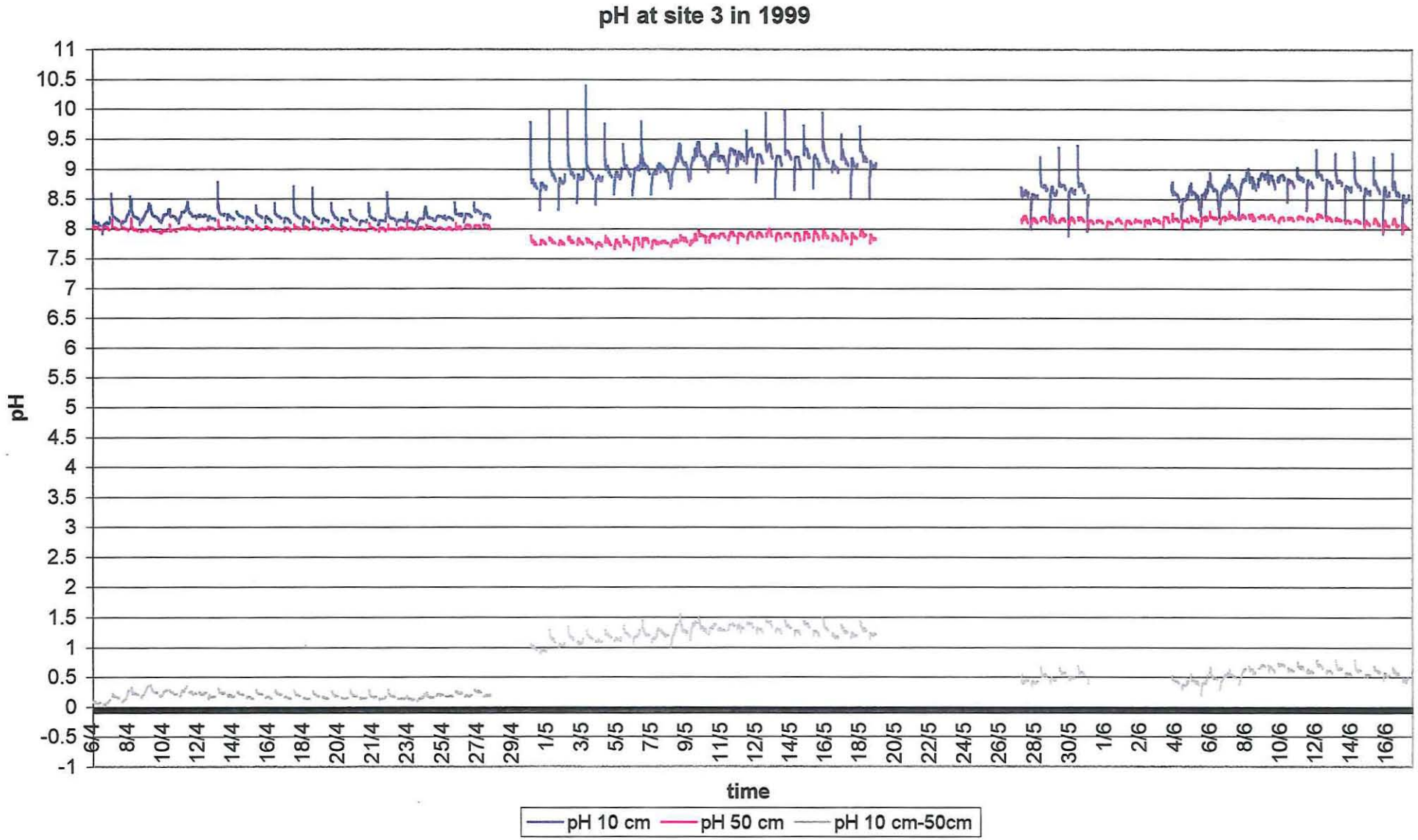
**Annex 8d.**


Temperature of on bottom culture at site 4 in 1999
temperature (C)
40
35
30
25
20
15
10
5
0
1/4
4/4
8/4
11/4
14/4
17/4
21/4
24/4
27/4
1/5
4/5
7/5
10/5
14/5
17/5
20/5
24/5
27/5
30/5
2/6
6/6
9/6
12/6
16/6
19/6
22/6
25/6
29/6
2/7
5/7
8/7
12/7
15/7
18/7
22/7
25/7
28/7
31/7
4/8
7/8
date
site 4 bottom

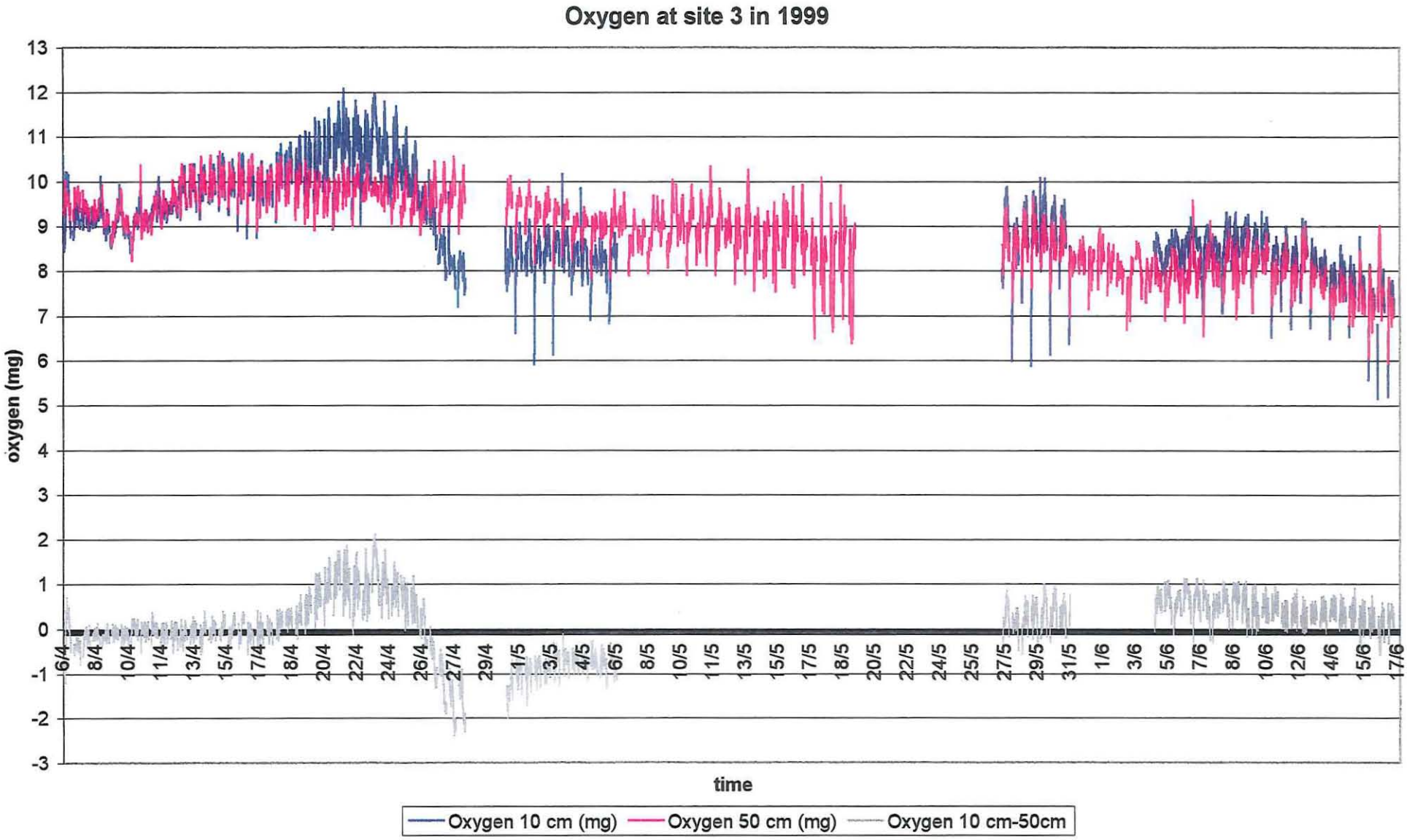
## Annex 9.


Water quality data at site 3 in 1998
40
38
36
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
30/4
3/5
5/5
7/5
10/5
12/5
15/5
17/5
20/5
22/5
25/5
30/5
1/6
3/6
5/6
7/6
10/6
14/6
17/6
20/6
24/6
27/6
29/6
2/7
4/7
6/7
8/7
12/7
15/7
17/7
20/7
22/7
25/7
28/7
30/7
1/8
3/8
5/8
8/8
11/8
15/8
18/8
20/8
23/8
26/8
28/8
30/8
1/9
4/9
6/9
date
salinity (ppt)
oxygen (mg)
pH

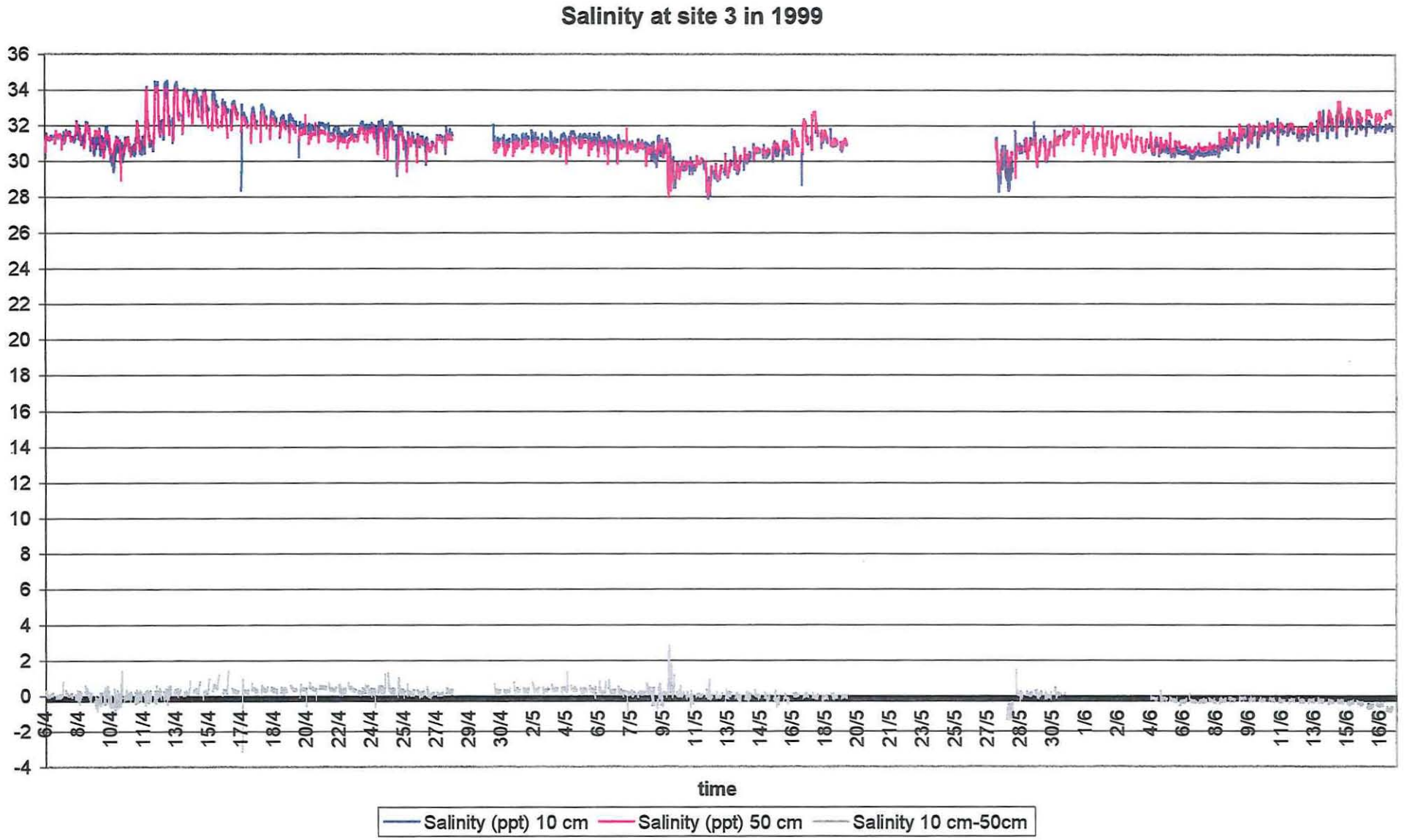
**Annex 10a.**


pH at site 3 in 1999
pH
11
10.5
10
9.5
9
8.5
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
-1
6/4
8/4
10/4
12/4
14/4
16/4
18/4
20/4
21/4
23/4
25/4
27/4
29/4
1/5
3/5
5/5
7/5
9/5
11/5
12/5
14/5
16/5
18/5
20/5
22/5
24/5
26/5
28/5
30/5
1/6
2/6
4/6
6/6
8/6
10/6
12/6
14/6
16/6
time
pH 10 cm
pH 50 cm
pH 10 cm-50cm

**Annex 10b.**


Oxygen at site 3 in 1999
oxygen (mg)
13
12
11
10
9
8
7
6
5
4
3
2
1
0
-1
-2
-3
6/4
8/4
10/4
11/4
13/4
15/4
17/4
18/4
20/4
22/4
24/4
26/4
27/4
29/4
1/5
3/5
4/5
6/5
8/5
10/5
11/5
13/5
15/5
17/5
18/5
20/5
22/5
24/5
25/5
27/5
29/5
31/5
1/6
3/6
5/6
7/6
8/6
10/6
12/6
14/6
15/6
17/6
time
Oxygen 10 cm (mg)
Oxygen 50 cm (mg)
Oxygen 10 cm-50cm

**Annex 10c.**


Salinity at site 3 in 1999
36
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
-2
-4
6/4
8/4
10/4
11/4
13/4
15/4
17/4
18/4
20/4
22/4
24/4
25/4
27/4
29/4
30/4
2/5
4/5
6/5
7/5
9/5
11/5
13/5
14/5
16/5
18/5
20/5
21/5
23/5
25/5
27/5
28/5
30/5
1/6
2/6
4/6
6/6
8/6
9/6
11/6
13/6
15/6
16/6
time
Salinity (ppt) 10 cm
Salinity (ppt) 50 cm
Salinity 10 cm-50cm

# Annex 11.

* means alive at the end of the experiment, number is the number of the oyster

| number | group | total weight (g) | day of experiment | date death |
|---|---|---|---|---|
| 1 | control | 116.4 | * | * |
| 2 | control | 89.7 | * | * |
| 3 | control | 81.3 | * | * |
| 4 | control | 81.9 | 20 | 28-5-00 morning |
| 5 | control | 115.6 | * | * |
| 6 | control | 125.5 | * | * |
| 7 | control | 115 | 30 | 7-6-00 morning |
| 8 | control | 86 | 46 | 23-6-00 morning |
| 9 | control | 91.9 | * | * |
| 10 | control | 111 | * | * |
| 11 | control | 98.1 | 18 | 26-5-00 morning |
| 12 | control | 103.3 | 30 | 7-6-00 morning |
| 13 | control | 73.6 | 28 | 5-6-00 morning |
| 14 | control | 81.1 | 28 | 5-6-00 morning |
| 15 | control | 87 | 28 | 5-6-00 morning |
| 16 | control | 86.7 | * | * |
| 17 | control | 98.8 | * | * |
| 18 | control | 66 | 23 | 31-5-00 morning |
| 19 | control | 115.9 | * | * |
| 20 | control | 110.9 | * | * |
| 21 | control | 109.8 | 38 | 15-6-00 morning |
| 22 | control | 75.9 | 34 | 11-6-00 morning |
| 23 | control | 98.3 | * | * |
| 24 | control | 100.6 | * | * |
| 25 | control | 100 | * | * |
| 26 | control | 71.3 | * | * |
| 27 | control | 99.9 | 30 | 7-6-00 morning |
| 28 | control | 103.1 | 50 | 27-6-00 morning |
| 29 | control | 77.7 | 32 | 9-6-00 morning |
| 30 | control | 83.7 | 34 | 11-6-00 morning |
| 31 | temp. | 112.2 | * | * |
| 32 | temp. | 121.5 | * | * |
| 33 | temp. | 107.8 | * | * |
| 34 | temp. | 78.2 | 11 | 19-5-00 morning |
| 35 | temp. | 107 | * | * |
| 36 | temp. | 92.4 | * | * |
| 37 | temp. | 80.7 | * | * |
| 38 | temp. | 100.2 | * | * |
| 39 | temp. | 117.4 | * | * |
| 40 | temp. | 86.4 | 19 | 27-5-00 morning |
| 41 | temp. | 104.8 | 47 | 24-6-00 morning |
| 42 | temp. | 92.3 | * | * |
| 43 | temp. | 102.4 | 32 | 9-6-00 morning |
| 44 | temp. | 84.9 | 34 | 11-6-00 morning |
| 45 | temp. | 101.7 | 45 | 22-6-00 morning |
| 46 | temp. | 88.9 | 20 | 28-5-00 morning |
| 47 | temp. | 94 | 38 | 15-6-00 morning |
| 48 | temp. | 105 | 30 | 7-6-00 morning |
| 49 | temp. | 110.5 | 38 | 15-6-00 morning |

| number | group | total weight (g) | day of experiment | date death |
|---|---|---|---|---|
| **50** | temp. | 98.3 | 49 | 26-6-00 morning |
| **51** | temp. | 109.6 | 28 | 5-6-00 morning |
| **52** | temp. | 94.9 | 34 | 11-6-00 morning |
| **53** | temp. | 91.2 | * | * |
| **54** | temp. | 95.8 | 34 | 11-6-00 morning |
| **55** | temp. | 102.1 | 38 | 15-6-00 morning |
| **56** | temp. | 85.5 | 45 | 22-6-00 morning |
| **57** | temp. | 79.6 | 26 | 3-6-00 morning |
| **58** | temp. | 89.8 | 28 | 5-6-00 morning |
| **59** | temp. | 82.6 | 34 | 11-6-00 morning |
| **60** | temp. | 94.9 | 38 | 15-6-00 morning |
| **61** | pH | 111.7 | 20 | 28-5-00 morning |
| **62** | pH | 109.8 | * | * |
| **63** | pH | 95.9 | * | * |
| **64** | pH | 87.3 | 3 | 11-5-00 morning |
| **65** | pH | 89.8 | * | * |
| **66** | pH | 84.3 | 18 | 26-5-00 afternoon |
| **67** | pH | 97.9 | 24 | 1-6-00 morning |
| **68** | pH | 70.3 | 7 | 15-5-00 morning |
| **69** | pH | 95.3 | 3 | 11-5-00 afternoon |
| **70** | pH | 106.9 | 18 | 26-5-00 afternoon |
| **71** | pH | 100.2 | * | * |
| **72** | pH | 108.9 | 25 | 2-6-00 morning |
| **73** | pH | 110.1 | 10 | 18-5-00 morning |
| **74** | pH | 77.1 | 34 | 11-6-00 morning |
| **75** | pH | 93.5 | 31 | 8-6-00 morning |
| **76** | pH | 123.1 | 18 | 26-5-00 afternoon |
| **77** | pH | 77.2 | 26 | 3-6-00 morning |
| **78** | pH | 76.5 | 6 | 14-5-00 morning |
| **79** | pH | 110 | 19 | 27-5-00 morning |
| **80** | pH | 98.8 | 2 | 10-5-00 midi |
| **81** | pH | 91.3 | 18 | 26-5-00 morning |
| **82** | pH | 73.4 | * | * |
| **83** | pH | 72.9 | 45 | 22-6-00 morning |
| **84** | pH | 87.2 | 49 | 26-6-00 morning |
| **85** | pH | 68.9 | * | * |
| **86** | pH | 86 | 9 | 17-5-00 midi |
| **87** | pH | 101.5 | 10 | 18-5-00 midi |
| **88** | pH | 73.9 | 39 | 16-6-00 morning |
| **89** | pH | 82.4 | * | * |
| **90** | pH | 83 | 18 | 26-5-00 afternoon |
| **91** | $O_2$ | 100.4 | 20 | 28-5-00 morning |
| **92** | $O_2$ | 81.3 | * | * |
| **93** | $O_2$ | 107.6 | * | * |
| **94** | $O_2$ | 129.4 | 45 | 22-6-00 morning |
| **95** | $O_2$ | 82.1 | 40 | 17-6-00 morning |
| **96** | $O_2$ | 95.5 | * | * |
| **97** | $O_2$ | 119.8 | * | * |
| **98** | $O_2$ | 74.6 | * | * |
| **99** | $O_2$ | 106.5 | 18 | 26-5-00 afternoon |
| **100** | $O_2$ | 74.4 | * | * |
| **101** | $O_2$ | 83.4 | 46 | 23-6-00 morning |

| number | group | total weight (g) | day of experiment | date death |
|---|---|---|---|---|
| **102** | $O_2$ | 83.8 | 6 | 14-5-00 morning |
| **103** | $O_2$ | 93.9 | 34 | 11-6-00 morning |
| **104** | $O_2$ | 108.4 | 1 | 9-5-00 afternoon |
| **105** | $O_2$ | 78.4 | * | * |
| **106** | $O_2$ | 94 | 38 | 15-6-00 morning |
| **107** | $O_2$ | 87.6 | * | * |
| **108** | $O_2$ | 87.1 | * | * |
| **109** | $O_2$ | 116.3 | 40 | 17-6-00 morning |
| **110** | $O_2$ | 105.6 | 19 | 27-5-00 morning |
| **111** | $O_2$ | 87.6 | 2 | 10-5-00 afternoon |
| **112** | $O_2$ | 82.7 | 31 | 8-6-00 morning |
| **113** | $O_2$ | 77.9 | 30 | 7-6-00 morning |
| **114** | $O_2$ | 98.9 | 34 | 11-6-00 morning |
| **115** | $O_2$ | 102.8 | * | * |
| **116** | $O_2$ | 70.6 | 41 | 18-6-00 morning |
| **117** | $O_2$ | 86 | * | * |
| **118** | $O_2$ | 84.4 | 31 | 8-6-00 morning |
| **119** | $O_2$ | 83.5 | 26 | 3-6-00 morning |
| **120** | $O_2$ | 69.6 | 26 | 3-6-00 morning |

| number | group | total weight (g) | wet meat weight (g) | dry meat weight (g) | shell weight (g) |
|---|---|---|---|---|---|
| **1** | $t_0$ | 115.5 | 15.84 | 3.596 | 68.8 |
| **2** | $t_0$ | 87.4 | 12.752 | 2.752 | 54.2 |
| **3** | $t_0$ | 96.1 | 11.382 | 2.195 | 58.3 |
| **4** | $t_0$ | 73.6 | 10.067 | 1.853 | 43.1 |
| **5** | $t_0$ | 106.3 | 10.832 | 2.718 | 72.8 |
| **6** | $t_0$ | 89.2 | 11.853 | 2.639 | 56.2 |
| **7** | $t_0$ | 141.5 | 22.699 | 5.561 | 89.7 |
| **8** | $t_0$ | 80.9 | 11.286 | 2.28 | 51.5 |
| **9** | $t_0$ | 68 | 12.3 | 2.733 | 39.5 |
| **10** | $t_0$ | 101.6 | 12.129 | 2.71 | 52.6 |
| **11** | $t_0$ | 74.1 | 9.386 | 1.9 | 49.0 |
| **12** | $t_0$ | 91.1 | 9.592 | 1.756 | 55.0 |
| **13** | $t_0$ | 75.2 | 10.539 | 2.304 | 43.3 |
| **14** | $t_0$ | 62.8 | 11.157 | 2.212 | 33.2 |
| **15** | $t_0$ | 85.4 | 9.046 | 1.83 | 51.2 |
| **16** | $t_0$ | 69.7 | 10.026 | 2.343 | 45.6 |
| **17** | $t_0$ | 91.7 | 13.255 | 2.789 | 59.3 |
| **18** | $t_0$ | 120.5 | 14.454 | 3.001 | 72.7 |
| **19** | $t_0$ | 95.4 | 13.324 | 2.877 | 54.0 |
| **20** | $t_0$ | 80 | 11.855 | 2.747 | 45.8 |
| **21** | $t_0$ | 116.9 | 13.911 | 3.04 | 72.6 |
| **22** | $t_0$ | 111 | 12.335 | 2.33 | 67.9 |
| **23** | $t_0$ | 81.9 | 12.148 | 2.253 | 44.7 |
| **24** | $t_0$ | 84.1 | 10.536 | 2.139 | 48.5 |
| **25** | $t_0$ | 92.7 | 10.113 | 2.087 | 58.5 |
| **26** | $t_0$ | 66.1 | 11.451 | 2.148 | 37.3 |
| **27** | $t_0$ | 70.7 | 10.192 | 1.858 | 38.3 |
| **28** | $t_0$ | 88.3 | 12.357 | 2.371 | 47.9 |
| **29** | $t_0$ | 121 | 13.385 | 2.151 | 68.4 |
| **30** | $t_0$ | 96.2 | 12.122 | 2.428 | 58.2 |

## Annex 12.

| number ($t_0$ group) | % lipids (per dry weight) | % carbohydrates (per dry weight) | % glycogen (per dry weight) |
|---|---|---|---|
| 1 | 13.7875993 | 18.7890316 | 17.2867396 |
| 2 | 17.5083166 | 14.0866879 | 13.2924747 |
| 3 | 16.6926041 | 7.99074897 | 6.84855586 |
| 4 | 15.6384872 | 5.37608536 | 3.73697938 |
| 5 | 17.1671622 | 11.1681634 | 11.6565903 |
| 6 | 13.8950871 | 15.3135097 | 9.76022069 |
| 7 | 17.0452804 | 17.4916678 | 14.8863996 |
| 8 | 16.6606807 | 8.58726144 | 7.36430721 |
| 9 | 17.8882959 | 8.82973043 | 7.74233953 |
| 10 | 14.6130321 | 16.5348174 | 12.6872881 |
| 11 | 14.5517873 | 8.9743939 | 6.47284461 |
| 12 | 13.1309358 | 11.1270244 | 9.53868903 |
| 13 | 14.1514581 | 15.3309552 | 11.4275006 |
| 14 | 14.1674812 | 13.0127838 | 11.1725838 |
| 15 | 10.7102476 | 9.30333641 | 6.11425194 |
| 16 | 17.0321924 | 10.9829314 | 7.40680294 |
| 17 | 12.673276 | 12.9289851 | 11.6463178 |
| 18 | 13.392091 | 18.2020151 | 17.2715558 |
| 19 | 15.7534391 | 13.0441139 | 11.5843772 |
| 20 | 20.8687806 | 13.014479 | 5.98261251 |
| 21 | 20.8591575 | 17.4825027 | 16.7642851 |
| 22 | 21.6082005 | 10.9823427 | 10.2726036 |
| 23 | 15.1498128 | 13.3840567 | 11.3974048 |
| 24 | 22.9850754 | 9.44465909 | 8.02268636 |
| 25 | 19.1564096 | 12.9534121 | 10.7553411 |
| 26 | 17.0671849 | 10.2217716 | 9.11728097 |
| 27 | 20.8972486 | 9.19561327 | 9.46697917 |
| 28 | 14.5957062 | 6.03044135 | 4.79802928 |
| 29 | 16.8667482 | 15.9533176 | 14.2236876 |
| 30 | 17.0132699 | 9.2889904 | 8.49074626 |

## Annex 13.

| number | group | tray of group | total weight at $t_0$ (g) | shell weight (g) | dry meat weight (g) |
|---|---|---|---|---|---|
| **34** | temp. | 1 | 78.2 | 48 | 3.533 |
| **64** | pH | 1 | 87.3 | 52.8 | 1.906 |
| **68** | pH | 1 | 70.3 | 44.4 | 1.704 |
| **69** | pH | 1 | 95.3 | 47.8 | 2.051 |
| **73** | pH | 2 | 110.1 | 62.5 | 2.49 |
| **78** | pH | 2 | 76.5 | 50.1 | 2.137 |
| **80** | pH | 2 | 98.8 | 51.4 | 2.173 |
| **86** | pH | 3 | 86 | 57.7 | 2.408 |
| **87** | pH | 3 | 101.5 | 53.5 | 2.641 |
| **102** | $O_2$ | 2 | 83.8 | 44.4 | 2.798 |
| **104** | $O_2$ | 2 | 108.4 | 62.3 | 2.862 |
| **111** | $O_2$ | 3 | 87.6 | 54.8 | 3.119 |

| group | tray | acc. mortality % |
|---|---|---|
| control | 1 | 0 |
| control | 2 | 0 |
| control | 3 | 0 |
| temperature | 1 | 10 |
| temperature | 2 | 0 |
| temperature | 3 | 0 |
| pH | 1 | 30 |
| pH | 2 | 30 |
| pH | 3 | 20 |
| oxygen | 1 | 0 |
| oxygen | 2 | 20 |
| oxygen | 3 | 10 |

# Annex 14.

day is day of laboratory experiment
alive means numbers of oysters alive

| day | control tray 1 alive | control tray 2 alive | control tray 3 alive | temp. tray 1 alive | temp. tray 2 alive | temp. tray 3 alive | pH tray 1 alive | pH tray 2 alive | pH tray 3 alive | oxygen tray 1 alive | oxygen tray 2 alive | oxygen tray 3 alive |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 17 | 10 | 10 | 10 | 9 | 10 | 10 | 7 | 7 | 8 | 10 | 8 | 9 |
| 18m | 10 | 9 | 10 | 9 | 10 | 10 | 7 | 7 | 7 | 10 | 8 | 9 |
| 18a | 10 | 9 | 10 | 9 | 10 | 10 | 5 | 6 | 6 | 9 | 8 | 9 |
| 19 | 10 | 9 | 10 | 8 | 10 | 10 | 5 | 5 | 6 | 9 | 7 | 9 |
| 20 | 9 | 9 | 10 | 8 | 9 | 10 | 4 | 5 | 6 | 8 | 7 | 9 |
| 21 | 9 | 9 | 10 | 8 | 9 | 10 | 4 | 5 | 6 | 8 | 7 | 9 |
| 22 | 9 | 9 | 10 | 8 | 9 | 10 | 4 | 5 | 6 | 8 | 7 | 9 |
| 23 | 9 | 8 | 10 | 8 | 9 | 10 | 4 | 5 | 6 | 8 | 7 | 9 |
| 24 | 9 | 8 | 10 | 8 | 9 | 10 | 3 | 5 | 6 | 8 | 7 | 9 |
| 25 | 9 | 8 | 10 | 8 | 9 | 10 | 3 | 4 | 6 | 8 | 7 | 9 |
| 26 | 9 | 8 | 10 | 8 | 9 | 9 | 3 | 3 | 6 | 8 | 7 | 7 |
| 27 | 9 | 8 | 10 | 8 | 9 | 9 | 3 | 3 | 6 | 8 | 7 | 7 |
| 28 | 9 | 5 | 10 | 8 | 9 | 7 | 3 | 3 | 6 | 8 | 7 | 7 |
| 29 | 9 | 5 | 10 | 8 | 9 | 7 | 3 | 3 | 6 | 8 | 7 | 7 |
| 30 | 8 | 4 | 9 | 8 | 8 | 7 | 3 | 3 | 6 | 8 | 7 | 6 |
| 31 | 8 | 4 | 9 | 8 | 8 | 7 | 3 | 2 | 6 | 8 | 7 | 4 |
| 32 | 8 | 4 | 8 | 8 | 7 | 7 | 3 | 2 | 6 | 8 | 7 | 4 |
| 33 | 8 | 4 | 8 | 8 | 7 | 7 | 3 | 2 | 6 | 8 | 7 | 4 |
| 34 | 8 | 4 | 6 | 8 | 6 | 4 | 3 | 1 | 6 | 8 | 6 | 3 |
| 35 | 8 | 4 | 6 | 8 | 6 | 4 | 3 | 1 | 6 | 8 | 6 | 3 |
| 36 | 8 | 4 | 6 | 8 | 6 | 4 | 3 | 1 | 6 | 8 | 6 | 3 |
| 37 | * | * | * | * | * | * | * | * | * | * | * | * |
| 38 | 8 | 4 | 5 | 8 | 4 | 2 | 3 | 1 | 6 | 8 | 5 | 3 |
| 39 | 8 | 4 | 5 | 8 | 4 | 2 | 3 | 1 | 5 | 8 | 5 | 3 |
| 40 | 8 | 4 | 5 | 8 | 4 | 2 | 3 | 1 | 5 | 7 | 4 | 3 |
| 41 | 8 | 4 | 5 | 8 | 4 | 2 | 3 | 1 | 5 | 7 | 4 | 2 |
| 42 | 8 | 4 | 5 | 8 | 4 | 2 | 3 | 1 | 5 | 7 | 4 | 2 |

| day | control tray 1 alive | control tray 2 alive | control tray 3 alive | temp. tray 1 alive | temp. tray 2 alive | temp. tray 3 alive | pH tray 1 alive | pH tray 2 alive | pH tray 3 alive | oxygen tray 1 alive | oxygen tray 2 alive | oxygen tray 3 alive |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **43** | 8 | 4 | 5 | 8 | 4 | 2 | 3 | 1 | 5 | 7 | 4 | 2 |
| **44** | 8 | 4 | 5 | 8 | 4 | 2 | 3 | 1 | 5 | 7 | 4 | 2 |
| **45** | 8 | 4 | 5 | 8 | 3 | 1 | 3 | 1 | 4 | 6 | 4 | 2 |
| **46** | 7 | 4 | 5 | 8 | 3 | 1 | 3 | 1 | 4 | 6 | 3 | 2 |
| **47** | 7 | 4 | 5 | 8 | 2 | 1 | 3 | 1 | 4 | 6 | 3 | 2 |
| **48** | 7 | 4 | 5 | 8 | 2 | 1 | 3 | 1 | 4 | 6 | 3 | 2 |
| **49** | 7 | 4 | 5 | 8 | 1 | 1 | 3 | 1 | 3 | 6 | 3 | 2 |
| **50** | 7 | 4 | 4 | 8 | 1 | 1 | 3 | 1 | 3 | 6 | 3 | 2 |