

# Digestive physiology and metabolism of green abalone *Haliotis fulgens* from postlarvae to juvenile, fed three different diatoms

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## Abstract

Growth, survival, digestive enzymes, ingestion rates, digestibility, fatty acid profile and energy budget were used to assess the nutritional quality of three diatoms as food for the first 3 months of age: *Navicula incerta* (NAV), *Amphiprora paludosa* (AMP), *Nitzschia thermalis* (NIT) and a combination of all three species (MIX). The highest growth was observed for postlarvae fed the MIX ( $51.37 \mu\text{m day}^{-1}$  and  $0.578 \pm 0.1 \text{ mg day}^{-1}$ ), but was not significantly different from the NAV treatments ( $46.60 \pm 3.4 \mu\text{m day}^{-1}$  and  $0.550 \pm 0.1 \text{ mg day}^{-1}$ ).

Abalone larvae, which are lecithotrophic organisms, seem to utilize proteins as a preferred energy substrate up to metamorphosis, since the relative lipid content increased from 15 to 30% from days 0 to 10. Thereafter, lipids are rapidly utilized and decreased to a level of 2% of the dry matter in the postlarvae whole soft tissue. Tissue fatty acid analysis indicated a similar trend among treatments, where relative fatty acid levels increased during the endogenous feeding period and started to decrease concomitant with the start of the exogenous feeding. Polyunsaturated fatty acids, reported on abalone tissue showed a similar trend, among treatments.

Based on the results reported here, it can be concluded that the type of diatom is an important factor for growth, where a high lipid and low ash content could be important to improve the performance in terms of growth and survival, in combination to stimulate protease activity and therefore better digestibility. Last but not the least important, the use of monoculture with NAV will be of importance when culturing abalone postlarvae. The estimated energy budget, back calculated from the digestibility figures, indicates that abalone requires from 25 to 38 cal day<sup>-1</sup> g abalone<sup>-1</sup> for adequate growth.

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**Keywords:** Abalone postlarvae; *Haliotis fulgens*; Diatoms; Growth; Apparent digestibility

## 1. Introduction

Abalone aquaculture still faces the problem of low survival and poor performance during the culture of the postlarvae stage (Uriarte et al., 2006). In an attempt to overcome this problem, a number of studies have been

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performed addressing one of the various aspects associated with the culture of postlarvae under controlled conditions, such as: natural chemical inductors of settlement (Slattery, 1992), dietary value of diatoms using proximate composition (Brown, 1991; Brown et al., 1997), the effect of culture methodology on diatom chemical composition, diatom density (Daume et al., 2004; Watson et al., 2004) and cell size (Kawamura et al., 1998a,b). Kawamura et al. (1995) reported that the dietary value of diatoms was related to the ability of abalone to break down the diatom cells using their radula during grazing. This ability was related to the radula strength overcoming the high adhesive capacity or the weakly silicified cell walls, but not to the digestive enzymes present in the abalone gut. However, in later studies and practice, the polyculture of diatoms has been emphasized by means that abalone can select their appropriated food (Martínez-Ponce and Searcy-Bernal, 1998; Gordon et al., 2006). Kawamura et al. (1998a) reported that the fast growth rates obtained using two diatom strains were related to the capacity of postlarvae to efficiently digest the diatom by breaking down cells walls during the passage through the gut, possibly influenced by diatom morphology, attachment strength, frustula hardness and postlarval age/size. When larvae are ready to metamorphose and culture conditions are adequate, metamorphosis is completed without problems (Hahn, 1989). If their requirements are not met, metamorphosis can be delayed resulting in a negative impact on future development (Takami et al., 2002).

Diatoms are the main diet for abalone postlarvae, not only as a source of nutrients, but also to adequately induce metamorphosis and promote good settlement in combination with other natural stimulants, such as  $\gamma$ -aminobutyric acid (GABA) among others (Takami et al., 1997). It has been reported that during the first 12 to 18 days after hatching, diatoms are more important as metamorphosis inductors than as nutrient sources, since differences in growth can be observed only after two weeks among different diatom strains (Kawamura et al., 1998a; Daume et al., 2004). Another critical period for the postlarvae development is the change from feeding on diatoms to feeding on macroalgae. This crucial period can have a significant economic impact on abalone production systems, since high mortalities are usually obtained (Searcy-Bernal et al., 1992). Some authors have suggested that radula hardening is the most important factor (Johnston et al., 2005), whereas others have blamed the digestive capacity of the postlarvae (Kawamura et al., 1998a). While the chemical composition between diatoms for postlarvae and macroalgae for juveniles is very different, abalone does not

suffer any metamorphosis to excuse these changes in nutrients.

Few studies to date evaluating abalone postlarvae feed utilization have combined information on growth, energy budgets, digestive enzyme activity and chemical composition of diatoms. Moran and Manahan (2003) studied the energy metabolism during larval development; however almost no information is given of the postlarvae stage. Combining these parameters can result in a more complete assessment of diatoms utilization, and under which conditions a beneficial effect is obtained on the overall performance under culture conditions.

Therefore, the objective of this study was to utilize a multidisciplinary approach to evaluate feed utilization by abalone (*Haliotis fulgens*) postlarvae, from fertilization to 3-month-old, using three species of diatoms and a mixture of the three.

## 2. Materials and methods

### 2.1. Experimental design

#### 2.1.1. Dietary treatments

Three benthic diatom strains isolated from the coastal area of Baja California (BC) and Baja California Sur (BCS), Mexico (Correa-Reyes et al., 2001) (Table 1) were used for this study: *Amphiprora paludosa* var. *hyaline* (AMP), *Navicula incerta* (NAV), *Nitzschia thermalis* var. *minor* (NIT) and a mixture of all three species (MIX). The diatoms were fed to the abalone from settlement to three months of life. Proximate composition and caloric content of the dietary treatments are presented in Table 1.

#### 2.1.2. Experimental units

A total of 2000 abalone postlarvae with an average shell length of  $909.21 \pm 130 \mu\text{m}$  and  $0.558 \text{ mg}$  (from one single batch) were selected for each bucket for use in the feeding trial that lasted a total of 90 days. Each experimental unit consisted of 20 L buckets in a batch-closed system (100% exchange per h) provided with supplemental air to maintain satisfactory oxygen ( $7.5 \pm 0.5 \text{ mg O}_2 \text{ L}^{-1}$ ). Temperature was maintained at  $20 \pm 1 \text{ }^\circ\text{C}$  throughout the experiment by submerging the buckets in a water-bath with a flow-through system connected to a heat pump. Each of the four treatments was run in triplicate for a total of 12 experimental units, and treatments were randomly assigned to buckets in the culture system. A photoperiod of 12L/12D was maintained throughout the experiment. Light was controlled using 75 W cool-white fluorescent lamps to provide  $150 \mu\text{E m}^{-2} \text{ s}^{-1}$ . Diatoms previously grown in separate tanks (see below), were added to each experimental unit, by

Table 1

Proximate composition in dry weight and energy content of four experimental diets used to feed postlarvae green abalone (*Haliotis fulgens*)

	AMP	NAV	NIT	MIX
Ash (%)	53.31±6.4 <sup>ab</sup>	41.80±4.8 <sup>b</sup>	57.64±4.2 <sup>a</sup>	53.77±1.0 <sup>ab</sup>
Crude protein (%)	16.46±0.6 <sup>a</sup>	15.97±6.7 <sup>a</sup>	16.23±3.6 <sup>a</sup>	18.73±2.7 <sup>a</sup>
Crude lipids (%)	3.28±1.0 <sup>a</sup>	7.46±1.3 <sup>b</sup>	4.44±1.0 <sup>ab</sup>	6.29±1.0 <sup>ab</sup>
NFE (%)	26.95±5.8	36.16±5.4	19.92±0.6	21.21±3.4
Calories (kcal g <sup>-1</sup> DW)	2.054±0.2 <sup>a</sup>	2.608±0.4 <sup>a</sup>	1.669±0.6 <sup>a</sup>	2.547±0.1 <sup>a</sup>

AMP = *Amphipora paludosa* var. *hyalina*, NAV = *Navicula incerta*, NIT = *Nitzschia thermalis* var. *minor* and MIX = mixture of previous strains.

NFE: Nitrogen free extract calculated by difference.

Calories are given as 1000 cal g<sup>-1</sup> of dry weight.Mean values in the same row with different superscript letters are significantly different ( $P < 0.05$ ).

supplying 150 mL broth culture to each monospecific treatment and 50 mL from each diatom to the mixture treatment.

## 2.2. Diatoms production

The three benthic diatom strains were grown under similar conditions. All diatoms were grown monospecifically in a non-axenic batch culture system using triplicate 18 L white plastic containers with 12 L of “F” medium (Guillard and Rhyter, 1962). The inoculums were produced in progressive volumes of 10 mL, 150 mL and 12 L. Cell densities of the inoculums used to grow each strain were based on cell size to homogenize the volume of food available. Seawater used in the media was disinfected using commercial chlorine (6%) as described by Hemerick (1973). Diatoms were grown at a constant room temperature (20±1 °C) and salinity (34±1‰) with a pH ranging from 7.8 to 8.7. Continuous light intensity of 150 μE m<sup>-2</sup> s<sup>-1</sup> was provided by cool-white fluorescent lamps. Cell density was calculated by direct count using a 0.1 mm hemacytometer observed under a compound Microscope Meiji model ML5100. To avoid possible cell aggregations, ultrasound (1 to 3 min) was applied to the sample prior to cell density determination. Every week inoculums with five-day-old diatoms were added to the experimental units. For the mixed treatment an estimate of equal volumes of each diatom was made and added to the experimental bucket.

## 2.3. Chemical analysis

### 2.3.1. Proximate analysis

Proximate composition of diatoms and abalone tissue was determined in triplicate according to standard procedures (AOAC, 1990). The moisture content of each sample was calculated from the samples (200 mg) dried to constant weight at 60 °C. Mean total nitrogen

content was determined by the micro-Kjeldahl method, and percent crude protein was then calculated as % N×6.25. Mean total lipid concentration was determined gravimetrically after being extracted with methanol–chloroform, eluted with dichloromethane, and vacuum evaporation of solvent according to the method of Folch et al. (1957). Mean ash content was determined by heating samples to 550 °C for 6 h. Nitrogen free extract was calculated by difference (% NFE=100–(% crude protein+% total lipid+% ash). Samples of the soft tissue of experimental abalone from each dietary treatment were collected at the end of the experiment, and frozen at –80 °C for subsequent analysis.

### 2.3.2. Enzyme analysis

Abalone postlarvae samples were taken once every week throughout the experimental period. Depending on abalone size, 1 to 10 organisms were sampled and pooled to provide adequate amounts for all enzyme analysis (Lazo et al., 2000). Abalone postlarvae were homogenized using an ultra-Turrax homogenizer in ice-cold physiological saline solution (NaCl 0.9%) and diluted in 1:3 (w/v). All homogenates were centrifuged at 8500 ×g for 15 min at 4 °C. The supernatant (crude extracts) samples were frozen and stored at –80 °C until enzyme assays.

### 2.3.3. Proteases

Alkaline protease activity was determined according to a modified method of Sarath et al. (1989). Briefly, the incubation mixtures consisted of 100 μL 0.05 M Tris–HCl with 10 mM CaCl<sub>2</sub> buffer at pH 8.1, 100 μL crude extracts and 150 μL 2% azocasein as substrate. The reaction was performed at 25 °C and terminated by adding 750 μL 10% TCA. The optical density of the supernatant was measured at 360 nm. Only the linear fraction of the reaction was used to estimate rates of enzyme activity for each enzyme assay. Enzyme activity

was expressed in units (U); 1 U of enzyme activity was defined as the increase of 0.001 U of absorbance/min. Activity was reported either as total activity (U/post-larvae) or specific activity (U/mg protein). All enzymatic assays were performed in three replicates.

Trypsin activity was determined according to a modified method of Erlanger et al. (1961). BAPNA (N $\alpha$ -benzoyl-L-arginine-4-*p*-nitroanilide hydrochloride) was used as substrate. The reaction was performed in 1 mM BAPNA in 50 mM Tris–HCl with 20 mM CaCl<sub>2</sub> buffer at pH 8.2, incubated at 25 °C and terminated by adding 200  $\mu$ L 30% acetic acid. The amount of *p*-nitroaniline liberated from BAPNA was determined at 410 nm. Enzyme activity was expressed in units (U); 1 U was defined as the increase of 0.01 U of absorbance/min.

#### 2.3.4. Lipases

Lipase activity was determined by the hydrolysis of 4-nitrophenyl-caproate (4-NPC) according to the modified method of Gjellesvik et al. (1992), but without bile salts. Briefly, the reaction was initiated by adding the enzyme extract to 0.5 M Tris–HCl (pH 7.4) buffer, 4-NPC (100 mM in ethanol, with a final concentration 0.35 mM in assay mixture). Temperature was maintained at 20 °C and the increase in absorbance was recorded at 400 nm. Enzyme activity was expressed in units (U); 1 U was defined as the increase of 0.001 U of absorbance/min.

#### 2.3.5. Carbohydrases

Amylase activity was determined by the method of Bernfeld (1955), with 1% starch as substrate. A solution of 3,5-dinitrosalicylic acid was used to determine the amount of reducing sugar produce as maltose by measuring absorbance at 546 nm. Enzyme activity was expressed in units (U); 1 U was defined as the increase of 0.01 U of absorbance/min.

#### 2.3.6. Protein content

To estimate specific activities of the homogenates, protein concentration was determined in the crude extracts according to Bradford (1976) using a bovine serum albumin (BSA) as standard and reported as mg protein equivalent to BSA.

#### 2.3.7. Fatty acids analysis

Aliquots of the lipid extracts of pool samples from eggs and muscle tissue of abalone postlarvae were initially refluxed for 3 min in a 0.5 M KOH solution in methanol and followed by methylation of fatty acids through additional refluxing (3 min) in 14% borontri-

fluoride in methanol (Metcalf et al., 1966). Fatty acid methyl esters (FAMES) were analyzed in a Hewlett Packard 5890II gas chromatograph equipped with a flame ionization detector (260 °C). FAMES were separated with capillary column (Omegawax™ 320 by Supelco Inc.; 30 m  $\times$  0.32 mm, film thickness 0.25  $\mu$ m) using hydrogen as the carrier gas. The initial oven temperature was 140 °C. Five minutes after injection of the sample (1  $\mu$ L), the temperature was increased to 240 °C at a rate of 4 °C/min. This temperature was maintained for an additional 10 min. Fatty acids were identified by comparison with the retention times of standards (37 Component FAME Mix, Supelco Inc.; GLC 87, Nu-Chek Prep) and well-characterized profiles of samples of marine oils (PUFA1 and PUFA3, Supelco Inc.). The concentration for each fatty acid was calculated from the corresponding area in the chromatogram with the help of an internal standard (23:0) using the software package HP ChemStation rev. A.06 for Windows. Results are given as percentage of total fatty acids.

### 2.4. Experimental procedure

#### 2.4.1. Growth in terms of length

Every week growth in terms of length was measured by sampling 30 postlarvae from each experimental unit and taking photographs using an inverted microscope ZEISS model Stemi 2000-C. The software “Motic Image 2000” ver. 1.3 was used to calculate the growth from the digital pictures.

#### 2.4.2. Feed intake

Feed intake for each dietary treatment was estimated using a modified technique from that reported by Martínez-Ponce and Searcy-Bernal (1998). Briefly, diatoms were grown in tissue culture dishes of 6 wells to completely cover the bottom, then 5 postlarvae were placed in the culture dishes and feeding was monitored in an optical microscope and recorded using a video camera. The software “Motic Image 2000”, ver. 1.3 was used to measure the cleared path at different times within 2 h. Feed intake expressed as percent of body weight (% BW) was estimated from the weight of cells ingested per organism per day as follows:

$$Fi = 100(DWC/wet\ weight\ of\ post\ larvae) \quad (1)$$

where DWC is the dry weight of the total number of ingested cells in mg after 1 h observation (extrapolated to 24 h) divided by the mean weight of the postlarvae. Diatom weight was calculated per strain by drying to a constant dry weight (60 °C) a known number of cells.

Feed intake expressed as mg diatoms was used to calculate the calories ingested per abalone using the theoretical values according to Gnaiger (1983): protein 5.73, lipids 9.43, and carbohydrates 4.18.

The feed conversion efficiency (FCE) was calculated using total wet weight gain per abalone and the corresponding feed intake is as follows:

$$\text{FCE} = 100[\text{wet weight gain(g)}]/[\text{feed intake(g)}] \quad (2)$$

### 2.5. Apparent digestibility

Apparent digestibility of the diatoms was calculated using a natural internal and inert marker (chlorophyll “ $\alpha$ ”). Chlorophyll has been previously used to determine forage digestibility in other species with good results (Shearer, 1963). Since chlorophyll is converted to pheophytin when exposed to oxygen (due to oxidation), both forms were quantified to show total amount of marker in the food and feces (Shearer, 1963).

Chlorophyll and pheophytin were measured as recommended by Arar and Collins (1997). Briefly, fluorescence is measured after extraction with chloroform and correction for pheophytin after acidification, using chlorophyll “ $\alpha$ ” (Sigma) as a standard.

Apparent dry matter digestibility (AD) was then calculated as follows:

$$\text{AD}(\%) = [1 - (\text{marker in fed}/\text{marker in feces})] \times 100 \quad (3)$$

where marker is the sum of chlorophyll and pheophytin.

### 2.6. Oxygen consumption

One to ten abalone depending on size were used to determine abalone oxygen consumption. Abalone were placed in a 2 mL acrylic chamber to register oxygen uptake, recorded using a computer-controlled polarographic oxygen sensors (Strathkelvin Instruments, Ireland), with six channels. Prior to use, the sensors were calibrated with a 0% oxygen solution (2% sodium sulfite in 0.01 M sodium borate as buffer) and 100% oxygen saturated water. The sensors were held in the solution/water ( $21 \pm 1$  °C) until variations were no longer recorded. The incubations lasted 1–1.5 h and were conducted at noon using a randomized block design per time (three consecutive days). The rate of oxygen consumption ( $\mu\text{mol O}_2 \text{ g}^{-1} \text{ h}^{-1}$ ) was calculated individually to obtain the slope of the  $\text{O}_2$  evolution curve, after subtraction the  $\text{O}_2$  depletion from the two chambers without animals (controls). The following equation was used to calculate oxygen consumption:

$$\text{VO}_{2e} = (\text{Cs} * m * 60) / (100\% * \text{Wwt}) \quad (4)$$

where  $\text{VO}_{2e}$  corresponds to the rate of oxygen consumption of the experimental organism ( $\mu\text{L O}_2 \text{ h}^{-1}$ ); Cs is the total amount of  $\text{O}_2$  in the incubation chamber at 100% saturation ( $\mu\text{L O}_2$ );  $m$  is the slope of the  $\text{O}_2$  evolution curve ( $\% \text{O}_2 \text{ min}^{-1}$ ); and Wwt is the sum of the live weight of experimental organisms in the incubation chamber. At the end of each period the weight of the experimental abalone was determined in an ultrabalance ( $\pm 1$   $\mu\text{g}$ ) and the net volume from the chambers was calculated by weight.

Table 2

Biological indices of postlarvae green abalone (*Haliotis fulgens*) measured before and after feeding the four different treatments

	AMP	NAV	NIT	MIX	P
Initial length ( $\mu\text{m}$ )			909.21 $\pm$ 130.6		
Initial weight (DW mg)			0.558		
Final length (mm)	4.66 $\pm$ 0.1 <sup>b</sup>	5.41 $\pm$ 0.4 <sup>ab</sup>	4.33 $\pm$ 0.7 <sup>c</sup>	5.92 $\pm$ 1.1 <sup>a</sup>	<0.001
Final weight (mg)	40.80 $\pm$ 16.6 <sup>a</sup>	52.84 $\pm$ 26.1 <sup>a</sup>	14.37 $\pm$ 5.9 <sup>b</sup>	55.50 $\pm$ 23.9 <sup>a</sup>	<0.05
Growth rate ( $\mu\text{m day}^{-1}$ )	39.38 $\pm$ 1.1 <sup>b</sup>	46.60 $\pm$ 3.4 <sup>ab</sup>	35.14 $\pm$ 6.0 <sup>c</sup>	51.37 $\pm$ 9.8 <sup>a</sup>	<0.001
Growth rate (DW mg day <sup>-1</sup> )	0.424 $\pm$ 0.1 <sup>a</sup>	0.550 $\pm$ 0.1 <sup>a</sup>	0.145 $\pm$ 0.0 <sup>b</sup>	0.578 $\pm$ 0.1 <sup>a</sup>	<0.001
Feed intake (% BW)	0.15 $\pm$ 0.01	0.16 $\pm$ 0.02	0.10 $\pm$ 0.04	0.09 $\pm$ 0.04	=0.053
Feed intake (cal g <sup>-1</sup> abalone)	3.03 $\pm$ 0.015 <sup>b</sup>	4.27 $\pm$ 0.53 <sup>a</sup>	1.61 $\pm$ 0.61 <sup>b</sup>	2.32 $\pm$ 0.94 <sup>b</sup>	=0.004
Feed conversion efficiency (% <sup>a</sup> )	706.6 $\pm$ 33.8	641.8 $\pm$ 77.3	1194.2 $\pm$ 576.3	1320.9 $\pm$ 670.6	=0.229
Mortality (%)	18.27 $\pm$ 1.5 <sup>b</sup>	16.77 $\pm$ 3.1 <sup>b</sup>	36.48 $\pm$ 0.2 <sup>a</sup>	12.08 $\pm$ 2.9 <sup>b</sup>	<0.0001
Apparent digestibility <sup>b</sup> (% DW)	46.53 $\pm$ 6.3 <sup>ab</sup>	57.42 $\pm$ 5.0 <sup>a</sup>	41.86 $\pm$ 3.9 <sup>b</sup>	45.73 $\pm$ 0.7 <sup>ab</sup>	<0.05

AMP = *Amphipora paludosa* var. *hyalina*, NAV = *Navicula incerta*, NIT = *Nitzschia thermalis* var. *minor* and MIX = mixture of previous strains. DW = dry weight.

Mean values in the same row with different superscript letters are significantly different. Initial weight corresponds to a batch total weight.

<sup>a</sup>Calculated according to daily growth rate estimated in the last period.

<sup>b</sup>Calculated as indicated in Eq. (3) in Materials and methods, using chlorophyll and pheophytin as the internal marker.

Table 3  
Energy budget for postlarvae green abalone (*Haliotis fulgens*) fed with the different treatments

	AMP		NAV		NIT		MIX	
	Value	%	Value	%	Value	%	Value	%
Expected intake <sup>a</sup> DW (cal day <sup>-1</sup> abalone <sup>-1</sup> )	1.74	100	1.34	100	4.22	100	1.95	100
Weight change DW (cal day <sup>-1</sup> abalone <sup>-1</sup> )	0.21	12.06	0.27	20.13	0.07	1.66	0.29	14.89
Respiration (cal O <sub>2</sub> day <sup>-1</sup> abalone <sup>-1</sup> )	0.6	34.48	0.5	37.27	1.7	40.24	0.6	30.81
Apparent digestibility		46.5		57.4		41.9		45.7
Feces		53.5		42.6		58.1		54.3
Respiration								
MR (μL O <sub>2</sub> h <sup>-1</sup> abalone <sup>-1</sup> ) <sup>b</sup>	5.27±1.4 <sup>a</sup>		4.55±0.4 <sup>a</sup>		14.54±4.2 <sup>b</sup>		4.97±1.0 <sup>a</sup>	

AMP = *Amphipora paludosa* var. *hyalina*, NAV = *Navicula incerta*, NIT = *Nitzschia thermalis* var. *minor* and MIX = mixture of previous strains. Energy balance is given per organism in relation to energy intake. Mean values in the same row with different superscript letters are significantly different ( $P < 0.05$ ).

<sup>a</sup> Expected intake calculated by difference between cost of growth and respiration in relation to apparent digestibility.

<sup>b</sup> Oxygen consumption from fed abalone.

### 2.7. Statistical analysis

To determine whether mean growth, expressed as final body weight (log transformed), and the shell length of abalone fed the different diets were significantly different, a two-way analysis of variance, dietary treatments × two time periods each day, was conducted. Diet and interaction were estimated using orthogonal contrasts. The daily feed intake was compared among treatments using a two-way analysis of variance (period × treatment). Enzyme activity data was compared among sampling dates using one-way analysis of variance and *a-posteriori* Tukey test to see possible differences among dietary treatments. All significance levels were set at  $P < 0.05$ . All the statistical analyses were performed using SAS-GLM procedures (SAS 8.2, 2001).

### 3. Results

Growth response in terms of length and weight of abalone postlarvae fed the different diets is presented in Table 2. Highest growth was observed for postlarvae fed the MIX ( $51.37 \pm 9.8 \mu\text{m day}^{-1}$  and  $0.578 \pm 0.1 \text{ mg day}^{-1}$ ), but was not significantly different from the NAV ( $46.60 \pm 3.4 \mu\text{m day}^{-1}$  and  $0.550 \pm 0.1 \text{ mg day}^{-1}$ ). However, NAV was similar to AMP ( $39.38 \pm 1.1 \mu\text{m day}^{-1}$  and  $0.424 \pm 0.1 \text{ mg day}^{-1}$ ) treatment. On the other hand, abalone fed the NIT diet resulted in significantly lower growth ( $35.14 \pm 6.0 \mu\text{m day}^{-1}$  and  $0.145 \pm 0.0 \text{ mg day}^{-1}$ ).

Feed intake, expressed as percentage of body weight (%DW), resulted in low values where no significant differences were observed, from 0.16 to 0.09% for NAV and MIX treatments, respectively: values that resulted in high feed conversion efficiency (FCEs) among dietary

treatments, with values ranging from 1320 to 641% for MIX and NAV, respectively. In a similar manner, the feed intake converted to calories per gram abalone resulted in low values from 4.26 to 1.61 for NAV and NIT, respectively, being significantly different for NAV.

Significant differences were observed in mortality among treatments. Highest mortality (36.48%) occurred in postlarvae fed the NIT diet, whereas the lowest was found in postlarvae fed the MIX (12.08%) diet. Similarly, significant differences in apparent digestibility (AD) were observed (Table 2) among dietary treatments. Highest AD was obtained for NAV diet (57.42%), while the lowest digestibility was observed for the NIT diet (41.86%).

Since feed intake estimates were not accurate and provided very low values of feed intake, total caloric intake was back calculated. The energy budget presented in Table 3 was estimated by back calculating the

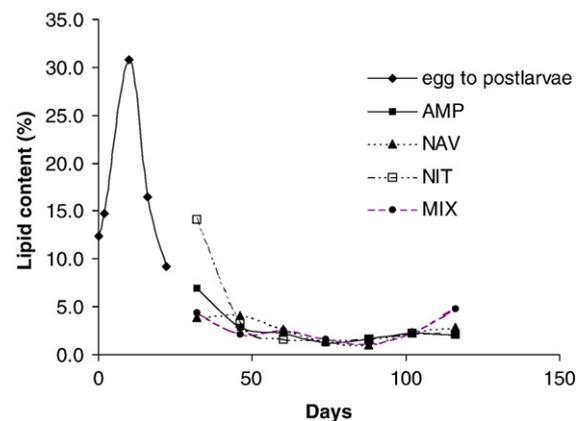


Fig. 1. Lipid content (%) in eggs, larvae and postlarvae of *Haliotis fulgens*. Postlarvae were fed three different diatoms and a mixture of all three (4 treatments) during 4 weeks.

Table 4  
Fatty acid composition (% of total fatty acids) in eggs, larvae and postlarvae of *Haliotis fulgens*

		Fatty acids																			
		14:0	15:0	16:0	16:1n-7	17:0	18:0	18:1n-9	18:1n-7	18:2n-6	18:3n-3	20:1n-11	20:1n-9	20:1n-7	20:4n-6	20:4n-3	20:5n-3	22:4n-6	22:5n-3	22:6n-3	n-3/n-6
Eggs		8.53	0.22	27.19	7.43	0.17	2.65	12.39	12.99	0.53	0.62	3.33	0.92	0.98	3.30	0.26	3.35	0.83	3.17	0.12	1.96
Larvae		8.00	nd	27.31	7.50	nd	2.25	11.97	12.52	0.44	0.49	4.88	2.46	7.88	2.36	0.21	2.88	0.52	2.03	1.03	2.37
Postlarvae newly settled		8.26	0.20	28.40	7.22	0.17	2.74	12.52	13.21	0.54	0.58	3.57	0.95	1.04	3.07	0.24	2.99	0.77	2.92	0.10	1.89
Postlarvae settled		3.35	nd	21.65	5.42	0.44	4.24	9.37	11.36	0.83	0.81	3.15	0.76	0.97	3.14	nd	6.47	0.69	5.00	0.39	3.19
Treatment	Week	Fatty acids																			
		14:0	15:0	16:0	16:1n-7	17:0	18:0	18:1n-9	18:1n-7	18:2n-6	18:3n-3	20:1n-11	20:1n-9	20:1n-7	20:4n-6	20:4n-3	20:5n-3	22:4n-6	22:5n-3	22:6n-3	n-3/n-6
AMP	1	4.54	nd	16.49	6.60	0.39	5.31	3.38	7.52	0.78	0.30	2.73	nd	0.33	0.91	0.39	16.84	nd	9.58	0.08	16.14
	2	3.65	0.29	18.45	10.51	0.40	5.51	2.25	9.14	0.82	nd	2.92	0.26	0.53	2.65	0.40	16.15	nd	8.44	0.67	7.41
	3	1.82	0.32	18.30	6.05	0.62	5.55	3.91	7.56	1.67	2.24	3.47	0.35	0.43	3.92	0.28	14.14	0.50	8.56	0.91	4.67
	4	1.84	0.45	19.60	2.85	0.84	7.63	3.96	7.46	1.90	2.77	3.12	nd	0.35	3.67	0.37	13.90	0.44	7.96	1.37	4.74
NAV	1	2.18	nd	15.69	11.18	nd	4.50	2.73	9.28	0.57	nd	2.28	nd	0.52	0.87	nd	12.10	nd	5.36	2.86	14.17
	2	1.18	nd	19.69	5.96	0.67	7.68	3.36	9.53	0.79	0.46	5.01	nd	0.53	3.31	nd	15.94	nd	10.75	1.27	6.93
	3	1.39	0.34	19.02	6.63	0.71	5.84	4.86	6.94	1.61	1.95	3.70	0.32	0.43	2.88	0.24	14.13	0.27	8.64	0.97	5.77
	4	1.73	0.48	18.92	2.14	0.94	6.01	4.01	7.54	2.45	3.85	3.29	nd	0.32	4.00	0.36	13.78	0.39	7.88	1.59	4.26
NIT	1	5.07	0.36	19.61	17.40	0.38	5.01	2.55	7.78	0.66	nd	2.41	nd	0.47	2.11	nd	14.21	nd	5.81	1.99	7.95
	2	2.17	0.30	18.64	11.32	0.48	5.96	2.44	10.48	0.77	0.33	3.87	nd	0.66	2.12	0.33	15.93	nd	8.23	0.89	8.93
	3	1.91	0.37	18.17	4.65	0.68	6.02	5.21	8.00	2.18	2.61	4.02	nd	0.37	3.32	0.35	13.49	0.33	8.56	0.92	4.72
	4	1.58	0.43	19.63	2.86	0.92	6.24	4.25	7.29	2.32	3.68	3.18	nd	0.32	4.52	0.49	14.25	0.42	7.06	2.74	4.12
MIX	1	2.82	nd	14.51	14.90	nd	4.04	1.54	8.27	0.54	nd	1.90	nd	0.41	0.53	0.40	14.53	nd	5.60	1.08	20.25
	2	1.65	nd	20.00	4.71	0.63	8.07	3.28	9.83	0.65	0.84	5.08	nd	0.48	2.18	0.31	15.85	nd	10.97	0.64	10.12
	3	1.85	0.34	19.65	6.77	0.66	6.17	4.72	8.59	1.58	1.78	3.83	nd	0.39	3.22	0.33	13.32	0.33	8.57	0.85	5.17
	4	1.83	0.33	18.81	3.39	0.71	5.71	3.41	7.65	1.82	2.83	3.05	nd	0.35	2.90	0.36	13.77	0.33	7.82	1.21	5.51

nd: not detected.

Postlarvae were fed three different diatoms and a mixture of all three (4 treatments) during 4 weeks.

Table 5

Analysis of variance and least square means from the digestive specific enzyme activity data of the green abalone postlarvae fed the four dietary treatments

Source	Trypsin		Alkaline proteases		Amylase		Lipase	
Treatment	(3) 0.184	<0.004	(3) 86	<0.006	(3) 0.409	<0.07	(3) 4.97	<0.25
Experimental unit (treatment)	(8) 0.018		(8) 23		(8) 0.117		(8) 3.04	
Day	(3) 0.373	<0.0001	(2) 800	<0.0001	(2) 7.342	<0.0001	(3) 32.15	<0.0001
Lineal	(1) 0.524	<0.0002	(1) 1567	<0.0001	(1) 14.621	<0.0001	(1) 63.50	<0.0001
Quadratic	(1) 0.013	0.4861	(1) 192	<0.0252	(1) 1.262	<0.02	(1) 17.00	<0.0139
Cubic	(1) 0.702	<0.0001					(1) 18.55	<0.0106
Treatment × day	(9) 0.082	<0.0162	(6) 66	<0.1083			(9) 1.611	<0.7162
Error	(21) 0.026		(13) 30		(17) 0.196		(21) 2.36	
R <sup>2</sup>	0.842931		0.891178		0.848965		0.751955	
Least square means								
AMP	0.6163 <sup>a</sup>		15.049 <sup>a</sup>		1.245 <sup>a</sup>		4.815 <sup>a</sup>	
MIX	0.5877 <sup>ab</sup>		13.033 <sup>ab</sup>		1.271 <sup>a</sup>		5.266 <sup>a</sup>	
NAV	0.3877 <sup>b</sup>		7.7175 <sup>b</sup>		0.778 <sup>a</sup>		3.276 <sup>a</sup>	
NIT	0.6940 <sup>a</sup>		9.5648 <sup>ab</sup>		1.194 <sup>a</sup>		4.453 <sup>a</sup>	

Different superscript letters within the same column indicate significant overall differences between treatments.

expected intake of calories to support the obtained growth utilizing the apparent digestibility values of the diets and the energy utilized for growth and respiration (Table 2). Thus, caloric intake required to maintain the observed growth must have been 4.22 cal for NIT, 1.95 for MIX, 1.74 for AMP and 1.34 cal for the NAV treatments. Based on the results obtained for the estimated cost of respiration, each abalone expend between 0.5 and 0.6 cal day<sup>-1</sup> (NAV, AMP, and MIX) corresponding to 37, 34 and 30% from the intake energy, respectively, whereas NIT utilized 1.7 cal (40% from the intake). The energy driven to grow for AMP, NAV and

MIX treatments was between 0.21 and 0.29 cal day<sup>-1</sup>, corresponding to 12, 20 and 15% from the intake, respectively, whereas NIT treatment utilizes only 0.07 cal day<sup>-1</sup>, corresponding to 1.66% from the intake energy. The oxygen consumption rates of abalone under the different feed regimes show significant differences with values ranging from 4.97 to 14.54 μL O<sub>2</sub> h<sup>-1</sup> abalone<sup>-1</sup> for MIX and NIT, respectively (Table 3).

Total lipid content from eggs to post-settle larvae prior to their establishment as feeding treatments is presented in Fig. 1, where differences could be observed throughout the development. Eggs had a lipid content of

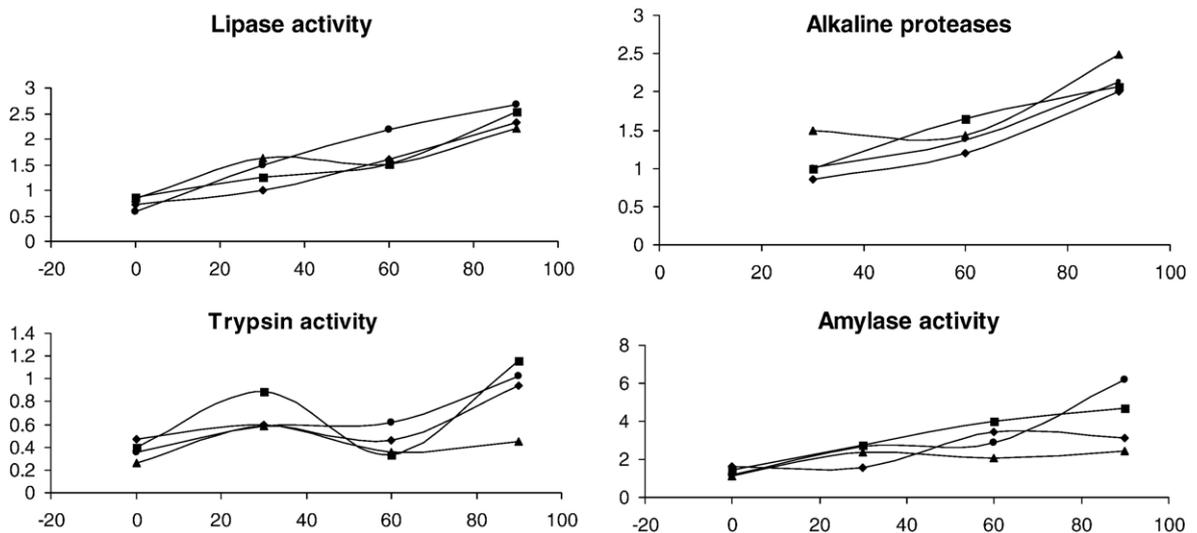


Fig. 2. Enzyme specific activity of lipases, alkaline proteases, trypsin and amylase from day 0 to 90 days from postlarvae fed different diets. NIT (■); AMP (◆); MIX (●); NAV (▲).

10% with an increase up to 30% 10 days after fertilization followed by a decrease of 5% just before exogenous feeding commenced. Thereafter, lipid levels were maintained close to 3% throughout the feeding trial (Fig. 1). Moreover, fatty acid analysis from eggs, larvae and newly settled postlarvae before feeding indicated that the fatty acid profile is conserved until settlement (Table 4). Fatty acids found in higher proportions in the egg were the saturated 14:00, 16:00, 16:1n-7, and the PUFAs 18:1n-9, 18:1n-7, 20:5n-3 and 22:5n-3. After settlement and before exogenous feeding initiated, the HUFAs 20:5n-3, 22:5n-3 and 22:6n-3 showed a relative increase in concentration as well as the n-3/n-6 ratio. Fatty acids, 18:3n-3, 18:2n-6, 20:4n-6, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3, tended to increase in postlarvae fed the different algal diets, resulting in a decrease of n-3/n-6 ratios in all treatments.

Specific enzyme activity expressed as units per mg protein resulted in a significant increase in activity throughout the experiment for all digestive enzymes evaluated (trypsin, alkaline proteases, amylases, and lipases (Table 5)). Significantly lower specific alkaline proteases and trypsin activities were observed for the postlarvae fed the NAV diet compared to the other dietary treatments. However, no significant differences among dietary treatments were found for lipase and amylase specific activities (Fig. 2).

#### 4. Discussion

To our knowledge, this is the first work performed evaluating diet utilization in abalone postlarvae using more than one or two response variables, such as growth or biochemical composition. Here a multidisciplinary approach was utilized to evaluate growth, survival, feed ingestion, digestibility, energy budget, feed impact on tissue fatty acids and digestive enzyme activities. The data presented here demonstrates that a single diatom (NAV) or a combination of all three diets (MIX) resulted in the highest growth and survival. This last is opposite to that reported by Gordon et al. (2006) where a mixture of microalgae resulted in better growth than single strains. Moreover Gordon et al. (2004) concluded that larvae settlement was not related to the biochemical composition of microalgae. These contradictory results could be due to differences in microalgae strains. Growth rates obtained in the present study are similar to those reported in earlier studies for other abalone postlarvae, ranging from 21.7 to 60.6  $\mu\text{m day}^{-1}$  (Takami et al., 1997; Kawamura et al., 1998a) even for the same species (Uriarte et al., 2006) with a quite good survival for MIX and NAV treatments (over 83%). The factors associated

to the higher growth and survival in the present work are related to several factors, like chemical composition, in particular ash and lipid content of the diatoms. NIT and AMP treatments resulted in a lower postlarvae growth and survival. These diets contained high ash and in the case of AMP a low lipid level. Moreover, high values of apparent digestibility were associated with the NAV diet, which contained the lowest content in ash and the highest lipid content, whereas NIT resulted in the highest ash content with the lowest digestibility.

The feed ingestion reported here resulted in extremely low values and much lower to those reported earlier for juvenile abalone (Gómez-Montes et al., 2003; Montañó-Vargas et al., 2005) being not enough to support the reported growth. It appears that the methodology utilized was not adequate for our purposes and significantly underestimated feed intake. The method used here requires the measurement of cleared patches by the abalone using fixed images from a video recorder (photographs) taken at different times and extrapolating to the complete plate (5 cm of diameter). Even if several areas are taken per plate, it seems that 5 postlarvae per plate are not enough to accurately represent what is occurring in the whole plate. Thus, it is recommended for future measurements, the registration from each single postlarvae following their feeding path along time in order to quantify the area clear by one postlarvae through time, from several postlarvae in each experimental unit per triplicate. This should give more accurate estimates of feed intake per organism while feeding. Additionally, our FCE values were exceptionally high and unrealistic, resulting from our feed intake underestimation.

Since an accurate estimate of feed intake was lacking in the present work, to build the energy budget it was decided to back calculate feed intake based on the minimum feed intake required to obtain the observed growth. In order to do this, digestibility values of each diet were combined with the caloric estimates of growth and respiration, assuming no other energy loss. In this way it was possible to estimate a caloric intake equivalent to 25 to 35  $\text{cal day}^{-1} \text{g abalone}^{-1}$  to sustain the best growth (NAV and MIX, respectively). In an earlier study with juveniles of the same species an estimated caloric intake of 60  $\text{cal day}^{-1} \text{g abalone}^{-1}$  was obtained utilizing more accurate feed intake values (Gómez-Montes et al., 2003). Nonetheless, in the latter study a certain amount of energy could not be explained (ranging from 7 to 28% of the total caloric intake). Additionally, the lower values obtained in the presented study (25 to 35  $\text{cal day}^{-1} \text{g abalone}^{-1}$ ) could be partially explained by the fact that here it was not considered energy lost due

to ammonia and mucus production by the organism. Montaña-Vargas et al. (2005) showed that ammonia and mucus in juvenile abalone accounted for 8% of that expended for respiration, energy that was not considered in the present work, and it could explain part of the underestimation of energy intake.

To achieve a maximum growth with a reasonable feed efficiency in terms of energy flow, it is expected that at least 10% of the energy ingested be directed to growth and 20% to respiration (Gómez-Montes et al., 2003). In the present study, the used energy estimated by back calculating the expected intake of calories in both treatments NAV and MIX, with the highest growth rate, resulted in energy values funneled to growth ranging from 20 to 15% of the total intake being NAV the most efficient diatom, whereas AMP resulted in 12% and lastly NIT only 1.7%. Moreover, the energy driven to respiration resulted in higher values than earlier reports (20 to 23%; Gómez-Montes et al., 2003), ranging from 31 to 40% of the calculated ingested energy.

The NIT treatment resulted in a negative impact in the energy flow where most of the energy was used for respiration compared to growth. This negative result is apparently due to the stress suffered by the postlarvae searching for optimal food. It has been demonstrated that abalone is strongly attracted by specific microalgae and it is possible that this particular *Nitzschia* was not suitable as the *Nitzschia laevis* reported by Gordon et al. (2004). Moreover, in this work it was not possible to estimate basal metabolism from the unfed group after 3 days with no food since those organisms expended more energy than the fed group. To reach a basal metabolism it has been recommended one week for juveniles (Gómez-Montes et al., 2003), however here it was considered that for such small animals more than three days would be critical.

Moreover, the apparent digestibility values of the diets were higher for the NAV, AMP and MIX treatment, whereas NIT resulted in a significantly lower digestibility compared to NAV. Kawamura et al. (1998a,b) found that growth and survival of postlarvae abalone *Haliotis iris* were highly dependent on diatoms digestibility, associated with diatoms morphology, attachment, frustula strength and postlarval age/size. Based on the results from the present study, we suggest that a combination of higher lipid content and digestibility of the diatoms is an important factor to promote adequate growth and survival with the concomitant energy flow efficiency. The methodology used by Kawamura et al. (1998b) to estimate the digestibility is different from the present study and was based on the estimation of the total broken and unbroken cells. NIT is among the

largest diatoms typically utilized as postlarvae diet (Mercado et al., 2004). This large size in combination with the high ash content can make it difficult for the postlarvae to ingest and digest the cells, as shown from our intake and digestibility data and high respiration rates expended searching for food.

As mentioned previously, our estimates of feed intake were extremely low and thus resulted in very high feed efficiency values. It is highly recommended for future studies to video tape one postlarvae at a time, during their feeding behavior and extrapolate to a 24-h period, assuming abalone postlarvae are constant grazers (Martínez-Ponce and Searcy-Bernal, 1998).

With respect to the fatty acid data, results obtained in the present study, show similarities in the fatty acid profile of eggs reported earlier for another species, *H. asinina* (Bautista-Teruel et al., 2001). In both studies, values for 14:0, 16:0 and 18:1n-7 correspond to the 50% of total fatty acids. Moreover, the fatty acid profiles found in the whole body are similar to earlier reports for the same species (Durazo-Beltrán et al., 2003a,b) and even other abalone species such as *H. rubra* and *H. laevigata* (Grubert et al., 2004). However, during the experiment it was possible to observe that the postlarvae showed a similar trend in fatty acid content along time and among diet treatments. In addition small differences could be observed between fatty acids. For instance, the 20:5n-3 was able to register the highest levels even during the first week of feeding. It seems that this fatty acid could readily be absorbed from the diatoms since it has been reported that diatoms are usually rich in this fatty acid (Gordon et al., 2006), whereas the 22:5n-3 was more or less conserved from 5 to 8% and the 22:6n-3 increased from 3 to 7 times more from the start to the end of the feeding period. Similarly, their precursor 18:3n-3 was conserved prior to the feeding period; once the postlarvae start grazing, this fatty acid increased up to 4 times. The fatty acids n-6 showed a similar trend, where the precursor 18:2n-6 increased up to 4 times along the feeding period, similar to the 20:4n-6. In general all diets had the tendency to decrease the n-3/n-6 ratio which means an accumulation of the n-6 fatty acids.

In general all enzyme activities showed a trend to increase through time as the postlarvae digestion system matures. The postlarvae fed the NAV treatment were the only ones showing the lowest specific trypsin activity, whereas they showed the highest specific total alkaline proteases. This performance is difficult to explain apart from considering that there is a probability that other alkaline proteases apart from trypsin are playing an important role on the overall digestion process when

NAV is fed to postlarvae. In addition to the high enzyme activity, digestibility was highest in the NAV treatment.

Lastly, it is important to mention that chlorophyll/pheophytin as internal markers could be an adequate method to measure apparent digestibility when only a small amount of feces are available. This method measures chlorophyll and pheophytin by fluorescence, which is highly sensitive and therefore only a few mg is needed. Additionally, this methodology has low cost, is simple and requires common laboratory equipment which makes it a good alternative to estimate apparent digestibility.

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