

Evaluation of microalgae diets for the spat of mangrove oyster *Crassostrea rhizophorae* (Gülding, 1828) and its growth in outdoor conditions

Evaluación de dietas microalgales para pre-semillas de la ostra de mangle *Crassostrea rhizophorae* (Gülding, 1828) y su crecimiento en condiciones exteriores

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ABSTRACT | In order to guarantee an adequate level of macromolecular reserves, and allow their successful transfer to the natural environment, we have studied the survival, growth and biochemical composition (carbohydrates, lipids and proteins) of the spat (initial length 3.77 ± 0.64 mm) of the mangrove oyster *Crassostrea rhizophorae*, fed with seven different combinations (diets) of three tropical microalgae: *Chaetoceros* sp. Araya strain (Ch-A), *Isochrysis galbana* (Ig) and *Tetraselmis chui* (Tc). The microalgae biochemical composition was also determined. In the indoor bioassay, each one of the seven diets was assigned three aquariums (replicates with 21 spat). In addition, three more replicates were arranged in an outdoor environment (control). After 36 days, the biochemical and biometric parameters of the juveniles in the indoor bioassay were determined, and they were transferred to the outdoor environment. The control treatment suffered considerably high mortality rates ($\approx 80\%$) during this period and it was not possible to obtain further data from it for the experiment. The transferred juveniles continued to be cultured in suspension for 30 days, after which their biochemical and biometric parameters were evaluated again. During this period, samplings of the environmental variables were taken weekly. In general, within the indoor period, the greatest biometric and biochemical values were obtained in the organisms fed with one monoalgal diet (Ch-A), bialgal diets (Ch-A+Tc; Ch-A+Ig) and the trialgal diet (Ch-A+Ig+Tc), a tendency that remained in the outdoor environment. This was attributed to the balanced contribution of biomolecules previously offered by these diets. These results suggest that the juveniles made use of the energy content found in carbohydrates and lipids once they were transferred outdoors; where these energy sources were probably catabolized to compensate for the scarce availability of food (low chlorophyll *a* and organic seston) observed in the outdoor culture site.

Palabras clave

Composición bioquímica
Bivalvos
Crecimiento
Requerimiento nutricional
Juveniles
Supervivencia

RESUMEN | Con el fin de garantizar un nivel adecuado de las macromoléculas energéticas, que permitan su transferencia exitosa al ambiente natural, se estudió la supervivencia, el crecimiento y la composición bioquímica (carbohidratos, lípidos y proteínas) de pre-semillas ("spat") (talla inicial 3.77 ± 0.64 mm) de ostra de mangle *Crassostrea rhizophorae*, alimentada con tres microalgas tropicales: *Chaetoceros* sp. Cepa Araya (Ch-A), *Isochrysis galbana* (Ig) y *Tetraselmis chui* (Tc), ofertadas como dietas unialgales o mezcladas en dietas bialgales y trialgales. La composición bioquímica de las diferentes cepas de microalgas fue también determinada. En el bioensayo bajo condiciones de laboratorio (hatchery), se utilizaron tres acuarios (réplicas con 21 semillas) para cada una de las dietas. Adicionalmente tres réplicas se confinaron en el medio ambiente (control). Después de 36 días, se analizó sus contenidos bioquímicos y registraron las variables biométricas, y los juveniles se transfirieron al ambiente exterior (tres réplicas) y se cultivaron en suspensión durante 30 días. Al final del cultivo en el medioambiente se obtuvieron de nuevo sus variables bioquímicas y biométricas. Sin embargo, debido a una alta mortalidad ocurrida en las

réplicas de control ($\approx 80\%$), no fue posible obtener más datos de estas últimas, después de 36 días. Durante el cultivo en el medio ambiente, se tomaron muestras semanales de las variables ambientales. En general, en el cultivo en el laboratorio, los mayores valores biométricos y bioquímicos se obtuvieron en los organismos alimentados con la dieta monoalgal (Ch-A), dietas bialgales (Ch-A + Tc y Ch-A + Ig) y la trialgal (Ch-A+Ig+Tc), una tendencia que se mantuvo en el cultivo en el medio ambiente. Lo anterior se atribuyó a la contribución equilibrada de las biomoléculas ofrecidas anteriormente por estas dietas. Estos resultados evidenciaron el uso de la energía contenida en carbohidratos y lípidos, por parte de las ostras juveniles, una vez que fueron transferidas al medio ambiente, donde estas fuentes de energía probablemente fueron catabolizadas para compensar la escasa disponibilidad de alimentos (baja Clorofila *a* y materia orgánica) observada en el sitio de cultivo en el medio ambiente.

INTRODUCTION

According to hatchery protocols, once the competent bivalve larvae have completed their metamorphosis, they enter a relatively more sessile stage, allowing cultivation in relatively more open nursery systems, where they can be maintained until they reach the minimum size to be placed in enclosures that allow their cultivation in the natural environment. This change to a much less controlled environment, in most cases, implies that the energy reserves obtained from their feeding in the nursery will be fundamental for their survival during the adaptation period, which confirms the importance of the food quality supplied before this phase arrives (Veniot *et al.*, 2003). It is also known that this impact should be even more severe in tropical areas due to heavier oligotrophic conditions. Transplantation of juveniles to outdoor conditions has been used as a recurrent technique for the grow-out phase, yet it usually yields variable results (mortality and/or poor growth) due to the complex interactions of environmental variables affecting the response and condition of the juveniles (Persoone and Claus, 1980, Lodeiros *et al.*, 2016). This happens even if the juveniles were held with an optimal combination of temperature and food availability (Claus *et al.*, 1983). Knowing this, the production of the juveniles in controlled environments that ensure good feeding conditions seems to be the right method to guarantee the nutritional quality necessary to support bivalve culture activity. Thus, the nutritional quality of the microalgae used as food and the response of each cultivated species to these diets, must be considered and investigated during the postlarval development (Albentosa *et al.*, 1997, Rivero-Rodríguez *et al.*, 2007).

The nutritional value of the microalgae used to feed the postlarval-juvenile phases of bivalves is one of the most important factors for the mollusks, since it can affect their growth and development; this can be improved by using mixtures of living microalgae, which together achieve a higher nutritional value. One of the most important characteristics when selecting a microalgal species for the initial and juvenile stages in hatcheries, is the biomolecules they contain, such as proteins, carbohydrates and lipids (Sánchez-Lazo and Martínez-Pita, 2012). Therefore, a precise knowledge of the composition of the microalgae species used in indoor or outdoor facilities is essential to provide juvenile bivalves with the correct diet and enhance the growth of hard and soft biomasses (Lodeiros *et al.*, 2016).

Certain Bivalve species have undergone high exploitation rates, as they have supported important fisheries in the Caribbean ecoregion for decades (Gil and Moreno, 2007). Today, these populations require urgent conservation actions (Carranza *et al.*, 2008, 2011), and among the oyster species that have been described as used to establish commercial productions in the Caribbean, the mangrove oyster *Crassostrea rhizophorae* (Guilding 1828) is one of the most promising candidates. Although some techniques have been established for grow-out production (Hernández *et al.*, 1998, Lodeiros and Freitas, 2008), its juvenile production is still undeveloped (Lovatelli and Sarkis, 2011, Velasco and Barros, 2007).

The present study evaluates the effect of different microalgae diets in the spat indoor culture and its posterior impact on juvenile outdoor culture, with the objective of developing technological culture protocols that optimize the mass production of mangrove oyster *C. rhizophorae*.

MATERIALS AND METHODS

Indoor juveniles culture

A total of 534 wild *C. rhizophorae* spat, with a shell height of ≈ 4 mm (measured as the length between the umbo and ventral shell) were harvested by hand from a natural cohort settled on mangrove roots in the La Restinga Lagoon reserve, Margarita Island, Nueva Esparta State, Venezuela ($10^{\circ}58'40.78''$ N; $64^{\circ}10'07.45''$ O). The oyster spat were transferred to the Instituto de Estudios Avanzados (IDEA) of the Ministerio del Poder Popular para la Ciencia, Tecnología e Innovación, a research station in Mochima Bay, Sucre State, Venezuela ($10^{\circ}20'47.30''$ N; $64^{\circ}20'42.10''$ O), and placed in insulated containers packed with moistened foam layers to maintain a cool environment and prevent stress.

Three replicate sets of 10 oyster spat (mean and Sd 3.77 ± 0.64 mm) were used for initial measures; the other 441 oyster spat were equally distributed in handmade polyethylene mesh baskets inside of 21 18-L carboys (resulting in 21 juveniles per carboy). These carboys were cut from the bottom and placed in an inverted position, and the mouth was covered with a plastic cap (see Lodeiros *et al.* 2016 to descriptions). Each carboy was filled with 7 L of filtered ($0.45 \mu\text{m}$) and UV-sterilized sea water, which was continuously aerated and maintained at a temperature and salinity of 28-30 °C and 35-37 PSU, respectively. The entire seawater volumes were fully changed daily. Triplicated sets were used to test seven microalgae diets using *IsochrYSIS galbana* (Ig, Haptophyceae), *Chaetoceros* sp. strain Araya (Ch-A; Bacillariophyceae) and *Tetraselmis chui* (Tc; Chlorodetrophyceae). *Chaetoceros* sp. strain Araya is catalogued (BGAUDO-35) in the germoplasm collection of the Instituto Oceanográfico de Venezuela, Universidad de Oriente, as a tropical clone isolated from the Araya Peninsula (Sucre State, Venezuela), whereas *I. galbana* and *T. chui* are temperate microalgae species that are well adapted to the conditions of our study site (Marín *et al.* 1994). The algal species were supplied as three monoalgal diets (Ig, Ch-A and Tc), three binary combinations (Ch-A+Ig, Ig+Tc and Ch-A+Tc) and one ternary mixture (Tc+Ch-A+Ig). The microalgae culture was performed in outdoor conditions with natural sunlight, considering 12-h light and 12-h dark photoperiod regime, using filtered ($0.45 \mu\text{m}$), UV-sterilized and fertilized (f/2 medium) sea water (Guillard and Ryther, 1962), with silica supplement for the diatom (*Chaetoceros* sp.), contained in 400 L transparent cylinders, at 24-30 °C and 34 PSU. When the microalgae in exponential growth phase were used as diets and to formulate the oyster dietary treatments, the methodology described by Lodeiros *et al.* (2016) was followed. Additionally, aliquots (5 mL) of each monoalgal culture were taken in triplicates; these were centrifuged for 15 min (5000 rpm), the supernatant discarded, and the resulting pellet was kept at -10 °C until the time of the biochemical analysis. Table 1 shows the microalgae characteristics (dry mass, size by Lodeiros *et al.*, 2016) and macromolecular biochemical components (proteins, lipids and carbohydrates), and Table 2 shows the formulated diets to feed pre-juveniles oysters in indoor conditions, based on cell dry mass of the different microalgae studied. As a control group, three replicates with 21 oysters each were placed directly in the uncontrolled outdoor environment of the intertidal zone of the Isla Larga mangrove (Fig. 1), located in front of the IDEA station (Mochima), (Site chosen due to the natural presence of *C. rhizophorae*), maintaining the replicates of the previous treatment diets. A total of 534 oysters spat were used in this study as follows: 30 for initial measurements, 441 for the dietary bioassay, and 63 for the control group.

Table 1. Dry mass, size and biochemical composition (% of dry mass) of the different microalgae used to feed the oyster spat of *C. rhizophorae*.

Microalgae	Dry mass (Pg/cel)	Size (μm)	Proteins (%)	Lipids (%)	Carbohydrates (%)
Ch-A	58.9 ± 1.6	5.3x7.5	42.2 ± 1.2	29.2 ± 2.2	11.5 ± 1.8
Ig	70.7 ± 2.8	5.1x3.5	42.3 ± 2.2	32.2 ± 1.2	12.6 ± 3.2
Tc	154.7 ± 1.9	16.1x8.2	54.2 ± 0.9	10.3 ± 1.9	17.2 ± 1.9

Table 2. Formulated diets to feed the oyster spat in indoor conditions, based on cell dry mass of the different microalgae studied.

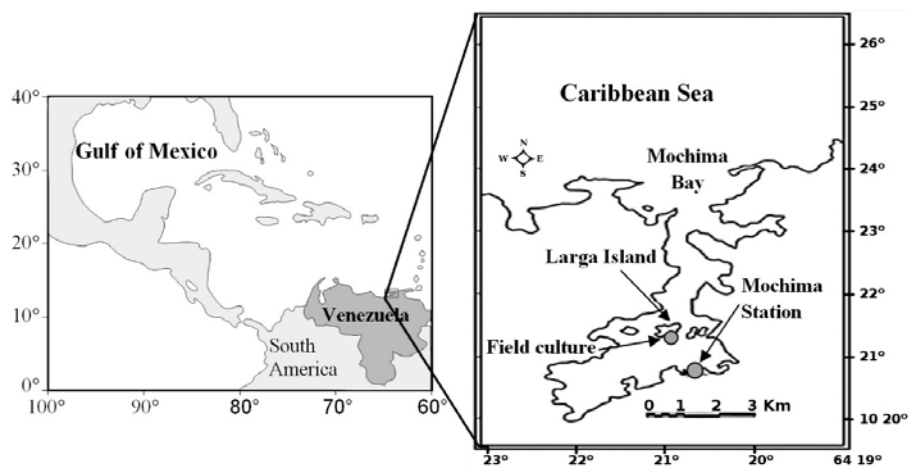
Diets	Volume to administer (mL)	Cell density (cel/mL)
Ch-A	450	500 000
Ig	231	300 000
Tc	180	187 000
Ch-A + Tc	225 + 90	500 000 + 187 000
Ch-A + Ig	225 + 115,5	500 000 + 1 300 000
Ig + Tc	105,5 + 90	1 300 000 + 187 000
Ch-A + Ig + Tc	150 + 60 + 77	500 000 + 187 000 + 1 300 000

After 30 days, the living oysters in each replicate were counted to determine the survival rate, and the length of the shells was determined with a digital caliper (0.1 mm precision); in addition, a representative batch (5 organisms per replicate) was dehydrated at 70 °C for 72 h, and later burned to ashes at 450 °C for 4 h in a furnace; the organic matter of soft tissue was estimated as the weight loss after burning. With regards to the final biochemical composition, proteins were quantified by the method of Lowry *et al.* (1951), modified by Herbert *et al.* (1971), while for the determination of total lipids, a quantitative carbonization test was performed (Marsh and Westein, 1966). Lipid extracts were obtained using the methodology described by Bligh and Dyer (1959). Carbohydrates were estimated by the phenol sulphuric method (Dubois *et al.*, 1956). Biochemical composition data were expressed as organic matter (OM) content (mg/g OM).

Outdoor juvenile culture

Juveniles fed with the different microalgal diets were placed in the same handmade polyethylene mesh baskets used in the indoor culture, with 10 juvenile oysters in each basket, which were deployed for 30 days in hanging culture. The outdoor culture was performed in the intertidal zone of the Isla Larga (Fig. 1), located in front of the IDEA station (Mochima), (site chosen due to the natural presence of *C. rhizophorae*), maintaining the replicates of the previous treatment diets. At the end of the experimental period, survival rate, shell height, dry mass and biochemical composition of the organisms were determined.

Temperature was recorded continuously with a SEALOG electronic thermograph (Vemco, Halifax) placed at a depth of 4 m in the same study site in Mochima Bay. Seawater samples were collected weekly in triplicates with a 5 L Niskin bottle, and the salinity was measured *in situ* using a hand refractometer ATAGO S/Mill (range 0–100 UPS). The water samples were pre- filtered (80 µm) to remove large particulate matter and zooplankton, and then transferred to the laboratory in a dark container. Seawater samples were filtered through precombusted (450 °C for 4 h) and weighed GF/C filters and later rinsed with isotonic ammonium formate (0.5 M).

**Figure 1.** Study area in the north-eastern area of Venezuela.

Total particular matter (TPM) was established by weighting the filters after being dried until they reached a constant mass (60 °C for 48 h). Particulate organic matter (POM) corresponded to the weight loss after burning at 450 °C for 4 h in a furnace. The phytoplankton biomass was estimated using the chlorophyll *a* measured by colorimetric procedures (Strickland & Parsons 1972).

Statistical analysis

Survival data (%), several growth parameters of the juvenile oysters (shell length and dry mass) and biochemical composition of both phases (indoor and outdoor culture) did not meet the assumptions of the analysis of variance (ANOVA). To determine if there were differences in the final biochemical composition, shell height and mass of juvenile oysters fed with the different diets (indoor culture), and the juveniles in the culture site at the sea (outdoor condition), these were contrasted with a Kruskal-Wallis (K-W) analysis, followed by Multiple Range Tests (Zar, 2014). A probability level of 0.05 was established for the test performed.

RESULTS

Growth

Before showing the results, it is necessary to indicate that in the case of the control group it was not possible to determine the biometric and lipid composition variables, as there were not enough individuals to carry out the respective samples. This is a consequence of the 80% mortality rate that these juveniles suffered.

All diets promoted shell height growth of the oysters in both indoor and outdoor environments, arriving at >14 mm, and showed significant differences between the microalgal diets. Spat fed with the Ch-A, Ch-A+Tc, Ch-A+Ig and Ch-A+Ig+Tc and diets presented the highest values, oscillating between 14.86 and 18.08 mm (Fig. 2A). The organisms fed with Ig diet presented intermediate growth value, showing 16.39 mm, and the lowest value were observed in the organisms fed with the Ig+Tc diet (14.09 ± 0.55 mm).

The shell height increment values of the juveniles in outdoor suspension culture were similar to those obtained in indoor culture (Fig. 2B) Therefore, significant differences were observed in juveniles that were previously fed with the Ch-A diet (18.28 ± 4.02 mm), or their combination Ch-A+Tc (17.9 ± 3.69 mm), Ch-A+Ig (18.88 ± 4.08 mm) and Ch-A+Ig+Tc (18.08 ± 3.75 mm), while those previously fed with the diets Ig (16.65 ± 3.61 mm), Tc (15.97 ± 2.71 mm) and Ig+Tc (15.62 ± 3.75 mm) showed significantly lower shell increment.

Soft tissues

As was observed in shell height, the soft tissue mass increment of the spat (Fig. 2C) showed significant differences in relation to the microalgal diets used. The tissue mass growth was significantly higher in those organisms fed with the diets containing Ch-A, the binary combinations of Ch-A+Tc (0.69 ± 0.04 g) and Ch-A+Ig (0.68 ± 0.04 g), and the monoalgal diet Ch-A (0.63 ± 0.05 g). The diets that contributed the least to the fattening of the organisms were the monoalgal Tc (0.40 ± 0.04 g) and the binary diet Ig+Tc (0.44 ± 0.03 g).

The juveniles that showed the highest mass obtained in the outdoor environment (Fig. 2D), were those that were previously fed with the monoalgal diets Ch-A (0.114 ± 0.017 g) and Ig (0.106 ± 0.016 g), and the binary diets Ch-A+Tc and Ch-A+Ig, with values that included between 0.105 ± 0.017 and 0.095 ± 0.019 g, respectively. The lowest values were observed in the organisms that had been fed with the binary diet of Ig+ Tc (0.065 ± 0.015 g).

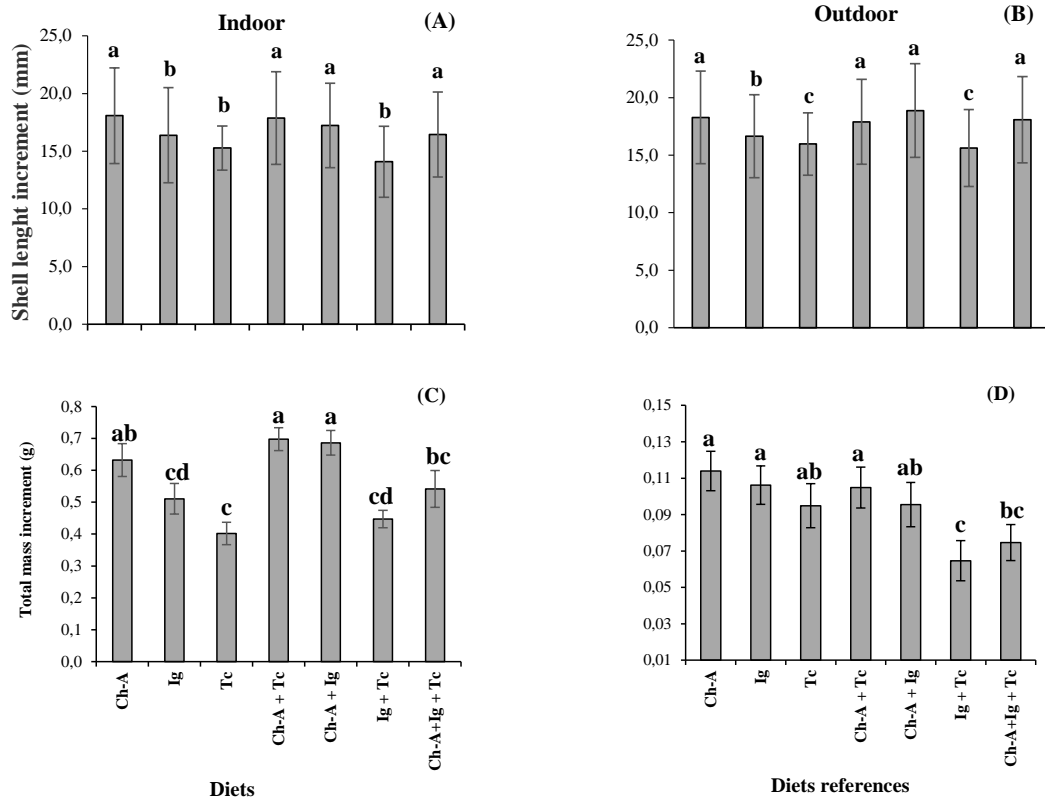


Figure 2. Increase in shell height and mass of soft tissues of the oyster spat (indoor conditions) and juveniles (outdoor conditions). (The vertical bars represent the standard deviations of the values) Note that in the outdoor condition treatments, the different diets are only shown as reference.

Survival

The survival rates of the juvenile oysters showed significant differences in relation to the different microalgal diets offered to them for 36 days (Fig. 3). The highest survival percentages were obtained in the organisms fed with the ternary diet (68.25 ± 5.50%); followed by the binary diets Ch-A+Tc (60.32 ± 11.51%) and Ig+Tc (58.73 ± 7.27%). By contrast, the rest of the diets presented percentages below 30 to 50%, with the control treatment presenting the lowest values for this parameter (19.60 ± 2.75%). Despite the differences in indoor survival, the diets had no effect on survival during the outdoor phase since this was around 90-100%.

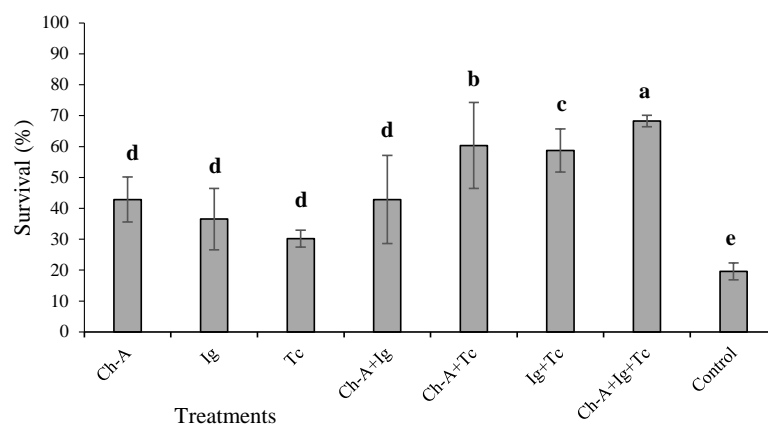


Figure 3. Survival of the oyster spat fed with different microalgal diets during indoor condition. (vertical bars represent the standard deviations of the values).

Macromolecular biochemical composition

Fig. 4 shows the results, in terms of proteins, lipids and carbohydrates content obtained by the spat that were fed with the different diets and later deployed under suspended culture conditions.

Proteins

In indoor laboratory conditions, the protein counts were significantly higher in the spat fed with the ternary diet Ch-A+Tc+Ig (529.82 ± 4.92 mg/g), followed by those consuming the monoalgal diets Ch-A (482.44 ± 10.49 mg/g) and Tc (471.13 ± 8.53 mg/g), which did not present significant differences between them (Fig. 4A), while the diet with the lowest protein count was Ig (271.79 ± 0.01 mg/g). Once the culture period in the outdoor environment passed, the situation changed; significantly higher protein concentrations were observed in the juveniles that were previously fed in indoor bioassay with the binary diet Ch-A+Tc (472.98 ± 8.91 mg/g), followed by the diet Ch-A+Ig (434.72 ± 24.08 mg/g) and Tc (400.61 ± 13.16 mg/g) (Fig. 4B). In addition, it was also possible to observe a decrease in the absolute protein content in those juveniles that were previously fed in the laboratory with the monoalgal diets Ch-A and Tc, and with the ternary diet Ch-A+Tc+Ig, while, for the rest of the individuals, the protein contents increased.

Lipids

Indoors, lipids showed the highest values in the spat fed with the ternary diet Ch-A+Tc+Ig (308.0 ± 7.75 mg/g) and the Tc monoalgal diet (306.64 ± 8.05 mg/g) (Fig. 4C). While for the juveniles grown in the natural environment, those previously fed with monoalgal diet Ig (334.03 ± 30.68 mg/g) showed significantly higher lipid contents, followed by those fed with the Tc diet (286.22 ± 36.48 mg/g) and those fed with the ternary diet Ch-A+Tc+Ig (285.02 ± 19.84 mg/g) (Fig. 4D); no significant differences were observed among individuals previously fed with the other diets. The only increases in lipid contents were observed in the juveniles that were previously fed in the laboratory with the monoalgal diet Ig and the binary Ig+Tc (Fig. 3D).

Carbohydrates

Carbohydrates were significantly higher in the oyster spat fed with the Ig diet alone (476.24 ± 8.82 mg/g) or the combined Ig+Tc (488.65 ± 13.85 mg/g) and Ch-A+Ig (455.35 ± 12.22 mg/g) diets (Fig. 4E), while the diet with the lowest carbohydrate values was the ternary diet Ch-A+Tc+Ig (161.38 ± 0.14). In the outdoor culture, carbohydrates reached their highest contents in the juveniles that were previously fed with the monoalgal diet Ch-A (542.88 ± 13.83 mg/g), followed by the ternary Ch-A+Tc+Ig (448.84 ± 30.68 mg/g) and the binary diet Ig+Tc (442.40 ± 12.01 mg/g) (Fig. 4F). No significant differences were observed with the other diets. Notably, there were marked decreases in the contents of those individuals previously fed with the Ig monoalgal and all the binary diets, and increases in those fed with the monoalgals Ch-A and Tc and the ternary diet (Fig. 4F).

Outdoor environmental variables

Environmental variables in the mangrove area where the juvenile oysters were cultured presented variations despite the short time interval between sampling (7 days). Temperature showed temporal variations (Fig. 5A) ranging between 29 and 30.4 °C (the maximum value, observed in the second sampling), while salinity fluctuated between 29.2 and 37 PSU, with the lowest value in August (Fig. 5B). Organic matter values varied between 3.3 and 5.7 mg/L (Fig. 5E), and the inorganic fraction of the total seston presented high values, ranging from 10.0 to 12.7 mg/L (Fig. 5C). Chlorophyll *a* values remained low throughout the sampling period with values below 1.5 µg/L (Fig. 5D). The dissolved oxygen content presented variations, observing that after the first sampling, the concentrations were below 5 mg/mL, presenting its lowest value in the last sampling during August (Fig. 5E).

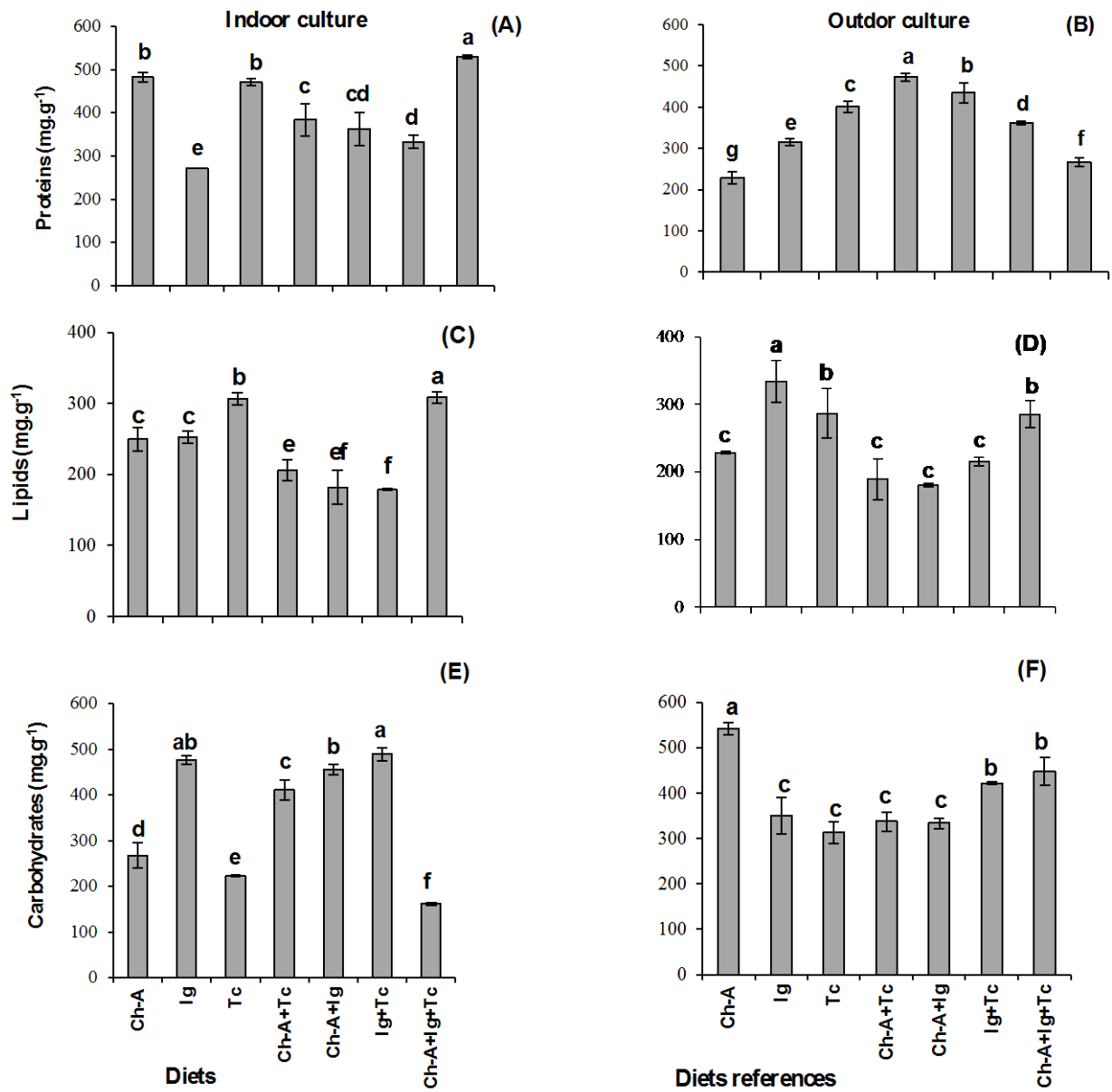


Figure 4. Contents of the different protein, lipid and carbohydrate macromolecules, of the oyster spat (microalgal diets) and juveniles (natural food) maintained in indoor and outdoor conditions. (vertical bars represent the standard deviations of the values). In the treatment of outdoor conditions, the different diets are only shown as reference.

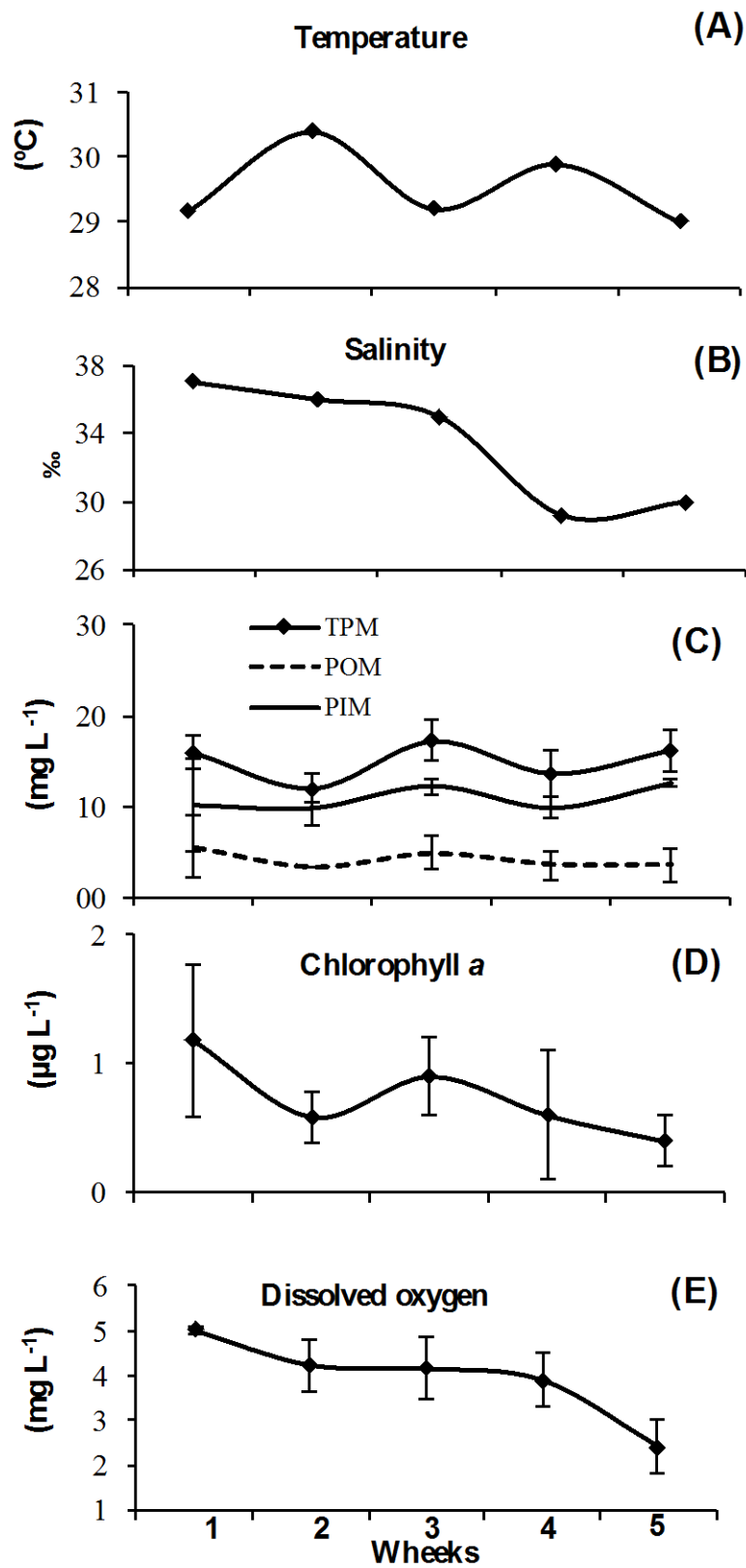


Figure 5. Fluctuations of the environmental parameters, temperatura (A), salinity (B), seston (C) clorophyll-*a* (D) and dissolved oxygen (E), during the outdoor experimental period. (vertical bars represent the standard deviations of the values).

DISCUSSION

The term juvenile or seed in the culture of bivalve mollusks has been used in literature in different ways and a specific definition does not exist. Though, its use in practice has been technically eloquent since, in all of the cases, these terms have implied an oyster with appropriate size for manipulation during cultivation, and that has the physiological condition to withstand the changes associated with these practices. For *C. rhizophore*, we consider that the size of 7-8 mm is a manageable size that could withstand the environmental changes and associated confinement of the culture outdoors. Our results show that, after one month, all the diets in the spat culture produced organisms >14 mm, which after one month of culture outdoors, had a survival rate of >90%. This corroborates that *C. rhizophorae* juveniles >10 mm fit the physiological robustness required for outdoor cultivation.

Although all diets successfully yielded individuals that could be considered juveniles (>90% survival rate) after one month, each diet produced juveniles with different physiological conditions in terms of energy reserves.

Of the three microalgal cultures used for the feeding bioassay, the one with the lowest yields in terms of growth in the outdoor environment was *T. chui*, reaching a maximum of 200,000 cells/mL (30,9 µg), whereas *I. galbana* and *Chaetoceros* sp. Araya strain reached high cell densities of 2,400,000 cells/mL (169,7 µg/mL) and 1,500,000 cells/mL (88,4 µg/mL), respectively. This is an aspect that should be considered when these microalgal species are chosen to be used in intensive bivalve cultures, since the highest costs of bivalve production are caused by microalgae cultivation (Cotteau and Sorgeloos, 1992; Uriarte *et al.*, 2002). However, this is not the only aspect that should be considered for the selection of a microalgae, since its biochemical composition and therefore the capacity to satisfy the nutritional requirements of the organisms is also important. *Tetraselmis chui* had higher concentrations in terms of proteins and carbohydrates, compared to the other two microalgae; but in the case of lipids, the values were higher in the other two species of microalgae (Ch-A & Ig). A similar result was observed by Pico-Segura *et al.* (2013), who reported values of proteins, carbohydrates and lipids for *T. chui* of 37.6%; 31.6% and 6.7%, respectively.

The growth of the shell and soft tissue mass for the spat of *C. rhizophorae* fed with the different microalgal diets showed that the presence of *Chaetoceros* sp. Araya strain in the diet promotes higher rates of growth and fattening for this bivalve. This suggests that this microalgae species provides enough energy to reach the seed stage with a high energy condition, which was better complemented when combined with *T. chui* (Tc) than with *I. galbana* (Ig).

A similar result was obtained with the oyster *Crassostrea corteziensis* (Rivero-Rodríguez *et al.*, 2007) and the scallop *Nodipecten subnodosus* (Saucedo *et al.*, 2013), with high nutritional contribution by some species of *Tetraselmis* sp., when combined in a binary diet with other *Chaetoceros* spp, such as *C. calcitrans* or *C. muelleri*, generating high growth rates. However, the nutritional value of microalgae *T. chui* continues to be widely discussed (Helm and Laing, 1987; Núñez *et al.*, 2002; Becker, 2004; Milke *et al.*, 2008). *Isochrysis galbana* has been indicated as a better complement in combined diets with diatom species for several bivalves (Albentosa *et al.*, 1996a, b; Marshall *et al.*, 2010; Oliva *et al.*, 2013; Sánchez-Lazo and Martínez-Pita, 2014). Regardless, *Tetraselmis* sp. has, among other biochemical components, high concentrations of retinol (> 0.8 µg g⁻¹) which supports the protection of tissues and their growth mechanisms, like the cholesterol produced by *C. calcitrans*, which has been found to be a limiting factor in the growth of mollusk larvae. Therefore, future trials that take these elements into account could help to clarify what other benefits are provided by the microalgae used as food for this bivalve (Brown *et al.*, 1999; D'Souza and Kelly, 2000).

By contrast, when *T. chui*, *I. galbana* and other species of the same genus were supplied in monoalgal form to other marine organisms, a low intake of energy was observed, reflecting low growth rates, which has been observed in larvae of the shrimp *Penaeus monodon* (Sivakumar *et al.*, 2001) and in larvae of the clam *Ruditapes philippinarum* (Yan *et al.*, 2006). However, in other species of bivalves it has been possible

to promote high growth rates in juveniles, such as with *Ruditapes decussatus*, *Venerupis pullastra* and *Crassostrea gigas*, specifically when the microalgae *T. chui* was combined with *I. galbana* (Albentosa *et al.*, 1996 a, b; Marshall *et al.*, 2010). These last results do not match those obtained with our study on *C. rhizophorae*, since this combination did not promote the best growth rates, reinforcing that the diets are species-specific and that they should not be extrapolated from one species to another (Rivero-Rodríguez *et al.*, 2007). In order to achieve optimal diets for a species, it is necessary to carry out individualized nutritional studies to ensure success in the raising of organisms and their subsequent development in the environment. With this in mind, criteria such as acceptability, digestibility, growth rate and biochemical composition with emphasis in the fatty acids, end up being the most considered aspects for the selection of microalgal diets (Southgate, 2003; Martínez-Fernández *et al.*, 2004; Marshall *et al.*, 2010).

The diet Ch-A+ Tc coincided with a greater contribution of proteins and lipids to the tissues of the organisms, compared to the other two binary mixtures, generating the necessary energy for growth. This has been described as an important element for postlarvae of bivalves that present specific energetic demands, where these organisms tend to retain much more of their protein compounds to satisfy anabolic demands (production of amino acids) with respect to the carbohydrates (Gabott, 1975; Kreeger *et al.* 1993; Uriarte and Farías, 1999; Matias *et al.*, 2010).

The analysis of the changes observed in the biochemical composition of the juveniles of *C. rhizophorae*, when subjected to culture in the marine environment, showed that the organisms that were fed with Ch-A+Tc and Ch-A+Ig showed a higher protein content than that obtained at the end of the indoor bioassay. However, a decrease can be observed in the other bioenergetic molecules (more specifically the carbohydrates and lipids, which was probably due to their contribution to growth), because the food availability in the culture site was low, such as can be observed in the concentrations of chlorophyll *a* and POM. Uriarte and Farias (1999) point out that bivalves require more carbon than nitrogen in their natural environment and that an increase in the availability of proteins in their diet can only be beneficial if they have enough energy to metabolize these biomolecules. By contrast, organisms with lower growth rates decreased their protein reserves, suggesting a possible mobilization of the protein for the formation of energetic molecules, a condition that has been reported in scallop bivalves (Bricelj and Shumway, 1991). Bivalve mollusks use these biomolecules as an energy source during periods of intense metabolic demand (gametogenesis or poor food availability) slowing down or limiting their growth. A fact that has also been observed in the mussel *Perna viridis* (Acosta *et al.*, 2009).

In outdoor environments, the growth of juvenile oysters varies greatly and depends on genetic characteristics, the juvenile's initial size, culture density, environmental factors, among other factors (Dégremont *et al.*, 2005a, b). However, food availability is one of the variables that has the highest incidence in these organisms (Pacheco *et al.*, 1983; Vera and Aldana, 2000; Helm and Bourne, 2006; Acosta *et al.*, 2009). The site of cultivation chosen in the Bay of Mochima (more specifically, in Isla Larga) had limited water circulation, mainly due to the tidal movements through the central channel of the bay, which were weakened by the interference of the island, which acted as a barrier (Roa, 1961, Caraballo, 1970). This caused the availability of food to be low throughout the culture period in this environment, with average concentrations of chlorophyll *a* around <0.7 µg/L, higher inorganic fraction of seston maintained between 10-12.7 mg/L, higher salinity variations (29.2-37.0 ups) and relatively high temperatures (29-30 °C), which are consistent with the conditions reported by other authors who indicated values of chlorophyll *a* between 0.5-1.3 µg / L (Mengual *et al.*, 2009, Expósito and Zoppi, 1999).

MacDonalds and Thompson (1985) indicated that shell formation in bivalves requires a lower energy input than tissue formation. This could explain why the growth observed in the environment (outdoor) was similar to that observed in the bioassay of microalgae diets (indoor), in a similar period of time (36 days). However, the high mortality (≈80%) observed in the control replicas initially placed in the environment, suggests that environmental conditions were stressful for the juveniles of *C. rhizophorae*, such as was previously described by Márquez *et al.* (2011) and Mengual *et al.* (2011) for the pearl oysters *Pteria colymbus* and *Pinctada imbricata*, respectively, which were grown in the same area of the present study.

Summarizing the aspects observed in the feeding bioassay, diets that included Ch-A strain, alone were the ones which had the highest growth rates, especially in those treated with the combination Ch-A+Tc (adequate combination of proteins and lipids), and Ig (high content of carbohydrates and lipids). These diets promoted higher growth and survival rates of the spat of *C. rhizophorae*: Therefore, they seem the best to overcome the transition from the hatchery to the prevailing conditions in the culture site outdoors. Given the results obtained in the present study and taking into account that numerous studies have demonstrated that the lipid levels and composition of marine bivalves can clearly reflect the biochemical and environmental conditions of their seed development (De Moreno *et al.*, 1980; Napolitano *et al.*, 1992; Fernández-Reiriz *et al.*, 1996; Freitas *et al.*, 2003), we recommend carrying out bioassays to study the dynamics of the different classes of lipids and fatty acids, especially those of recognized metabolic importance in bivalves. From this standpoint, the influence of contrasting or extreme environmental conditions, such as those presented by the study site, and the lipid composition of bivalves distributed in such environments would be an interesting study.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical use of animals

All the procedures followed the guidelines for ethical and responsible research using in vivo animals for experiments (Kilkenny, *et al.*, 2010).

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