International Council for Exploration of the Sea

C.M.1987/F:21

A BIOTEST SYSTEM FOR OPTIMALIZATION OF ENVIRONMENTAL PARAMETERS FOR PRODUCTION OF HALIBUT FRY

by

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Abstract.

A system for controlled testing of different environmental parameters in seawater was made. Both the biotest system and an experimental setup using larvae of the Atlantic Halibut (Hippoglossus hippoglossus L.) are described.

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Introduction

The problem of successful farming of halibut has for some time been attended at several norwegian research institutions as well as at private companies. The different areas of research have been broodstock handling, egg quality, the storage period and first feeding. During the storage period, between hatching and first feeding, the larvea is assumed to remain inactive in terms of physical activity, only undergoing embryological developemnt. A very frequent occuring problem in the propagation of halibut have been the gross mortality which is observed among the larvae during the storage period that lasts for about 40 days depending on the rearing temperature. Several systems for keeping larvae have been developed, but has so far given poor results in terms of surviving fryes.
Halibut larvae are assumed to be hatched at great depts in the north sea which have very stable environmental conditions in terms of temperature and light (Haug et al., 1984). This knowledge has been adapted to the rearing systems without the desired results.

High densities of larvae produce much organic wastes from decay, and thus increase the liability for bacterial growth. In some systems bacterial infections are obviously a major cause of larval mortality (Rabben and Jelmert 1986; Rabben et al. ,1986), but this is not the only factor that have to be concidered in the work of optimazing the rearing systems. Both light and temperature conditions seems to be of vital importance (op. cit.).
Also conditions that increase physical activity among the larvae may be concidered.
At present the information available on the biologi of the halibut larvae under natural conditions is limited. However, the experience from the last years experimental work indicate that environmental conditions like light, temperature, bacterial activity and stress stimuli should be further investigated.
The aim of this study was to make a system for controlled testing of environmental parameters for rearing of halibut larvae. Four strategies were used to attack the problem connected to bacterial activity. In addition temperature was selected as environmental variable.

MATERIAL AND METHODS

Eggs and sperm were stripped from ripe specimens of the Atlantic halibut, Hippoglossus hippoglossus. Eggs, milt and water were mixed in ratio 100 : 1 : 100, respectively.
The inseminated eggs were transferred to dedicated incubators supplied with a flow-through systems based on upwelling current (Jelmert and Rabben, 1987). The incubators were regulary inspected and dead eggs removed. 2-3 days before hatching the eggs were transferred to the biotest system.

The Biotest System

The biotest system was arranged according to fig. 1. The three main vessels are separately temperature controlled by extrernal coolers.
Each temperature contolled vessel contained 16 incubators. The test incubators (Fig. 2) were made from 10 l glass bottles ("A") (bottoms removed) mounted upside down on a machined PVC-collar ("B") glued to the bottom of the vessel.
A tube of clear PVC ("D") with inner diameter 20 mm was fitted to the bottom of the collar, and penetrated the bottom of the vessel. A transparrent rubber tube were used as a link between the PVC-tube and a scintillation vial. The rubber tube could be constricted by a pair of arterial tweezers when the incubator was tended.
The incubators made a watertight fitting to the vessels at the same time as they could be drained through the bottom. Single incubators were removable without draining the vessel. The incubators were supplied with a system for air bubbling through the bottom vial (not showed at the Fig.) Air bubbling was restricted to the time before hatching.
The inside of the collars were mashined conical. This design allowed the bulk of the sedimenting material to be collected in the scintillation vial ("E").

Water supply.

Sea water was pumped from 55 m depth outside the Austevoll Marine Aquaculture Station. The water was successively filtered through cartridges of 10 um, 0.45 um and 0.2 um pore size. A time-controlled pump filled a 60 l header tank at 15-minutes intervals.
Water was continuously supplied from the header tank and injected near the bottom ("G", Fig.2) in 24 of the incubators. Water was removed through a over-flow ("F", Fig. 2) system that set the volume of each incubator to 8.0 l.
The water flow was regulated by gravity and an intravenous drop counter device. The flow could easily and accurately be contolled in the range 0 to 20 ml/min.
The biotest system was located in a separate lab in total darkness. For making visual observations in the incubators, a hand held flash-light covered with several layers of blue film were used as light source. One layer of film had a absorbtion maximum of approx. 1 absorbanc unit in the range from 600-700 nm, compared to air.
The tender had to acclimate to the low light intensity for several minutes before observations were possible.

Bacterial grazers.

The bacterial grazers were introduced to reduce the amount of bacteria growing in the incubation water and on the surface of eggs/larvae.
A nanoflagellate Paraphysomonas sp. was isolated from the seawater tubing system at the Austevoll Marine Aquaculture Station. A known non-pathogen bacteria Vibrio natriegens were

grown in 10 l glucose-limited batch cultures. This specific bacterium will not grow at temperatures below 11-13 °C (T. Lien, IMP, Univ. of Bergen, pers.comm.).
The grazer was added to the glucose-deprived bacterial culture and grown to a density of 5.7*1E5 grazers/ml. The density of V. natriegens was 8.3*1E6 in this culture. The culture was filtered trough a folded cellulose filter (Scleicher & Schüll 597 1/2) to remove aggregates of bacteria and grazers.
At day 5, 200 ml of filtered bacteria/grazer-culture was added to 4 incubators in each of the three vessels. The denstiy of grazers and bacteria in the filtrate was 2.5*1E4 cells/ml and 5.0*1E6 cells/ml, respectively.
The density of grazers in the incubators were thus 6.3*1E2 cells/ml after the culture had been supplied.

Sampling and preservation of samples.

20 ml samples were collected from the surface water in scintillation vials. A 2 ml subsample was removed for measurement of bacterial activity, and 2 ml of a saturated HgCl2-soloution was added to the rest of the sample. The samples were stored at 5°C in the dark until counted.

Bacterial activity, counting of bacteria and grazers.

Bacterial activity (Somville and Billen, 1983) was monitored in each incubator at regular intervals. Bacterial activity was measured immidately in the 2 ml subsamples (see above). Counting of microorganisms were performed by epifluorescence microscopy (Porter and Feig, 1980) of the fixed samples.
15 ml sample were stained with DAPI and collected on a pre-stained (Irgalan Black) Nuclepore filter with a negative pressure not exeeding 150 mm Hg.

Particulate organic matter.

Particulate organic carbon and nitrogen was measured on a Carlo Erba Strumentazione model 1106 CHN analyzer.
20 eggs (5*2 + 10) were dried to constant weight at 60°C.
Triplicate volumes of 20 - 200 ml of the supplied grazer culture were filtered onto precombusted Whatman GF/F glass fibre filters.
The filters were dried to constant weight in 60 °C and stored in a desicator in room temperature until analysed. In the analysis we used benzimidazol as standard.

Experimental conditions

Eggs were collected from the hatchery 2-3 days before hatching and incubated in the biotest system. The biotest set up and the lab was exposed to approx. 600 ppm ethylene oxide for 24 h. before the vessels and incubators were filled asepticaly with freshwater and seawater, respectively.

The temperature were set to 6°C in all the vessels when the eggs were introduced.

The conditions to be tested were:

1. Stagnant water + 60 ppm Oxytetracycline-HCl
2. Stagnant water + bacterial grazers
3. Flow (10 l pr. day)
4. Flow (10 l pr. day) + Oxytetracycline-HCl supplied every 7.day to an end concentration of 60 ppm.

Temperature

By the time of hatching, the temperatures in the three vessels were slowly adjusted to be 4,6 and 8 centigrades. The temperature was controlled to ± 0.1°C. The temperatures were selected according to the temperatures for the larvae in the natural biotops (Haug et al., 1984)

Stagnant systems

Groups A and D were kept stagnant throughout the experiment. In group A, bacterial grazers were administrated to check if they were able to reduce the bacterial load. Group A were kept stagnant without any additions. Both groups were supplied with a bottom layer of salter water ("salt plug"), in order to prevent the larvae from mixing with the precipitating wastes.

Circulating systems

Groups B and C were supplied with a system for continous renewal of the incubation water as indicated in fig. 2. Group B were supplied with oxytetracycline to end concentration 60 ppm every 7.day. The oxytetracycline was diluted by the inflowing water in the period between each supply.

Waterflow, current patterns.

The flow systems in group B and C were constructed to serve two functions. Firstly to reduce total bacterial number, and secondly to give the negative bouyant larvae a positive lift in the water. To achieve this the water supply tubing ("G") was inserted into the bottom vial ("E") in order to make the water current laminar.

In a pilot experiment, red dye wase mixed into the water supply of a test incubator and the movement of the dye was monitored. The water flow was laminar until the water front reached the lower part of the coned bottle neck. After this point a micro turbulent pattern were observed, and the dye

was distributed unregulary in the water coloumn.

Survival rates

Survival of larvae were regulary monitored in the incubators. Dead larvae were removed and counted at regular intervals.

Behaviour

Since the larvae are assumed to be non-active in the storage period, it was nessessary to monitor their behaviour in small water volumes with or without water currents. A parallel group of two sylinders; one stagnant, and one with upwelling water flow, were used as observation chambers for larval behaviour. A total of 5 larvae were used for each registration period. Activity were registered as swimming bursts both horizontally and verically. Each registration period lasted for 5 min.
The flow was tested for laminaerity by using red dye in the input water, and observation of ascading dye front in the sylinder.

RESULTS

Amount of carbon and nitrogen in supplied eggs and grazer culture.

The supplied eggs had a carbon content of 0.74 mg ± 0.015 mg, n=10. The CV was thus 2% and within the precicion of the method. The nitrogen content was 0.185 mg ± 0.005 mg. The CV was thus 2.8%.

The molar ratio of carbon to nitrogen was 4.66 : 1. The filtered grazer culture had a carbon content of 0.15 ugC/ml and a nitrogen content of 0.07 ugN/ml. The molar ratio of Carbon to nitrogen was 2.5 : 1.

The total amount of carbon supplied to the incubators with the grazer cultures was: 0.15 ug/ml * 200 ml = 30 ug. This is 4 % of the amount of carbon in one halibut egg (0.74 mg = 740 ug).
The total amount of nitrogen supplied to the incubators with the grazer cultures was: 0.07 ug/ml * 200 = 14 ug. This is 7.6 % of the amount of nitrogen in one halibut egg (0.185 mg = 185 ug).

The bacterial numbers (Fig. 5a; B,D and F) showed a maximum at may 19th, and a decline towards may 26th. This decline was due to grazing pressure as can be infered from Fig. 5b; B,D and F.
The bacterial numbers and activity was low in the incubators with antibiotics throughout the period (Fig. 4; C,E and G and Fig. 5b; C,E and G).

Survival rates

The mortality rates of the halibut larvae were monitored regulary as shown in fig. 3 a and b. The mortality in group A shows a steady increase until day 20 after incubation for the lowest temperature. The marked effect of temperature on survival in terms of days after incubation is seen in both the stagnant systems. 100% mortality is reached about the time of complete yolksac absorbance. When survival time in the groups A and D are recalculated from days after hatching to daydegrees after hatching, the survival times is close to similar for all three temperatures.

As a response to the flowing water in group B and C, the larva gathered in the scintillation vial in which they were trapped. This behaviour of the larvae in the circulating systems will be treated later. However, the larvae in groups B and C showed a much faster decline in survival than the larvae in group A and D.
Due to the very rapid death, bacterial numbers and activity were not monitored in these groups.

Behaviour

The observed behaviour of the larvae in group B and C in contrast to the stagnant systems in group A and D indicated that water flow or current was the promoting factor for this behaviour. A experimental setup using a transparent PVC sylinder mounted vertically as flow-through incubator was constructed to quantify the swimming activity in relation to water current. The sylinder contained 3 cm of small glass beads in the bottom over the water inlet to ensure laminar water current. The results from this experiment are shown in table 1. The data demonstrate that water current affects the swimming behavior of the larvae. It is however not clear whether the larvae swim counter current (positive rheotaxis) or just downwards in responce to water movement. The table shows that the total number of swimming bursts were higher in the stagnant systems. The quality of the bursts, however, was very different. In the stagnant systems a swimming burst very quickly ceased and the larvae then became inactive. In the system with water flow, a swimming burst was very violent and lasted for a long period of time, often bringing the larvae far from the starting point.
The values in the table are thus somewhat misleading. The total swimming activity of the larvae in the waterflow systems was much higher than in the stagnant systems.

TABLE 1: Swimming activity of halibut larvae in 2 different incubators. Flow rate was 5.5 ml/h

system	bursts	n	direction	age
Stagnant	1.3 ± 1.6	8	vertical	5
stagnant	14.6 ± 6.7	8	horizontal	5
flow	2.6 ± 2.1	8	vertical	5
flow	8.3 ± 4.9	8	horizontal	5
stagnant	2.0 ± 1.4	4	vertical	7
stagnant	22.0 ± 6.5	4	horizontal	7
flow	3.5 ± 2.8	4	vertical	7
flow	16.7 ± 6.1	4	horizontal	7

DISCUSSION

The biotest system

The biotest system proved to be a good system for several types of biotests. The temperature stability over a long period of time eliminates the problem of determinations of developalmental age in daydegrees. In terms of successful rearing of halibut larvae, the system will hopefully give further information about environmental demands. Using several separate small incubation units has the advatage of better statistical control over the conditions tested.

The water renewal system proved to be very reliable in terms of constant flow over a long period of time. In this set up, no moving mechanical parts were used, thus minimizing the risk of breakdowns. The system is also inexpensive and easy to mount compared with a traditionally dosage systems based on peristaltic pumps. The "drop-counter system" is independent of water pressure in the header tank, since each dosage unit has a separate pressure chamber.

The advantage of using bottles with coned neck as test incubators is dual. Firstly, the glass bottles may be autoclaved separately if wished. Secondly it minimizes particular organic matter sedimenting on the inner suface of the chamber. Wastes from decay is easily drained out through the bottom tubing.

Biological experiments

We find the carbon to nirogen ratio of 4.7 : 1 of the analyzed halibut eggs quite interesting. If this ratio is a general feature, the halibut eggs are rich in nirogen compared to carbon. It is likely that the bulk of an egg consists of proteins and nucleic acids, with very little energy reserves as fat or carbohydrates available. This is in accordance with the observations of death-rates of stressed

halibut larvae.
On the other hand, if this low C/N-ratio is a feature typical of the analyzed egg batch, the analysis performed could be a method for determination of egg quality. This problem deserves further study.

The survival rates monitored in group A and D demonstrate a positive effect of the bacterial grazers. This is also reflected in the data of bacterial activity (fig. 4).
The data of bacterial and grazer numbers (fig. 5 a and b) is reasonable in accordance with the measurements of bacterial activity. It should be noted that there was a shift towards seath-growing bacteria in the last sample, probably a response to the grazing pressure.

It should be noted that the data given in figs. 4,5 a and b are mean values of results from 4 incubators. The range of the data is omitted for clarity of the figures. The 4 incubators should not be considered to be parallel incubations with respect to bacterial and grazer numbers, bacterial activity and mortality.
One egg loosing its integrity will start a bacterial bloom and an eventually death due to microbial activity would be a time dependent selfcatlytic process. This could be infered from the development of mortality in some of the incubators, where an early larval death was followed by a massive mortality. These incubators were omitted when mean survival rates were calculated.

Due to the unexpected larval behaviour in groups B and C, it is impossible to compare flow-through systems with satgnant systems. However, larval rearing experiments conducted in larger conical tanks supplied with a upwelling water reneval system (Rabben et al., 1987) indicate positive effects of slow water renewal during the storage periode.

The temperature effects as demonstrated in fig. 3 is not an indication of higher performance of the larvae in the low temperatures, since the mortalities are similar in all temperatures when recalculated to daydegrees after hatching.
It can however be deduced from Fig. 3b that the effect of the combination of bacterial grazers/bacterial seem to have optimum conditions at low temperatures.

The behaviour experiments fully explained the gathering of live larvae in the bottom vial in the circulating groups (B and C). Although the total number of swimming bursts in the stagnant and circulating groups was similar, the type of activity was clearly more violent in the latter. This type of activity increased the risk of being trapped in the bottom vial which was intended to be a collector for dead material. Once the larvae get into the vial, they will be further activated by frequent collisions with the vial wall. This phenomenon was easily observed, and is suspected to be the main reason for the high mortality rates in these groups. Administration of plugs into the bottom tubing prevented the larvae from entering the bottom vials, but at that point the larvae were already dying.

The biological experiments that were carried out in the biotest system this season was limited by the end of the spawning season. Only late eggs of assumed poor quality were available for incubation in the biotest group. The incubation served however as a good pilot experiment, and several of the features of the biotest system were optimized for further experimental work.

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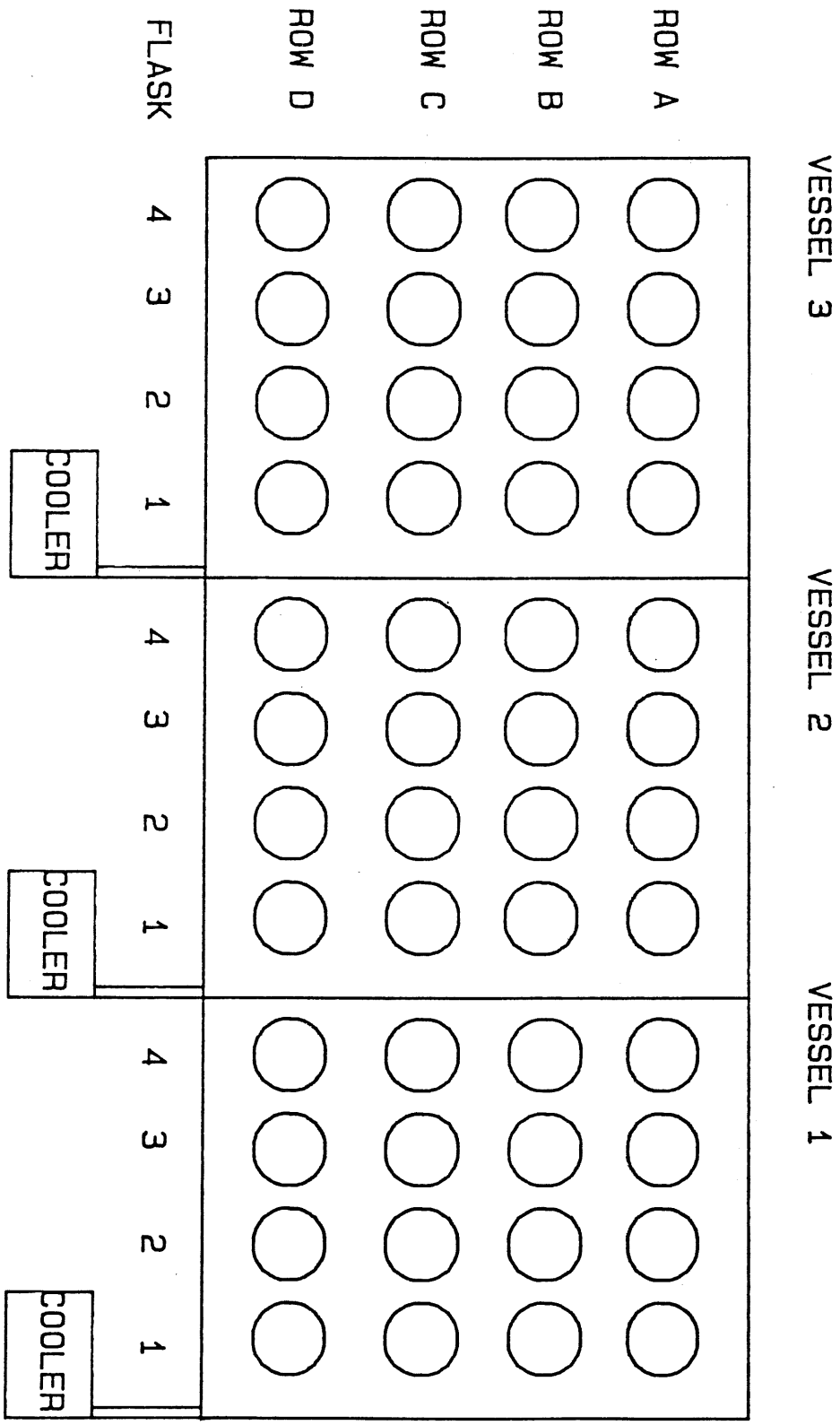


FIGURE 1. Schematic dorsal view of the biotest system. The system consists of three 500 liters temperatur controlled vessels each containing 16 incubators. The coolers were placed in a separat room to remove heat for the lab.

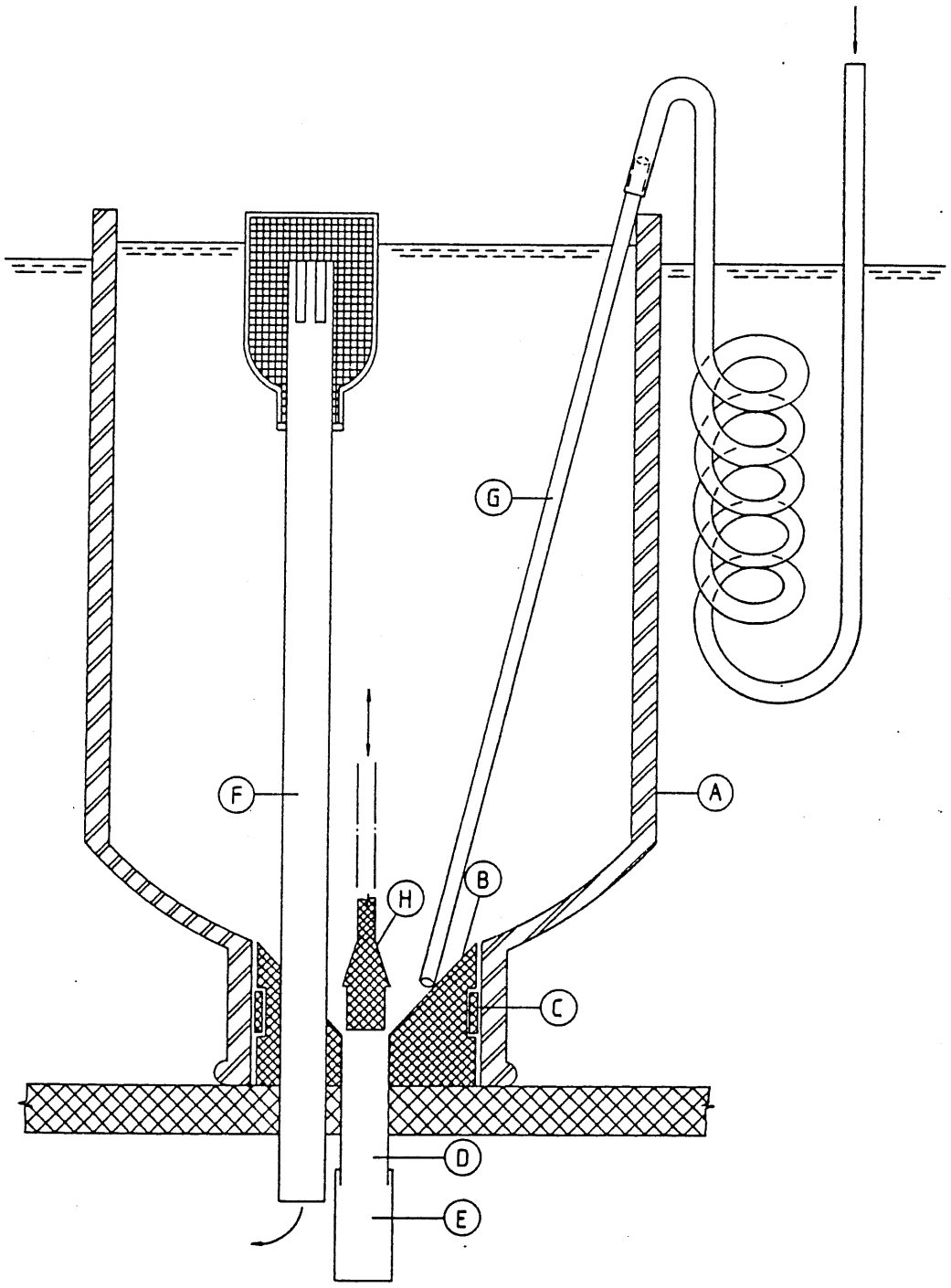
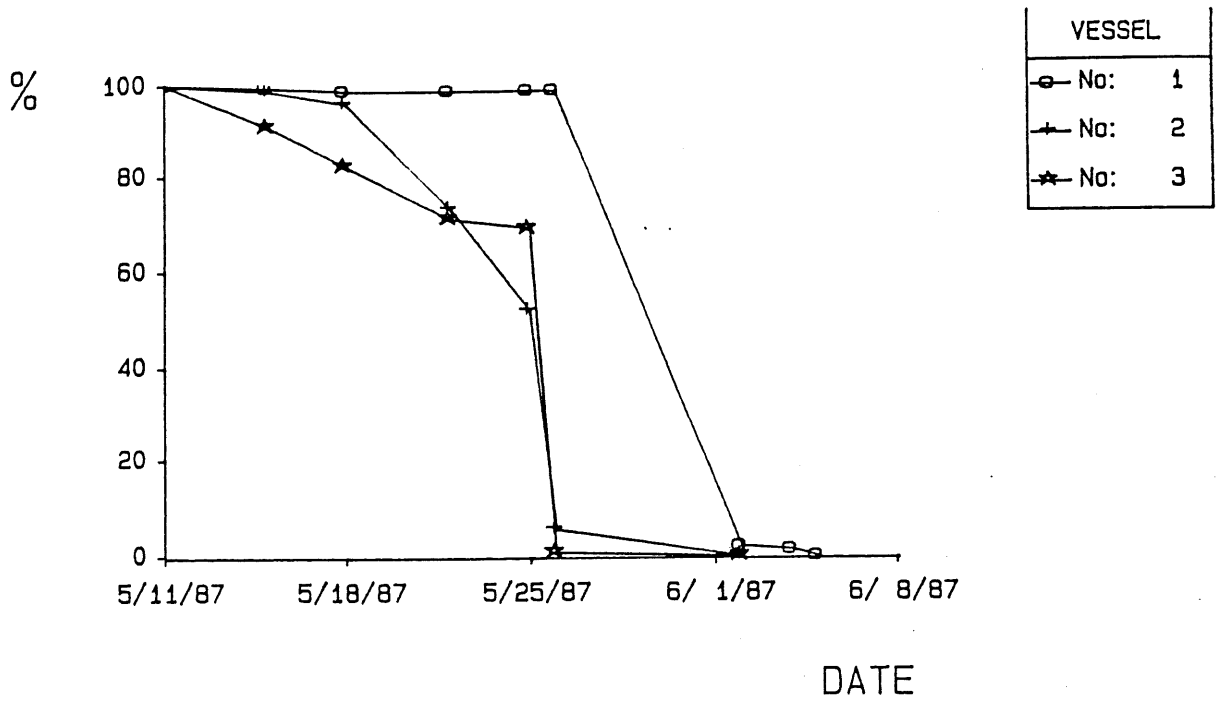


FIGURE 2. Schematic drawing of a single incubator mounted in the temperature controlled vessel. A : 10 l glass flask where the bottom is removed. B : PVC-collar that supplied with an gasket ("C") makes a water-tight fitting to the vessel bottom. D : PVC tube for incubator tending. E : scintillation vial for collection of wastes presipitating form the incubator. F : "munk" overflow system. G : Water inlet. H : PVC-plug.



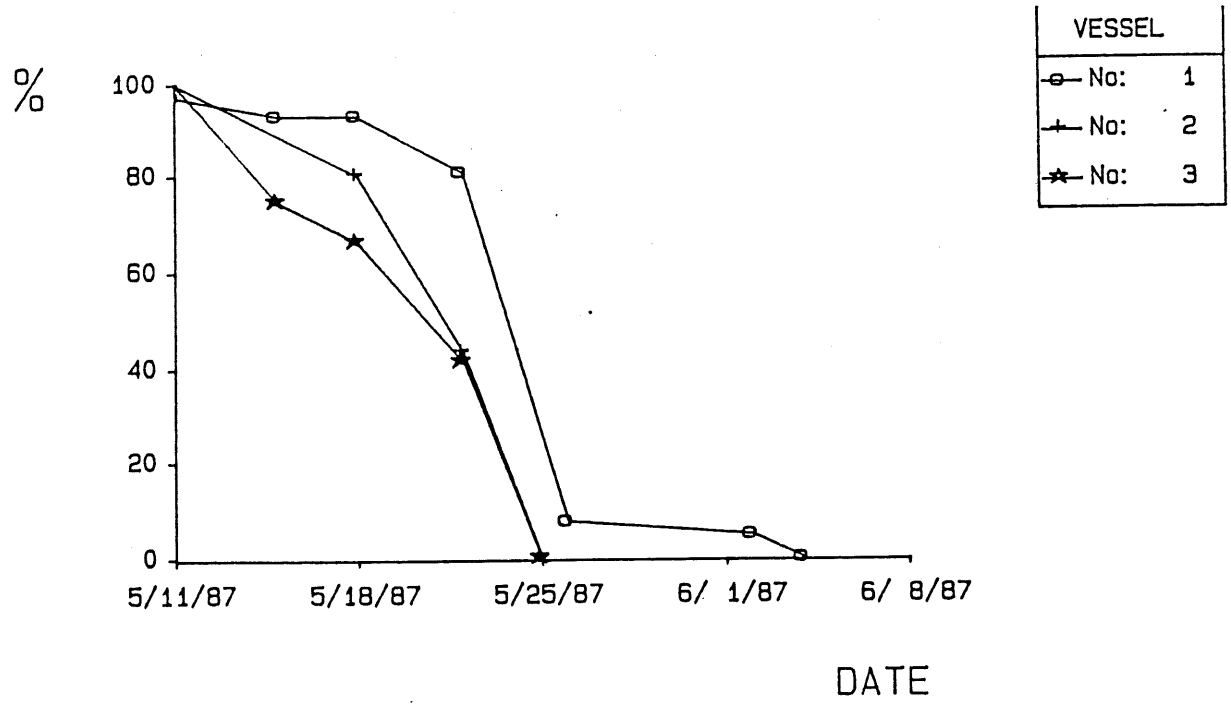


FIGURE 3. Survival rates for halibut larvae at 4, 6 and 8 centigrades incubated in stagnant sea water. In set up a) the bacterial grazer (Paraphysomonas sp.) were introduced in the incubators at day 7 after incubation. In set up b) 60 ppm oxytetracyclin was administrated to each incubator.

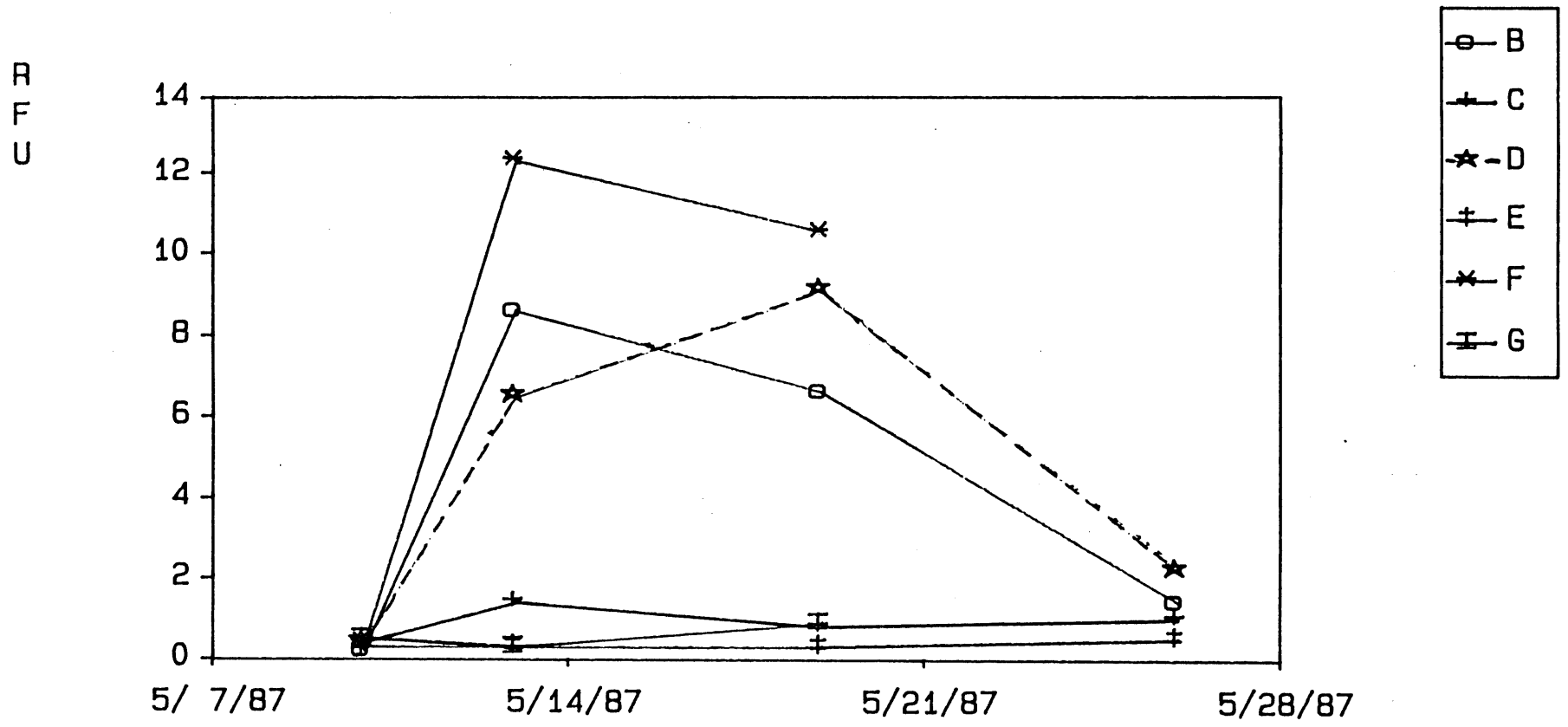


FIGURE 4. Bacterial activity in the incubators (mean). B, D and F; 4, 6 and 8°C, respectively, grazers added. C, E and G; 4, 6 and 8°C, respectively, antibiotics added.

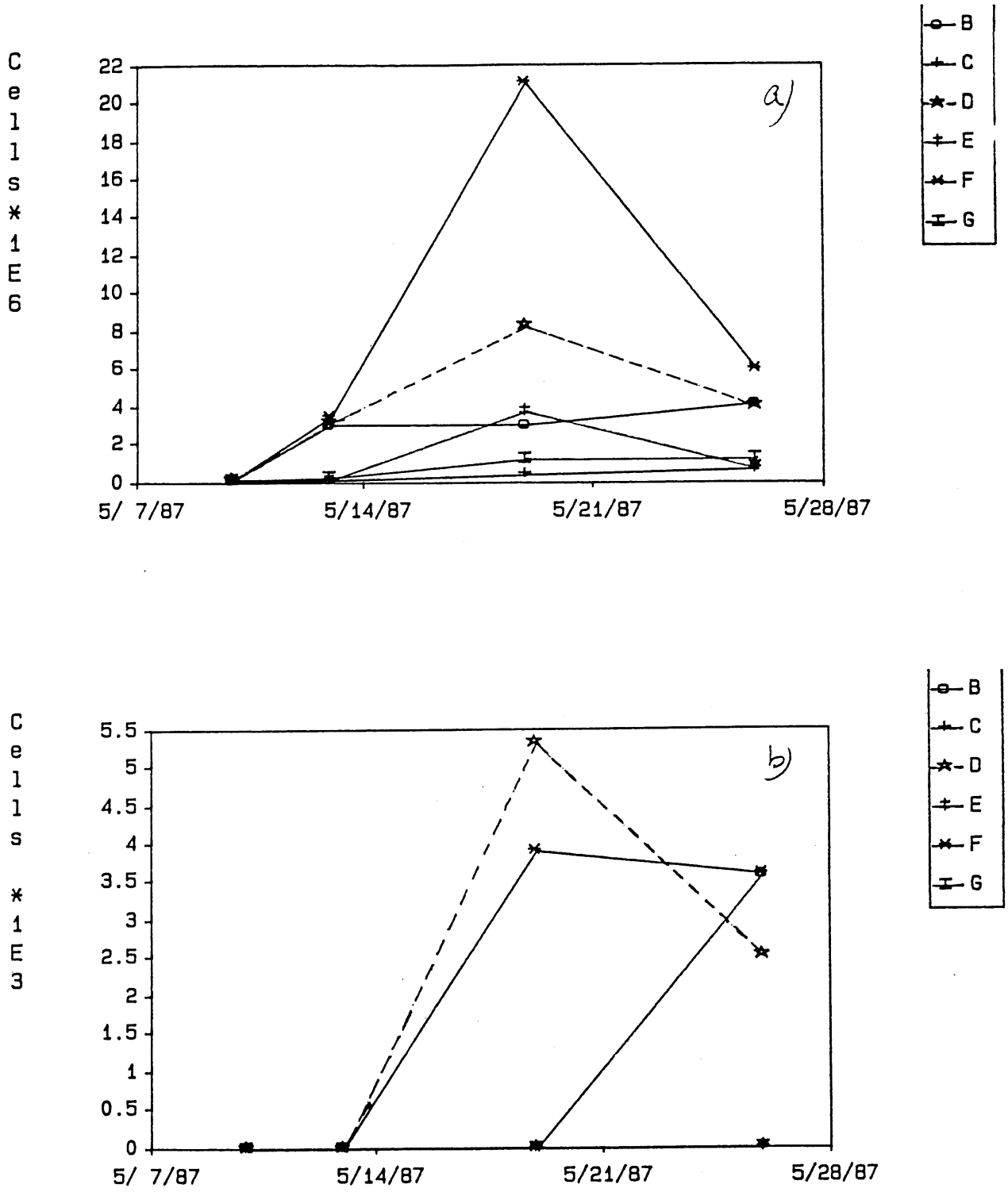


FIGURE 5. Cell numbers (mean) in incubators. Figure a) Bacterial number/ml, legends as figure 4. Figure b) Grazer number/ml, legends as figure 4.