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Evaluation of IMTA-produced seaweeds (*Gracilaria*, *Porphyra*, and *Ulva*) as dietary ingredients in Nile tilapia, *Oreochromis niloticus* L., juveniles. Effects on growth performance and gut histology

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Abstract The present study evaluated the effects of the inclusion of three seaweeds, *Gracilaria vermiculophylla* (GRA), *Porphyra dioica* (POR), and *Ulva* spp. (ULV), as dietary ingredients for Nile tilapia (*Oreochromis niloticus*) juveniles, on the growth performance, body composition, and gut histology. Three experimental diets (GRA, POR, and ULV) were formulated to replace 10 % of whole diet by each of the three seaweeds. A control diet (CTRL) was used, without inclusion of any seaweed. Diets were fed to triplicate groups of 25 Nile tilapia juveniles, with an average body weight (ABW) of 12.1 g, in an 84-day trial. At the end of the trial, growth performance was significantly reduced ($P<0.05$) in fish fed the GRA diet, whereas the feed conversion ratio increased significantly in those fish. None of the treatments caused adverse effects on body composition. The inclusion of the three seaweeds in the diet led to evident changes in the fish digestive system morphology with significant reduction of *villi* length on GRA diet. The results obtained in this study suggest the usefulness of *P. dioica* and *Ulva* spp. to partially replace fishmeal in practical diets for tilapia juveniles up to 10 %, as no negative consequences on growth performance or body composition were observed. However, the inclusion of 10 % *G. vermiculophylla* seems to have a negative effect in diet palatability, reducing fish feed intake and growth performance.

Keywords Nile tilapia · Alternative feed ingredients · Seaweeds · *Gracilaria vermiculophylla* · *Porphyra dioica* · *Ulva* spp.

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Introduction

Aquaculture is the world's animal food production sector with the fastest growth. However, the production rate is predicted to slow mainly due to water restraints, limited availability of suitable production locations, and the rising costs of fish meal and oil. Aquaculture is also responsible for the consumption of great amounts of fish and environmental degradation (FAO 2012), so developing an environmentally sustainable aquaculture is a major factor to ensure the sustainability of this industry (Troell et al. 2003; Neori et al. 2004). It is crucial to utilize ecological engineering tools, such as integrated multitrophic aquaculture (IMTA), to mitigate environmental problems, and to turn aquaculture more sustainable (Buschmann et al. 2008; Chopin et al. 2008; Nobre et al. 2010). In such balanced systems, aquaculture effluents can be converted into crops of commercial value that reduce biofiltration costs (through the restoration of water quality) and can be used for human consumption (mainly for food and health), as a feed ingredient or a supplement to other

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aquaculture species (Chopin et al. 2001; Neori et al. 2004; Troell et al. 2009; Nobre et al. 2010).

Nile tilapia tolerate a wide range of environmental conditions, being one of the most successfully farmed fish species and constituting an important protein source for human consumption (Rinchard et al. 2002). Fish feeding represents the major operational cost for most commercial farms (Naylor et al. 2000; Güroy et al. 2007), with protein being the most expensive dietary component (Lovell 2002). Fish meal is a high-quality protein source, containing all amino acids essential for fish, but its exploitation has already been pushed to the limit (FAO 2012). It is vital for the future of aquaculture to find available and more sustainable protein sources to include in aquafeeds which might reduce the dependency on fish meal (Alexis 1997; Tacon et al. 2006). Alternative protein sources should meet important requirements (e.g., protein levels and fatty acid profiles), and critical limitation of the plant protein sources used in fish feeds is that they are deficient in certain amino acids such as lysine, methionine, threonine, and tryptophan (Mai et al. 2006a, b; Li et al. 2009). Protein sources of plant origin do not represent the ultimate alternative to fish meal, and the need to find new ingredients is a challenging goal. Numerous algae appear as promising alternative since they generally contain all the essential amino acids (although with significant variation among species) and may be used, at least, as partial substitute for fishmeal (Dawczynski et al. 2007; Lupatsch 2009). Moreover, the use of IMTA-produced seaweeds to replace fishmeal in aquafeeds is advantageous mainly due to reduced operational costs (Nobre et al. 2010) and protein content.

Seaweeds have high nutritional value due to their high protein and mineral content and are also natural sources of water-soluble and liposoluble vitamins and essential fatty acids from the omega-3 family, which can present benefits for human health and animal nutrition (Norziah and Ching 2000; Khotimchenko et al. 2002). Seaweeds with high protein content and production rates are receiving more attention as an alternative plant protein source (Fleurence 1999; Buschmann et al. 2001; Marinho et al. 2013). IMTA-produced seaweeds generally present higher productivity levels and less variability in protein content than seaweed from the natural environment due to the continuous supply of nutrients and the minimum disturbance by grazers or epiphytes (Schuenhoff et al. 2003; Mata et al. 2010; Abreu et al. 2011). Several authors have previously shown that different species of seaweed, such as *Ascophyllum nodosum* (Nakagawa et al. 1997), *Gracilaria cornea* and *Gracilaria bursa-pastoris* (Valente et al. 2006), *Porphyra* (Soler-Vila et al. 2009), and *Ulva lactuca* (Wassef et al. 2001) can be used as partial substitutes (up to 10 %) of dietary fishmeal. Nevertheless, their protein content and/or quality (amino acid profile) could not fulfill the nutritional requirements of fish. Despite representing a potential food alternative, seaweeds can hold certain substances with some level of toxicity and anti-nutrient activity, which can contribute to the reduction of its nutritional quality and negative effect on fish growth (Oliveira et al. 2009). Anti-nutritional factors (such as lectins, protease inhibitors, goitrogens, allergens, anti-vitamins, saponins, tannins, phytate, and toxins) are widely distributed in plants and algae, and several negative effects caused by their incorporation in fish diets have been reported. Hence, the wide selection of such new food sources needs to previously consider the presence of anti-nutritional factors (ANFs) (Liener 1994; Francis et al. 2001; Bajpai et al. 2005).

Gracilaria is one of the seaweed genera most exploited worldwide (Yarish and Pereira 2008), and its economic importance comes from the phycocolloid industry, being the main source of agar (Peng et al. 2009). *Gracilaria vermiculophylla* is a non-indigenous Asian red algae (Nyberg et al. 2009) naturalized in the Ria de Aveiro, Portugal, where it is the dominant *Gracilaria* species. This species is well adapted to shallow soft-bottom bays, lagoons, estuaries, harbors, and inlets (Thomsen and McGlathery, 2007); is highly resistant to various stressful factors (darkness, sedimentation, desiccation, different nutrient conditions); grows well under an extensive range of environmental conditions; and is reproductive throughout the year (Thomsen and McGlathery, 2007; Nyberg and Wallentinus 2009; Abreu et al. 2011). *Gracilaria* species are also efficient biofilters due to their good capacity to remove ammonia and nitrate from the water (Neori et al. 2000; Yokoyama and Ishihi 2010; Abreu et al. 2011).

Porphyra/Pyropia species are among the most important maricultured seaweeds, with a constant increase in world production since 1990, reaching approximately 1,000,000 t in 2010. In China, Japan, and Korea, these red algae are extensively cultivated and possess a high economic importance worldwide, being widely used for human consumption (under the common name of nori) (Fleurence 1999; FAO 2012). They are rich in iron, zinc, sodium, potassium, and calcium (Dawczynski et al. 2007) and, due to their high surface to volume ratio, are fast growing species, capable of a quick assimilation of nutrients (Neori et al. 2004). All these facts suggest that this genus is one of the most promising for bioremediation and integrated aquaculture (Chopin et al. 1999, 2001; Carmona et al. 2006). *Porphyra dioica* is the most common *Porphyra* species in the North of Portugal (Pereira et al. 2006), inhabits the intertidal zone of rocky beaches throughout the year; and is able to grow within a wide range of temperatures, photoperiod, and light intensity (Pereira et al. 2004).

Ulva spp. are green algae found in a variety of habitats and on several different substrates (Bunker et al. 2010). They have a good vitamin and mineral profile and are especially rich in glutamic and ascorbic acid, alanine, and iron (Briand and Morand 1997; Ortiz et al. 2006; García-Casal et al. 2007).

Despite not having the economic value of *Gracilaria* and *Porphyra* species, *Ulva* spp. were already studied as an ingredient for herbivorous aquatic animals (Dworjanyn et al. 2007) and several fish species like European sea bass, *Dicentrarchus labrax* L. (Valente et al. 2006), common carp, *Cyprinus carpio* L. (Diler et al. 2007), Nile tilapia, *Oreochromis niloticus* L. (Güroy et al. 2007; Ergün et al. 2009; Pereira et al. 2012; Marinho et al. 2013), and rainbow trout, *Oncorhynchus mykiss* Walbaum (Güroy et al. 2011, 2013). Moreover, according to Pereira et al. (2012), Nile tilapia (*O. niloticus*) seems to digest and utilize better *G. vermiculophylla*, *P. dioica*, and *Ulva* spp. than other tested seaweeds (*Sargassum muticum*).

The aim of the present study was to evaluate the effect of inclusion of three seaweeds, *G. vermiculophylla* (GRA), *P. dioica* (POR), and *Ulva* spp. (ULV), as alternative vegetal protein sources on growth performance and feed efficiency, body composition, and gut histology of the intestinal mucosa in Nile tilapia (*O. niloticus*) juveniles.

Materials and methods

Ingredients and experimental diets

Gracilaria vermiculophylla and *Ulva* spp. were collected from wild populations established in Ria de Aveiro (40° 37′ 56′ N, 8° 44′ 36″ W), while *Porphyra dioica* from natural populations of Mindelo (41° 18′ 35′ N, 8° 44′ 27′ W). The three seaweeds were then produced free-floating in 1200 L polyethylene circular tanks, with a surface area of 1.5 m^2 and bottom aeration, in an IMTA system established in a land-based commercial fish aquaculture (Aquacultura Coelho & Castro, Lda. Póvoa de Varzim, Portugal), for 3 to 4 months. More information about this IMTA system can be found in Abreu et al. (2011).

Seaweeds were collected, dried in an oven at 45 °C, for 48 h, and ground to 2-mm size particles. Their proximate composition is given in Table 1.

Four experimental diets, with similar protein and energy values, were formulated (control (CTRL), *G. vermiculophylla* (GRA), *P. dioica* (POR), and *Ulva* spp. (ULV)) according to the known nutritional requirements of tilapia (NRC 1993), containing only either fishmeal as animal protein source (CTRL diet) or 10 % of each seaweed meal as partial replacement of the fish meal (Table 2). Main ingredients were ground (<250 μm), mixed in a horizontal helix ribbon mixer (Mano, 100-L capacity, CPM, San Francisco, USA), and dry pelleted using a laboratory pellet press (CPM, C-300, San Francisco, USA) with a 2.4-mm die. Diets were dried at 37 °C for 24 h and stored in a refrigerator until use. Throughout the duration of the trial, experimental feeds were stored at room temperature. Samples of each diet were taken for proximate composition analysis.

Table 1 Proximate composition (% DM) of the IMTA-produced dry seaweeds *Ulva* spp. (ULV), *Porphyra dioica* (POR), and *Gracilaria vermiculophylla* (GRA)

	ULV	POR	GRA
Dry matter (%)	91.7	91.4	94.1
Crude protein	26.7	41.2	49.2
Crude fat	2.7	4.9	2.1
Ash	28.3	18.6	27.4
Organic matter	68.8	79.5	70.0

Table 2 Ingredients and proximate composition of the experimental diets control (CTRL), *Ulva* spp.(ULV), *Porphyra dioica* (POR), and *Gracilaria vermiculophylla* (GRA)

	Dietary treatments			
	CTRL	ULV	POR	GRA
Ingredients (g kg^{-1})				
Fish meal 65 (LT)[a]	250	150	150	150
Aquatex 15.6[b]	250	150	160	160
Soybean meal 48[c]	150	250	220	220
Wheat meal	270	270	290	290
Vitamin and mineral mix[d]	15	15	15	15
Binder[e]	10	10	10	10
Coline	10	10	10	10
Rapeseed oil	20	20	20	20
Dicalcium phosphate	25	25	25	125
Ulva spp. meal	0	100	0	0
P. dioica meal	0	0	100	0
G. vermiculophylla meal	0	0	0	100
Proximate composition (% DM)				
Dry matter	91.9	89.6	89.4	89.7
Crude protein	36.1	34.7	34.5	35.6
Crude fat	7.5	7.6	7.8	7.3
Ash	8.5	11.6	8.9	9.4
Gross energy (kJ g^{-1} DM)	16.8	15.9	16.2	16.3

LT low temperature

[a] Peruvian fishmeal, Exalmar, Peru

[b] Aquatex (15.6 crude protein; Sotexpro, Bermericourt, France)

[c] Solvent extracted dehulled soybean meal, Sorgal SA, Portugal

[d] Vitamins (in mg or IU kg^{-1} diet): vitamin A (retinyl acetate), 20,000 UI; vitamin D_3 (DL-cholecalciferol), 2000 UI; vitamin E (Lutavit E 50), 100 mg; vitamin K_3 (menadione sodium bisulfite), 25 mg; vitamin B_1 (thiamine hydrochloride), 30 mg; vitamin B_2 (riboflavin), 30 mg; calcium pantothenate, 100 mg; nicotinic acid, 200 mg; vitamin B_6 (pyridoxine hydrochloride), 20 mg; vitamin B_9 (folic acid), 15 mg; vitamin B_{12} (cyanocobalamin), 100 mg; vitamin H (biotin), 3000 mg; vitamin C (Lutavit C35), 1000 mg; inositol, 500 mg; coline chloride, 1000 mg; and betaine (Betafin S1), 500 mg. Minerals (mg or % kg^{-1} diet): Co (cobalt carbonate), 0.65 mg; Cu (cupric sulfate), 9 mg; Fe (iron sulfate), 6 mg; I (potassium iodide), 0.5 mg; Mn (manganese oxide), 9.6 mg; Se (sodium selenite), 0.01 mg; Zn (zinc sulfate) 7.5 mg; Ca (calcium carbonate), 18.6 %; KCl, 2.41 %; and NaCl, 4.0 %

[e] Lignin sulfate

Fish and rearing conditions

Experiments were carried out by trained scientists, according to the Federation of European Laboratory Animal Science Associations (FELASA) category C recommendations, and conducted according to the European Directive 2010/63/EU (http://eur-lex.europa.eu/homepage.html?locale=en) on the protection of animals used for scientific purposes.

About 1000 juvenile Nile tilapia (Natural Male Tilapia® NMT; initial body weight: 0.5 g) were purchased from a commercial fish farm (Til-Aqua, Netherlands) and transported to the Experimental Research Station of University of Trás os Montes e Alto Douro (UTAD; Vila Real, Portugal). Fish were adapted to these new conditions for about 3 months, during which they were fed a commercial feed. At the beginning of the trial, fish were acclimated to the rearing conditions for 15 days, during which they were fed the control diet once a day (10:00 am).

Growth trial

Homogeneous groups of 25 juvenile Nile tilapia (average body weight of 12.1±0.04 g) were randomly distributed among 12 square glass tanks (four diets, n=3; volume 90 L), in a recirculation freshwater system (water flow rate, 120 L h^{-1}; temperature, 25±1 °C; dissolved oxygen, 75 % saturation level (around 6.5–7 mg L^{-1}); and pH, 6.9±0.1 with natural day/night photoperiod). Ammonia and nitrite levels were monitored weekly using commercial kits (SERA) and never exceeded 0.03–0.08 mg L^{-1} NH_3 (at pH=8–8.5) and 0.5 mg L^{-1} NO_2^-, respectively. Experimental diets were randomly assigned to the tanks establishing triplicate groups of fish per treatment that were hand-fed twice a day (10:00 am and 5:00 pm) until apparent satiation, during 84 days. Total feed consumption and mortality data were daily recorded for each tank. Every 4 weeks, fish were group weighed and biomass was used to calculate feed intake.

At the end of the trial, all fish were fasted for 24 h and individually weighed. Six fish from each tank were anaesthetized (MS-222 Sigma; 0.1 g L^{-1}), euthanized by severing their spinal cord, and stored at−20 °C for subsequent whole-body analysis. Additionally, two fish per tank were sampled for histological analysis. Whole fish and tissue samples were stored at−80 °C for chemical analysis.

Analytical methods

Nile tilapia were collected at the beginning and the end of the trial and freeze-dried, finely milled, and homogenized prior to analysis. All chemical analyses were performed according to AOAC (2006), and samples were run in duplicates. Frozen whole-body samples were pooled, minced without thawing, and moisture content was determined (105 °C for 24 h). Samples were analyzed for ash content by combustion in a muffle furnace (550 °C for 6 h), for crude protein with a nitrogen determinator (N × 6.25, Leco nitrogen analyzer, Model FP-528, Leco Corporation, USA), for crude lipid content by petroleum ether extraction (Soxtherm Multistat/SX PC, Germany; 40–60 °C), and for gross energy in a adiabatic bomb calorimeter (C-2000, IKA-Werke, Germany).

Histological analysis

The digestive tracts were removed, and a proximal intestinal segment (2 cm) was isolated and preserved in 4 % buffered formalin for histological analyses. Histological samples were transversally sectioned, dehydrated, and embedded in paraffin according to standard histological procedures. Three paraffin sections (3 μm thickness) of each animal were stained with hematoxylin–eosin and observed under a light microscope (Nikon Eclipse E600) in a blind trial. Histological images of each section were recorded using a Nikon digital camera DXM 1200. For each fish intestine section, the length of ten *villi* was measured from the submucosa to the base of the enterocytes (Pirarat et al. 2011), and the diameters of three intestine transverse sections (only the circular sections) were measured from serosa to serosa, both using ImageJ 1.44o software. For each experimental group, villi and diameter mean standard deviation was determined.

Calculations

Growth performance and feed utilization were studied in terms of final body weight (FBW, g), specific growth rate (SGR, % body weight day^{-1}), feed intake (FI, % initial body weight (IBW) day^{-1}), feed efficiency (FE), protein efficiency ratio (PER), and feed conversion ratio (FCR). All formulae are presented in Table 3. In the used formulae, W_0 corresponds to the initial fish mean weights, W_1 to the final fish mean weights, and ln to the natural logarithm.

Statistical analysis

Data are presented as mean standard deviation, and statistical analysis followed the methods outlined by Zar (1999). Data were tested for normality and homogeneity of variances using Kolmogorov–Smirnov and Levene's test, respectively. Then, all data were subjected to one-way analysis of variance (ANOVA) to test differences between dietary treatments. When these tests showed significance (P<0.05), individual means were compared using Tukey's multiple comparison test. Significant differences were considered when P≤0.05. All statistical tests were performed using the Statgraphics Centurion XV statistical software (Statgraphics Inc., USA).

Table 3 Growth performance and feed utilization in juvenile Nile tilapia (*Oreochromis niloticus*) fed experimental diets control (CTRL), *Ulva* spp.(ULV), *Porphyra dioica* (POR), and *Gracilaria vermiculophylla* (GRA)

	Dietary treatments			
	CTRL	ULV	POR	GRA
IBW (g)	12.1±0.05	12.1±0.05	12.1±0.05	12.1±0.05
FBW (g)	34.96±1.56 a	33.97±1.35 a	33.73±1.66 a	26.16±1.00 b
SGR (% day^{-1})	1.27±0.06 a	1.22±0.06 a	1.23±0.06 a	0.91±0.04 b
FI (% IBW day^{-1})	0.86±0.04 a	0.81±0.03 ab	0.86±0.02 a	0.77±0.02 b
FCR	1.41±0.04 a	1.37±0.15 a	1.44±0.08 a	2.03±0.09 b
PER	1.81±0.05 a	1.91±0.22 a	1.81±0.10 a	1.24±0.05 b

Values are means±standard deviation (n=3). In each row, values with different letters indicate significant differences between treatments (P<0.05). DWG=100 × [$(W_1-W_0)/W_0$)]/days; SGR=100 × [(ln W_1 (g)−ln W_0 (g))]/days; FI=(dry feed intake/W_0)/days; PER=wet weight gain (g)/crude protein intake (g); FCR=dry feed intake (g)/weight gain (g)

IBW initial body weight, *FBW* final body weight, *SGR* specific growth rate, *FI* feed intake, *FCR* feed conversion ratio, *PER* protein efficiency ratio

Results

Fish mortality during the present experiment was zero for all tanks. Experimental diets had the following crude protein (% dry matter (DM)) levels: CTRL=36.1 %, GRA=35.6 %, POR=34.5 %, and ULV=34.7 %).

Results obtained for growth performance and feed utilization are shown in Table 3. Diets were readily accepted by fish, indicating that there were no problems relating to the palatability of algae-supplemented diets, except for GRA diet, in which fish showed a poor acceptance, resulting in a lower voluntary food intake (with significant differences when compared to CTRL and POR groups). Excluding GRA diet, all fish almost tripled their IBW and mean body weight after 84 days of feeding. FCR ranged between 1.37 and 1.44, which is in general accordance with the range of values reported for tilapia with similar body size. FBWs of the fish fed ULV and POR diets were not significantly different from the control diet (P>0.05). At the end of the experimental period, fish fed GRA diet had a significantly lower weight (P<0.05) than fish of all other groups. In fact, FBW, specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER), and FCR were significantly lower in fish fed GRA diet compared to all other treatments (P<0.05). Although, feed intake in GRA diet was similar to the ULV group (P>0.05).

The whole-body composition of the differently fed fish did not show any significant variation between treatments (P>0.05, Table 4).

The intestinal villi length and intestine diameter at the end of the experimental period are shown in Figs. 1 and 2 and Table 5. The histological measurements of the intestinal folds showed a significant reduction in villi length in fish fed POR and GRA diets when compared with the CTRL treatment (P<0.05). The GRA diet showed significantly lower villi values regarding all the other diets on study (P<0.05). The observed ULV diet villi length was not significantly different from the villi lengths of CTRL and POR fed fish. Length of intestinal villi from POR fed fish was significantly different to CTRL (longest villi) and GRA (shortest villi) treatments. Regarding diameter values, the GRA diet presented lower values than the other treatments, being only significantly different from the CTRL diet (P<0.05). For both parameters, villi length and intestine diameter, results are distributed as CTRL > ULV > POR > GRA.

Discussion

The IMTA-produced seaweeds can represent multiple advantages for the aquaculture sector acting as nutrient biofilters, providing a possible mechanism for fish farmers to reduce the nitrogen loads from the cultivation effluents and being valuable additional crops (that use nutrients and other by-products from fish production) with commercial interest and with improved protein content (Holdt and Edwards 2014).

Table 4 Whole-body composition in Nile tilapia (*Oreochromis niloticus*) fed experimental diets control (CTRL), *Ulva* spp. (ULV), *Porphyra dioica* (POR), and *Gracilaria vermiculophylla* (GRA)

	Dietary treatments			
	CTRL	ULV	POR	GRA
Whole-body composition (% WW)				
Moisture	75.2±0.1	75.6±0.6	75.4±0.3	74.9±0.1
Ash	4.1±0.3	3.7±0.2	3.8±0.1	3.9±0.2
Protein	16.2±0.1	15.8±0.2	15.6±0.2	15.7±0.1
Lipids	3.5±0.1	3.8±0.1	3.7±0.1	3.6±0.1
Energy (kJ g^{-1})	5.2±0.0	5.1±0.1	5.0±0.2	5.1±0.2

Values are the means±standard deviation (n=3)

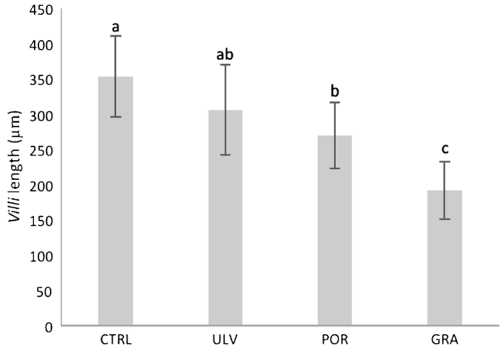

Fig. 1 Length of intestinal *villi* (µm) in Nile tilapia (*Oreochromis niloticus*) fed experimental diets control (*CTRL*), *Ulva* spp. (*ULV*), *Porphyra dioica* (POR), and *Gracilaria vermiculophylla* (GRA). Values are presented as the mean standard deviation of six tilapia sampled for each experimental diet (n=6). *Bars with different letters* differ significantly (P<0.05)

Seaweeds are traditionally used in alimentation of Asian countries, but their utilization by western consumers is moderated. The addition of seaweeds to the diet of aquatic animals produced by aquaculture regimes would be an opportunity to introduce seaweeds into the human food in western countries chain (Fleurence et al. 2012). The edible seaweed consumption in Europe is close to 70 t versus 97,000 t in Japan (Darcy-Vrillon 1993). These factors can contribute to a more cost-effective and sustainable activity, helping the development of a social acceptance of the aquaculture industry. In fact, the nutritional value of seaweed differs according to the species and family but, in general, seems to be a suitable source of proteins for fish and human nutrition.

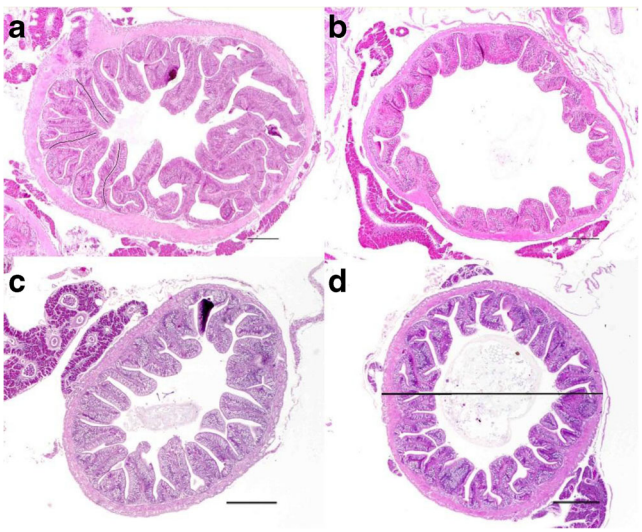

Fig. 2 Sections of the proximal intestine of Nile tilapia (*Oreochromis niloticus*) feed experimental diets. **a** Control diet. The *black lines* represent the *villi* measurement method followed in all assays. **b** *Gracilaria vermiculophylla* diet. Note the significant smaller dimensions of the villi. **c** *Ulva* spp. diet. **d** *Porphyra dioica* diet. The *black line* represents the measurement method of the diameter followed in all assays. Hematoxylin and eosin staining. *Bar* **a**, **b**=200 µm; **c**, **d**=300 µm

Economically, cichlids are the third most important group of farmed fish, after carps and salmonids (FAO 2012). Several studies evaluated alternative dietary protein sources for Nile tilapia (*O. niloticus*), such as plant proteins (El-Saidy and Gaber 2003; Fiogbé et al. 2004; Azaza et al. 2009; Kumar et al. 2012) or animal proteins (El-Sayed 1998; Thompson et al. 2012). However, not many studies focused on the use of seaweeds as a potential protein source for this important finfish species (Marinho et al. 2013; Stadtlander et al. 2013). The use of seaweeds in fish feeds depends on their cost and nutritional quality. Seaweeds like *Palmaria palmata* contain a high concentration of methionine, but other species, belonging to the brown seaweeds (e.g., *Laminaria digitata*), show lower concentration in this amino acid (Fleurence 2004). Seaweeds used in the present work had a crude protein content (% DM) of 49.2, 41.2, and 26.7 % for GRA, POR, and ULV, respectively. These contents are similar to the values previously reported for the IMTA production of these species (Neori et al. 2000; Schuenhoff et al. 2003; Valente et al. 2006; Mata et al. 2010), but higher than the ones reported for naturally occurring algae populations. Wild growing *Gracilaria* crude protein content was 19.1 % in *Gracilaria lemaneiformis* (Xuan et al. 2013); wild *Porphyra* spp. had between 25.8 and 31.3 % (Patarra et al. 2011; Dawczynski et al. 2007), whereas crude protein of natural growing *Ulva rigida* ranged between 8.0 and 16.0 % (Diler et al. 2007; Güroy et al. 2007, 2011). For seaweeds as a by-product of a fish farm effluent, crude protein content was higher: in *Gracilaria bursa-pastoris* was 30.2 % (Valente et al. 2006) and for *Gracilaria conferta* was 33 % (Neori et al. 2000); regarding *Ulva* species, protein content for *Ulva rigida* was 29.5 % (Valente et al. 2006), while for *Ulva lactuca*, the protein content was 37.5 % (Schuenhoff et al. 2003) and 28 % (Neori et al. 2000).

According to some studies, partial substitution of fishmeal by seaweeds can induce positive effect on growth, feed utilization, body composition, and resistance to stress and diseases (Diler et al. 2007; Güroy et al. 2007; Ergün et al. 2009; Pereira et al. 2012; Marinho et al. 2013; Güroy et al., 2013). Nevertheless, the existence of adverse effects on fish metabolism caused by long-term utilization of such ingredients has been reported as well (Davies et al., 1997).

The results obtained in the present work indicate that the inclusion of seaweed meal, at levels of up to 10 % in practical diets, did not cause noticeable negative effects on growth performance and FE of Nile tilapia. In fact, the values obtained (DGI, FCR, and SGR) are in agreement with Abdel-Tawwab et al. (2010). After 84 days, fish body weight almost tripled in all treatments, except for the GRA diet, which was significantly lower comparing to the other three treatments. Despite all diets being similar in composition, the poor results obtained for the GRA diet can be related to poor voluntary feed intake (feed palatability) or some ANFs. Unfortunately, seaweeds contain polysaccharides (e.g., xylans, agar, and

Table 5 Measurements of *villi* length (μm) and intestine diameter (μm) for Nile tilapia (*Oreochromis niloticus*) fed experimental diets control (CTRL), *Ulva* spp. (ULV), (*Porphyra dioica* (POR), and *Gracilaria vermiculophylla* (GRA)

	Dietary treatments			
	CTRL	ULV	POR	GRA
Length	352.92±56.92 a	305.37±64.05 ab	269.26±47.23 b	191.25±41.34 c
Diameter	2148.2±343.4 a	1697.3±493.7 ab	1629.3±289.8 ab	1386.1±172.6 b

Values are presented as the mean standard deviation of six tilapias sampled for each experimental diet ($n=3$). Within a row, means with different letters differ significantly ($P<0.05$)

alginates) which are ANFs limiting their digestibility (Horie et al. 1995). Saponins, tannins, or phytic acid also can reduce the tastiness of a diet by their bitterness and interference with the absorption of dietary lipids and bile salts (Tacon 1995; Francis et al. 2001), causing a reduction of growth performance and feed utilization (Yldirim et al. 2009). Other authors inferred that the existence of ANFs in algae meals can reduce its nutritional quality, interfere with the efficiency of digestive processes, and reduce growth rates (Dallaire et al. 2007; Oliveira et al. 2009).

Walker and Berlinsky (2011) observed that both feed intake and fish growth decreased when algal level was increased in diets affecting feed palatability, which reduced fish feed intake. Sáez et al. (2012) reported that *Ulva* meal contains antinutritive substances able to inhibit digestive proteases of *Sparus aurata* juveniles.

Benevides et al. (1998) and Oliveira et al. (2009) demonstrate the presence of several ANFs in *Gracilaria* species. Also regarding the GRA diet, this was significantly less ingested by fish when compared to the other treatments. This resulted in protein ingestion probably below minimum recommended levels for an adequate growth as indicated by the poorest PER (Table 3). It is known that lectins, who are present in other *Gracilaria* species (Benevides et al. 1998), are resistant to proteolytic hydrolysis by digestive enzymes and harmful to the gut, which may affect the nutritional quality of the marine algae, making them one of the most important ANFs (Bardocz et al. 1995). In general, ANFs can lead to a decrease in growth performance and FE (Olvera-Novoa et al. 2002), can affect the digestive activity (Robaina et al. 1995), or can cause the formation of complexes with minerals (Sugiura et al. 1999) and proteins (Moyano et al. 1999), modifying digestion processes.

Previous studies with Nile tilapia have shown that up to 20 % dietary inclusion of *U. rigida* did not affect the growth performance (Güroy et al. 2007; Azaza et al. 2008), confirming the present results. However, Stadtlander et al. (2013) reported that the 15 % replacement of fish meal by *Porphyra yezoensis* meal increased the growth parameters of Nile tilapia, but the same result was not verified with the 10 % inclusion of POR, although POR and *P. yezoensis* are different species and the nutritional content varies with species, age, and habitat (Ortiz et al. 2006). Similarly, in other finfish species, Valente et al. (2006) reported that the 10 % inclusion of *U. rigida* and *G. bursa-pastoris* meal and 5 % inclusion of *G. cornea* meal had no negative effects on the growth performance and feed utilization efficiency of juvenile European sea bass (*D. labrax*). Soler-Vila et al. (2009) demonstrated that POR could be included up to 10 % in rainbow trout (*Oncorhynchus mykiss*) diet, without significant negative effects on the growth performance. In Xuan et al. (2013), the use of *G. lemaneiformis* up to 15 % inclusion did not affect the growth of black sea bream (*Acanthopagrus schlegelii*) juveniles. Different results were obtained by Davies et al. (1997), who evaluated the use of *Porphyra purpurea* at higher inclusion levels (16.5 and 33 %) as fish meal replacement for juvenile thick-lipped gray mullets (*Chelon labrosus*), with both diets suppressing the growth performance and feed utilization efficiency. The response of fish to dietary seaweed inclusion seems to be dose- and species-dependent, since nutritional composition and digestibility differ between seaweeds (Mustafa and Nakagawa 1995; Pereira et al. 2012). The nutritive value of the used *Gracilaria* meal, IMTA-produced versus wild (Abreu et al. 2011), can partly explain the different growth observed in GRA treatment. Moreover, algal compounds (mainly polysaccharides, such as carrageenan, fucoidan, alginates, and β-glucans) and heavy metals (such as lead, arsenic, and mercury) may modulate the fish growth (Feldlite et al. 2008), also playing a role in disease resistance (Díaz-Rosales et al. 2005; Reyes-Becerril et al. 2013).

Regarding the morphological aspect of the intestine, we observed a significant reduction in villi length in tilapia fed experimental diets, especially in the GRA diet. The intestine diameter, also for the GRA diet, was significantly shorter than that for the CTRL diet ($P<0.05$). Villi length and intestine diameter are related with the number of epithelial cells (Caspary 1992), resulting in the shortening of villi length in less cell surface and total intestinal surface that is able to absorb nutrients (Moldal et al. 2014). The reduction of the diameter could be related with the villi shortness too. Both of these parameters can be associated with the lower growth of the animals fed GRA diet. The villi length and muscular layer

thickness are connected with the local microbiota and the diet composition (Peinado et al. 2012). Alterations in diet composition can affect gut morphology which play a key role in the digestion and absorption of nutrients (Klurfeld 1999). Previous studies showed that algal bioactive compounds may modulate gut morphology in rainbow trout (Heidarieh et al. 2012) and Nile tilapia (*O. niloticus*) (Merrifield et al. 2011). Moreover, in salmonids (Krogdahl et al. 1999) and sea bream (Sitjà-Bobadilla et al. 2005), ANFs lead to histological changes in the intestine which could impair intestinal absorption or lead to immune instability.

Regarding the final body composition, it was found that the 10 % inclusion of the three seaweeds did not influence whole-body protein or lipid content of juvenile Nile tilapia. Nevertheless, the utilization of such ingredient in fish diets must be complemented with sensorial studies to evaluate the effects on the flesh quality.

The use of seaweeds in the diet of marine animals produced by aquaculture appears to be an interesting prospect especially to increase the growth of fish and to improve the treatment of wastewater generated by this activity. However, the economic feasibility of this approach needs to be determined (Darcy-Vrillon 1993). The results obtained in this study suggest that POR and ULV can be considered as potential ingredients to include in diets for tilapia juveniles, up to 10 %, as no negative consequences on growth performance or body composition were observed. However, the inclusion of 10 % GRA decreased feed intake and growth performance.

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