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ORIGINAL RESEARCH

Effect of four diets based on three microalgae on the growth performance and quality of Mediterranean mussel flesh, *Mytilus galloprovincialis*

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Abstract The objective of this study was to assess the biologic impact of four diets based on three species of microalgae on the Mediterranean mussels *Mytilus galloprovincialis.* For this purpose, flesh weight, linear growth, survival rate and the biochemical composition of mussel flesh have been evaluated. Mussels fed with a mixture of the three species; *Nannochloropsis* sp., *Dunaliella* sp., and *Phaeodactylum* sp., exhibited the highest lipid level (7.66%). This suggests that mixing several species in the same diet favors lipid production. Protein level was significantly higher in mussels fed with *Dunaliella* sp. (28.92%) compared to *Phaeodactylum* sp. (24.52%), *Nannochloropsis* sp. (22.94%) and a combination of the three species (26.07%). Wet weight gain was significantly higher in mussels fed with *Dunaliella* sp. (7.76 g) compared to mussels fed with the mixture of the three species (6.87 g). However, mussel shell length was not different among the four groups, and ranged between 41.7 mm and 42.3 mm. In conclusion, the data from this study suggest that a diet based on *Dunaliella* sp. can efficiently cover the protein requirements of the Mediterranean mussel, *Mytilus galloprovincialis* during the growth cycle.

Keywords *Mytilus galloprovincialis* **.** Microalgae **.** Biochemical composition **.** Diets **.** Growth performance

Introduction

In recent years, mussels have become a targeted product for human consumption. To satisfy this immense need, the production of mussels by different breeding programs has also increased. The natural deposits available to produce the necessary quantity of spat to be sown became a real problem in some countries. Therefore, the production of mussel seeds from hatchery sources is now a primary necessity (Albentosa et al. 1994; Albentosa et al. 1999; Harel et al. 2002; Martínez-Fernández et al. 2004). Microalgae have been generally used in aquaculture feeds. They are included in the rearing of several animal species related of mariculture production, including bivalves as live feeds used directly for all their growth cycles (Pettersen et al. 2010) and fish during some larval cycles as the diet for live prey, such as rotifers and artemia to maintain high fish larval growth and survival rates (Liu et al. 2002; Ferreira et al. 2009). Besides, marine species are considered as a source of lipids with a higher concentration of DHA and are used to replace fish oil in gilthead seabream larvae microdiets (Atalah et al. 2007; Miller et al. 2007; Eryalçın et al. 2013). Microalgae diets have been confirmed to be the best diet for bivalve hatcheries and laboratory tests,

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because of their intrinsic qualities (Martínez-Fernández et al. 2004). Therefore, the optimal production of bivalves in hatcheries requests a large mass-production of microalgae that was considered the nutritional source for these organisms, from the larval stage to the adult stage. The selection of appropriate diets for the experimental feeding method is very important in aquaculture, especially during the preparation and conditioning of these organisms for later reproductions. It has been reported that the application of a mixed algal diet for bivalves ensures obtaining a balanced diet (Muller-Feuga et al. 2003; Rico-Villa et al. 2006). Currently, isolation of microalgae from the marine environment and their culture under well-controlled conditions are the most common techniques for feeding bivalves (Robert and Trintignac 1997). Furthermore, bivalves separate from crustaceans and larvae fish by using microalgae directly. Consequently, the microalgae using as food must exhibit a suitable quantity and quality. According to Laing (1987), the choice of microalgae intended for feeding bivalves must follow some specific characteristics such as digestive efficiency, absence of toxic components and appropriate cell size of microalgae. To ensure adequate feeding for bivalves, microalgae diets used, need to have suitable size, easy to digest, rich biochemical composition, without toxicity, optimal growth (Laing 1987; Rico-Villa et al. 2006; Pettersen et al. 2010). These factors have been advised because of the differentiation in the nutritional values between various species of microalgae and the subsequent physiological responses registered in bivalves fed with different algae diets (Tremblay et al. 2007; González-Araya et al. 2012; Gonzales et al. 2013).

In general, it is difficult to determine suitable diets for each bivalve species (Rico-Villa et al. 2006). As for the intrinsic quality of diets, one can have different species or even different strains of the same species of microalgae with very different nutritional values. Also, depending on the conditions under which microalgae are produced; a microalgae species may be considered an excellent or poor food source. If the culture conditions are similar, the nutritional effect changes depending on the consumer organisms (Cordero Esquivel 1994). Therefore, the diets prepared with several species of algae increase the chances to provide a well-balanced diet, for the best growth and development of bivalves (Muller-Feuga et al. 2003; Rico-Villa et al. 2006). Previous studies have analyzed the various aspects of growth and feeding at different stages of the development of bivalve mollusks by using single-species diets or mixtures of seaweeds such as *Chaetoceros calcitrans*, *Pavlova lutheri*, *Isochrysis galbana (ISO)*, *Tetraselmis suecica* (*T*) and *T-ISO* as a feed for *Pteria sterna* larvae (Martínez-Fernández et al. 2004). Other studies were interested in *Rhodomonas* sp. species which was considered a high-performance scheme, and therefore, it was among the most successful species to ensure good nutrition during the different stages of scallop *pecten maximus* larvae development (Tremblay et al. 2007). While the experimental diets based on *Chaetoceros muelleri*, *Thalassiosira weissflogii*, and the diatom *Chaetoceros calcitrans forma pumilum* have a good nutritional property for *Ruditapes decussates* larvae, *M. galloprovincialis*, and *Crassostrea gigas* larvae, respectively (Rico-Villa et al. 2006; Pirini et al. 2007; Aranda-Burgos et al. 2014).

In this context, our study aimed to assess the biological performances of the Mediterranean mussel, *Mytilus galloprovincialis* in response to four diets; three diets consisted of a single microalgae species (*Nannochloropsis* sp., *Dunaliella* sp., or *Phaeodactylum* sp.), and the fourth diet consisted of a mixture of the three preceding species. In this study, to determine the best microalgae diet that optimizes the growth performances and the biochemical composition of the flesh of *Mytilus galloprovincialis* mussels, weight, linear growth, and the quality of the biochemical components of mussel flesh were evaluated during the three month-diet.

Materials and methods

Mussel culture

At the M'diq Bay, located in the western part of the Mediterranean coast of Morocco, near a fish farm, between Cape Sebta (35 ° 54'N, 5 ° 17'10 "W) to the North and Cape Negro (35° 40'N, 5° 16'40"W) to the South, *Mytilus galloprovincialis* mussels were collected. Shell length and body weight were between 30±1.03 mm and 40±1.10 mm, and between 3±1.42 g and 9±1.17 g, respectively. The individual samples were collected and transferred to the Aquaculture Station of the Center Specialized in Zootechnics and Marine Aquaculture Engineering of the National Institute of Fisheries Research (M'diq, Morocco).

After a thorough cleaning to eliminate the epiphytes, mussels underwent ten days-phase of acclimatization to the new conditions of life in the laboratory. During the acclimation phase, all individual samples were

kept in a 1 m^3 tank during 10 days in order to adapt to the new farming conditions and to ensure the stability of the environmental conditions, temperature, salinity, and diet. Mussel samples received a diet based on *Nannochloropsis gaditana* species. Then, they were randomly distributed in twelve similar plastic aquariums of a volume of 10 L with 95 mussels per aquarium.

Microalgae culture

The microalgae species used during this study were isolated then identified in the PROTEE laboratory at the University of Toulon, France (*Nannochloropsis* sp., *Dunaliella* sp.), and in the National Institute for Fisheries Research-Morocco (*Phaeodactylum* sp.). They are cultured by passing from 3 mL in test tubes (mother cell) to 5 L of Guillard F/2 medium culture in Erlenmeyer flasks, which is rich on micro, macro-elements, and vitamins that are necessary for the growth and development of these microalgae during exponential phase (Fernández-Reiriz et al. 2015) to a final amount of 64.44×10^6, 12×10^6 and 25×10^6 cells mL^{-1} for *Nannochloropsis* sp., *Dunaliella* sp., and *Phaedactylum* sp., respectively.

In each aquarium, *Mytilus galloprovincialis* mussels received 7 L of seawater, filtered with a sand filter. *Nannochloropsis* sp. (group A), *Dunaliella* sp. (group B), *Phaeodactylum* sp. (group C), and the mixture of the three species *Nanno/Duna/Phae* (group D) were used for mussel diets.

Physical conditions

Compressed air has been continuously injected into all aquariums to ensure the oxygenation of water and maintain a homogeneous distribution of algal cells of each diet in the aquariums for the well-been of mussels. Lighting throughout the aquarium was provided by two fluorescent lamps of 36W operated on a light/dark cycle of 16/8 h. Daily water renewal was done manually to adjust the quantity of seaweed diet to the algal cell density required (number of cells in mL) which is 10^4 of algae cells mL^{-1} for each mussel. To ensure proper water quality, all aquariums used in this study were daily cleaned by removing pseudo-feces, feces, other debris, and dead mussels. During the whole experiment process, pH was maintained at 7-8, temperature and salinity of seawater were kept at 16-18 °C and 35 - 36 ‰, respectively.

Sampling procedure

The experiment process was conducted as follows; twelve aquariums divided into four groups; three groups fed with one species each, either *Nannochloropsis* sp. alone, *Dunaliella* sp., *Phaeodactylum* sp., and a group fed with a combination of the tree species. Every two weeks, the biochemical composition of the mussel flesh, including lipids and proteins was evaluated for five mussels from each aquarium.

Body weight and linear growth

Every two weeks, the shell length, and body weight of 30 mussels from each aquarium were measured, using a caliper, and a lab balance, respectively.

Biochemical composition of microalgae and mussel body

To evaluate the effect of isolated microalgae (Table 1) on the biochemical quality of Mediterranean mussel flesh, total protein and lipid were determined every two weeks for five mussels of each aquarium. To measure the protein content, the Lowry method was used as follow; 0.8 mL of microalgae samples were centrifuged at 10000 rpm for 10 min. Then, 4 mL of cupro-alkaline reagent was added and mixed with the pellet. 0.4 mL of 1 N Folin reagent was added after 10 min of incubation at room temperature. Finally, the mixture was incubated again for 30 min at room temperature, and the protein level was measured at 750 nm using a Rayleigh UV-1800 spectrophotometer and the calibration curve; $y=0.797x+0.021$, $R^2=0.977$. The same protocol was used to determine the protein level in mussel flesh with an additional step, that requires rinsing the flesh with 10 ml sterilized water, followed by 2 min of homogenization, before the centrifugation step (González López et al. 2010). Bligh and Dryer method was used for lipid extraction

Table 1 Content of total lipids, total proteins in the three microalgae species used in this study

Characteristics	*Nannochloropsis* sp.	*Dunaliella* sp.	*Phaeodactylum* sp.
Cell density ($\times 10^6$ mL^{-1})	22^c	6.5^a	10^b
Total lipid (%)	36^a	32^a	45^b
Total protein (%)	24.10^a	33^b	29.11^b

The different letters indicate significant differences in lipid levels between diets (Tukey HSD, $p < 0.001$).

procedures. 1 g of dried biomass was placed in a test tube, then 8 mL methanol and 4 mL chloroform (*V/V*: 2/1) were added. After agitation for 10 min in a Stomacher, another 4 mL of chloroform was added to the mixture, and the tube was mixed for 3 minutes. To achieve a better extraction of lipids, the mixture was incubated for 24 hours in the dark, then 4 mL of distilled water was added, and the tube was centrifuged for 15 minutes at 3400 rpm to obtain an aqueous supernatant and an organic phase composed of chloroform and lipids extracts (Safi et al. 2014). The organic phase was filtered and dried at the next step. The sample was weighed before and after to determine the total lipid content. This method was used to measure the total lipids of four grams of flesh mussels (Bligh and Dyer 1959).

Statistical analysis

All the experiments were carried out in triplicate and all the data were tested for the normality and the homogeneity of the variances. All statistical tests were performed using SPSS statistical software (SPSS software version 16.0 IBM). One-way analysis of variance (ANOVA) was used to test the differences between the diets for each variable (survival rate, biochemical composition, weight, and linear growth), and were considered significant for $P < 0.05$. Post-hoc Tukey test was used to correct for multiple comparisons of the means between the groups.

Results

Growth performance of microalgae

The three species used as diets were cultured at the following starting cell densities; 0.8×10^6, 0.2×10^6, and 1.25×10^6 cells mL^{-1} for *Nannochloropsis* sp., *Dunaliella* sp., and *Phaeodactylum* sp., respectively. After 15 days of culture, the three species reached the maximum algal concentrations of 64.44×10^6, 12×10^6, and 25×10^6 cells mL^{-1} for *Nannochloropsis* sp., *Dunaliella* sp., and *Phaeodactylum* sp., respectively (Fig. 1).

Lipid levels of the flesh mussels

The initial lipid content in the mussels tested was as follow; 3.19±0.03%, 3.28±0.04%, 3.18±0.07%, and

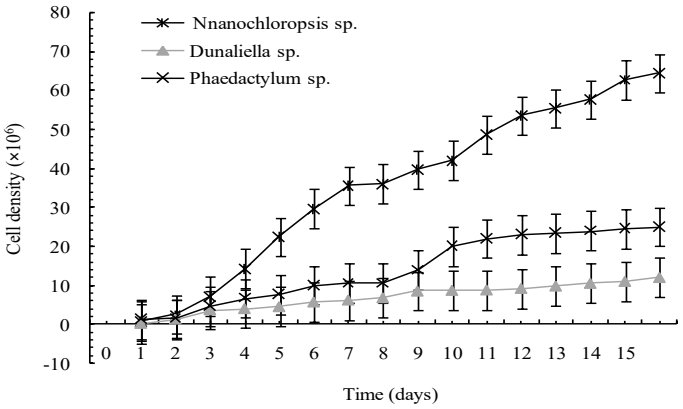

Fig. 1 Growth kinetics of the three algae species (*Nannochloropsis* sp., *Dunaliella* sp., and *Phaeodactylum* sp.). Vertical bars denote a standard error (SE).

3.26±0.10% from groups A, B, C, and D, respectively. Group D that received a mixed diet of three species, reached a higher lipid content after 30 days of diet compared to the other groups fed by one-microalgae species; *Dunaliella* sp., *Nannochloropsis* sp., and *Phaeodactylum* sp.. After 60 days with the algae diet, a decrease in lipid level was observed for the four groups and was recovered gradually thereafter (Fig. 2).

Proteins in the flesh mussels

The protein levels increased gradually during the first thirty days of the feeding and decreased during the following fifteen days. Subsequently, after this period, another increase of mussel protein level was observed for all the tested groups; with the greatest protein level was registered in group B fed with *Dunaliella* sp. alone and group D fed with the mixture of three species *Nannochloropsis* sp., *Dunaliella* sp., and *Phaeodactylum* sp..

The initial average protein values in the four batches of mussel ranged between 4.69±0.06% and 4.82±0.03%. The rate increased during the last 90 days of the experiment reaching a value of 28.92±0.02, 26.07±0.01 per 100 g, in the mussels fed with *Dunaliella* sp. diet, and mussels fed with the mixture of three species (*Nannochloropsis* sp., *Dunaliella* sp., and *Phaeodactylum* sp.), respectively. These values were higher than those from mussels fed with the *Phaeodactylum* sp. (24.52±0.02%) or *Nannochloropsis* sp. (22.94±0.06%). The protein level in mussels was significantly different between the four groups, starting from day 15 of the diet, throughout day 90 of the experiment (Fig. 3).

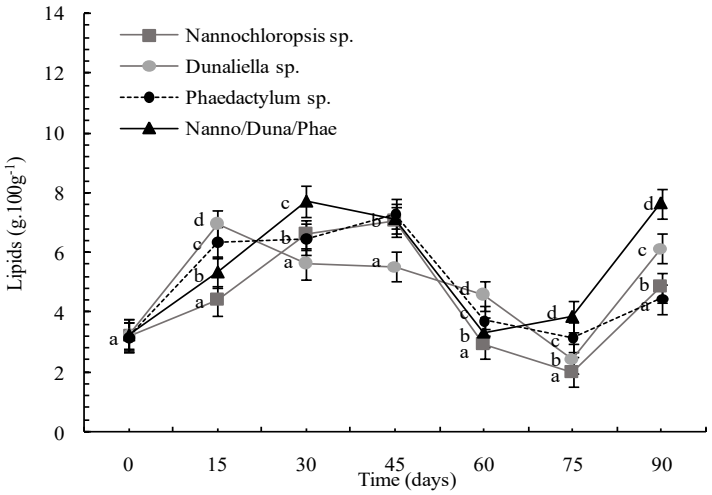

Fig. 2 Changes in lipid levels in the Mediterranean mussels during the algae diets (n=3). Vertical bars denote a standard error (SE). The different letters indicate significant differences between diets (Tukey HSD, $P < 0.001$).

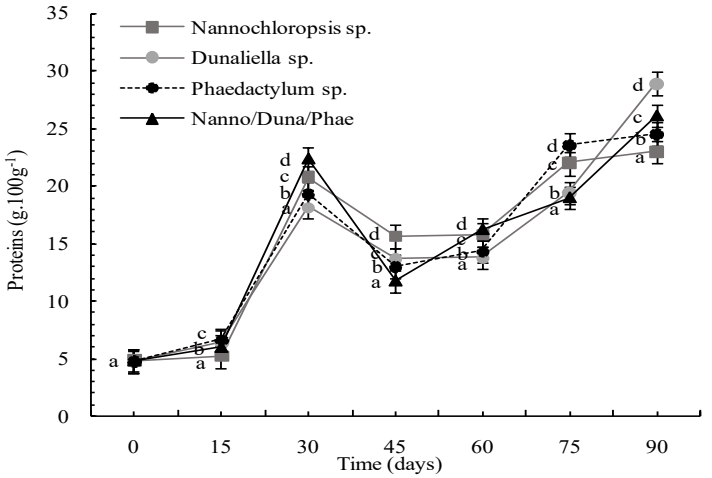

Fig. 3 The dose of proteins in the Mediterranean mussels fed by four microalgae regimes (n = 3). Vertical bars denote a standard error (SE). The different letters indicate significant differences between diets (Tukey HSD, $P < 0.001$).

Mussel wet weight changes during growth

The evolution of mussel in the four tested groups showed that their wet weight increased gradually from 5.39±1.42, 5.66±1.32, 5.71±1.17, and 5.6±1.03 g to 7.64±2.48, 7.76±1.96, 7.58±2.21, and 6.87±1.38 g for groups; A, B, C, and D, respectively. Group D exhibited the lowest mussel wet weight from the first month. Groups A and B experienced a fall in average mussel wet weight from day 45 until day 60 of the experiment before recovering at day 70 of the experiment (Fig. 4).

Linear growth

The evolution of the shell length of the mussels fed with different diets showed that linear growth has a similar trend during the experiment between the four groups. The average initial shell lengths were of 36.7±0.38, 37.0±0.32, 37.7±0.28, and 37.3±0.18 mm for groups A, B, C, and D, respectively. At day 90 of the experiment, groups A, B, C, and D recorded mean values of 42.4±0.26, 42.3±0.28, 42.3±0.26, and 41.7±0.23 mm, respectively (Fig. 5).

Survival rate

The lowest survival rate for the four diets was recorded during the last fifteen days of the experiment. As for the overall survival rate, mussels fed with *Dunaliella* sp., *Phaeodactylum* sp. and with a combination

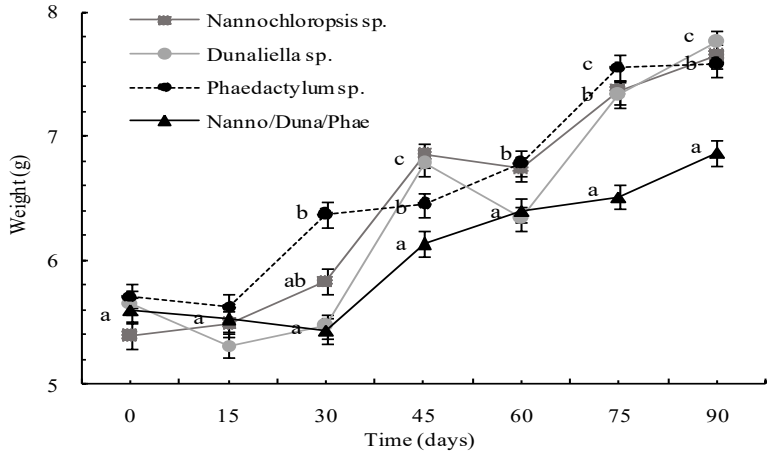

Fig. 4 Evolution of the average weight of mussels fed with the four microalgae diets. Vertical bars denote a standard error (SE).The different letters indicate significant differences between diets (Tukey HSD, $P < 0.001$).

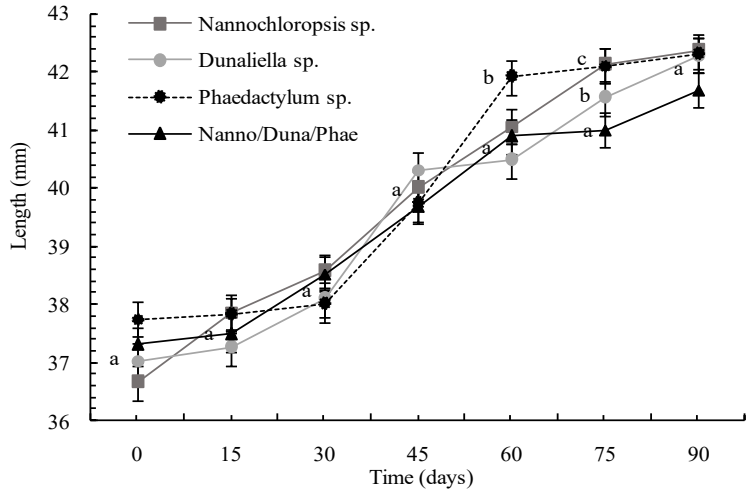

Fig. 5 Evolution of the average shell length of mussels fed by four diets. Vertical bars denote a standard error (SE). The different letters indicate significant differences between diets (Tukey HSD, $P < 0.001$).

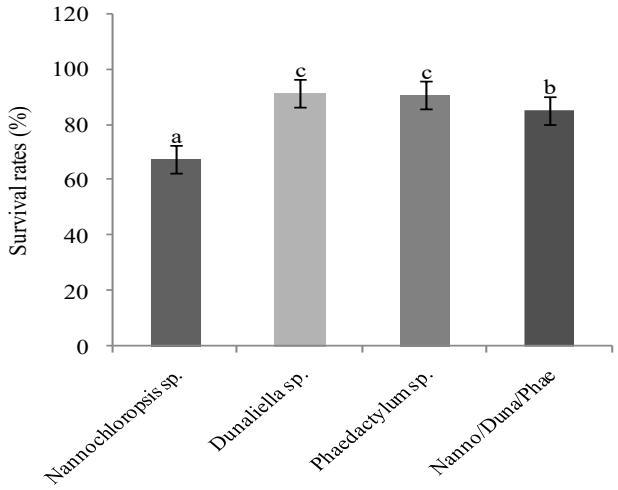

Fig. 6 Survival rates of mussels fed by four diets at the end of the experiment. Vertical bars denote a standard error (SE). The different letters indicate significant differences in survival rates between diets (Tukey HSD, $P < 0.05$).

of the three microalgae species diet (*Nannochloropsis* sp., *Dunaliella* sp., *Phaeodactylum* sp.), showed the highest average survival rates; 95%, 95%, and 94% respectively, but mussels fed with *Nannochloropsis* sp. species exhibited the lowest average survival rate (89%) (Fig. 6).

Discussion

Microalgae selected to feed bivalves must meet certain criteria related to size, cell morphology, and nutritional value (Albentosa et al. 1996; Brown et al. 1997; Martínez-Fernández et al. 2004; González-Araya et al. 2012; Gonzales et al. 2013). Some microalgae have a good nutritional profile for bivalves but are difficult to ingest (Fernández-Reiriz et al. 2015). The growth and the assimilation of biochemical components (lipids and proteins) by *M. galloprovincialis* depend considerably on the microalgae diet administrated (Brown et al. 1997; Seixas et al. 2009). In this study, the highest lipid levels were recorded in group D, fed with a combination of the three microalgae species; *Nannochloropsis* sp., *Dunaliella* sp., and *Phaeodactylum* sp., which confirms the results reported by Robert and Trintignac (1997) for *Tetraselmis suecica*, *Chaetoceros calcitrans* and *Isochrysis galbana* as fed for *M. galloprovincialis*. These data together suggest that diversified diets provide an adequate biochemical composition and a better nutritional regime than mono-algal diets. The four groups experienced an important reduction in lipid levels during the two samplings (T60-T75); this reduction in lipid accumulation was explained by gonad development and gamete release during this period. These phenomena are known to consume biochemical reserves (Orban et al. 2002, Nevejan et al. 2007). Furthermore, Ibarrola (1996) reported that the increase in the digestive effort that happens in the digestive gland may enhance the lipid loss through metabolic fecal losses (Ibarrola et al. 1996). In the other hand, some discrepancies were observed between different published studies that could be due to the different experimental parameters; such as the duration of the experiments, the physicochemical conditions, altering diet, and the experimental installations (Joseph 1982; Rico-Villa et al. 2006; Pettersen et al. 2010).

Proteins are necessary for the production of enzymes and the biosynthesis of tissues. Thus, the dietary components should be rich in protein levels for the metabolic processes and tissue development (Gatenby et al. 2003). The three microalgae used in this study; *Nannochloropsis* sp., *Phaeodactylum* sp., and *Dunaliella* sp. contain 24%, 29%, and 33% protein rate, respectively. Furthermore, the highest protein content was recorded in mussels fed with the single-algal dietary regimen. For example, for the mono-algal diet with *Dunaliella* sp., the final protein level was 28.92 per 100 g of mussels compared to 26.07 per 100 g in mussels fed with the mixed diet of three species. Moreover, in the framework of a project developed by Salinalgue in 2013, they have used the same species of microalgae as a source rich in protein. Proteins are very important for the growth of juveniles and adults undergoing gametogenesis for *Crassostrea virginica* oyster, and *Mytilus trossulus* mussel with protein levels higher than 15.6% and 57.4%, respectively (Langton

et al. 1977; Wikfors et al. 1992; Kreeger and Langdon 1994). The majority of proteins are degraded for optimal feeding of mussels (Kreeger et al. 1995; Kreeger et al. 1996). On the other hand, some species like *Tetraselmis suecica* have complex polysaccharides and proteins which explain the difficulty in digestion by mollusks (Epifanio 1979).

Our data about mussel weight and linear growth showed that *Dunaliella* sp. was the best mono-algal diet for the *M. galloprovincialis* mussel. However, mussels fed with a diet based on the combination of the three species (*Nannochloropsis* sp., *Dunaliella* sp., and *Phaeodactylum* sp.) exhibited the lowest meat wet weight and linear growth levels. Growth in shell length was constant during the experimental period, but weight growth fluctuated during the study period. The difference in physiological and biochemical growth rates was expressed by the difference in dimension (size, volume, and weight) of the three species of microalgae (Fernández-Reiriz et al. 2015). In our study, the difference in weight and linear growth between the different lots could not be explained by the difference in the size of diet cells, since the most successful strain in terms of growth was the strain with large cell size (*Dunaliella* sp.) and a small cell density of 6.5×10^{6} mL^{-1}. These results can be explained by the effect of rate, protein quality, and dietary lipids. A specific daily growth rate showed that group A fed with *Nannochloropsis* sp. has the highest daily values; 0.07 mm per day during the first 75 days and then 0.06 mm per day from until the 90 days.

For the survival rate, all four groups maintained high survival rates during the first 60 days of the diet. Subsequently, the group fed by *Nannochloropsis* sp. experienced a dramatic decline that lasted until the end of the experiment. This significant decrease in the survival rate could be related to the depletion of lipid reserves during the breeding period caused due to difficult digestion metabolites. Based on Martínez-Fernández study, *Nannochloropsis* sp. (group A) was very easy to ingest probably because of their small size (2 to 3 µm), but they were difficult to digest (Martínez-Fernández et al. 2004). Moreover, when the larvae of *A. ventricosus circularis* mussels were fed with *Nannochloris oculata*, the cells were ingested but they were not digested (Lora-Vilchis and Maeda-Martinez 1997). Based on these data, we conclude that in our study, the increased mortality observed in group A was caused by a failure to recover the digestive efficiency after the spawning phase.

Conclusion

In this study, *Dunaliella* sp. Diet was associated with the ideal protein profile. Moreover, the data from this study showed that this species of microalgae covers the nutritional requirements of *Mytilus galloprovincialis* mussel, from the protein level to the lipid level of the flesh, mussel weight, and the linear growth. Therefore, *Dunaliella* sp. could be considered one of the best mono-algal diets for mussels. However, further investigations in nutrient requirements are still needed to promote a well-balanced diet for aquatic mollusk farming.

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Conflict of interest The authors declare that they have no conflict of interest.

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