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## A flow-through rearing system for ecophysiological studies of Pacific oyster *Crassostrea gigas* larvae

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### Abstract:

While literature is relatively abundant on adult shellfish, ecophysiological studies at the larval stage are scarce because of both technical difficulties and inadequate methodology. A tool dedicated to provide basic information for larval ecophysiology was accordingly developed. Two steps were followed: first a flow-through method of *Crassostrea gigas* larval culture was perfected during a set of experiments in which rearing systems and larval density were assessed. Then a continuous hydrobiological data recorder was adapted with modifications to comply with our experimental aim. SILO (Système d'Instrumentation des Larves en flux Ouvert) allowed the successive acquisition of hydrobiological parameters in ten 150 l larval tanks in which larvae were reared using flow-through techniques under controlled environmental conditions. An automated system enabled sequential measurement of hydrobiological parameters from one larval culture to the next. A chamber system contained several probes for measurement of temperature, salinity, pH and fluorescence. The electronic system allowed real time acquisition, storage and transfer of data. SILO was successful as a larval rearing device, reducing larval disturbance that could result from transfer and handling for measurement. It was also efficient as a tool to provide basic information for larval ecophysiology research. The effect of temperature (17, 22, 25, 27, 32°C) on growth and ingestion of microalgae was studied to test SILO. Maximum growth occurred at 27 °C ( $16 \pm 2 \mu\text{m d}^{-1}$ ) and 32 °C ( $15 \pm 3 \mu\text{m d}^{-1}$ ) whereas larvae reared at 22 and 25°C showed lower development ( $8.6 \pm 3.1$  and  $11.7 \pm 2.5 \mu\text{m d}^{-1}$ , respectively). Moreover, metamorphosis exhibited no significant difference at 27 or 32 °C with  $87.5 \pm 7.1$  and  $85.9 \pm 9.6\%$  respectively, while at 22 or 25 °C lower metamorphosis was recorded with  $55.2 \pm 8.3$  and  $57.6 \pm 9.6\%$ , respectively. The lowest temperature (17 °C) strongly inhibited ingestion whereas the highest temperature (32 °C) stimulated maximum feeding activity over the whole larval rearing period.

**Keywords:** Flow-through; *Crassostrea gigas*; Bivalve larvae; Ecophysiology; Temperature

## 1. Introduction

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The Pacific oyster (*Crassostrea gigas* Thunberg) is cultured in many countries, depending substantially on juvenile collection from the natural environment (Helm et al., 2004). However, spat production in hatcheries is increasing due to the high seasonality and variation in recruitment from year to year of this activity (Robert and Gérard, 1999). In the last decade French hatchery spat production of *C. gigas* has regularly increased from 10% to 20–25% (Ponis et al., 2006b). However, the demand for triploid oysters suddenly boosted hatchery spat production, estimated nowadays to 50% (Robert, unpublished data). Despite an indisputable know-how in commercial mollusc hatcheries, some biological aspects (e.g. control of sex ratio, estimation of gamete quality, larval feeding requirements) are still unknown because knowledge has been mainly achieved by an empirical approach. Optimisation in the hatchery process is accordingly necessary and involves a better understanding of molluscan physiological requirements. A major goal in hatchery production is to improve larval and post-larval survival by attempting to maximize larval growth and success at metamorphosis. For this reason, feeding has been considered as the most important aspect in hatchery operations focusing mainly on the type of the microalgae used as feed (Brown et al., 1998; Knuckey et al., 2002; Ponis et al., 2006a). In contrast, *C. gigas* larval rearing systems did not receive particular attention.

In the Ifremer experimental mollusc hatcheries, techniques for bivalves larval culture followed a standard practice protocol (Helm et al., 2004), involving a static system with full exchange of water, three times a week, and feeding once a day. This method employed by Rico-Villa et al. (2006) led to satisfactory results in terms of growth ( $13\text{--}14\ \mu\text{m day}^{-1}$  at  $25\ ^\circ\text{C}$  and  $5\ \text{larvae ml}^{-1}$ ) and final yield (45–55%). However, due to frequent renewal of seawater and tank cleaning, the labour demand is high. A strategy which would considerably reduce these constraints is the development of simplified larval rearing techniques using flow-through systems for bivalve larvae (Malouf and Breese, 1977; Southgate and Ito, 1998; Magnesen et al., 2006).

In addition, the improvement of rearing methods should allow a new approach for the study of various environmental factors affecting larval physiology. Indeed many studies on *C. gigas* larvae have generally been focused on assessing larval growth, development, metamorphosis and settlement by varying one or two environmental combined factors (Helm and Millican, 1977; His et al., 1989; Abdel-Hamid et al., 1992), while only limited studies have involved the quantification of physiological functions such as ingestion (Gerdes, 1983a; Baldwin and Newell, 1995) or respiration (Gerdes, 1983b; Mona et al., 1993; Gouilletquer et al., 2004). Moreover, most measurement practices in larval physiology have been based on the transfer and handling of larvae from rearing tanks to measurement chambers. This procedure can induce stress for the tested organisms and might have some repercussions on measured data. Flow-through system should provide required and constant experimental conditions with reduced larval handling.

For these reasons, a tool dedicated to provide basic information for larval ecophysiology was searched, developed and thoroughly tested at Ifremer, resulting in SILO (**S**ystème d'**I**nstrumentation des **L**arves en flux **O**uvert). This technology consisted of a flow-through rearing system allowing the maintenance of a constant flow of phytoplankton enriched seawater at desired temperature conditions, as well as, a tracking system consisting in a continuous hydrobiological parameters recorder. Moreover, SILO paid careful attention to avoid disturbance of the larvae during the whole rearing period, thus allowing the collection of valid measurements throughout larval and metamorphosis stages.

The present study describes (1) a flow-through system of *Crassostrea gigas* larval culture based on preliminary tests in which rearing device and larval stocking density were assessed, (2) an autonomous hydrobiological measurement system capable of monitoring continuously key parameters during the whole larval period ( $\pm 3$  weeks), and (3) the whole device, corresponding to the combination of both systems, to study the effects of temperature on growth and ingestion of microalgae by larvae.

## 2. Materials and methods

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All larval experiments were run on two-day-old D shaped larvae obtained from *Crassostrea gigas* broodstock maintained in cultivated area in Aber Benoit, western Brittany (France). The oysters were then conditioned at  $19^\circ\text{C}$  in the Ifremer experimental hatchery located at Argenton, Brittany (France) according to a well settled method consisting of a flow-through system enriched with phytoplankton

mixture. The daily mixed diet consisted of *Isochrysis affinis galbana* (T-ISO), *Chaetoceros gracilis* and *Skeletonema marinoi* (1:1:1 in dry weight) at a ratio of 6% of the oyster dry weight.

Six weeks later, gametes from 3 males and 6 females, obtained by stripping, were mixed on the basis of 50 spermatozoa per oocyte in 5 l beakers. Forty embryos  $\text{ml}^{-1}$  were transferred to complete development to D-stage over two days in 150-l tanks filled with 1  $\mu\text{m}$  filtered seawater at 25 °C and salinity of 34.

## 2.1. Preliminary larval rearing studies

Initial experiments were run to get basic data on larval performance (growth, survival and competence) in a flow-through rearing device. Experiment 1 consisted of comparing a static system (S) and a flow-through system (F) both in 150-l cylindrical tanks at 5 larvae  $\text{ml}^{-1}$  while Experiment 2 reported the effects of three stocking densities (5, 50 and 100 larvae  $\text{ml}^{-1}$ ) in a F rearing system on larval performance. Experimental conditions run in two replicates were similar for both rearing systems (S and F): 1  $\mu\text{m}$  filtered seawater, 25 °C, mean salinity of 34.5, aeration of 0.5  $\text{l min}^{-1}$ . Larvae were fed a bispecific diet of T-ISO and *C. calcitrans* forma *pumilum* (1:1 in cells) because this mixture has been reported to be particularly efficient (Rico-Villa et al., 2006). In the S system, larvae were fed once a day and feed quantity adjusted as the larvae grew while tanks were drained and cleaned three times a week. In the F system a continuous phytoplankton enriched seawater flow was provided at 18  $\text{l h}^{-1}$ . A submerged “banjo” sieve was attached to the outflow pipe and was cleaned daily to avoid clogging and overflowing.

Each experiment ended when  $\geq 50\%$  of the whole population as eyed larvae after draining the total tank volume. This criterion was used to estimate the percentage of morphological competence for metamorphosis. Larval size was assessed by measuring the initial and final shell length of 200 individuals using image analysis (WinMager 2.0 and Imaq Vision Builder 6.0 software for image capture and analysis, respectively). Final survival was estimated by subtracting total cumulated mortalities (number of empty shells) from the number of larvae initially stocked.

## 2.2. Flow-through rearing system design

In the Argenton hatchery, coarse seawater filtration consists of a 55  $\mu\text{m}$  polyvinyl chloride (PVC) disc filter system (Arkal Spin Klin, Israel). Before its distribution in the experimental room, incoming seawater passed through a 30-l cartridge equipped with a 1  $\mu\text{m}$  cotton bag filter and was then supplied to each outlet of experimental tanks at the appropriate temperature. Water temperature was continuously controlled by means of thermo-regulated automatic valves type EA20 (+GF+, Switzerland) connected to digital controllers type PE65 (+GF+).

A diagram of our flow-through rearing system is shown in Fig. 1a. The complete system consisted of a set of ten 150-l cylindro-conical fibreglass tanks. Each experimental tank inlet was equipped with a flowmeter type SK52 (+GF+, max. flow 100  $\text{l h}^{-1}$ ). However, when the experimental design was complete (ten 150-l tanks), it was difficult to maintain a minimum flow lower than 40  $\text{l h}^{-1}$  and accordingly a continuous seawater flow of  $0.66 \text{ l min}^{-1} \pm 0.11 \text{ l min}^{-1}$  was provided in each experimental tank (including phytoplankton supplied directly in the seawater line). This flow allowed a complete water exchange in each tank every 4 hours equivalent to  $6.4 \pm 0.5$  renewals over a 24 h period. Continuous outflow drained from the top side of the tank after passing through a totally submerged rectangular sieve attached to the outflow pipe. The sieve was fitted on both sides with a nylon mesh screen, offering a total surface area of 1050  $\text{cm}^2$ , thus reducing the suction pressure across its surface and preventing larvae being stuck against the sieve or discharged with the water flow. Moreover, mesh sieve sizes ranged from 40, 60 and 80  $\mu\text{m}$  as larvae grew throughout the experiment. The sole technical intervention consisted of daily sieve cleaning to avoid clogging and overflowing. Seawater in tanks was aerated from the main hatchery airline and was fitted to the bottom of each tank to maximise circulation of water to the whole tank. Moreover, air bubbles helped prevent larvae from sticking to the outflow sieve and reduced the settling of debris on the bottom of tanks during the rearing period, thus limiting a potential source of bacterial contamination.

Outflow from each tank discharged into a secondary tank named “reservoir” of 70-l, in which seawater was pumped from the bottom side for hydrobiological survey. Pumped seawater passed through a sieve to avoid large debris entering the measurement system. This reservoir was used to avoid any disturbance directly to larval culture when seawater was sampled for hydrobiological survey. Finally, the overflow discharged from the side of the reservoir to waste (Fig. 1a).

### 2.3. Description of hydrobiological tracking system

Seawater from reservoir was continuously pumped to the measurement system for hydrobiological survey. The measurement system, using the Ifremer MAREL technology (Blain et al., 2004), allowed the real time data acquisition, transfer and storage to a central database during the entire larval rearing period. Moreover, it enabled alternate pumping from one experimental treatment to the next with an automated pump and valve system.

This technology consisted of a hydraulic circuit equipped with temperature, salinity, fluorescence and pH sensors, all fitted in a unique measurement chamber with a volume of 2.5 l (Fig. 1b). The internal circuit and sensors were cleaned between each sampling using an automatic water pump (Jabsco PAR-MAX2, range 0 to 8.7 l min<sup>-1</sup>) and *in situ* chlorination of seawater to avoid fouling in the chamber. From each reservoir, 64 ± 2 l was sampled at a flow of 3.2 ± 0.1 l min<sup>-1</sup> (Fig. 1b) and pumping lasted 20 minutes with 19 min for measurement chamber rinsing and stabilization of measurements. Data acquisition, transfer and storage, with suppression of extreme values and averaging the three remaining values, occurred during the last minute. The sampling cycle provided a total time of 200 min for a complete cycle of 10 rearing tanks. Each tank was accordingly surveyed 6 to 7 times per day.

The electronic system for measurement and control was a multitask processor, which managed actuation of valves and pump, the real time data acquisition, transfer and storage. Intranet transmission system was used for a bi-directional communication with the measurement system at any time. Data transfer and storage to the central database, located in Ifremer Brest, occurred each hour.

The physicochemical sensors were calibrated at Ifremer, Brest, before experimentation. The salinity-temperature sensor (CLS 52, Endress+Hauser, E+H) measured conductivity (range 0 to 600 mS cm<sup>-1</sup>) and temperature of seawater (-35 to 250 °C). The pH sensor (range 6.5 to 8.5) was equipped with an electrode CPS 11 (E+H). The fluorescence sensor (Sea Point SFC fluorimeter, Oceano instrument Ltd., range 0 to 150 FFU) had an excitation wavelength of 470 nm and emission wavelength of 685 nm. Consequently fluorescence data were measured in Fluorescein Fluorescence Units (FFU).

### 2.4. Experiments with SILO

The combination of the flow-through rearing system and the measurement device resulted in a tool designed for ecophysiological studies and was named SILO (Système d'Istrumentation des Larves en flux Quvert: Fig. 2). This technology allowed the maintenance of a constant flow of phytoplankton enriched seawater and appropriate temperature conditions, as well as, a continuous hydrobiological parameters recorder for ecophysiological measurements. Moreover, by using a secondary reservoir, SILO avoided any disturbance to larval rearing tank. To assess the feasibility of SILO, a set of experiments was carried out by measuring the effects of temperature on growth, metamorphosis and ingestion of *C. gigas* larvae. Owing to technical procedures and complexity of rearing, the replications were carried out in time (June 2005 and July 2006).

Larvae were reared at a density of 30 larvae ml<sup>-1</sup> in 1 µm filtered seawater at a mean salinity of 34.5 and at 0.5 l min<sup>-1</sup> aeration. Feeding during larval and settling stages depended on larval size. It began accordingly with the supply of microalgae at 40 cells µl<sup>-1</sup> and ended with supply of 200 cells µl<sup>-1</sup>. Ration consisted of a bispecific diet (1:1 in cells) of T-ISO and *C. calcitrans* forma *pumilum* (Cp, week 1) or *C. gracilis* (Cg, from week 2). Cg was used when it suited the size of the larvae (≥120 µm). Moreover, this latter diatom was produced efficiently, in batch culture in 300-l cylinders allowing satisfactory cellular densities (4–5 millions ml<sup>-1</sup>), for its use in the flow-through rearing system with a high demand for food. Temperature was maintained and continuously regulated at five different levels: 17, 22, 25, 27 and 32°C in the same experimental room. Each rearing condition consisted of a test tank containing larvae and food and a control tank with only constant flow of enriched phytoplankton seawater, both at the desired experimental temperature. D-stage larvae, obtained 48 h after fertilization at 25 °C were then reared at the appropriate condition by progressive 2 °C step increase or decrease each 4h. Low larval mortalities (≤5%) were recorded on days 2 and 7, which corresponded for this latter to the first draining of the total test tank volume (see below).

In order to evaluate larval ingestion rate (IR), fluorescence data were analysed from pumped reservoir seawater (test and control) at each different temperature. Data collected by the fluorimeter were sent to a data acquisition system. Six to seven fluorescence recordings per day and per tank were standardized to phytoplankton density, expressed in number of cells µl<sup>-1</sup>, by means of an electronic particle counter (Multisizer III) equipped with a 100-µm aperture tube. IR was estimated following the equation:  $IR = [(C_c - C_t) * f] / nb$ , where  $C_c$  and  $C_t$  are the phytoplankton densities (number of cells µl<sup>-1</sup>) in the control and test tanks respectively,  $f$  is the water flow through each tank and  $nb$  is the number of

larvae in test tank. IR data was averaged on a daily basis and expressed in cells per larvae and per day.

In each test tank, larvae were sampled (1 ml) once a day to observe larval development throughout the experiment while three precise samples (100 µl) were made each 2-3 days to measure larval shell length at each temperature. This sampling was operated from a 5 l seawater sample collected in each larval test tank and by concentrating larvae in a 1 l graduated cylinder. The remaining larvae were put back to the test tank. Shell length data were acquired following similar techniques previously described (image analysis). Moreover, once a week, the test tank was drained to achieve total number of larvae remaining and IR was accordingly adjusted. By daily observation of larval development, the occurrence of competent larvae was precisely detected for each experimental condition. When ≥50% of the population were competent, plastic disks (15 cm diameter) were placed in test tanks and used as collectors. After four days, metamorphosis was evaluated by counting the number of remaining larvae and subtracting them from the total number of larvae initially stocked. An estimation of spat attached to collectors and tank walls was performed to confirm results.

## 2.5. Statistical analysis

Normality and homoscedasticity were initially evaluated with a Kolmogorov–Smirnov and Cochran test, respectively. For preliminary studies, mean (SD;  $n = 2$  replicate tanks per rearing system and larval stocking density, respectively) of shell length, survival and competence were analysed using one-way analysis of variance (ANOVA) while significant differences ( $P < 0.05$ ) between treatments were determined using the Scheffé's pairwise multiple comparison test (Statview 5.0). For experiments with SILO, experimental trials in time (June 2005 and July 2006) were used as replicates ( $n = 2$ ). Data of growth rate and metamorphosis were analysed using one-way ANOVA while pairwise differences between treatments were detected by Scheffé's multiple comparison test ( $P < 0.05$ ). Data expressed in percentage were transformed ( $\arcsin[\sqrt{x/100}]$ ) before statistical analysis.

## 3. Results

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### 3.1. Preliminary larval rearing studies

Initial larval shell length, on day 2, was  $81.8 \pm 0.1 \mu\text{m}$  and  $79.8 \pm 0.3 \mu\text{m}$  for experiments 1 and 2 respectively. On day 18, mean shell length in F system was  $295.8 \pm 45.0 \mu\text{m}$  and was significantly higher than in S system with  $262.5 \pm 58.4 \mu\text{m}$  (>86%: Table 1). Moreover, on day 18, competence was found to be significantly higher in F system (71% vs. 55% in S system) but survival was similar (Table 1). Lastly, a fractionation of the larval population was observed in the static system leading to size heterogeneity from the second week of rearing, which increased thereafter (CV= 12, 21, 29% on day 7, 14 and 18, respectively).

No significant differences in shell length ( $302.7 \pm 33.6 \mu\text{m}$  to  $308.1 \pm 36.2 \mu\text{m}$ ) and competence (82 to 84%) were detected at densities of 50 and 100 larvae  $\text{ml}^{-1}$  on day 16 in a flow-through system (Table 2). In contrast, larval performances recorded at 5 larvae  $\text{ml}^{-1}$  on day 16 revealed a significant lower shell length ( $296 \pm 30 \mu\text{m}$ ) and competence (72%: Table 2). Mean survival was high in all treatments (≥90%: Table 2).

### 3.2. Hydrobiological environment survey

In this study, the hydrobiological environment was controlled in experimental tanks for phytoplankton concentration and temperature. Other parameters, such as pH and salinity, were recorded but generally followed the variations of the seawater from the natural surroundings. Since environmental conditions can influence significantly the physiology of larvae, we report here the data acquisition concerning seawater temperature, salinity, pH and phytoplankton density during a complete period of larval rearing.

There were no major fluctuations in temperature in test and control tanks during 35 consecutive days and the average seawater temperature was  $31.8 \pm 0.1 \text{ }^\circ\text{C}$ ;  $27.2 \pm 0.2 \text{ }^\circ\text{C}$ ;  $25.2 \pm 0.2 \text{ }^\circ\text{C}$ ;  $22.0 \pm 0.2 \text{ }^\circ\text{C}$  and  $17.4 \pm 0.1 \text{ }^\circ\text{C}$  for 32, 27, 25, 22 and 17  $^\circ\text{C}$  desired in the experimental design. There were no

major fluctuations in salinity and pH throughout the experimental rearing period with a mean value of  $34.0 \pm 0.5$  psu and  $\text{pH} = 8.20 \pm 0.01$ .

For the fluorescence survey, data expressed in Fluorescein Fluorescence Units (FFU) were standardized and used to calculate larval ingestion rate. For example, at 32 °C fluorescence in the control tank showed increasing values, according to demand and related to larval size, up to a maximum value on day 11 followed by a decrease during metamorphosis whereas in the test tank fluorescence showed constant values (Fig. 3a). At 17 °C, fluorescence in control and test tanks showed similar values during the first two weeks; then from day 16 a clear separation occurred between values recorded in test and control tanks because of a noticeable increase of larval ingestion (Fig. 3b). Whatever the temperature, the test tanks exhibited a constant value, equivalent to 30 cells  $\mu\text{l}^{-1}$ , corresponding to the desired phytoplankton concentration during the whole rearing period. At both temperatures, fluorescence values were lower during the first six days because of a difference in diet composition based on the mixture T-ISO + *C. calcitrans* forma *pumilum* in week 1. In week 2, *C. gracilis* substituted *C. calcitrans* forma *pumilum* (Figs. 3a and b).

### 3.3. Experiments with SILO

No significant differences were detected in terms of growth rates between larvae reared at 27 and 32 °C with  $16.3 \pm 2.4$  and  $15.5 \pm 3.1$   $\mu\text{m d}^{-1}$  respectively (Table 3). Metamorphosis followed a similar trend with  $87.5 \pm 7.1$  and  $85.9 \pm 9.6\%$  respectively (Table 3). Moreover, metamorphosis occurred in less than 15 days with the first competent larvae detected on day 11. Compared to the higher temperatures, larvae reared at 22 and 25 °C exhibited significantly lower growth rates ( $8.6 \pm 3.1$  and  $11.7 \pm 2.5$   $\mu\text{m d}^{-1}$ , respectively) and metamorphosis ( $55.2 \pm 8.3$  and  $57.6 \pm 9.6\%$ , respectively: Table 3). While no differences in growth rates were found at 22 and 25 °C, metamorphosis was delayed at 22 °C on day 25 (vs. day 20 at 25°C). At 17 °C, larvae showed significantly lower growth rates ( $4.8 \pm 1.5$   $\mu\text{m d}^{-1}$ ) resulting in low metamorphosis ( $15.8 \pm 2.6\%$ ) on day 35 (Table 3) despite high survival (70%).

Algae ingestion by larvae increased with temperature and three phases for larvae reared between 22 to 32 °C can be distinguished (Fig.4). At 27 and 32 °C, a slight consumption (4 000 to 20 000 cells per larvae per day) occurred during the first week followed by a sharp increase up to day 11 with values as high as 45 000 to 65 000 cells followed by a decrease ranging from 25 000 to 35 000 cells during metamorphosis. Larval ingestion at 22 and 25 °C showed similar response with 4 000 to 8 000 cells per day consumed during the first week, followed by an increased uptake of 35 000 cells up to day 11 with a small decrease on day 12 and recovery soon after. At 17 °C larvae exhibited poor ingestion over the whole rearing period with a consumption ranging from 1 000 to 5 000 cells per day (Fig. 4).

## 4. Discussion

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The dedicated tool described in the present paper was designed to provide information for larval ecophysiology study. This system, which included a flow-through rearing system coupled to a tracking device, was developed over the course of three years. A number of preliminary studies were conducted to get basic data on flow-through rearing system (e.g. water flow and circulation, mesh size sieve and constant supply of algal food).

Significant growth and competence improvements were obtained in the flow-through system compared to static system. The lower larval performances recorded in the latter should be related to underfeeding because no adjustment of phytoplankton supply was made at night, larvae being fed once a day in the morning. Moreover, in the static system a fractionation of the larval population was observed from the second week of rearing with smaller larvae, which may be inhibited by larger ones. This heterogeneity, increasing with time, may be related to the way the larvae were fed. In the static system food became limited quite rapidly after some hours (Robert, unpublished data) and there was probably a trophic competition between larvae inducing such intra population size differences. On the other hand, the use of a flow-through system with a constant water exchange and a daily cleaning of the sieve facilitated the circulation in the whole tank and removal of catabolic products promoting favourable conditions for larval development. Moreover, as recommended in the literature (Southgate and Ito, 1998; Magnesen et al., 2006; Sarkis et al., 2006) extreme care was taken in maintaining a constant flow of phytoplankton over the course of the rearing to ensure an optimal food provision to larvae. This led to rapid shell growth and were associated with an earlier and homogenous metamorphosis for the whole larval population.

According to the present study, it was clearly demonstrated that high stocking densities can be used in the larval culture of *C. gigas*. In contrast to standard static methods used in our laboratory, with densities of 1 to 10 larvae  $\text{mL}^{-1}$ , it became possible to initiate larval cultures with stocking densities of up to 100 larvae  $\text{mL}^{-1}$ . This was possible because of the continuous renewal strategy, which maintained constant quality of water in cultures by eliminating larval metabolites and allowed permanent food supply. Nevertheless, the significant performance improvement recorded here in the flow-through system with high stocking densities contrast with those reported by Malouf and Breese (1977) for *C. virginica* larvae, Andersen et al. (2000) and Sarkis et al. (2006) for scallop larvae (*Pecten maximus* and *Argopecten gibbus*). These authors found that an increase in larval stocking density resulted in inhibitory effect on growth and competence, which may be explained by low water flow renewal and insufficient food ration in their rearing systems. For *C. gigas* larval densities may undoubtedly be increased to 300 larvae  $\text{mL}^{-1}$  because preliminary trials run during two weeks in our laboratory showed similar growth performances compared to 5 larvae  $\text{mL}^{-1}$  (14.2 vs. 14.5  $\mu\text{m d}^{-1}$ ). Nevertheless, the definition of optimal larval stocking density in such flow-through rearing system was not the aim of the present contribution but sufficient biomass was needed to induce phytoplankton consumption detectable by the fluorescence probe. The present sets of data underline however the interest of flow through rearing system concept for commercial hatcheries.

Results from preliminary flow-through larval rearing led us to select 150 l as a reference volume at a density of 30 larvae  $\text{mL}^{-1}$ . This density was chosen as a good compromise between high larval performances, sufficient larval biomass to allow interpretable ecophysiology data recording and phytoplankton availability in the hatchery. Thus to allow a set of recording in SILO, a daily production of 100–150 l phytoplankton was needed. On the other hand, these initial results reported that a flow rate  $\geq 20 \text{ l h}^{-1}$  was suitable, and 40  $\text{l h}^{-1}$  was chosen to prevent a draining of the compact pump and accordingly the loss of data during the continuous process of acquisition.

In its present form, the application of an automated hydrobiological data recorder system led to good results from the managerial and biological point of view. This device showed that the five seawater temperatures desired were effectively constant throughout the whole rearing period thus maintaining stable experimental conditions. Similarly, fluorescence data were consistent with measurements made with an electronic particle coulter counter (Multisizer III) to evaluate the recorder system accuracy and to standardize the required units for larval ingestion. Another feature of the system was the chlorination *in situ* which removed fouling organisms, resulting in clean equipment and stable measurements during the complete hydrobiological survey experiments.

This work showed the tolerance of *C. gigas* larvae to temperature in a range of 17 to 32 °C during its whole larval development (from D-stage to young post-larvae). Optimal larval growth and metamorphosis were recorded at highest temperatures, which is in agreement with previous observations in the literature (Helm and Millican, 1977; Henderson, 1982; His et al., 1989). However, larval production is currently run at 25–28°C in most commercial hatcheries. In these conditions, satisfying larval performances are generally achieved but an improvement in larval stocking density and larval cycle production turnover might be expected when using a flow through larval rearing system.

Grazing results allowed clear identification of three periods previously reported for *C. gigas* larvae (Gerdes, 1983a; Rico-Villa et al., 2006). In summary, a low but progressive consumption was noted from D-stage veliger to early hinged larvae (young umboned larvae) when larval metabolism is partially sustained by reserves (His and Seaman, 1992; Laing and Earl, 1998). During the second phase, microalgae ingestion increased sharply from hinged to eyed competent larvae, until a sudden decrease was noted prior to metamorphosis. These three periods were clearly distinguishable for larvae reared at 27 and 32 °C leading to a total rearing period of 15 days. In contrast, at 22 and 25 °C larvae exhibited the first two periods but on day 12 a lesser decrease occurred. This brief reduction may be related to a transient poor quality of microalgae. Indeed, feeding activity at both temperatures recovered the next day and allowed to continue larval development and achieve high metamorphosis (>50%). In the meantime, ingestion also decreased at 27 and 32 °C but this event interfered with the decrease of ingestion occurring prior to metamorphosis without affecting final yield (>85%).

Increased ingestion rate at higher temperatures suggests a biological strategy for larvae to satisfy energetic requirements (Newell and Branch, 1980) and appears to be related to an increase in cilia beat for feeding and a high metabolic regulation (Loosanoff and Davis, 1963; Bayne and Newell, 1983; Beiras et al., 1994). On the other hand, at the lowest temperature (17 °C) larvae showed a constant but weak ingestion resulting in slow growth despite food availability. The incapability of larvae to grow at 17°C could be due to inability to assimilated ingested food at low temperature (Manoj and Appukuttan, 2003). However, larvae were quite tolerant because high survival (70%) was recorded on

day 35 while only 15% of metamorphosis was achieved. Therefore, 17 °C could be considered as an inadequate temperature from a commercial standpoint.

The use of the flow-through rearing method is beneficial because this technique reduces labour demand, allows a high larval stocking density, reduces space requirements, eases larval handling, and improves larval development and metamorphosis. It was also demonstrated that its assemblage with a tracking system allows an efficient and continuous hydrobiological survey providing basic information for larval ecophysiology study in terms of growth, survival, competence, metamorphosis and ingestion of *C. gigas* larvae under different environmental parameters. Moreover, the relatively high sampling frequency gives confidence to the ingestion measurements reported by such a device.

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## Tables

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**Table 1**

Effects of rearing system (Experiment 1) on mean (SD;  $n = 2$ ) shell length, survival and competence on day 18. Larvae were reared in a flow-through (F) or standard static (S) techniques.

Rearing system	Shell length ( $\mu\text{m}$ )	4.1. Survival (%)	Morphological competence (%)
F	295.8 (45.0) <sup>a</sup>	85.7 (2.2) <sup>a</sup>	70.7 (3.2) <sup>a</sup>
S	262.5 (58.4) <sup>b</sup>	88.7 (1.1) <sup>a</sup>	54.5 (7.3) <sup>b</sup>

Values within the same column with a common superscript letter are not significant at  $P > 0.05$ .

**Table 2**

Effects of larval stocking density in a 150 l flow-through rearing system (Experiment 2) on mean (SD;  $n = 2$ ) shell length, survival and competence on day 16.

Larval density (ml <sup>-1</sup> )	Shell length (µm)	4.2. Survival (%)	Morphological competence (%)
5	296.0 (30.0) <sup>a</sup>	92.3 (1.3) <sup>a</sup>	72.2 (4.3) <sup>a</sup>
50	308.1 (36.2) <sup>b</sup>	92.8 (2.1) <sup>a</sup>	84.5 (5.3) <sup>b</sup>
100	302.7 (33.6) <sup>b</sup>	90.4 (2.0) <sup>a</sup>	82.7 (4.2) <sup>b</sup>

Values within the same column with a common superscript letter are not significant at  $P > 0.05$ .

**Table 3**

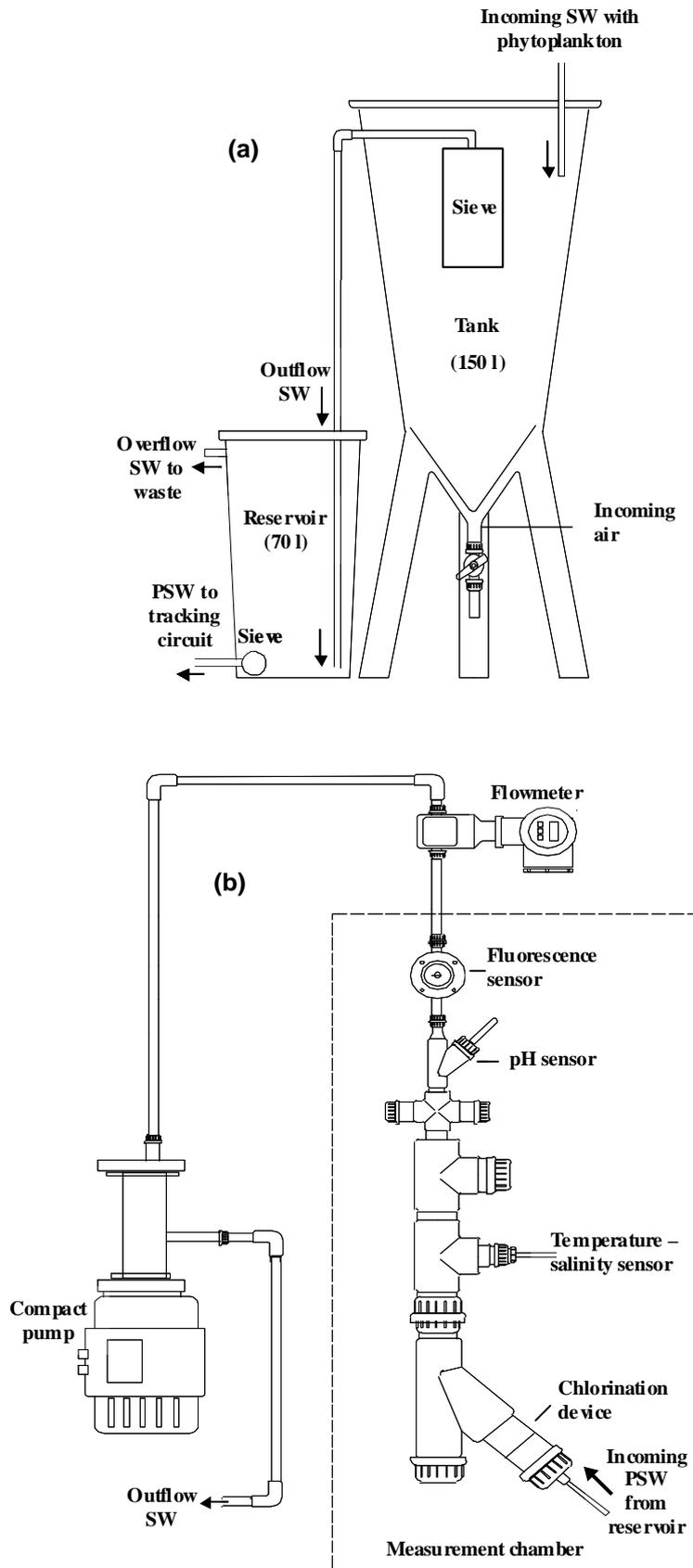
Effects of temperature on growth and metamorphosis (mean ± SD;  $n = 2$  replicate experimental trials in time) of larvae reared in SILO. Day of occurrence of competent larvae is reported for each temperature condition.

Temperature (°C)	Growth (µm d <sup>-1</sup> )	Occurrence of competence (day)	Metamorphosis (%)
17	4.81 (1.53) <sup>a</sup>	31	15.88 (2.60) <sup>a</sup>
22	8.64 (2.97) <sup>b</sup>	21	55.20 (8.30) <sup>b</sup>
25	11.73 (2.48) <sup>b</sup>	16	57.60 (9.60) <sup>b</sup>
27	16.26 (2.37) <sup>c</sup>	11	87.45 (7.11) <sup>c</sup>
32	15.50 (3.11) <sup>c</sup>	11	85.88 (9.60) <sup>c</sup>

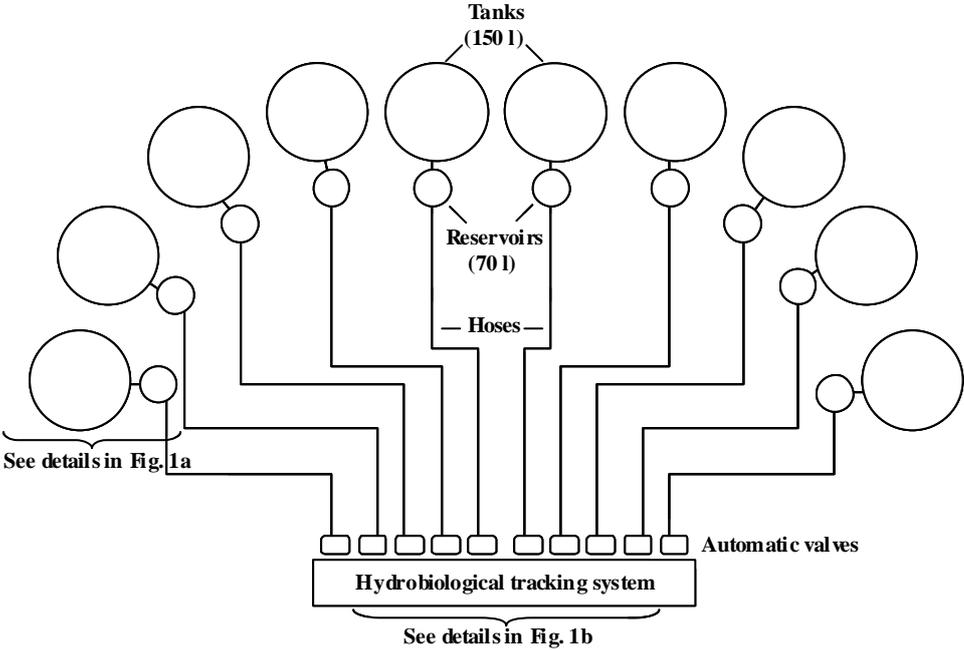
Values within the same column with a common superscript letter are not significant at  $P > 0.05$ .

## Figures

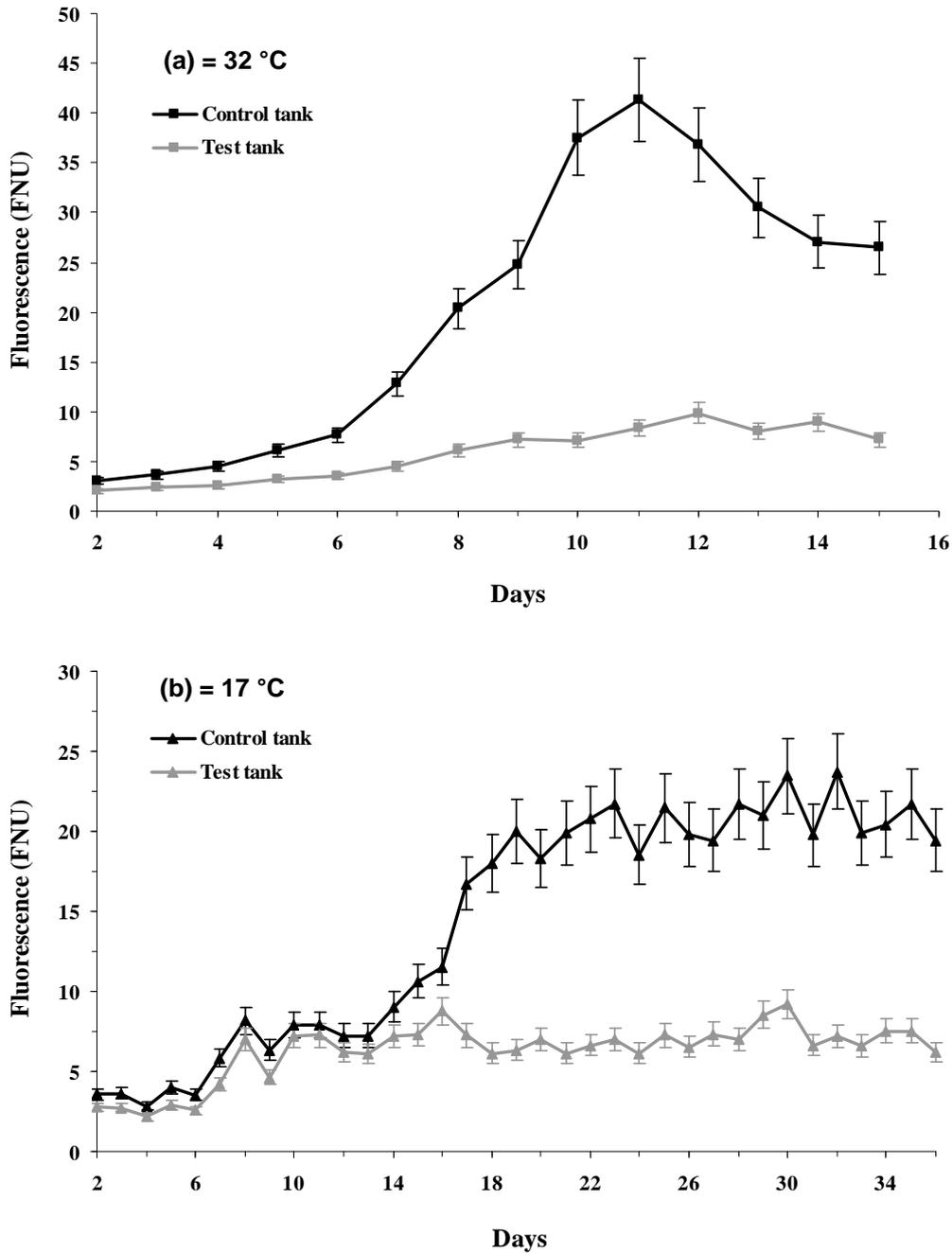
**Fig. 1.** Details of: **(a)** flow-through rearing system and **(b)** hydrobiological tracking system. SW = seawater; PSW = pumped seawater.



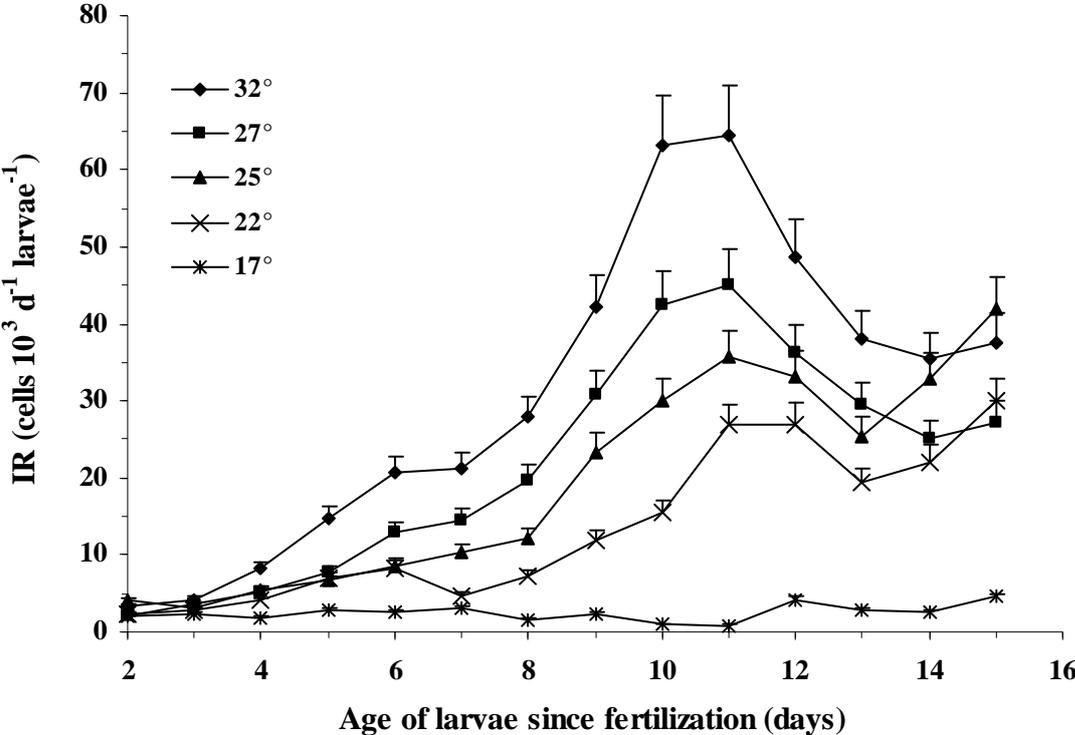
**Fig. 2.** Overview of SILO in the experimental room: ten tanks (150 l) with ten reservoirs (70 l) connected to measurement tracking system for hydrobiological parameters survey.



**Fig. 3.** Fluorescence data (Fluorescein Fluorescence Units, FFU) recorded in SILO at 32 °C **(a)** and 17 °C **(b)** in control tanks (black line) and test tanks (grey line) during the larval rearing period. Each point represents a daily mean  $\pm$  SD ( $n = 6-7$  data). Note that y-axis scale ranged from 0 to 50 for **(a)** while it ranged from 0 to 25 for **(b)**. Note also that the x-axis (time) corresponds to a longer rearing period at 17 °C (35 days vs. 15 days at 32 °C).



**Fig. 4.** Ingestion rate (IR) expressed in cells  $10^3$  per day per larvae reared at five different temperatures through the whole larval life. Each point represents daily mean + SD ( $n = 6-7$  data) at each temperature.



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