

# **Evaluation of freeze-dried microalgal diets for the seed culture of *Ruditapes decussatus* using physiological and biochemical parameters**

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## **Abstract**

This study evaluates the nutritional value of several species of freeze-dried microalgae used to feed the seed of *Ruditapes decussatus*. These microalgae were: *Isochrysis galbana*, clone T-ISO; *Tetraselmis suecica*; and *Phaeodactylum tricornutum*. The addition of antibiotics, kaolin and fresh microalgae to the freeze-dried diet was also evaluated, and a study was made of the nutritional value of one species of microalga in each of the four stages of preparation of the freeze-dried diet: culture; concentration; freezing and freeze-drying. Both physiological and biochemical criteria were used to evaluate the different diets: availability; acceptability; digestibility; and biochemical composition.

The highest seed growth rate were achieved with a diet of *Isochrysis*, followed by *Tetraselmis*, whilst the growth rates achieved with a dried diet of *Phaeodactylum* were much lower. The differences observed between diets of *Isochrysis* and *Tetraselmis* were due to a greater ingestion of the cells of *Isochrysis*. The minimal nutritional value of *Phaeodactylum* appears to be related to the low digestibility of its cell wall. The growth rates of seed fed on a diet of dried microalgae were significantly lower than those achieved with fresh diets (Albentosa et al., 1996b) for each of the species of microalgae that were tested, and this appears to be related to the lower digestibility of the microalgae after they have been freeze-dried. The addition of antibiotic or kaolin to the freeze-dried diet did not result in a major increase of seed growth rates. The substitution of a small proportion of freeze-dried microalgae with fresh microalgae produced a significant increase in seed growth rates, although these were still lower than those obtained with a completely fresh diet. The loss of nutritional value of microalgae when

they are freeze-dried may be due to some kind of process which occurs during freeze-drying, altering the digestibility of the microalgae.

Keywords: Freeze-dried microalgae; Seed: *Ruditapes decussatus*; Growth

## 1. Introduction

The availability of alternative diets to fresh phytoplankton for the culture of bivalves would be a major step forward in the development of commercial hatcheries (Coutteau and Sorgeloos, 1992). The search for an inert food has mainly been centred around various species of dried microalgae which have been cultured heterotrophically (Laing, 1989); yeasts (Epifanio, 1979; Coutteau et al., 1994); microcapsules of different kinds (Langdon, 1983) or bacterial proteins (Nell and Wisely, 1983, 1984; Doulliet and Langdon, 1993, 1994). A total substitution of fresh phytoplankton by any of these different foods has not as yet been achieved. At present partial substitutions of between 40-50% of the fresh diet have been achieved, according to the different authors.

The necessary requisites of an alternative diet to fresh phytoplankton for bivalves are as follows: an appropriate particle size for the filtration mechanism of these animals; stability in the culture system; ease of digestibility; zero toxicity; and a biochemical composition that covers all the nutritional needs of these animals (Laing, 1989). In accordance with these criteria, the low nutritional value of these diets has been variously related to the deterioration of the water in the culture due to the proliferation of bacteria caused by these diets (Urban and Langdon, 1984); a lower stability of the culture system due to higher rates of sedimentation than those produced by fresh phytoplankton (Foe and Knight, 1986); low acceptability (Urban and Kirchman, 1992); poor digestibility (Epifanio, 1979; Nell, 1985); or deficiencies in their biochemical composition (Doulliet and Langdon, 1993).

The objective of the present study was to evaluate the use of inert diets composed of microalgae which have been cultured autotrophically and then freeze-dried, i.e. diets that are identical to fresh diets in size, shape and biochemical composition and differ only in their inert nature, for the seed culture of the little-neck clam, *Ruditapes decussatus*. Physiological parameters (ingestion, absorption and growth) and

biochemical parameters (biochemical composition of the diets and the seed) were used to evaluate the different diets. Furthermore, a comparison was made between the results obtained with these inert microalgae diets and those obtained with fresh diets of the same microalgae. The latter have been published in a separate article (Albentosa et al., 1996b).

## 2. Material and methods

### 2.1. Experimental conditions

#### 2.1.1. Experiment 1

This experiment was designed to ascertain the most appropriate daily food ration in diets of inert microalgae. The microalga used in this experiment was the clone T-ISO of *Isochrysis galbana*. The rations fed, expressed in organic matter, were 2% (the optimal ration for seed of *R. decussatus* fed fresh microalgal diets, Albentosa et al., 1996a); 4% and 6% of the live weight of the seed.

#### 2.1.2. Experiment 2

This study evaluated the nutritional quality of different species of microalgae: *I. galbana*, clone T-ISO; *Tetraselmis suecica* and *Phaeodactylum tricornutum*, when fed to the seed after undergoing a freeze-drying process.

#### 2.1.3. Experiment 3

The effect of the addition of an antibiotic (cloramphenicol,  $8 \text{ mg l}^{-1}$ ) or kaolin to the seed cultures fed a freeze-dried diet was studied (a). In addition, the substitution of a small proportion of the daily freeze-dried diet (20%) with fresh microalgae was evaluated. Both experiments were carried out with the microalga *I. galbana*, clone T-ISO (b).

#### 2.1.4. Experiment 4

This study analyzed the effect of each of the different stages in the preparation of the freeze-dried diet on the nutritional value of the microalgae. The diets tested were fresh, concentrated, frozen and freeze-dried microalgae, using the species *Isochrysis galbana*, clone T-ISO.

## 2.2. Seed

The seed used in the experiments was obtained from broodstock conditioned at the Instituto Español de Oceanografía. Both spawning induction and larval culture were carried out according to the methodology described for this species by Pérez Camacho et al. (1977).

Initial seed size was less than 1.5 mm, with slight differences in each experiment. Table 1 shows the initial characteristics (size, live and dried weight and organic content) of the seed used in each experiment.

The seed was randomly distributed between the different experimental cultures. The cultures were grown in plastic vessels with a maximum capacity of 6 l, and the seed was placed on the bottom of the vessel. The culture vessels were lightly aerated in order to minimize the sedimentation rates of the diets. The daily ration of each diet was 2%, the optimum diet according to the results of Experiment 1. The initial biomass placed in each culture was calculated in such a way that the initial concentration of food after adding the daily ration would be less than 2 mg of organic matter l<sup>-1</sup> (less than 100 T-ISO, or 10 *Tetraselmis* or 100 *Phaeodactylum* cells μl<sup>-1</sup>), below the threshold of production of pseudofaeces for this bivalve (Albentosa et al., 1996a). In Experiment 1, the initial biomass was lower for the higher rations in order to keep the initial food concentration at the same level. Each diet was tested in triplicate and a fourth culture without animals was also used to determine the availability of food in the culture system, using the sedimentation rate as a basis for calculation.

## 2.3. Diets

The microalgae were cultured in 6 l glass jars inside an isothermic room maintained at a constant temperature of 18°C and with permanent lighting at 9900 lux. Salinity was

maintained at a constant 33%. The culture medium used was that described by Walne (1966). The microalgae were harvested at the beginning of the stationary growth phase.

The concentrated diets were obtained by passing the harvested microalgae through a centrifuge. The resulting concentrate was re-suspended in seawater and maintained at a temperature of 5°C for a maximum period of 10 days. The concentrated microalgae were observed daily through a microscope to check their mobility. The frozen diets were obtained from cultured microalgae that had been concentrated in a centrifuge and then frozen at -30°C. The freeze-dried diets were obtained in the same manner as the frozen ones, and were then subsequently freeze-dried. The inert microalgae-frozen and freeze-dried-were re-suspended in seawater by a process of agitation just before being added to the seed culture.

The dry weight and organic content of the fresh phytoplankton cells-in suspension and in concentrate-and that of the inert cells-frozen and freeze-dried-were calculated by filtration through Whatman GF/C glass-fibre filters that had been previously ashed. The filters were washed in a solution of 0.5 M ammonium formate and were then dried to a constant weight at 100°C and ashed at 450°C.

Samples of each of the diets administered during each experimental period were collected and subjected to biochemical analysis. Samples of the microalgal cultures, the concentrates and suspensions obtained from the frozen and freeze-dried products were concentrated in a centrifuge, washed with a solution of 0.5 M ammonium formate and stored at -30°C until they were freeze-dried. These freeze-dried samples were then stored at -30°C.

#### 2.4. Parameters for assessing the diets

Daily ingestion was calculated from the number of cells cleared in each experimental culture after each feeding period (24 h) by means of the following expression:  $I = V/n[C_{e0} - (C_{e0} * Sed) - C_{ef}]$ , where I is the ingestion expressed in cells ind<sup>-1</sup> day<sup>-1</sup>; V is the volume of the seed culture (ml); n is the number of clams, and Sed is the sedimentation rate which was calculated as:  $(C_{b0} - C_{bf})/C_{b0}$ . Initial and final

concentrations of the control culture (without animals) were noted as  $C_{bo}$  and  $C_{bf}$ , and initial and final concentrations of the experimental cultures as  $C_{eo}$  and  $C_{ef}$ , respectively.

Increases in live, dry (100°C, 24 h) and ash-free dry weight (450°C, 24 h) for each experimental culture were registered weekly, and at the end of the 4 week experimental period length of the seed were also determined. In Experiment 2, only live weight was checked weekly, meanwhile dry, ash-free dry weight and length were only measured at the beginning and at the end of the experiment. After these measurements, the remainder of the seed was used to perform the biochemical analyses.

Gross growth efficiency ( $K_1$ ) was taken to be the proportion of organic matter ingested which is used in growth, according to the equation  $K_1 = G/I$ , where  $G$  is the increase in organic matter in the seed over a period of time and  $I$  is the quantity of organic matter ingested in the same period. Net growth efficiency ( $K_2$ ) was estimated as the proportion of organic matter absorbed, since they are sexually immature animals the organic matter absorbed only includes the organic matter used in growth and that consumed in the respiration process, which is incorporated as organic matter in the seed, that is to say,  $K_2 = G/(G + R)$ , where  $R$  is the organic matter equivalent to oxygen consumption by the seed due to respiration. We considered the same respiration rate for all treatments,  $2.45 \mu\text{g O}_2 (\text{mg seed AFDW})^{-1} \text{h}^{-1}$ , data obtained in our laboratory with seed of the same species and same size (Albentosa et al., 1996a). The following conversion factors were used: 1 mg  $\text{O}_2$  equivalent to 0.6998 ml  $\text{O}_2$  (Ansell, 1973) and 1 mg AFDW equivalent to 1.2 ml  $\text{O}_2$  (Walne, 1965, cited by Laing and Millican, 1986). Absorption efficiency was estimated as the proportion of ingested organic matter which is absorbed, following the equation  $AE = (G + R)/I$ .

## 2.5. Analytical methods

Proteins were determined by the method of Lowry et al. (1951) after alkaline hydrolysis with 0.5 N NaOH for 24 h at 30°C. Total carbohydrate was quantified as glucose by means of the phenol-sulphuric method (Strickland and Parsons, 1968). Lipids were extracted by a modification of the Bligh and Dyer method (Bligh and Dyer, 1959; Fernandez-Reiriz et al., 1989): lipid material was extracted by means of chloroform-methanol (1:2); after centrifugation, the sediment was extracted again with

chloroform methanol (2:1). In order to purify the extract, both supernatants were washed with a mixture of chloroform, methanol and water (8:4:3), following Folch et al. (1957). Total lipids were gravimetrically determined through evaporation of the solvent in the purified extract on aluminium sheets at 60-80°C.

## 2.6. Statistical methods

The results were analyzed with the statistical package Statgraphics. Comparison between the different parameters of evaluation was carried out by an ANOVA with a level of significance of  $P < 0.05$ . Data were expressed as means  $\pm$  SD. Percent composition data and efficiencies were transformed by the angular transformation ( $\arcsin \sqrt{\text{percentage}}$ ) prior to analysis to ensure normality. The homogeneity of variances was tested by means of the Bartlett test. Differences between each one of the treatments, in the case of multiple comparisons, were analyzed using the Student-Newman-Keuls multiple range test. Comparison between regression lines was made by means of covariance analysis (Snedecor and Cochran, 1971; Zar, 1974).

## 3. Results

### 3.1. Experiment 1

#### 3.1.1. Growth

The growth of the seed during the experiment was similar for the three food rations tested. The final dry weights achieved were  $0.276 \pm 0.009$  mg dry weight (DW) per individual for the seed cultured with the 2% ration;  $0.290 \pm 0.021$  mg DW ind<sup>-1</sup> with the 4% ration; and  $0.282 \pm 0.026$  mg DW ind<sup>-1</sup> with the 6% ration. These weights were not significantly different ( $P > 0.05$ ). The percentages of organic matter relative to the dry weight of the seed were different for each of the rations, although the differences were not significant ( $P > 0.05$ ). The results obtained were 13.3%, 12.5% and 11.6% of the total dry weight for the 2%, 4% and 6% rations respectively.

#### 3.1.2. Efficiency of food use

The increase in the food ration resulted in an increase in the ingestion of the freeze-dried diet, giving a total ingestion of 108.3  $\mu\text{g}$  ash-free dry weight (AFDW)  $\text{ind}^{-1}$  in the seed cultured with a 2% ration, 165.8  $\mu\text{g}$  AFDW with a 4% ration and 203.0  $\mu\text{g}$  AFDW with a 6% ration over the 4 week experimental period (Table 2). In spite of the differences observed in ingestion, the growth of the seed was similar in all cases, which indicates that the efficiency of use of the freeze-dried diet decreased as the ration increased. As a result, the overall growth efficiency,  $K_1$ , was 20.4 for the lowest ration, dropping to 13.2 and 9.3% for the middle and highest rations respectively, due to the reduction in efficiency with which the ingested food was absorbed (Fig. 1). It should be noted that both the  $K_1$  and the absorption efficiency were significantly different (ANOVA) between the 2% ration and either of the other two; the differences between the latter were not significant.

## 3.2. Experiment 2

### 3.2.1. Growth

The growth of the seed differed according to the species of microalgae with which it was fed; the seed fed freeze-dried *Isochrysis* gave a final weight of  $0.344 \pm 0.016$  mg DW  $\text{ind}^{-1}$  and that fed *Tetraselmis* a weight of  $0.286 \pm 0.021$  mg DW  $\text{ind}^{-1}$ , whilst the seed fed *Phaeodactylum* gave a final weight of  $0.187 \pm 0.013$  mg DW  $\text{ind}^{-1}$ , these weights were significantly different. Organic percentage was significantly higher in the seed fed *Tetraselmis* (16.7%) whereas no difference was observed between the other two diets ( $P > 0.05$ , 14.7% in both cases).

### 3.2.2. Ingestion

Ingestion rates (IR) expressed in  $\mu\text{g}$  AFDW ingested  $\text{ind}^{-1} \text{ day}^{-1}$  were related to the live weight (LW) of the seed according to the following equations:

$$\text{Isochrysis: IR} = (20.74 \pm 0.04) \text{ LW}^{(1.30 \pm 0.04)} \quad r = 0.97 \quad P < 0.001 \quad n = 73$$

$$\text{Tetraselmis: IR} = (16.18 \pm 0.07) \text{ LW}^{(1.16 \pm 0.05)} \quad r = 0.93 \quad P < 0.001 \quad n = 73$$

$$\text{Phaeodactylum: IR} = (20.46 \pm 0.21) \text{ LW}^{(1.33 \pm 0.16)} \quad r = 0.71 \quad P < 0.001 \quad n = 71$$



The covariance analysis performed on these equations only shows significant differences between the diets of Isochrysis and Tetraselmis.

### 3.2.3. Efficiency of food use

The gross growth efficiencies ( $K_1$ ) were greater for the cultures fed Isochrysis (25.8%) and Tetraselmis (32.5%) than for Phaeodactylum, where  $K_1$  was 14.6% (Table 3). Differences between Phaeodactylum and each of the other two diets were significant according to the variance analysis ( $P < 0.05$ ). The efficiencies of absorption were significantly different ( $P < 0.01$ ) between each of the inert diets tested, the highest being Tetraselmis (53.2%), followed by Isochrysis (42.9%) and finally Phaeodactylum (31.1%).

### 3.2.4. Biochemical composition

Table 4 shows the results of the biochemical analyses of the different dried microalgae used as food. As far as the main components are concerned, the cells of Isochrysis and Phaeodactylum have a very similar composition, with protein accounting for approximately 13% of total organic matter, carbohydrate for over 20% and lipid for a relatively high proportion of about 30%. On the other hand, the cells of Tetraselmis have a lipid content which was noticeably lower than that of the other two microalgae at 11.6%, while the proportion of carbohydrate was double that of the other species at 45.6%.

The biochemical composition of the seed at the beginning and at the end of the experiment for each of the different inert diets tested is shown in Table 5. The variance analysis carried out on the data of the biochemical composition of the seed showed no significant difference ( $P > 0.05$ ) between the lipid content of the seed for the different diets. However, significant differences were found for protein ( $P < 0.05$ ) and carbohydrate ( $P < 0.05$ ) content. With regard to the protein content of the seeds, significant differences were noted between the diet of Tetraselmis (26.4%) and Phaeodactylum (37.8%). The carbohydrate content of Tetraselmis (21.5%) was significantly higher than that of Isochrysis (15.2%) or Phaeodactylum (14.3%); the difference between the two latter diets was not significant.

### 3.3. Experiment 3

#### 3.3. I. Growth

The addition of an antibiotic or kaolin made no difference in the growth of seed fed a freeze-dried diet. The seed cultured without the antibiotic gave a final weight of  $59.49 \pm 0.87 \mu\text{g DW ind}^{-1}$  whilst the weight of the seed cultured with the antibiotic was  $59.07 \pm 1.60 \mu\text{g DW ind}^{-1}$ . The growth of seed fed an inert diet was slightly greater when kaolin was added, giving a final dry weight of  $64.68 \pm 5.77 \mu\text{g DW ind}^{-1}$ , although this difference was not significant ( $P > 0.05$ ). No significant differences were observed in the organic content of the seed at the end of the experiment for each of the different diets tested.

When a small proportion of the freeze-dried microalgae was replaced with fresh microalgae this resulted in a slight increase in the growth of the seed. The weight reached by seed fed a totally freeze-dried diet was  $0.276 \pm 0.009 \text{ mg DW ind}^{-1}$ , but when 20% of this diet was replaced by fresh microalgae the weight was  $0.318 \pm 0.015 \text{ mg DW ind}^{-1}$ , value significantly higher than the former. On the other hand, the seed fed a totally fresh diet reached a significantly higher weight,  $0.576 \pm 0.015 \text{ mg DW ind}^{-1}$ . No significant differences were observed in the organic content of the seed.

#### 3.3.2. Efficiency of food use

The addition of the antibiotic or kaolin to the seed cultures fed the freeze-dried diet produced an increase in the gross growth efficiency,  $K_1$  (Table 6), due to the increased efficiency of absorption of the dried microalgae that occurred when the antibiotic or kaolin were added. This effect was greater in the case of kaolin, and on comparing cultures with and without kaolin the growth was  $9.0 \mu\text{g}$  of organic matter  $\text{ind}^{-1}$  as compared with  $7.2 \mu\text{g}$ , with an ingestion of  $21.0 \mu\text{g}$  of organic matter  $\text{ind}^{-1}$  and  $25.2 \mu\text{g}$ , respectively, which gives an increase of almost 50% in efficiency of absorption of the dried diet (66.6% with kaolin and 46.1% without).

The addition of fresh microalgae to the inert diet produced a substantial improvement in the efficiency of food use (Table 7). The gross growth efficiency,  $K_1$ , was 30% for the mixed diet as compared with 20.4% for the inert diet. Nevertheless, the  $K_1$  for the fresh diet, 53.8%, was higher than either of the other two, with a significant difference in each case ( $P < 0.001$ , ANOVA, test SNK). It is therefore apparent that substituting 20% of the inert diet for fresh microalgae significantly increased the absorption of the diet ( $AE = 50.4\%$ ) over that of the totally inert diet (36.4%), although it was still significantly lower than that of the fresh diet (78.3%) (Table 7).

### 3.4. Experiment 4

#### 3.4.1. Growth

The growth of the seed varied according to the process by which the microalgae was preserved. At the end of the experimental period, the highest weight was reached by seed fed the diet in suspension,  $3.07 \pm 0.28$  mg DW ind<sup>-1</sup>, followed by those fed the concentrated diet at  $2.48 \pm 0.24$  mg DW ind<sup>-1</sup>, whilst the seed fed frozen and freeze-dried diets only reached  $1.58 \pm 0.05$  and  $1.40 \pm 0.11$  mg DW ind<sup>-1</sup>, respectively. Dry weights of the seed fed either of the fresh diets were significantly different from the weights of seed fed either of the inert diets. No significant differences were observed, however, between one inert diet and the other. The organic content of the seeds fed fresh diets (13.1% and 12.7%, respectively, for the suspension and concentrated diets) was significantly higher than that of the seed fed an inert diet (11.6% and 12.1%, respectively, for the frozen and freeze-dried diets).

#### 3.4.2. Efficiency of food use

The gross growth efficiencies,  $K_1$ , varied from a maximum of 53.9%, which corresponds to the seed fed the fresh diet, to a minimum of 25.1% for the seed fed the freeze-dried diet (Table 8). The efficiency of the other two diets came somewhere between these two; 45.6% for the concentrated diet and 32.5% for the frozen diet. The differences between the various  $K_1$  were significant ( $P < 0.05$ ) for each of the diets tested. The efficiency of absorption was greatest in the seed fed the diet in suspension, with an average value of 76.6%, whilst the lowest value was recorded by the seed fed

the freeze-dried diet, 44.0%. Absorption efficiencies were significantly different according to the type of diet applied ( $P < 0.05$ , ANOVA), and there was a significant difference between each of the diets and the other three tested ( $P < 0.05$ , SNK test).

#### 4. Discussion

Inert microalgal diets have scarcely been used for feeding molluscs, at least in the case of traditionally cultured microalgae (autotrophic cultures). The most important experiments in this field are those of Hidu and Ukeles (1964) with the larvae of *Mercenaria mercenaria*, and Masson (1977) and Cordero Esquivel and Voltolina (1996) with the larvae and juveniles of *Mytilus galloprovincialis*. Other authors have used heterotrophically cultured microalgae, especially the species *T. suecica*, as for example Laing et al. (1990) with the larvae of *Tapes philippinarum* and Laing and Millican (1991); Laing and Gil Verdugo (1991); Laing and Millican (1992) and Curatolo et al. (1993) with seed of the same species. All these experiments showed the need to add a certain proportion of fresh microalgae to the inert diets in order to achieve comparable growth rates to those of fresh diets.

In this study it was observed that the inert microalga which produced the best seed growth rates in *R. decussatus* was *Isochrysis*, followed by *Tetraselmis*, whilst the lowest growth rates was achieved by the seed fed *Phaeodactylum*. The ration of dried microalgae which proved to be the most appropriate for the seed of *R. decussatus* was the same as that obtained for fresh microalgae (Albentosa et al., 1996a): 2% of the live weight of the seed. An increase in the ration produced no change in the growth rates of the seed, and although an increase in ingestion was noted for seed cultured with the higher rations this resulted in a drop in the efficiency of absorption. This fact may be related to a saturation of the digestive gland leading to an increase in the quantity of undigested food which is eliminated directly in the form of faeces (Griffiths and Griffiths, 1987).

The differences observed in the ingestion of the various species of freeze-dried microalgae may provide an explanation for the differing growth rates obtained. If this is so, then the lower acceptability of the cells of *Tetraselmis* would explain the lower growth rate obtained with this diet in comparison with *Isochrysis*. On the other hand,

the seed fed *Phaeodactylum* showed ingestion rates higher than those of seed fed both *Isochrysis* and *Tetraselmis*, yet its growth was less than that of either of the latter.

The efficiency of absorption, an indicator of the digestibility of the microalgae, differed according to the microalgae used. The most easily digested species, according to our test results, was *Tetraselmis*, followed by *Isochrysis* and *Phaeodactylum*. The lower efficiency of absorption of the microalgae *Isochrysis* when compared with *Tetraselmis* is compensated for by a higher ingestion rate in *Isochrysis*, and consequently a larger amount of energy available for growth. The low growth rates obtained with *Phaeodactylum* could be due its poor digestibility, since ingestion rates were highest with this diet.

The differences in the biochemical composition of the various inert diets, especially that between *Tetraselmis* on one hand and *Phaeodactylum* and *Isochrysis* on the other, provide no explanation for the different growth rates observed. In fact, previously mentioned parameters such as ingestion and digestibility would appear to be of greater importance in determining the nutritional quality of the inert diets used.

In a previous study (Albentosa et al., 1996b) which evaluated the nutritional quality of various diets of fresh microalgae, the growth rates observed were significantly greater than those obtained in this study with inert microalgal diets and the same seed stock. Nevertheless, the growth rates obtained with the inert diets of *Isochrysis* and *Tetraselmis* were comparable with that of the fresh diet of *Phaeodactylum*. The covariance analyses of the curves that relate ingestion to the weight of the seed fed dried diets were significantly different ( $P < 0.05$ ) from the curves obtained with a fresh diet of the same microalgae. When the results of the ingestion rates obtained in this study are compared with those obtained in the previous study, a different alimentary pattern emerges. With the inert diets the highest ingestion rate was achieved by the seed fed *Isochrysis*, whilst with the fresh diets this was achieved by *Tetraselmis*. The larger cell size of *Tetraselmis* could impede the retention of the microalgae in the branchia resulting in lower efficiency of retention than in *Isochrysis*. In the case of a fresh diet, the movement of the flagellae would overcome this difficulty. The majority of the studies undertaken on inert algal diets give no data about the ingestion of the algae. The study by Laing and Gil Verdugo (1991) described similar ingestion rates for fresh and

inert *T. suecica* in juvenile clams (*R. decussatus* or *M. mercenaria*), whilst in another species of clam (*T. philippinarum*) the acceptability of the fresh diet was significantly higher than that of the inert diet. The ingestion rates calculated by these authors do however show a great dispersion, so these results should be interpreted with caution.

Given that the acceptability of the dried microalgae was comparable with or even greater than that of fresh microalgae, the lower growth rates observed in this study of inert diets could be due to the inferior efficiencies of absorption of this type of diet, which are approximately half those of fresh diets (Albentosa et al., 1996b). The process of preserving the microalgae may in some way alter the cell wall or compact the cell, thus making it more difficult to digest. These data contrast with those obtained by Laing and Gil Verdugo (1991) with seed of the same species of bivalve, *R. decussatus*. According to these authors, the efficiency of use of the dried diet is similar to or even higher than that of the same diet when fresh, which leads these authors to conclude that the drying process aids digestion of the microalgae cells. However, the low growth rates reported in the previous study may indicate the existence of a factor that limited the growth of the seed in the experiment, independently of the diet administered.

According to Urban and Langdon (1984) inert diets can encourage the development of bacteria in the culture vessels, and this in turn, according to Masson (1977) and Langdon and Bolton (1984), could increase the sedimentation rates of inert particles. In our study, no variation was observed in the sedimentation rates of the freeze-dried microalgae when the antibiotic was added. The average sedimentation rate of the freeze-dried diet, with or without the added antibiotic, was less than 10% of the initial concentration after 24 h. Other authors point out that the development of bacteria may have a positive effect on growth by providing a food source and/or aiding the digestion of food particles (Langdon and Siegfried, 1984; Urban and Langdon, 1984). In our study, the use of an antibiotic in the seed cultures fed inert diets showed no influence on the seed growth rates. These results are similar to those described by Langdon and Bolton (1984).

The addition of inorganic particles to the seed cultures fed freeze-dried microalgae had no significant effect on the growth rates of the seed. The only differences observed concern ingestion and the efficiency of absorption; the addition of kaolin to the freeze-

dried diet of microalgae produced a slight decrease in ingestion and an increase in the efficiency of absorption compared with the same diet without kaolin. These results, as far as ingestion is concerned, contradict the findings of Kiorboe et al. (1981) and Winter (1976). Ali (1981), on the other hand, questioned the increase in ingestion of algae cells in the presence of inorganic particles. According to this author, when sedimentation occurs the inorganic particles could have microalgae attached to them, and this would lead to an overestimation of the ingestion rates. Another explanation given in the bibliography (Murken, 1976) for the increase in growth of bivalves when inorganic matter is added to the food, refers to a greater digestibility of the algal diets. The increase in the efficiency of absorption observed in this study could be due to the fact that when kaolin is added to the diet it may have a beneficial effect on the trituration of the freeze-dried microalgae or on the efficiency of the enzymes in the digestive system of these animals. Nevertheless, this improvement in the absorption of inert microalgae was not sufficient to bring the growth rates up to the levels of those obtained with a fresh diet.

The addition of a small proportion of fresh microalgae to the freeze-dried diet led to an increase in growth rates when compared with the wholly inert diet. Epifanio (1979) Urban and Langdon (1984) and Coutteau et al. (1994) all describe substitutions of up to 50% of fresh microalgal diets with yeasts. Laing and Gil Verdugo (1991); Laing and Millican (1991) and Curatolo et al. (1993) substituted various proportions of fresh microalgae with dried microalgae. The proportion of fresh microalgae (20%) used in the present study was not large enough to achieve the same growth rates as with fresh diets, although there was a significant increase in growth rates compared with those of totally inert diets. The ingestion rates of both inert and mixed diets were higher than that of the fresh diet, and therefore the acceptability of the diets provides no explanation for the difference in growth observed between them. The efficiency of absorption of the mixed diet was significantly higher than that of the inert diet, but significantly lower than that of the fresh diet, which indicates that substituting 20% of the diet does not completely resolve the problems of digestibility found in inert diets.

The different means of preservation of microalgae examined in this experiment, following the sequence of the transition from fresh cells to inert (fresh, concentrated, frozen and freeze-dried) made it possible to evaluate the suitability of each of these diets

for achieving maximum growth in clam seed. The different growth rates observed for each of the different means of preserving the microalgae cannot be explained by the different ingestion rates recorded. However, on analyzing the digestibility of the algae cells, significant differences were observed according to the type of diet. The efficiency of absorption of the microalgae was highest for microalgae in suspension, and diminished according to whether the microalgae were administered as a concentrate, frozen or freeze-dried. The difference between the diet of microalgae in suspension and as a concentrate was notably less (the EA decreases by under 9%) than that of the other two diets (over 20 or 30% in relation to the fresh diet). According to the results of our study, the nutritional quality of the microalga *Isochrysis* for the seed of *R. decussatus* was not dramatically altered when it was centrifuged to produce a concentrate. Watson et al. (1986) with larvae of *Crassostrea virginica* and Nell and O'Connor (1991) with oyster larvae of the species *Saccostrea commercialis* obtained similar results with other microalgae in concentrate. The nutritional value of these concentrates was comparable with that of the microalgae in suspension. When frozen microalgae were used as a food source, the growth rate was only half that of the fresh diet in suspension, which indicates a large loss of nutritional quality in the microalgae during the freezing process. The findings related to the efficiency of absorption indicate that freezing has a negative effect on the digestibility of the microalgae. Similar results were obtained for the freeze-drying process. To sum up, our results would appear to indicate that the lower nutritional value of the freeze-dried diet is mainly due to a change in the digestibility of the microalgae that occurs as the result of the freezing process. As a result of this change, different growth rates of the seed occur depending on whether the microalgae are fresh (in suspension or as a concentrate) or inert (frozen or freeze-dried).

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Table 1

Initial live (LW) and dry (DW) weights, expressed as  $\text{mg ind}^{-1}$ , and initial length, expressed as  $\text{mm ind}^{-1}$ , of the *R. decussatus* seed used in each of the experiments. Data are expressed as means of three replicates  $\pm$  SD

	LW ( $\text{mg ind}^{-1}$ )	DW ( $\text{mg ind}^{-1}$ )	Length ( $\text{mm ind}^{-1}$ )
Experiment 1	$0.207 \pm 0.004$	$0.116 \pm 0.001$	$0.845 \pm 0.101$
Experiment 2	$0.151 \pm 0.003$	$0.084 \pm 0.001$	$0.769 \pm 0.093$
Experiment 3			
(a)	$0.038 \pm 0.001$	$0.021 \pm 0.001$	$0.483 \pm 0.045$
(b)	$0.207 \pm 0.004$	$0.116 \pm 0.001$	$0.845 \pm 0.101$
Experiment 4	$0.963 \pm 0.014$	$0.606 \pm 0.008$	$1.380 \pm 0.160$

Table 2

Gross ( $K_1$ ) and net ( $K_2$ ) growth efficiencies and absorption efficiency (AE) of the *R. decussatus* seed fed the three different rations of the freeze-dried microalga *I. galbana*, clone T-ISO. Daily rations are expressed as food organic weight in relation to the seed live weight. Data of ingestion (I), growth (G) and respiration (R), expressed as  $\mu\text{g}$  ash-free dry weight (AFDW)  $\text{ind}^{-1}$  used in the efficiencies calculations are also shown. Data represent means  $\pm$  SD from three replicates. The same superscript in each column indicates means which do not differ significantly (ANOVA, SNK test,  $P < 0.05$ )

Ration	$(\mu\text{g AFDW ind}^{-1})$			( $\%$ )		
	I	G	R	$K_1$	$K_2$	AE
2%	$108.3 \pm 5.7^b$	$22.2 \pm 2.4^a$	$17.2 \pm 0.4^a$	$20.4 \pm 1.2^a$	$56.2 \pm 2.2^a$	$36.4 \pm 1.0^a$
4%	$165.8 \pm 16.7^a$	$21.9 \pm 6.9^a$	$17.8 \pm 1.4^a$	$13.2 \pm 3.4^b$	$54.5 \pm 5.3^a$	$23.9 \pm 4.0^b$
6%	$203.0 \pm 41.0^a$	$18.2 \pm 3.7^a$	$16.9 \pm 0.7^a$	$9.3 \pm 3.0^b$	$51.5 \pm 4.3^a$	$17.9 \pm 4.7^b$

Table 3

Gross ( $K_1$ ) and net ( $K_2$ ) growth efficiencies and absorption efficiency (AE) of the *R. decussatus* seed fed three different freeze-dried microalgae diets *I. galbana*, clone T-ISO, *T. suecica* and *P. tricornutum*. Data of ingestion (I), growth (G) and respiration (R), expressed as  $\mu\text{g}$  ash-free dry weight (AFDW)  $\text{ind}^{-1}$  used in the efficiencies

calculations are also shown Data represent means  $\pm$  SD from three replicates. The same superscript in each column indicates means which do not differ significantly (ANOVA, SNK test,  $P < 0.05$ )

Diet	$(\mu\text{g AFDW ind}^{-1})$				$(\%)$	
	I	G	R	K <sub>1</sub>	K <sub>2</sub>	AE
Isochrysis	148.0 $\pm$	38.3 $\pm$	25.2 $\pm$	25.8 $\pm$	60.1 $\pm$	42.9 $\pm$
	4.4 <sup>a</sup>	5.4 <sup>a</sup>	0.9 <sup>a</sup>	2.9 <sup>a</sup>	2.8 <sup>a</sup>	3.0 <sup>b</sup>
Tetraselmis	109.7 $\pm$	35.6 $\pm$	22.7 $\pm$	32.5 $\pm$	61.0 $\pm$	53.2 $\pm$
	2.7 <sup>b</sup>	4.0 <sup>a</sup>	1.3 <sup>b</sup>	3.0 <sup>a</sup>	1.4 <sup>a</sup>	3.7 <sup>a</sup>
Phaeodactylum	106.1 $\pm$	15.3 $\pm$	17.5 $\pm$	14.6 $\pm$	46.3 $\pm$	31.1 $\pm$
	5.4 <sup>b</sup>	3.5 <sup>b</sup>	0.7 <sup>c</sup>	4.1 <sup>b</sup>	4.8 <sup>b</sup>	5.5 <sup>c</sup>

Table 4

Biochemical composition of the three freeze-dried microalgae (*I. galbana*, clone T-ISO, *T. suecica* and *P. tricornutum*) used as food for *R. decussatus* seed. Data are expressed as  $\text{pg cell}^{-1}$  and as relative percentage to the total organic matter

Diet	Dry	Organic	Protein		Carbohydrate		Lipid	
	weight ( $\text{pg cell}^{-1}$ )	weight (pg $\text{cell}^{-1}$ )	(pg $\text{cell}^{-1}$ )	(%)	(pg $\text{cell}^{-1}$ )	(%)	(pg $\text{cell}^{-1}$ )	(%)
Isochrysis	16.9	16.2	2.2	13.8	3.3	20.6	5.1	31.4
Tetraselmis	148.0	144.6	17.3	12.0	65.9	45.6	16.7	11.6
Phaeodactylum	11.6	11.2	1.4	12.9	2.8	25.4	3.4	30.1

Table 5

Biochemical composition of *R. decussatus* seed fed on freeze-dried microalgal diets: *I. galbana*, clone T-ISO, *T. suecica* and *P. tricornutum*. Data are expressed as  $\mu\text{g ind}^{-1}$  and as relative percentage to the total organic matter. Data represent means  $\pm$  standard deviation from three replicates. The same superscript in each column indicates means which do not differ significantly (ANOVA, SNK test,  $P < 0.05$ )

Diet	Dry	Organic	Proteins	Carbohydrate	Lipid
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	weight (mg ind <sup>-1</sup> )	weight (mg ind <sup>-1</sup> )	( $\mu$ g)	(%)	( $\mu$ g)	(%)	( $\mu$ g)	(%)
Initial	0.085	0.012	4.7	38.3	2.0	16.4	2.3	18.7
Isochrysis	0.344	0.051	16.8	33.2 <sup>ab</sup>	7.6	15.2 <sup>b</sup>	11.1	21.9 <sup>a</sup>
	$\pm 0.016$	$\pm 0.005$	$\pm 1.4$	$\pm 2.2$	$\pm 0.4$	$\pm 1.6$	$\pm 2.4$	$\pm 4.1$
Tetraselmis	0.286	0.048	12.7	26.4 <sup>b</sup>	10.3	21.5 <sup>a</sup>	8.1	16.9 <sup>a</sup>
	$\pm 0.021$	$\pm 0.004$	$\pm 1.4$	$\pm 0.8$	10.9	$\pm 0.8$	$\pm 0.7$	$\pm 0.6$
Phaeodactylum	0.187	0.028	11.0	37.8 <sup>a</sup>	4.2	14.3 <sup>b</sup>	5.2	17.7 <sup>a</sup>
	$\pm 0.013$	$\pm 0.004$	$\pm 1.0$	$\pm 6.5$	$\pm 0.6$	$\pm 3.2$	$\pm 0.2$	$\pm 2.1$

Table 6

Gross ( $K_1$ ) and net ( $K_2$ ) growth efficiencies and absorption efficiency (AE) of the *R. decussatus* seed fed the *I. galbana* freeze-dried diet with the addition of the antibiotic or kaolin. Data of ingestion (I), growth (G) and respiration (R), expressed as  $\mu$ g ash-free dry weight (AFDW) ind<sup>-1</sup> used in the efficiencies calculations are also shown. Data represent means  $\pm$  SD from three replicates. The same superscript in each column indicates means which do not differ significantly (ANOVA, SNK test,  $P < 0.05$ )

Diet	( $\mu$ g AFDW ind <sup>-1</sup> )			(%)		
	I	G	R	$K_1$	$K_2$	AE
Freeze-dried	25.2 $\pm$ 1.4 <sup>a</sup>	7.2 $\pm$ 0.3 <sup>a</sup>	4.5 $\pm$ 0.1 <sup>a</sup>	25.8 $\pm$ 0.6 <sup>c</sup>	61.750.5a	46.1 $\pm$ 1.3 <sup>c</sup>
Freeze-dried + antibiotic	23.0 $\pm$ 0.3 <sup>ab</sup>	8.0 $\pm$ 0.6 <sup>a</sup>	4.9 $\pm$ 0.1 <sup>a</sup>	34.7 $\pm$ 2.3 <sup>b</sup>	62.2 $\pm$ 1.7 <sup>a</sup>	55.7 $\pm$ 2.1 <sup>b</sup>
Freeze-dried + kaolin	21.01 $\pm$ 2.2 <sup>b</sup>	9.0 $\pm$ 2.1a	5.0 $\pm$ 0.3a	42.6 $\pm$ 5.5 <sup>a</sup>	63.8 $\pm$ 3.8 <sup>a</sup>	66.6 $\pm$ 4.6 <sup>a</sup>

Table 7

Gross ( $K_1$ ) and net ( $K_2$ ) growth efficiencies and absorption efficiency (AE) of the *R. decussatus* seed fed the *I. galbana* freeze-dried diet with the addition of 20% of the food ration of fresh microalgae. Data of ingestion (I), growth (G) and respiration (R), expressed as  $\mu$ g ash-free dry weight (AFDW) ind<sup>-1</sup> used in the efficiencies calculations

are also shown. Data represent means  $\pm$ SD from three replicates. The same superscript in each column indicates means which do not differ significantly (ANOVA, SNK test,  $P < 0.05$ ).

Diet	( $\mu\text{g AFDW ind}^{-1}$ )			(%)		
	I	G	R	K <sub>1</sub>	K <sub>2</sub>	AE
Fresh	126.6 $\pm$	68.0 $\pm$	31.0 $\pm$	53.8 $\pm$	68.7 $\pm$	78.3 $\pm$
	6.2 <sup>a</sup>	4.1 <sup>a</sup>	0.7 <sup>a</sup>	3.3 <sup>a</sup>	0.9 <sup>a</sup>	4.0 <sup>a</sup>
Freeze-dried	108.3 $\pm$	22.2 $\pm$	17.2 $\pm$	20.4 $\pm$	56.2 $\pm$	36.4 $\pm$
	5.7 <sup>b</sup>	2.4 <sup>b</sup>	0.4 <sup>c</sup>	1.2 <sup>c</sup>	2.2 <sup>c</sup>	1.0 <sup>c</sup>
Freeze-dried + 20% fresh	97.5 $\pm$	29.3 $\pm$	19.8 $\pm$	30.0 $\pm$	59.6 $\pm$	50.4 $\pm$
	2.3 <sup>b</sup>	2.3 <sup>b</sup>	1.00 <sup>b</sup>	1.7 <sup>b</sup>	0.5 <sup>b</sup>	2.7 <sup>b</sup>

Table 8

Gross (K<sub>1</sub>) and net (K<sub>2</sub>) growth efficiencies and absorption efficiency (AE) of the *R. decussatus* seed fed the *I. galbana* microalgae preserved by different processes: culture, concentration, freezing and freeze-drying. Data of ingestion (I), growth (G) and respiration (R), expressed as  $\mu\text{g ash-free dry weight (AFDW) ind}^{-1}$  used in the efficiencies calculations are also shown. Data represent means  $\pm$  SD deviation from three replicates. The same superscript in each column indicates means which do not differ significantly (ANOVA, SNK test,  $P < 0.05$ )

Diet	( $\mu\text{g AFDW ind}^{-1}$ )			(%)		
	I	G	R	K <sub>1</sub>	K <sub>2</sub>	AE
Fresh	616.6 $\pm$	333.2 $\pm$	139.1 $\pm$	53.9 $\pm$	70.5 $\pm$	16.6 $\pm$
	40.1 <sup>a</sup>	32.7 <sup>a</sup>	6.9 <sup>a</sup>	2.1 <sup>a</sup>	1.4 <sup>a</sup>	2.2 <sup>a</sup>
Concentrated	542.5 $\pm$	247.2 $\pm$	119.5 $\pm$	45.6 $\pm$	67.4 $\pm$	67.8 $\pm$
	65.0 <sup>a</sup>	25.7 <sup>b</sup>	9.1 <sup>b</sup>	1.4 <sup>b</sup>	0.7 <sup>b</sup>	2.3 <sup>b</sup>
Frozen	350.9 $\pm$	112.9 $\pm$	80.7 $\pm$	32.5 $\pm$	58.5 $\pm$	55.5 $\pm$
	4.3 <sup>b</sup>	5.5 <sup>c</sup>	2.1 <sup>c</sup>	1.7 <sup>c</sup>	0.7 <sup>c</sup>	2.2 <sup>c</sup>
Freeze-dried	405.5 $\pm$	101.8 $\pm$	76.3 $\pm$	25.1 $\pm$	57.1 $\pm$	44.0 $\pm$
	38.5 <sup>b</sup>	10.5 <sup>c</sup>	2.1 <sup>c</sup>	0.7 <sup>d</sup>	2.1 <sup>c</sup>	1.4 <sup>d</sup>



Fig. 1, Effect of daily food ration on ingestion rate (solid line) and absorption efficiency (broken line) of seed of *R. decussatus* fed the freeze-dried microalgae *I. galbana*, clone T-ISO.