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Assimilation of dietary nitrogen supplied by fish meal and microalgal biomass from *Spirulina* (*Arthrospira platensis*) and *Nannochloropsis oculata* in shrimp *Litopenaeus vannamei* fed compound diets

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Abstract

The biomass of several microalgae species represents one of many single cell-derived products with great potential as dietary ingredients for the aquaculture feed industry. The present study compares the assimilation of dietary nitrogen and total dry matter supplied by fish meal and biomass from *Spirulina* (*Arthrospira platensis*) and *Nannochloropsis oculata* in postlarval Pacific white shrimp, *Litopenaeus vannamei*. The natural isotopic signatures of the ingredients were used as biomarkers to assess their respective nutritional contributions. Three control diets were manufactured with each of the main ingredients to establish shrimps' isotopic control values (initial mean weight 51 mg). Four mixed diets were formulated with varying proportions of ingredients on a dietary nitrogen basis (33:33:33 and permutated 50:25:25 proportions). Diets were supplied ad libitum four times daily for 22 days. Shrimp reared under most dietary treatments showed high growth rates. Final mean weight was significantly higher (268 ± 64 mg) in shrimp grown under diets containing only fish meal and those formulated with 50% fish meal (239 ± 64 mg) and 50% *Spirulina* (234 ± 57 mg). Isotopic assessments of ingredients and shrimp indicated significantly different nutrient assimilations. A higher incorporation of dietary nitrogen (43 to 52%) and dietary carbon (44 to 57%) from *Spirulina* was observed, while *N. oculata* and fish meal supplied lower proportions of dietary nitrogen to shrimp growth (4 to 33% and 25 to 51%, respectively). Results indicate a fast digestion and assimilation of *Spirulina*-derived nutrients, while *N. oculata* did perform poorly as a fish meal replacement ingredient.

Keywords *Litopenaeus vannamei* · Shrimp · *Arthrospira platensis* · *Nannochloropsis oculata* · Fish meal · Stable isotopes · Nutrient assimilation

Introduction

Finding substitutes for fish meal in aquaculture diets has important economic and ecological advantages given that this is an expensive commodity manufactured from small pelagic fish. Many marine fish populations are currently overexploited and the increased production of different aquaculture species continues to exert a high demand for fish meal. Among these mass-produced marine animals, the Pacific white shrimp *Litopenaeus vannamei* has become the main shrimp species produced through aquaculture practices, with a current production of 4 Mt annually (FAO 2018). Shrimp farming has thus become a major aquaculture sector requiring increasingly higher amounts of feeds and other commodities. Given the global importance of shrimp farming and its continued growth, finding sustainable alternatives to fish meal remains an outstanding challenge.

Fish meal has been the traditional protein source used in such aquafeeds, but has been partially substituted by plant proteins (NRC 2011). More recently, however, research has focused on the nutritional performance of different microbial-derived ingredients such as microalgal and bacterial biomass (Sarker et al. 2016; Qiu et al. 2018). Although research has shown these ingredients to have several nutritional benefits for aquatic organisms and different species of microalgae are extensively produced as part of larviculture operations, the application of microalgal biomass as a dietary ingredient remains relatively unexplored. This is

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mainly due to economic reasons as most of the current production technologies still demand a high level of resources. In addition, several technical difficulties in the production processes (photobioreactor efficiencies, energy costs, carbon dioxide, and fertilizer costs) are yet to be resolved (Nasseri et al. 2011; Acién et al. 2012), and safety and regulatory concerns related to the application of final products still remain (Shah et al. 2018). Despite these constraints, microalgal proteins have been compared to conventional plant proteins used in animal nutrition (Becker 2007; Teimouri et al. 2013) and it is worth exploring their applications as dietary ingredients in aquafeeds. Of note, microalgal biomass can be highly digestible (Tibbetts et al. 2017a) and studies on the use of microalgae meals as ingredient in aquaculture diets have indicated positive effects on growth, survival, pigmentation, and the immune response of crustaceans and fish (Gamboa-Delgado and Márquez-Reyes 2018). Therefore, as plant protein continues to be demanded for other uses and arable land becomes less available, it is reasonable to forecast a higher dietary inclusion of microalgal biomass in aquaculture feeds. Moreover, biorefineries and companies producing high-value microalgal compounds (e.g., astaxanthin) generate microalgal by-products (defatted microalgae biomass) that have been positively tested as aquafeed ingredients (Ju et al. 2012; Kiron et al. 2012). Specifically, microalgae species belonging to the genera *Haematococcus*, *Spirulina* (*Arthrospira*), *Chlorella*, and *Schizochytrium* have been tested as additives in aquaculture diets.

Nannochloropsis has been traditionally used to enrich zooplankton due to its high lipid and vitamin content, while *Spirulina* biomass has been deemed a highly nutritious item. Pakravan et al. (2017) reported that full replacement of fish meal with *Spirulina* in diets for in *L. vannamei* promoted growth rates as high as those observed in animals fed control diets only fish meal. Similarly, Macias-Sancho et al. (2014) successfully replaced 75% of fish meal with *Spirulina* without compromising shrimp growth. Additional benefits of *Spirulina* on crustaceans and fish include significant improvements in diet palatability (Silva-Neto et al. 2012), higher digestive protease activity (Pakravan et al. 2017), and enhanced immunological response (Watanuki et al. 2006). Despite these important advances, few studies have estimated the real, relative assimilation of dietary nitrogen and carbon supplied by microalgae meals and their contribution to the growth of the organisms that consume them.

One of the most reliable techniques to estimate nutrient assimilation is the determination of isotopic signatures in ingredients and animal tissue. In aquaculture nutrition, nitrogen and carbon isotope ratios ($^{15}N/^{14}N$ and $^{13}C/^{12}C$, expressed in delta notation as $\delta^{15}N$ and $\delta^{13}C$) are used as natural biomarkers to infer the origin (dietary ingredients) and fate of nutrients (tissue accretion, metabolic use, effluent losses) (Jomori et al. 2008; Gamboa-Delgado et al. 2016; Roussel et al. 2018). Integrating isotopic data into mathematical models has made it possible to convert isotopic information into dietary contributions (Phillips 2012). In order to increase the accuracy of interpretations, such studies require that the different dietary resources show distinctive isotopic values. As many of the ingredients used in aquaculture are derived from animal, plant, or microbial sources, and because each of these originates from a different environment with particular characteristics in terms of nutrient flows, they frequently show different isotopic signatures (Gamboa-Delgado 2014). The present study employed the natural isotopic differences found in fish meal and two types of massively cultured microalgae to assess the relative nutrient contributions supplied by such ingredients to the growth of postlarval Pacific white shrimp.

Materials and methods

Experimental meals and diets

Diets used in this study relied on three main nutrient sources: fish meal, *Spirulina* (*Arthrospira platensis*), and *Nannochloropsis oculata*. Fish meal (prime Mexican sardine, 68% protein) was used as a source of animal-derived nutrients and was previously analyzed for proximal content and isotopic values. *Nannochloropsis oculata* was obtained as a highly concentrated cellular paste (Reed Mariculture, Campbell, California, USA), while *Spirulina* (*A. platensis*) biomass was acquired as a dry fine powder (Pronat, Iztapalapa, Mexico). Proximate analysis and amino acid profiles of these three main ingredients were analyzed (Agricultural Experiment Station of the University of Missouri: AOAC 2006) and are shown in Table 1. Seven isonitrogenous (36% crude protein) and isoenergetic (4.7 kcal g^{-1}) experimental diets were formulated with different proportions of fish meal, *Spirulina*, and *N. oculata* biomass (Table 2). Diets were not manufactured with the objective of setting an ingredient-substitution study. Rather, they were formulated using ingredients with contrasting isotopic values in order to explore dietary contributions to shrimp growth. Three diets were formulated with only one source of dietary nitrogen: 100% fish meal (diet 100F), 100% *Spirulina* (diet 100S), and 100% *N. oculata* (diet 100N). These diets were used as isotopic controls to estimate and correct for the isotopic differences frequently observed between diets and consumers (isotopic discrimination factors, $\Delta^{15}N$ and $\Delta^{13}C$) after equilibrium was reached. The other four diets were formulated with varying proportions of the three main ingredients. One included 33% of each ingredient, estimated on a nitrogen content basis (diet 33FSN). The additional three diets included a permutated proportion of 50:25:25 of each ingredient (diets 50FSN, 50SFN, and 50NFS).

Table 1 Proximate, isotopic, and essential amino acid composition g (100 g)$^{-1}$ protein of fish meal and microalgal biomass derived from *Spirulina* and *N. oculata*. Ingredients were incorporated in experimental diets to conduct an assimilation study using stable isotope values as biomarkers. $n = 3$

	Fish meal	*Spirulina*	*Nannochloropsis*
Crude protein (g kg^{-1})	762	594	422
Lipids (g kg^{-1})	86	22	56
Ash (g kg^{-1})	83	73	218
Fiber (g kg^{-1})	1.0	12.0	6.0
Moisture (%)	5.9	7.9	11.1
δ^{15}N (‰)	16.50	9.97	2.10
δ^{13}C (‰)	− 19.50	− 23.29	− 40.37
Amino acid			
Arginine	6.43	7.20	5.18
Isoleucine	4.53	5.94	4.68
Phenylalanine	4.33	4.80	5.51
Histidine	3.49	1.61	1.74
Leucine	7.79	9.29	9.05
Lysine	8.60	4.91	5.69
Methionine	2.80	2.30	2.12
Threonine	4.51	5.09	4.95
Tryptophan	1.16	0.47	0.33
Valine	5.41	6.72	6.02

Before manufacturing the diets, fish meal was finely ground using a Pulvex 200 grinder fitted with a size #35 mesh. Additional sieving allowed separation of appropriate particle sizes. The concentrated *N. oculata* paste was freeze-dried for 48 h and the dry material was ground and homogenized. Dietary micronutrients were weighed to the nearest mg, hand-mixed, and added to the macronutrient mixture in a commercial blender. Water was added to the mixture and the dough was extruded through a die plate (1.4 mm orifices). Strands were collected and dried in a convection oven (20 min, 80 °C), after which they were broken down and refrigerated until used. Adequate formulation of the experimental diets was validated through proximate analyses including moisture content (method 930.15), crude protein (Dumas method, LECO), and lipid contents (Soxhlet system HT-1045, method AOAC 996.06) (Tecator 1983). The ingredients' available energy was estimated using a semi-micro bomb calorimeter (Parr 1425 PIC, USA).

Experimental animals and sampling

Pacific white shrimp (*Litopenaeus vannamei*) postlarvae were obtained from a commercial hatchery (AcuaMar, Baja California Sur, Mexico), and after transport, animals were acclimated to the following bioassay room conditions: seawater temperature 30.2 ± 0.7 °C, salinity 34.4 ± 0.7 g L^{-1}, pH 8.4 ± 0.1, and saturated dissolved oxygen. Total ammonia nitrogen (1.2 ± 0.2 mg L^{-1}), nitrite (1.2 ± 0.6 mg L^{-1}) and nitrate (11.7 ± 4.6 mg L^{-1}) were maintained within recommended ranges for penaeid shrimp. Most parameters were maintained within these values over the experimental feeding period. Before the assay, animals were fed for 20 days on a commercial compound diet (35% protein, Grupo Costamar, Hermosillo, Mexico) previously analyzed for isotopic values (δ^{15}N = 10.1‰ and δ^{13}C = − 23.5‰). This diet established initial reference isotopic values in shrimp before the start of the experiment. From this baseline, exponential isotopic changes were expected as each dietary treatment gradually transferred its distinctive isotopic signature to shrimp bodies.

At the beginning of the bioassay, shrimps (initial mean wet weight 51 ± 16 mg) were distributed in 21, 60-L glass fiber tanks. Twenty animals were placed in triplicate tanks and were previously selected in order to allocate animals with the same size distribution pattern in each unit. Tanks were individually fitted with air lifts and were connected to a recirculation system holding artificial seawater (Instant Ocean®, USA). Seawater was treated with cartridge and UV filters, protein skimmers, and a bubble bead biological filter. Experimental diets were supplied ad libitum at 8:00, 12:00, 16:00, and 20:00 h for 22 days. Uneaten feed, feces, and molts were siphoned out daily before first feeding. Sampling dates were defined according to the rates of isotopic change previously observed in fast-growing postlarval shrimps. On experimental days 0, 2, 4, 8, and 15, one or two shrimp were randomly collected from each triplicate tank. Animals were euthanized in ice/water slurry and were stored in sealed bags until sample pretreatment. As an estimate of growth (k) is required for the exponential model of isotopic change, the individual wet weight of five animals per replicate was determined on each sampling day. At the end of the experiment (day 22), all remaining animals were euthanized and weighed, and three shrimps per replicate tank (9 per treatment) were treated as previously described.

Sample pretreatment and stable isotope analysis

Sample pretreatment of diets and shrimps consisted of dehydration in a convection oven (50 °C) until a constant weight was achieved, followed by grinding with mortar and pestle. Diet samples and dry shrimp bodies contained different lipid contents and, as lipids are usually depleted in ^{13}C relative to carbohydrates and protein (De Niro and Epstein 1978; Stenroth et al. 2006), samples were delipidated to reduce variability of measured δ^{13}C values. Ground dry samples were suspended in a 50:50 solution of chloroform–methanol as described by Beaudoin et al. (2001) but considerably increasing the immersion time by 12 h. The procedure was repeated and lipid-extracted samples were oven-dried (50 °C until constant weight), homogenized again, and kept in a desiccator. Diet

Table 2 Formulation (g ingredient kg)$^{-1}$ diet proximal, and isotopic composition of seven experimental diets used to evaluate the contribution of nutrients supplied by fish meal (F), *Spirulina* (S), and *N. oculata* (N) biomass to the growth of postlarval *Litopenaeus vannamei*

Ingredient	100F	100S	100 N	50FSN	50SFN	50NFS	33FSN
Fish meal[a]	434.7	0	0	218	109	110	147
N. oculata biomass	0	0	853	213	213	426	285
Spirulina biomass	0	584	0	145	291	145	192
Wheat starch[b]	462	247	6	302	245	185	242
Monocalcium phosphate[c]	0	35.0	35.0	14.2	24.7	24.6	21.1
Calcium chloride[c]	0	11	11	0	5	5	5
Lecithin[d]	35.5	30	30	35.5	35	35	35
Alginate[c]	20	20	20	20	20	20	20
Cellulose[c]	19.0	15.3	18.1	19.0	17.7	18.3	18.5
Fish oil	19.6	47.5	16.2	23.6	29.5	21.7	25.1
Vitamin mix[a]	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Mineral mix[a]	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Cholesterol[e]	0	1.11	1.40	0	0.49	0.56	0.26
Choline chloride[c]	2	2	2	2	2	2	2
Vitamin C[a]	1	1	1	1	1	1	1
Antioxidant[a]	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Antifungic agent[a]	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Total	1000	1000	1000	1000	1000	1000	1000
Proximal and isotopic analyses							
Crude protein (g kg^{-1})	362	371	354	366	357	379	371
Lipids (g kg^{-1})	85	90	79	85	79	79	82
Gross energy (kcal g^{-1})	4.7	4.8	4.5	4.4	4.8	4.5	4.7
Moisture (%)	4.4	5.2	6.2	5.2	7.7	6.7	6.9
Ash (g kg^{-1})	92	88	229	129	131	168	142
δ^{15}N (‰)	17.38	9.98	2.04	10.88	9.36	7.43	9.46
δ^{13}C (‰)[f]	− 21.34	− 23.08	− 38.29	− 25.73	− 25.8	− 29.35	− 26.92

[a] Alimentos Costamar (Sonora, Mexico)

[b] Almidones y gluten S.A. (Monterrey, Mexico)

[c] Sigma-Aldrich (St. Louis, MO, USA)

[d] Ragaza Industrias Proteínas Naturales S.A. de C.V. (Monterrey, Mexico)

[e] Solvay Pharmaceuticals (Houston, TX, USA)

[f] After lipid extraction

and muscle tissue samples (1 ± 0.15 mg) were packed in tin cups (D1008 Elemental Microanalysis Ltd., UK) and organized in plastic microplates. Ingredient, diet, and shrimp samples were analyzed at the Stable Isotope Facility of the University of California (Davis, CA, USA). An elemental analyzer (PDZ Europa Scientific Roboprep) coupled to a stable isotope ratio mass spectrometer (PDZ Europa Hydra 20/20, UK) was used to determine carbon and nitrogen concentrations and their respective stable isotope values. Isotopic results are expressed in delta notation (δ), which is defined as *per mil* (‰) deviations from the δ^{15}N and δ^{13}C value of isotopic standard reference materials (atmospheric nitrogen and Pee Dee belemnite, respectively). Repeated measurements of calibration standards (nylon and BSA) indicated that the analytical precision (SD) was 0.12‰ for δ^{15}N values and 0.20‰ for δ^{13}C. Different terms exist to refer to the isotopic difference frequently observed between diets and animal consumers after equilibrium has been reached. In the present study, we use the term "discrimination factor" (Δ^{15}N or Δ^{13}C) to describe such isotopic differences.

Contribution of fish meal and microalgal biomass to growth

The relative contributions of dietary nitrogen, carbon, and total dry matter supplied by fish meal, *Spirulina*, and *N. oculata* biomass to shrimp growth were estimated using a mass-balance, concentration-dependent, isotope mixing model (Phillips and Koch 2002). The model's equation integrates the isotopic differences between the sources (ingredients in this study) and the mixture (shrimp bodies). Assumptions associated with the application of isotopic mixing models and

the validation of results were also met or taken into consideration (Gannes et al. 1997; Post 2002; Martínez del Rio and Wolf 2005; Martínez del Rio et al. 2009). Instead of using average, literature values, isotopic discrimination factors were obtained from the isotopic differences observed between control diets and control shrimp. Integration of isotopic corrections into the mixing models tends to increase the precision of results (Phillips 2012). $\delta^{13}C$ and $\delta^{15}N$ values determined in control and experimental shrimp were applied to the model to estimate each main ingredients' relative nutritional contributions. An indicator of treatment-specific variability of the nutritional contributions was obtained by introducing isotopic values measured in individual animals into the model. Preliminary analysis indicated that elemental contents in fish meal, *Spirulina*, and *N. oculata* biomass were significantly different (N = 10.5 ± 0.7, 9.9 ± 0.9, and 7.2 ± 0.4%, respectively, and C = 39.3 ± 0.4, 50.2 ± 1.7, and 41.6 ± 1.2%, respectively). Elemental concentrations are taken into account to estimate dietary contributions to growth in terms of dry matter.

Elemental residency times in shrimp bodies

$\delta^{15}N$ and $\delta^{13}C$ values were determined in shrimp sampled on experimental days 0, 2, 4, 8, 15, and 22. Isotopic data were then introduced into an exponential model (Hesslein et al. 1993, Eq. 1) that is able to separate the isotopic change caused by growth (k) from that caused by metabolic turnover (m). Additionally, the equation can generate predicted $\delta^{15}N$ and $\delta^{13}C$ values that can in turn be used in mixing models when organisms do not fully reach isotopic equilibrium with their respective diets.

$$C_{\text{sample}} = Cn + (Co - Cn)e^{-(k+m)t} \quad (1)$$

where C_{sample} is the isotope value in shrimp tissue at time t, Co is the isotope value of shrimp tissue in equilibrium with the initial diet, and Cn is the isotope value reached when animals are in equilibrium with a new diet. In turn, coefficients k and m provide an indicator of the time period necessary for half of the constituent nitrogen or carbon to be replaced after animals consume a new diet (half time, t_{50}) (MacAvoy et al. 2005, Eq. 2).

$$t_{50} = ln2/m + k \quad (2)$$

Statistical analyses

Mean survival and final weight, elemental contents of ingredients, and their isotopic values were analyzed by one-way ANOVA after normal distribution and data homoscedasticity were verified. Tukey's pairwise comparisons were used to detect significant differences. The dietary levels of nitrogen, carbon, and total dry matter available in the main ingredients (expected proportions) were compared to the estimated amounts of nutrients incorporated in shrimp bodies (observed proportions) by means of chi-square goodness of fit tests (χ^2). Parameter m (metabolic turnover rate) required by the exponential model of isotopic change was estimated by iterative non-linear regression. All tests were done using SPSS 17.0 software (SPSS Inc.) at a significance level of $P < 0.05$.

Results and discussion

Shrimp growth and survival

Experimental animals readily accepted and consumed all seven diets even though it has been reported that high dietary levels of microbial biomass can render diets unpalatable (Rumsey et al. 1991). By the end of the experiments shrimp reared under the different treatments showed significantly different survival rates ($P < 0.022$) and mean final wet weights ($P < 0.001$). During the experimental feeding period, water quality parameters remained within the optimal values for penaeid shrimp (Alcaraz et al. 1999). However, a fluctuation in the biological filter led to a temporary (36 h) increase of the total ammonia nitrogen (2.07 mg L^{-1}), which slightly affected the overall survival over the second week of the bioassay. All other parameters remained stable, and as the experimental tank array is designed to ensure that water quality variations affect all tanks simultaneously, it is likely that this transitory effect equally affected all organisms.

Final mean weight was significantly higher in shrimp grown under a 100% fish meal diet (100F) and those formulated with 25 and 50% *Spirulina* (diets 50FSN and 50SFN, Table 3). Previous studies have reported on nutritional benefits after varying levels of *Spirulina* biomass are added to shrimp diets. For example, Macias-Sancho et al. (2014) replaced fish meal with *S. platensis* in practical diets for *L. vannamei*.

Table 3 Final mean wet weight (mg), weight gain (%), specific growth rate, and survival rate (%) of postlarval shrimp *L. vannamei* fed diets formulated with different levels of fishmeal and microalgal biomass derived from *Spirulina* and *N. oculata*. Mean values ± S.D.

Diet	Final weight	Weight gain	SGR	Survival rate
100F	268 ± 64^{a}	435	8.23	93 ± 3^{a}
100S	187 ± 76^{bc}	274	6.60	80 ± 5^{b}
100N	127 ± 36^{d}	154	3.85	74 ± 11^{b}
50FSN	239 ± 64^{ab}	378	6.86	88 ± 3^{a}
50SFN	234 ± 57^{ab}	368	6.79	85 ± 0^{a}
50NFS	166 ± 47^{cd}	232	5.71	82 ± 6^{b}
33FSN	214 ± 44^{bc}	327	6.40	90 ± 3^{a}

Different superscripts indicate significant differences for that particular column

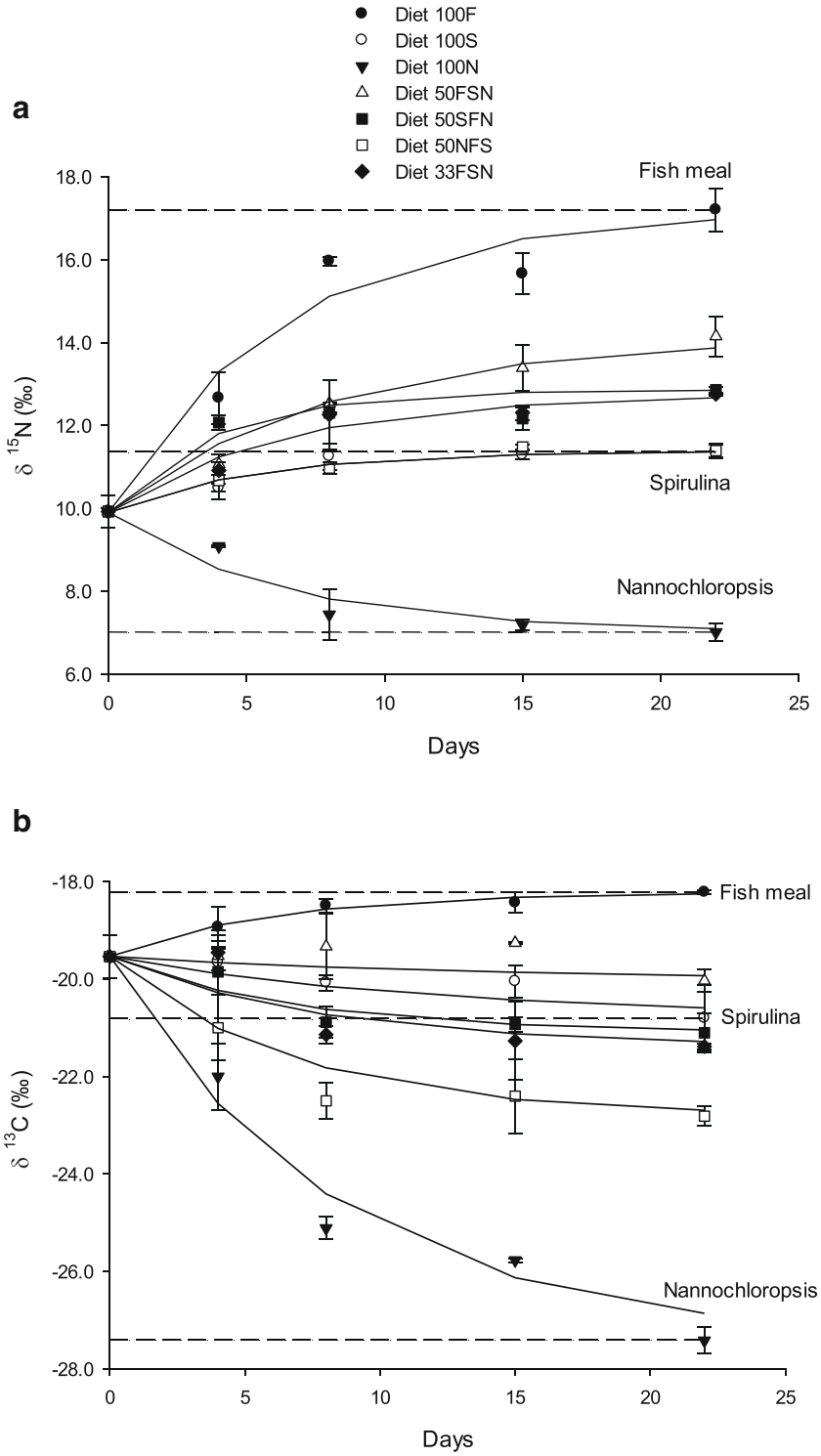

Fig. 1 Diet-elicited changes in nitrogen (**a**) and carbon (**b**) stable isotope values in postlarval Pacific white shrimp *L. vannamei* reared on diets formulated with different proportions of fish meal and microalgae biomass from *Spirulina* and *Nannochloropsis*. Dotted lines indicate corrected isotopic values of each main ingredient used as a protein source. Solid lines indicate predicted isotopic values generated by an exponential model of isotopic change and show the best fit to observed data

Replacement levels as high as 75% did not reduce the growth performance when compared to a control diet containing fish meal (40%), soy bean (5%), and yeast (5%). In this study, final mean weight and survival were significantly lower in shrimps on 50NFS diets and those containing only *Spirulina* or only *N. oculata* as nitrogen sources. The lowest growth rates were observed in animals on 100% *N. oculata* diet. The latter might be explained by a poorer ability to digest or assimilate this microalgal biomass. It can be argued that the less favorable amino acid profile of *N. oculata*, together with its relatively high ash content, both contributed to a poorer nutritional performance in shrimp.

Previous studies have reported that adding low levels (2.5 to 10%) of microalgal biomass to practical diets for crustacean

Table 4 Mean growth rates (k), estimated half times (t_{50}), and isotopic discrimination factors between whole bodies of postlarval Pacific white shrimp *L. vannamei* and diets containing fish meal (F), *Spirulina* (S), and *N. oculata* (N) biomass as sources of protein. Mean values ± S.D.

Diet		Nitrogen			Carbon		
	k	t_{50}	r	$\Delta^{15}N$	t_{50}	r	$\Delta^{13}C$
100F	0.082 ± 0.012^{a}	4.4 ± 0.7^{ab}	91	-0.2 ± 0.1^{a}	4.1 ± 0.4^{a}	41	3.1 ± 0.2^{a}
100S	0.065 ± 0.009^{bc}	3.6 ± 0.6^{a}	66	1.4 ± 0.2^{b}	8.4 ± 1.1^{b}	63	2.3 ± 0.1^{a}
100N	0.040 ± 0.011^{d}	4.3 ± 1.3^{ab}	73	5.0 ± 0.3^{d}	5.7 ± 0.4^{a}	78	10.8 ± 0.5^{c}
50FSN	0.069 ± 0.004^{ab}	5.6 ± 1.1^{b}	80	3.3 ± 0.2^{c}	–	–	5.7 ± 0.2^{b}
50SFN	0.069 ± 0.008^{ab}	2.7 ± 1.3^{a}	68	3.5 ± 0.4^{c}	4.7 ± 1.7^{a}	43	4.7 ± 0.3^{b}
50NFS	0.058 ± 0.014^{cd}	–	–	4.0 ± 0.5^{cd}	4.6 ± 1.1^{a}	66	6.5 ± 0.3^{b}
33FSN	0.064 ± 0.007^{bc}	4.4 ± 1.0^{ab}	66	3.3 ± 0.3^{c}	5.3 ± 0.5^{a}	44	5.5 ± 0.4^{b}

Different superscripts indicate significant differences for that particular column

and fish can positively affect growth and survival (Li et al. 2015; Teimouri et al. 2016; Pacheco-Vega et al. 2018). However, successful substitution of high levels of dietary fish meal using microalgae meals has also been reported for penaeid shrimps. For instance, Ju et al. (2012) replaced fish meal protein with a defatted microalgae meal (*Haematococcus pluvialis*) in diets for Pacific white shrimp (*L. vannamei*). A replacement of up to 50% did not negatively affect animals' growth rate, and in turn, diets positively impacted the final product as shrimps appeared redder and contained higher free and esterified astaxanthins than shrimp on the control diet. As with other sources of single cell-derived biomass, the digestibility of untreated microalgae cells might represent a limiting factor as some microalgae species have complex, thick cell walls that can hinder nutrient digestibility (Tibbetts et al. 2017a; Shah et al. 2018). In this study, the high ash content in *N. oculata* might have accounted to lower digestibility. On the other hand, ingredients derived from microbial biomass contain significantly higher proportions of nucleic acids than plant- and animal-derived ingredients (Gamboa-Delgado and Márquez-Reyes 2018). Although it has been demonstrated that low nucleic acid levels can improve microalgae's attractability and palatability, higher levels might promote feeding-deterrent activity (Skrede et al. 2009).

Isotopic influence of microalgal biomass on shrimps

Over the experimental period, shrimp body weight increased between 232 and 435%, except those on diet formulated with *N. oculata* as protein source (154%). The observed growth rate, characteristic of this species at this particular life stage, exerted a rapid dietary influence on shrimps. The isotopic values of the respective diets were thus rapidly reflected on the experimental animals, and by day 22, animals had reached or approached isotopic equilibrium ($\delta^{13}C$ values of diet 100N) with their respective feeds (Fig. 1a, b). Due to their different origins and/or production methodologies, fish meal (manufactured from wild fish populations) and both microalgae biomass types (grown on inorganic fertilizers with atypical isotopic values) showed contrasting isotopic values among them. The effect of fertilizers used to produce commercial microalgal biomass imprinted $\delta^{15}N$ values in *Spirulina* of 9.97 ± 0.13‰ and 2.10‰ ± 0.16 in *N. oculata*, which differed greatly to the $\delta^{15}N$ value in fish meal (16.50 ± 0.27‰). Similarly, $\delta^{13}C$ values in fish meal (− 19.50‰) and *Spirulina* (− 23.29‰) and *N. oculata* biomass (− 40.37 ± 0.10‰) were contrasting. The very negative value can be attributed to the use of supplementary CO_2 in the *N. oculata* culture vessels. Previous studies have reported on such significant isotopic influences in cells of other microalgae species (*Isochrysis*) (Gamboa-Delgado et al. 2008). These contrasting isotopic values of the main ingredients affected the values of each of the different diets and, as a result, these differences were reflected in the experimental shrimps.

The changing isotopic values observed in shrimp under most treatments were mainly attributed to their fast growth rate (k), although metabolic turnover (m) also played a significant role. For example, k value for animals grown on diet 100F was 0.082 day^{-1}, while their respective m value (for N) was 0.075 day^{-1}. Slow-growing animals showed the opposite trend. Isotopic changes describing exponential time trends are ideal for estimating elemental turnover rates and tissue residency times. However, after applying correction factors for isotopic discrimination, the isotopic values of some diets approached those of shrimp at the beginning of the bioassay and thus did not generate clear exponential isotopic changes (Fig. 1a, b). $\Delta^{15}N$ and $\Delta^{13}C$ values between animals and their respective control diets differed significantly (Table 4). $\Delta^{15}N$ values between shrimps and diet 100F were small and negative (− 0.2‰), while values observed in shrimp fed on diets 100S and 100N were significantly larger (1.4 and 4.9‰, respectively). The mixed diets resulted in $\Delta^{15}N$ values ranging from 3.3 to 3.9‰. In contrast, there was greater variation in $\Delta^{13}C$ values, which ranged from 2.3 to 10.9‰. It is worth mentioning that control diet 100N promoted significantly lower weight and larger $\Delta^{15}N$ and $\Delta^{13}C$ values in shrimp. There are studies indicating that nutrient scarcity triggers

Table 5 Estimated relative proportions of dietary nitrogen, dietary carbon, and total dry matter supplied from fish meal, *Spirulina* biomass (S), and *N. oculata* biomass to the somatic growth of postlarval Pacific white shrimp *L. vannamei* (mean ± SD, $n = 9$ per diet)

	Expected (dietary level)	Observed (shrimp bodies)	
		Mean	SD
Nitrogen			
50F:25S:25N			
Fish meal	49.6[a]	50.7[b]	± 8.9
Spirulina	24.9	45.3	± 6.8
Nannochloropsis	25.5	4.0	± 5.8
50S:25F:25N			
Fish meal	25.5[a]	35.0[b]	± 7.1
Spirulina	50.0	52.3	± 6.2
Nannochloropsis	24.5	12.7	± 7.6
50N:25F:25S			
Fish meal	25.0[a]	24.8[b]	± 6.4
Spirulina	25.5	42.6	± 9.9
Nannochloropsis	49.5	32.6	± 5.0
33F:33S:33N			
Fish meal	33.6[a]	35.2[b]	± 9.7
Spirulina	33.0	49.6	± 7.6
Nannochloropsis	32.4	15.2	± 5.5
Carbon			
50F:25S:25N			
Fish meal	46.1[a]	42.8[b]	± 9.8
Spirulina	29.6	52.1	± 4.3
Nannochloropsis	24.3	5.1	± 6.9
50S:25F:25N			
Fish meal	21.7[a]	27.9[b]	± 9.4
Spirulina	55.6	56.6	± 7.0
Nannochloropsis	22.7	15.5	± 8.7
50N:25F:25S			
Fish meal	22.8[a]	18.7[b]	± 5.6
Spirulina	29.2	43.6	± 10.0
Nannochloropsis	48.0	37.7	± 8.5
33F:33S:33N			
Fish meal	30.0[a]	27.9[b]	± 8.2
Spirulina	38.5	53.6	± 7.6
Nannochloropsis	31.5	18.5	± 6.0
Total dry matter			
50F:25S:25N			
Fish meal	37.9[a]	48.5[b]	± 9.3
Spirulina	25.2	46.0	± 8.6
Nannochloropsis	36.9	5.5	± 7.5
50S:25F:25N			
Fish meal	17.8[a]	32.2[b]	± 5.4
Spirulina	47.5	50.9	± 6.6
Nannochloropsis	34.7	16.9	± 4.3
50N:25F:25S			
Fish meal	16.1[a]	21.0[b]	± 5.6
Spirulina	21.3	38.5	± 10.2
Nannochloropsis	62.6	40.5	± 7.1
33F:33S:33N			
Fish meal	23.5[a]	32.1[b]	± 9.1
Spirulina	30.8	47.8	± 7.2
Nannochloropsis	45.7	20.1	± 2.6

Total dry matter contributions were estimated after correcting for elemental concentrations (C and N) available in the main ingredients. Different superscripts indicate significant differences between mean expected and observed dietary contributions

higher metabolic cycling and this, in turn, increases the isotopic discrimination factors (Fantle et al. 1999; Robbins et al. 2005). Such increments can be explained by higher proportions of heavy isotopes (^{13}C, ^{15}N) being incorporated in tissues of animals facing nutritional restrictions (Martínez del Rio and Wolf 2005). In this context, the higher discrimination factors ($\Delta^{15}N$ and $\Delta^{13}C$) observed in shrimp fed diets having 50 to 100% *N. oculata* as a protein source could be related to the comparatively lower availability of some essential amino acids (i.e., arginine, tryptophan) in this microalgae biomass.

Nitrogen and carbon half times in tissue

Nitrogen and carbon isotopic shifts in animals followed an expected pattern characterized by an exponential trend. For most treatments, predicted isotopic values fit well on the observed data, and from these data, parameter *m* (metabolic turnover) was estimated through an iterative non-linear regression. Although isotopic differences between conditioning and experimental diets were significant, it was not possible to estimate the nitrogen half times in animals under all dietary treatments because not all feeds resulted in exponential isotopic shifts (Fig. 1a, b). Parameters *m* and *k* indicated that estimated nitrogen half times in shrimp bodies ranged from 2.7 days under diet 50SFN to 5.6 days under diet 50FSN (Table 4). Such values are considered relatively short and are consistent with those observed in similar experiments on postlarval shrimp fed diets with high levels of torula yeast (2.3 to 8.0 days) and soy protein isolate (2.8 to 4.0 days) (Gamboa-Delgado and Le Vay 2009; Gamboa-Delgado et al. 2016). In contrast, estimated carbon half times in shrimp bodies ranged from 4.1 to 8.4 days. No apparent correlation was found between dietary levels of microalgal biomass and estimated carbon half times.

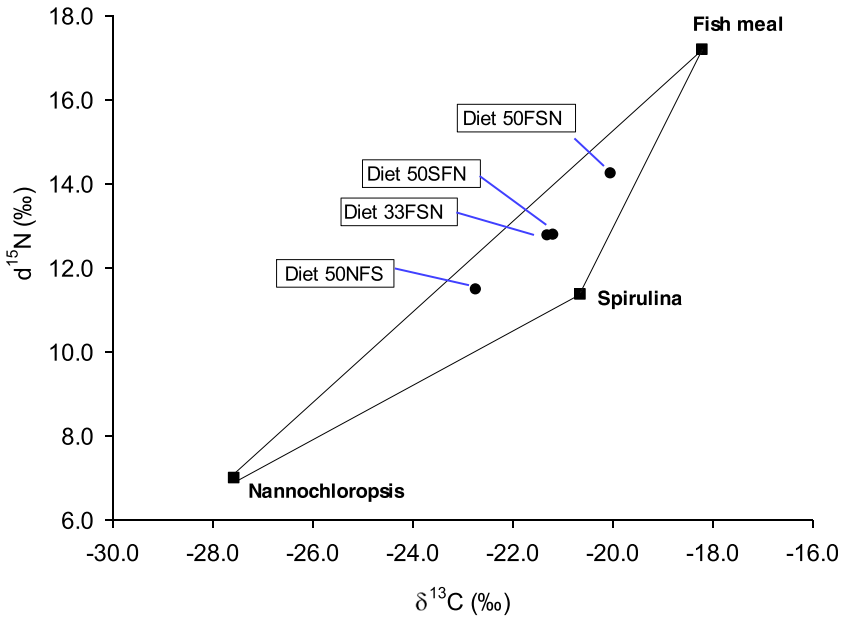

Fig. 2 Carbon and nitrogen stable isotope values of experimental diets (corrected for isotopic discrimination factors) and dual isotopic profiles of postlarval shrimps *L. vannamei* fed on experimental diets containing different proportions of fish meal and microalgae biomass from *Spirulina* and *Nannochloropsis*. Closer distances between shrimps and ingredients indicate higher nutritional contributions from that ingredient on a dry matter basis

Nutrient allocation from fish meal, *Spirulina*, and *Nannochloropsis* biomass

The use of whole bodies for isotopic analysis canceled any isotopic routing effects (i.e., dietary nitrogen and carbon were not differently allocated to specific tissues). Integration of isotopic values into the mixing models indicated that contributions of dietary nitrogen and carbon from fish meal and the two types of microalgal biomass were significantly different to the respective proportions of dietary nitrogen ($\chi^2 = 19.5$, $P < 0.001$ for diet 33FSN) and carbon ($\chi^2 = 12.3$, $P = 0.002$ for diet 33FNS) available in the dietary formulations (Tables 2 and 5). Most differences were attributed to the fact that shrimps fed on all mixed diets incorporated significantly higher amounts of dietary nitrogen (43 to 51%), dietary carbon (44 to 57%), and total dry matter (38 to 51%) from *Spirulina* than from *N. oculata* biomass (Fig. 2). In most diets, fish meal nitrogen was incorporated in similar proportions to those established in the experimental feeds, except in diet 50SFN, where fish meal contribution was higher (35%) than the available dietary proportion (25.5%). In this case, fish meal seems to have compensated for the significantly lower assimilation of dietary nitrogen from *N. oculata* biomass (12.7%).

The diet formulated with ingredients supplying equivalent amounts of dietary nitrogen (33FSN) ended up contributing 35.2, 49.6, and 15.2% of nitrogen from fish meal, *Spirulina*, and *N. oculata*, respectively. There were also significant differences between the dietary and assimilated proportions of dietary carbon and dry matter. The different nutritional contributions might be explained by differences in the digestibility and the nutritional composition of the different ingredients. The high ash content in *N. oculata* biomass (Table 1) might have accounted for the lower nutritional performance of diets containing this ingredient. On the other hand, the apparent digestibility coefficient (ADC) for the available protein in the fish meal used in the present study has been determined as 78% when fed to *L. vannamei* (Villarreal-Cavazos 2011). In contrast, Tibbetts et al. (2017a) reported a moderate in vitro protein ADC for *Nannochloropsis granulata* for *L. vannamei* (69 to 76%). *Spirulina* has higher protein digestibility coefficients, although these have been reported for aquatic vertebrates only. Sarker et al. (2016) estimated an ADC of 86% for tilapia when 30% *Spirulina* was included in a reference diet, while Palmegiano et al. (2008) measured ADCs ranging from 80 to 87% in sturgeon fed compound diets having 40 to 60% *Spirulina*. High levels of dietary microalgae tend to increase the ash and fiber content of the practical feeds (Tibbetts et al. 2017b; Pacheco-Vega et al. 2018) which in turn affects the digestibility for crustacean and fish; however, through extrusion processing, it has been demonstrated that the ADC for ash can be significantly improved in feeds containing microalgal biomass (Gong et al. 2018).

Conclusions

The use of carbon and nitrogen isotopic values as natural biomarkers showed that a substantial proportion of dietary nitrogen and carbon contributing to shrimp growth can be supplied from *Spirulina* biomass. Substituting fish meal with *Spirulina* (25 and 50%) still promoted growth and survival rates similar to those elicited by diet containing 100% fish meal as protein source. In contrast, *Nannochloropsis* biomass resulted in poor shrimp growth and nutrient assimilation, thus making it an unsuitable dietary ingredient for aquafeeds.

Isotopic estimations revealed that *Spirulina* nutrients were assimilated fast and, in some cases, at higher proportions than those supplied by fish meal under the experimental dietary combinations. Current microalgae production methods have greatly diversified and profitability has increased. Therefore, it is reasonable to predict that in the near future, microalgal biomass production will increase and its manufacturing costs will decrease. Additional studies can help elucidate whether further treatment to the microalgal biomass (e.g., cell wall disruption, removal of nucleic acids) can lead to higher growth and nutrient assimilation. The isotopic techniques can yield complementary information when conducting dietary assessments of the nutrients available in novel, sustainable ingredients for marine organisms.

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Compliance with ethical standards

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

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