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Effect of dietary phosphorus supplementation on utilization of algae in the grow-out diet of Nile tilapia *Oreochromis niloticus*

Ebtehal El-Sayed Hussein[1,2], Konrad Dabrowski[1], Deyab Mohammed Saad Deyab El-Saidy[2] & Bong-Joo Lee[1,3]

[1]School of Environment and Natural Resources, The Ohio State University, Columbus, OH, USA

[2]Department of Poultry Production, Minufyia University, Shebin El-Kom, Egypt

[3]Aquafeed Research Center, National Fisheries Research and Development Institute, Pohang, Gyeongbuk, South Korea

Correspondence: K Dabrowski, School of Environment and Natural Resources, The Ohio State University, Columbus, OH 43210, USA. E-mail: dabrowski.1@osu.edu

Abstract

This research involved testing the replacement of corn gluten protein concentrate in the diet of 1.5 g Nile tilapia with biofuel algae-based or algae supplemented with phosphorus diets. Experimental diets were formulated to replace 50% of the gluten meal protein in the control diet with biofuel algae protein (Alga50) or Spirulina (Spirulina50) protein. In addition, dicalcium phosphate (P) was added to the Alga50 at a concentration of 3.8% or 7.74% to mitigate aluminium (Al) toxicity and designated as Alga50 + P, and Alga50 + PP respectively. After 9 weeks of the feeding experiment, fish fed diets supplemented with P, Alga50 + P and Alga50 + PP differed significantly ($P < 0.05$) with respect to the feed conversion and protein deposition from the control. The mineral composition of the fish body showed a decrease in levels of Al and iron (Fe) due to the P supplements. The mineral composition of the faeces indicated that Al and Fe were excreted in the faeces and were not accumulated in the fish body. The effect of dicalcium P supplementation on the neutralization of Al in the diet resulted in improved fish growth and histological integrity of the gastrointestinal tract.

Keywords: aluminium, biofuel algae, Spirulina, tilapia, phosphorus supplementation

Introduction

Fish meal protein and plant protein concentrates are the most expensive ingredients in fish diets; minimizing the use of these ingredients is likely to decrease production costs and expand the success of the aquaculture industry (Naylor, Hardy, Bureau, Chiu, Elliott, Farrell, Forster, Gatlin, Goldburg, Hua & Nichols 2009). Several products have been examined as possible substitutes for fish meal in fish diets such as animal by-products (Sugiura, Dong & Hardy 2000; Lee, Dabrowski & Blom 2001), single cell proteins (Lunger, Craig & Mclean 2006; Lunger, Mclean & Craig 2007) and plant protein concentrates (Barrows, Gaylord, Stone & Smith 2007). Several studies have examined the replacement of fish meal with plant proteins including soybean meal (El-Sayed 2004), cottonseed meal (Rinchard, Mbahinzireki, Dabrowski, Lee, Garcia-Abiado & Ottobre 2002), pea protein concentrate (Penn, Bendiksen, Campell & Krogdahl 2011) and distillers dried grains with solubles (Coyle, Mengel, Tidwell & Webster 2004). However, the inclusion of plant protein in fish diets has been limited because of the anti-nutritional factors in the material such as trypsin inhibitors, lectins and glucosinolates (Gatlin, Barrows, Brown, Dabrowski, Gaylord, Hardy, Herman, Hu, Krogdahl, Nelson, Overturf, Rust, Sealey, Skonberg, Souza, Stone, Wilson & Wurtele 2007), as well as their unbalanced composition of amino acids (National Research Council (NRC) 2011). The search for using unconventional protein products in fish diets has been concentrated on several protein sources including torula yeast (Olvera-Novoa, Martinez-Palacios & Olivera-Castillo 2002), single cell proteins (Lim, Lam & Ding 2005) and

algae meal proteins (Takeuchi, Jeong & Watanabe 1990; Nandeesha, Gangadhara, Varghese & Keshavanath 1998).

Increasing attention has been given to algae protein as a replacement of fish meal and plant protein concentrates in fish diets due to its high content of dietary protein, carotenoids, chemical feeding attractants and sources of vitamins and minerals. Besides these different compounds, algae have been examined for synergistic effects between vitamins, protection against vitamin degradation, antioxidants and a binder for feed preparation (Liao, Takeuchi, Watanabe & Yamaguchi 1990; Mustafa, Umino, Miyake & Nakagawa 1994; Olvera-Novoa, Dominguez-Cen & Olivera-Castillo 1998). In addition, algae have been used as a feed additive that resulted in improved growth, feed efficiency, body constituents, carcass quality, physiological characteristics, stress responses and disease resistance in several fish species (Mustafa & Nakagawa 1995; Li & Tsai 2009).

One of the potential problems of algae as a dietary ingredient in animal feed might be their mineral content, particularly aluminium (Al) and iron (Fe). The nutritional value of alum-flocculated algae meal containing a high level of Al has been examined in broiler chicks and laying hens. The results showed that the depression of phosphorous utilization contributed to high levels of Al accumulation in the body (Sandbank 1979). It was reported that increasing algae levels in the diet of chicks increased the need for dicalcium phosphate in the diet two-fourfold (Lipstein & Hurwitz 1981). The detrimental effects of water-borne Al in fish include gill impairment, prevention of the absorption of such elements as K, Ca, Mg or replacement of these elements which affects vital processes in fish (Murungi & Robinson 1992).

Inorganic elements or minerals are required by all forms of aquatic animals for their normal life processes (Lall 2002). Phosphorus (P) is considered the most important, because of its significance in growth and bone mineralization, as well as lipid and carbohydrate metabolism (Watanabe 1988). Murungi and Robinson (1992) suggested that sodium hydrogen phosphate may mitigate the toxic effect of aluminium. It is also possible that phosphate combines with aluminium to form an insoluble compound that removes toxic ions from solution. That would explain the beneficial effect of phosphorus supplementation, however, dietary interaction between P and Al in fish, has not been examined.

In the present study, we analysed the effect of P supplementation in algae-based diets that contained high levels of aluminium and iron on juvenile Nile tilapia. We evaluated growth performance, carcass composition and the histology of the digestive tract. An important economic incentive of this research was to evaluate microalgae produced for biofuels in diet formulations for grow-out phases of Nile tilapia. Finally, we examined the mineral composition of the diets, fish body and faeces to establish the budget for major mineral elements.

Materials and methods

Fish, facility and feeding trial

The feeding trial was performed in the indoor installation of the Aquaculture Laboratory at the School of Environment and Natural Resources, The Ohio State University with juvenile tilapia (*Oreochromis niloticus*) averaging 1.5 ± 0.05 g in initial weight. Tilapia, breds for several generations in the Aquaculture Laboratory, were collected from brooding females as embryos.

Prior to the feeding trial, larvae/juveniles were fed a commercial diet (Tetra Min®, Tetra Holding US, Blacksburg, VA, USA) to allow for acclimation to the experimental conditions. Fish were randomly distributed into 15 glass tanks (three replicate tanks per each of the five treatments) at a density of 25 fish per tank. Each experimental diet was administered at a rate ranging between 6% of body weight at the beginning of the trial to 4% at the end (National Research Council (NRC) 2011). The fish were fed the experimental diets three times a day at 10:00, 13:00 and 16:00 and the trial lasted 9 weeks. All procedures and handling of animals were conducted in compliance with the guidelines of the Institutional Laboratory Animal Care and Use Committee, The Ohio State University. The feeding trial was conducted in 40 L glass tanks supplied with dechlorinated city water set up in a semi-recirculated system (at a rate of 0.25 L min^{-1}). The water-borne mineral concentrations ($n = 6$) in the system water were analysed in accordance with the methods described in AOAC (Association of Official Analytical Chemists) (1995). Water temperature was maintained at 28.1 ± 0.8°C. Supplemental aeration was also provided to maintain dissolved oxygen levels near saturation. Total fish weight in each tank was determined every 3 weeks to check growth and to

adjust the feeding rate. Feeding was stopped for 24 h prior to weighing.

Experimental diets

Five experimental diets were formulated to be isonitrogenous (crude protein, 38 ± 0.5%) and isolipidic (crude lipids, 12.9 ± 3.5%) (Table 1). Cod liver oil and soybean lecithin (to provide a phospholipid and linoleic acid source) were used as a lipid source. Corn gluten meal was the protein source in the control diet with 1.5% lysine and 0.5% methionine supplementation based on the requirements of juvenile tilapia (Dabrowski & Portella 2005). Fifty per cent of the gluten protein in the control diet was replaced either with algae produced to be a biofuel (Alga50) (Algamaxx, Independence Bioproducts, OH, USA) or Spirulina (Spirulina50) (Ocean Star International, Snow Ville, UT, USA). Additional diets were created by adding di-calcium phosphate to the Alga50 at levels making up 3.8% and 7.74% of the diet (designated as Alga50 + P and Alga50 + PP respectively). Supplemental P (3.8%) was needed to neutralize dietary Al (with 0.76 g P required to neutralize 1 g of Al, according to Lipstein & Hurwitz 1981). Experimental diets were cold-pelleted into 2.0 mm diameter in size, freeze-dried, crushed into a desirable size (0.85–2.00 mm) and stored in sealed plastic bags at −20°C until use. At the end of the feeding trial, samples from the experimental diets were prepared for proximate and mineral analysis.

Samples collection and analysis

At the beginning of the feeding trial, fifteen fish (30 g) were weighed, sacrificed by submersion in ice-slurry, and then stored in the freezer at −80°C as initial samples for proximate analysis. At the end of the feeding trial (9^{th} week) all fish were counted and weighed to calculate per cent weight gain (WG; [final biomass – initial biomass] × 100/initial biomass), feed conversion ratio (FCR; dry feed consumed/wet weight gain), protein efficiency ratio (PER; weight gain/protein intake), specific growth rate (SGR; [ln final biomass−ln initial biomass] × 100 $days^{-1}$) and survival (%) (Lee, Dabrowski, Blom, Bai & Stromberg 2002). Thirteen fish from each tank (39 fish per treatment) were sacrificed as described above, packed in sealed plastic bags and then frozen at −80°C for proximate analysis. Approximately 15–20 g of fish biomass was freeze-dried and ground into a powder for proximate chemical analysis. Samples were submitted to the Ohio State University Research

Table 1 A composition of the experimental diets fed to Nile tilapia for 9 weeks

Ingredients %	Control	Alga50	Alga50 + P	Alga50 + PP	Spirulina50
Corn gluten meal*	43.34	21.67	21.67	21.67	21.67
Algamaxx	–	38.74	38.74	38.74	–
Spirulina	–	–	–	–	21.80
Casein (vitamin free)	4.92	7.03	7.03	7.03	5.40
L-methionine	0.50	0.50	0.50	0.50	0.50
L-lysine	1.50	1.50	1.50	1.50	1.50
Wheat flour†	22.70	7.30	7.30	7.30	19.00
CMC	2.00	2.00	2.00	2.00	2.00
Vitamin Mix‡	2.00	2.00	2.00	2.00	2.00
Mineral Mix§	4.20	4.20	4.20	4.20	4.20
Stay-C 35¶	0.06	0.06	0.06	0.06	0.06
Choline chloride	0.17	0.17	0.17	0.17	0.17
Cod liver oil	7.41	4.67	4.67	4.67	6.83
Lecithin	3.71	2.34	2.34	2.34	3.42
α-cellulose	7.50	7.83	3.96	0.09	11.46

*Corn gluten meal protein was replaced at 50% with Algamaxx or with Spirulina (Ocean Star International, Snow Ville, UT, USA).
†Wheat flour is used to balance protein/lipid ratio differences between corn gluten meal and its replacer in the diets.
‡Vitamin mixture (mg kg^{-1} diet) sources were Rovimix series: retinyl acetate, 2.00; cholecalciferol, 0.10; DL-α-tocopheryl acetate, 125.00; menadione niacinamide bisulfite, 5.00; nicotinic acid, 25.00; riboflavin, 20.00; pyridoxine hydrochloride, 15.00; D-calcium pantothenate, 50.00; biotin, 1.00; folic acid, 5.00; cyanocobalamin, 0.05; myo-inositol, 500.00; thiamine mononitrate, 10.00 (Aquaculture Research Group, DSM Nutritional Products France, Animal Nutrition & Health Research, Saint-Louis, France).
§Five mg Se in the form of sodium selenite per kg Bernhart Tomarelli salt mixture (MP Biomedicals, LLC, Solon, OH, USA).
¶L-Ascorbic acid monophosphate (35%).

Extension Analytical Laboratory, Wooster, Ohio. Analyses of crude protein, moisture and ash were performed by standard procedures (AOAC (Association of Official Analytical Chemists) 1995).

The mineral composition of diets and the whole body of the fish were measured utilizing the inductively coupled plasma (ICP) emission spectrophotometric method with the use of an ARI-3560 spectrometer (Applied Research Labs, Valencie, CA, USA) following Watson and Issac (1990) methods. Samples were prepared for analysis using standard procedures outlined in the AOAC (Association of Official Analytical Chemists) (1995) manual. Lipids were extracted with a chloroform and methanol mixture (v/v, 2/1) using the procedure described by Folch, Lee and Stanley (1957).

Faeces collection

During the ninth week of the experiment, faeces were collected three times a day from the bottom of each tank by siphoning, cooled immediately on ice, frozen, freeze-dried and ground into a powder. Samples were then used for mineral analysis as described above.

Feed consumption (satiation) test

At the end of the second week of the experiment, a one day satiation feeding test was conducted to determine maximum voluntary intake. Fish were fed *ad libitum* three times. The amount of consumed feed was measured by weighing the container before and after feeding. The consumed feed was calculated as percentage of biomass after weighing all the fish in each tank 12 h after the last meal. Feed consumption (%) was calculated as 100 × dry feed consumption (g)/wet total biomass (g).

Histological analysis

For the histological examination, two randomly selected fish from each tank were sacrificed ($n = 6$ per treatment) at the end of the feeding experiment (9 weeks). The head and tail of each fish were cut off and the viscera were dissected and preserved in 10% neutral buffered formalin (Thermo Fisher, Kalamazoo, MI, USA) for 48 h. The following day, the viscera were washed with water several times and preserved in 75% ethyl alcohol for further processing. The intestine was separately dissected, straightened and parts of anterior and posterior intestine were examined. Tissues were routinely dehydrated in ethanol, equilibrated in xylene and embedded in paraffin according to standard histological techniques. All tissues were transversely and longitudinally sectioned. Sections were cut to 5 μm increments, mounted on glass slides and stained routinely with haematoxylin and eosin (H&E) or with Alcian-Blue-Periodic Acid-Schiff stain. After staining, the sections were immersed in xylene and set in a Permount medium.

Organic and inorganic metal compounds extraction

Two samples of algae and two diets were extracted with three different solvents: distilled water (DW), 2 *M* HCl and 0.33 *M* $LaCl_3$ (Hargrove & Thomas 1984). First, 50 mL of each solution was added to 1.0 g dry dietary matter, shaken for 24 h at 25°C, and then separated by centrifugation at 20000 × *g* for 15 min. Supernatants were collected after centrifugation in plastic tubes and sent to the Ohio State University Research Extension Analytical Laboratory, Wooster, Ohio. The amount of metals in the organic matter and inorganic fractions were determined by comparison of total metal content in the sample to that dissolved in the HCl (organic and inorganic fraction) and distilled water (inorganic). Minerals were analysed as described above (AOAC (Association of Official Analytical Chemists) 1995).

Statistical analysis

The experimental design was completely randomized for the distribution of fish and experimental diet. Differences among dietary treatments were tested by one-way ANOVA using the spss statistical package (version 18, SPSS, Chicago, IL, USA). Significant differences among treatments were identified using Duncan's multiple range test. The percentage of weight gain data and specific growth rate were arcsine transformed before the analysis. Differences were considered significant at $P < 0.05$.

Results

Growth performance

No mortality was observed during the entire feeding trial. The effects of the experimental diets on

fish growth and feed utilization throughout the 9-week period are presented in Table 2. The fish fed P-supplemented diets did not differ significantly ($P > 0.05$, Table 2) in final weight, weight gain and specific growth rate in comparison to the control group. No significant differences in fish body weight were found among the treatments except for the fish fed the Spirulina50 diet ($P < 0.05$) (Table 2). Fish fed the Spirulina50 diet increased their body weight by 13-fold during the 9-weeks experiment and their resulting mean weight was significantly higher than the fish fed the other experimental diets (Table 2, $P < 0.05$). This was the result of the best feed conversion ratio observed in fish fed Spirulina50 (1.1) (Table 2). However, FCR did not differ significantly ($P > 0.05$, Table 2) compared with the fish fed the control diet. Fish fed diets supplemented with algae, Algae50, Alga50 + P and Alga50 + PP differed significantly ($P < 0.05$) from the control diet in terms of feed intake, feed conversion ratio, protein intake and PER (Table 2). Although, supplementation of phosphorus in the Algae-based diet did not show significant growth enhancement in fish, a beneficial trend of P supplementation was observed in a dose-dependent manner in the results of growth, FI and FCR ($P > 0.05$) (Table 2). Therefore, algae meal protein can replace approximately 50% of gluten protein concentrate in diets for juvenile Nile tilapia without any adverse growth effects.

Feed consumption (satiation) test

Food acceptance was examined after two weeks of the feeding trial when the mean weight of fish was 2.4 g (Table 2). Fish accepted the experimental diets well and the daily feed intake was more than twice compared with the restricted feeding rate used in this experiment. However, feeding rate was adjusted to the least feeding group rate and be equal in all treatments (expressed as% biomass). The fish fed with algae or Spirulina-based diets consumed significantly more feed than the control group ($P < 0.05$), while there were no differences among the remaining treatments ($P > 0.05$, Table 2).

Proximate body composition

No significant differences were detected among all treatments in the whole body composition for moisture and lipid content ($P > 0.05$, Table 3). The content of ash in the whole body was affected

Table 2 Final body weight (FBW), Weight gain (WG), specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR), protein intake (PI), protein efficiency ratio (PER), net protein utilization (NPU) and daily satiation ratio (DSR) of juvenile Nile tilapia (initial wt, 1.5 ± 0.05 g) fed the experimental diets for 9 weeks. Values are mean ± SD

Treatment*	Control	Alga50	Alga50 + P	Alga50 + PP	Spirulina50
FBW (g)	14.1 ± 0.7^{a}	14.4 ± 0.1^{a}	16.1 ± 0.4^{a}	16.1 ± 0.8^{a}	21.8 ± 3.5^{b}
WG (%)	808 ± 48^{a}	839 ± 45^{a}	920 ± 12^{a}	999 ± 184^{a}	1306 ± 180^{b}
SGR (%)	3.5 ± 0.1^{a}	3.6 ± 0.1^{a}	3.7 ± 0.0^{a}	3.8 ± 0.3^{a}	4.2 ± 0.2^{b}
FI (g)	13.9 ± 0.7^{a}	17.8 ± 0.6^{b}	18.7 ± 0.4^{b}	19.4 ± 1.0^{b}	22.7 ± 2.3^{c}
FCR	1.1 ± 0.0^{a}	1.4 ± 0.1^{b}	1.3 ± 0.0^{b}	1.3 ± 0.0^{b}	1.1 ± 0.1^{a}
PI (g)	5.4 ± 0.3^{a}	6.7 ± 0.2^{b}	7.1 ± 0.2^{b}	7.4 ± 0.4^{b}	7.4 ± 0.8^{b}
PER	2.3 ± 0.1^{b}	1.9 ± 0.1^{a}	2.0 ± 0.0^{a}	2.0 ± 0.0^{a}	2.7 ± 0.3^{c}
NPU (%)	30.1 ± 1.8^{a}	26.8 ± 0.9^{a}	25.8 ± 4.7^{a}	28.0 ± 0.2^{a}	37.9 ± 4.4^{b}
DSR (%)	7.5 ± 0.5^{a}	11.8 ± 0.8^{b}	12.1 ± 0.2^{b}	11.3 ± 0.4^{b}	10.8 ± 1.4^{b}

*Values in the same row with the same superscript are not significantly different ($P > 0.05$).

Table 3 Body composition of juvenile tilapia fed the experimental diets for 9 weeks

Composition (% wet basis)	Initial	Control	Alga50	Alga50 + P	Alga50 + PP	Spirulina50
Moisture	76.9	75.4 ± 0.6^{a}	75.3 ± 1^{a}	76.8 ± 3.9^{a}	74.8 ± 0.7^{a}	74.9 ± 0.4^{a}
Protein	56.2	52.3 ± 2.0^{a}	56.2 ± 2.6^{b}	54.9 ± 0.8^{ab}	55.5 ± 1.3^{ab}	54.8 ± 1.8^{ab}
Lipid	6.8	7.7 ± 0.8^{a}	7.7 ± 0.6^{a}	7.4 ± 0.5^{a}	7.6 ± 0.2^{a}	8.0 ± 0.5^{a}
Ash	17.6	17.4 ± 0.4^{a}	19.4 ± 0.6^{bc}	20.0 ± 0.5^{cd}	20.5 ± 0.5^{d}	18.6 ± 0.7^{b}

Values are mean ± SD. Values followed by the same superscript letters in the same row are not significantly different ($P > 0.05$).

by the phosphorus supplementation of the diets. The values of ash content differed significantly ($P < 0.05$) among all treatments with higher ash content observed in fish fed the Alga50 + PP diet (DMRT test).

Mineral composition of water, diets and fish

The water-borne mineral concentration in the system was measured at the end of the feeding experiment and the analysed content of Ca, S, Na, Mg, K, P and Zn was 24.33 ± 1.91, 17.42 ± 1.81, 14.04 ± 1.97, 5.08 ± 0.95, 2.97 ± 0.47, 0.38 ± 0.07 and 0.25 ± 0.03 mg L^{-1} respectively.

The algae used in this study contained high levels of Al and Fe (Table 4) and resulted in higher levels of some metals in the body of fish fed those diets in comparison to fish fed the control diet (Fig. 1). Despite the high levels of Al in the algae used in this experiment, the concentration of Al in body tissues was less than 69.8 μg g^{-1} in all the groups fed diets with algae protein, i.e. below the detection level of the method used. The levels of Fe in the body of fish fed Alga50, Alga50 + P and Alga50 + PP diets were 202.6, 193.8 and 190.4 μg g^{-1}, respectively, and revealed a decreasing trend (Fig. 1). It is apparent that the demand for the essential element, phosphorus, affected the utilization of algae containing high levels of Al and Fe.

Other metals that were detected in fish fed the Alga50 protein included Mo (5.6 ± 2.1 μg g^{-1}) and Cu (252.2 ± 425.3 μg g^{-1}). There were no significant differences among all treatments in the concentration of metals such as Zn, S, Na and Mg in the fish body ($P > 0.05$, Fig. 1). Ca has shown a linear increase in its concentration in the fish body (Fig. 1) that paralleled the metal levels in the experimental diets (Table 4).

Mineral composition in faecal material

There were significant differences ($P < 0.05$) in ash and mineral composition of the faeces between fish fed different experimental diets for 9 weeks (Table 5). Fish fed the Alga50 + PP diet exhibited the highest value (24%) of ash in their faeces, whereas the lowest value was observed with fish fed the Spirulina50 diet (8%). The P levels in faeces increased significantly from those fed the control diet due to dietary P supplementation in Alga50 + P and Alga50 + PP diets (Table 5). This was because of the increasing level of P in the Alga50 + PP diet. Concentrations of calcium in the experimental diets, whole body and faeces increased significantly when additional $CaHPO_4$ was included in diet formulations and followed the same trend as the phosphorus concentrations.

Interestingly, significant differences ($P < 0.05$) in Al and Fe concentrations in the faeces between treatments were observed (Table 5) suggesting that Al and Fe were excreted and were not accumulated in the fish body. Moreover, the highest value of Al and Fe was observed with fish fed Alga50 + P. The decreased levels of Al were observed with additional $CaHPO_4$ in Alga50 + PP diet.

Table 4 Ash and mineral composition of the experimental diets fed to Nile tilapia for 9 weeks

Mineral	Control	Alga50	Alga50 + P	Alga50 + PP	Spirulina50
Ash (%)	5.2^{a}	9.7^{c}	13.2^{d}	16.8^{e}	6.7^{b}
P (%)	1.0^{a}	1.2^{c}	1.9^{d}	2.7^{e}	1.2^{b}
K (%)	0.36^{a}	0.58^{b}	0.56^{b}	0.58^{b}	0.68^{c}
Ca (%)	0.85^{a}	0.99^{b}	1.72^{b}	2.50^{d}	0.95^{b}
Na (μg g^{-1})	2.3^{a}	2.7^{c}	2.5^{b}	2.5^{b}	4.2^{d}
Mg (μg g^{-1})	484^{a}	927^{c}	887^{b}	890^{b}	1088^{d}
S (μg g^{-1})	4989^{b}	4905^{b}	4974^{b}	5282^{c}	4559^{a}
Al (μg g^{-1})	30^{a}	1792^{d}	1688^{c}	1681^{c}	117^{b}
B (μg g^{-1})	4.1^{a}	10.3^{c}	10.2^{c}	9.7^{c}	5.6^{ab}
Cu (μg g^{-1})	22.5	25.4	27.0	27.1	28.0
Fe (μg g^{-1})	156^{a}	3059^{d}	2887^{c}	2940^{c}	378^{b}
Mn (μg g^{-1})	145^{a}	250^{c}	241^{b}	244^{bc}	148^{a}
Mo (μg g^{-1})	1.7^{c}	1.5^{b}	1.2^{a}	1.6^{b}	1.5^{b}
Zn (μg g^{-1})	41^{a}	121^{b}	120^{b}	122^{b}	37^{a}

Values are mean ± SD of two samples. Values in the same row with different letters are significantly different ($P < 0.05$).

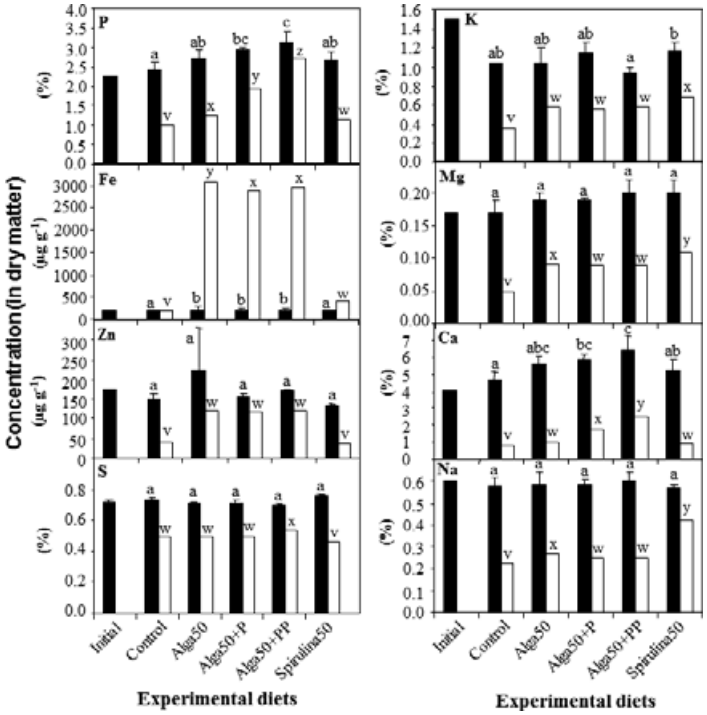

Figure 1 Mineral compositions of experimental diets and tilapia juveniles after 9-week feeding experiment. Solid and open bars indicate minerals in whole body and the experimental diets respectively. Values of the whole body are mean ± SD of triplicate groups; diets are means of two samples. Superscripts having different letters are significantly different ($P < 0.05$).

Table 5 Ash and mineral compositions of faeces of the fish fed the experimental diets for 9 weeks

Mineral	Control	Alg50	Alga50 + P	Alga50 + PP	Spirulina50
Ash (%)	–	14.7 ± 0.9^{b}	20.5 ± 1.0^{c}	24.0 ± 3.4^{d}	8.0 ± 0.2^{a}
P (%)	1.00 ± 0.38^{a}	0.98 ± 0.20^{a}	2.11 ± 0.11^{b}	3.12 ± 0.30^{c}	1.82 ± 0.07^{b}
K (%)	0.10 ± 0.09^{ab}	0.12 ± 0.00^{b}	0.13 ± 0.01^{b}	0.09 ± 0.01^{ab}	0.03 ± 0.00^{a}
Ca (%)	1.6 ± 0.7^{ab}	1.4 ± 0.3^{a}	3.0 ± 0.3^{c}	4.5 ± 0.5^{d}	2.4 ± 0.1^{bc}
Na (%)	0.13 ± 0.03^{b}	0.07 ± 0.02^{a}	0.05 ± 0.00^{a}	0.05 ± 0.00^{a}	0.06 ± 0.01^{a}
Mg (μg g^{-1})	1385 ± 178^{ab}	1399 ± 52^{ab}	1467 ± 48^{b}	1389 ± 224^{ab}	1147 ± 32^{a}
Fe (μg g^{-1})	1233 ± 243^{a}	8271 ± 373^{c}	8630 ± 668^{c}	6905 ± 1430^{b}	1118 ± 55^{a}
Al (μg g^{-1})	580 ± 110^{a}	4822 ± 240^{c}	5156 ± 231^{c}	3836 ± 84^{b}	374 ± 15^{a}
Mn (μg g^{-1})	191 ± 115	217 ± 59	253 ± 27	237 ± 20	203 ± 60
Cu (μg g^{-1})	37.4 ± 6.0^{a}	48.3 ± 12.0^{ab}	41.0 ± 5.1^{ab}	39.5 ± 10.0^{ab}	27.0 ± 1.2^{b}
Mo (μg g^{-1})	2.7 ± 1.3^{a}	1.6 ± 0.2^{a}	1.8 ± 0.1^{a}	1.8 ± 0.2^{a}	2.0 ± 0.1^{a}
S (μg g^{-1})	2530 ± 259^{a}	3374 ± 105^{b}	3588 ± 126^{b}	3855 ± 542^{b}	2259 ± 179^{a}
B (μg g^{-1})	129 ± 106^{a}	39 ± 1^{a}	39 ± 2^{a}	38 ± 1^{a}	36 ± 3^{a}
Zn (μg g^{-1})	1173 ± 165^{b}	1257 ± 194^{b}	1185 ± 204^{b}	1153 ± 293^{b}	747 ± 176^{a}

Values are mean ± SD of triplicate groups; values in the same row with different letters are significantly different ($P < 0.05$).

Organic and inorganic metal compounds extraction

Mineral compositions of two experimental diets and two algae samples extracted with different methods are presented in Table 6. Minerals dissolved in distilled water or dissolved in 2 *M* hydrochloric acid, are referred to as being associated with organic or combined, organic or inorganic fractions respectively. The higher concentrations of Al and Fe were extracted by HCl method from the experimental diets and algae used in the experiment. The same results were observed with the other minerals except K and Na, where the higher proportions were extracted from the diets by DW method. These data indicate that significant proportion of Al (85%) and Fe (70%) was in insoluble fraction (removed with the sediment during

Table 6 Mineral composition of experimental diets ($n = 2$) and algae ($n = 2$) extracted with different methods (DW; dissolved in distilled water, HCl; dissolved in 2 *M* hydrochloric acid). Minerals extracted with distilled water will be equivalent of inorganic proportion whereas those dissolved in HCl will be representing both inorganic and organic fraction. The remaining portion will be insoluble sediment.

	Expressed as per cent of total content			
Mineral	DW diets	DW Algae	HCl diets	HCl Algae
P	33.8 ± 10.7	42.9 ± 7.3	90.0 ± 37.2	77.8 ± 1.3
K	81.6 ± 9.4	59.6 ± 31.2	76.2 ± 16.5	96.9 ± 4.1
Mg	59.1 ± 7.2	48.0 ± 7.0	92.1 ± 45.1	78.1 ± 3.4
Al	2.4 ± 0.9	0.1 ± 0.2	19.6 ± 27.7	14.3 ± 13.6
Fe	1.1 ± 0.5	0.6 ± 0.3	40.2 ± 22.3	28.3 ± 0.1
Mn	44.4 ± 13.3	37.3 ± 21.7	105.1 ± 22.4	84.8 ± 6.4
Na	88.3 ± 11.2	79.6 ± 1.5	80.7 ± 18.4	84.1 ± 5.9

separation). This type of mineral characterization in fish faecal material was used for the first time to our knowledge and provides very useful data for evaluation of new dietary ingredients.

Intestinal histology

No significant differences were observed in the anterior intestine of fish fed Alga50 + PP diet (Fig. 2a–d). However, histological changes were noted in the posterior intestine samples from Alga50 + PP diet group (Fig. 3c and d) compared with control (Fig. 3a and b). The changes were characterized by increased enterocyte vacuolization accompanied by increased variation in vacuole size, fusion of the simple mucosal folds by areas along folds. Shortened, and the widening of lamina propria with concomitant leucocyte infiltration was observed (Fig. 3c and d). Enterocytes exhibited increased absorptive vacuolization and displacement of nuclei.

Discussion

Algae used in the current study contained high levels of Al and Fe (Table 4) which might have affected fish growth rate. After 9 weeks of feeding trial, there was, however, no effect on the growth and no mortality was observed among experimental groups. This is the first report on the effect of diets containing algae with high concentrations of Al and Fe in fish nutrition. Our results are similar to those of Lipstein and Hurwitz (1981) who used di-calcium phosphate supplementation with algae-containing diet to prevent a deleterious influence of high aluminium level algae in poultry diets. They found no effect of the diets containing 0.5% Al on the laying hens when supplemented with P. The need for source of P increased two-fourfolds with increasing level of algae in the diets. However, the dietary Al can differently affect aquatic animals. In Atlantic salmon (*Salmo salar*), a high concentration of dietary Al (2000 mg Al kg^{-1} diet, as $AlCl_3 \cdot 6H_2O$) did not affect the fish growth and vertebrae Al concentrations (20–27 mg kg^{-1}) compared to the fish fed the control diet

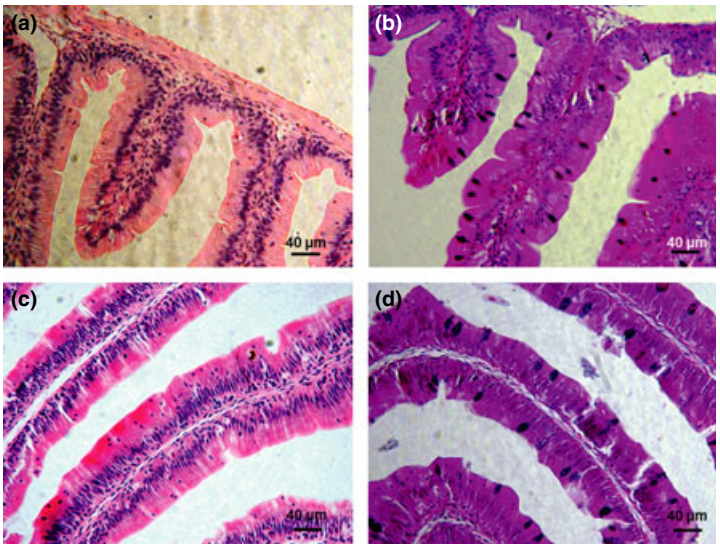

Figure 2 Longitudinal section of the anterior intestine of Nile tilapia fed corn gluten-based diet (Control, a, b) or Alga50 + PP diets (c, d). a, c (H&E staining), and b, d (Alcian-Blue-Periodic Acid-Schiff staining; scale bars = 40 µm).

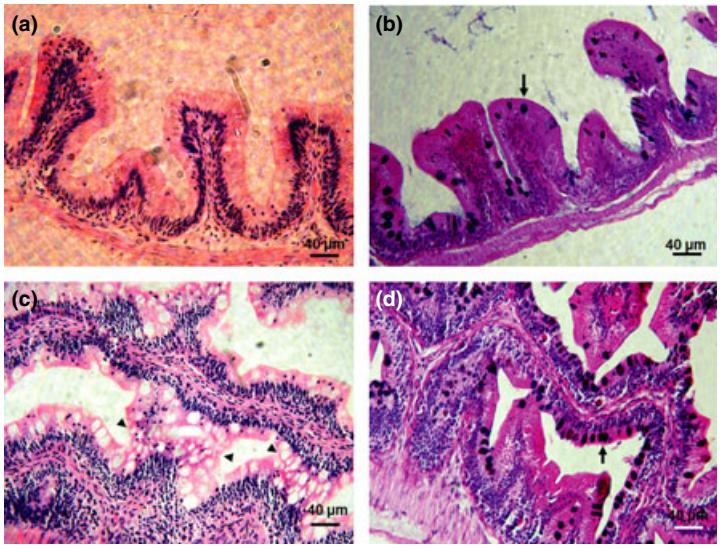

Figure 3 Longitudinal section of the posterior intestine of Nile tilapia fed corn gluten-based diet (Control, a, b) or Alga50 + PP diets (c, d). (Arrows indicate goblet cells; arrowheads point to fusion of mucosal folds). a, c (H&E staining), and b, d (Alcian-Blue-Periodic Acid-Schiff staining; scale bars = 40 µm).

(25 mg Al kg^{-1} diet) (Poston 1991). To the contrary, a positive effect of dietary Al on Japanese eel (*Anguilla japonica*) growth was observed when supplemented at 15 mg kg^{-1} of Al in comparison to control treatment. However, the highest level of dietary Al (100 mg kg^{-1}) was not toxic to fish (Park & Shimizu 1989).Likewise, in the current study, as in earlier observations in Atlantic salmon and Japanese eel, we confirmed that there was no compromising effect of high concentration of dietary Al on growth and body composition of Nile tilapia in grow-out diet containing 38% algae. In our previous experiment, the growth of tilapia larvae was compromised when algae containing high level of Al and Fe were used as a sole protein source (Hussein, Dabrowski, El-Saidy & Lee 2012).

In current study, fish fed Spirulina50 diet showed significantly better growth, feed utilization (the lowest FCR) and protein efficiency ratio as compared with fish fed control diet (Table 2). These results are in agreement with the findings by Hirahashi, Matsumoto, Hazeki, Saeki, Ui and Seya (2002), who reported that feeding Spirulina to fish and poultry improved growth and the survival rates. In the current study, the significantly higher PER in Spirulina50 indicates that algal protein is of high quality. It is in line with Olvera-Novoa *et al.* (1998) observations where the higher value of PER (2.0) in diets containing 60% Spirulina was found when fish meal was replaced in Mozambique tilapia (*Oreochromis mossambicus*) diets. In contrast, poor growth with Spirulina was found by Atack, Jauncey and Matty (1979) when used as a sole protein source in diets fed to rainbow trout and common carp.

The positive effect of dietary P supplement was linked to the neutralization of Al toxicity in poultry. It has been shown that 1,500 mg Al kg^{-1} diet depressed growth, feed intake, feed efficiency, bone ash and plasma P and Ca of young chicks. Increased dietary P decreases these negative effects of aluminium (Lipstein & Hurwitz 1981; Hussein, Cantor, Johnson & Yorkel 1990). The beneficial effect of dietary P supplement was reported in silver perch when the growth and feed efficiency was increased as dietary phosphorus level was increased from 0.24 to 0.72% (Yang, Lin, Liu & Liou 2006). In guppy (*Poecilia reticulata*) Shim and Ho (1989) demonstrated that fish weight doubled by increasing dietary P from 0.03 to 0.53% whereas dietary Ca had no effect. However, it was reported that the presence of phosphate reduces the Al uptake and results in an insolubility of aluminium phosphates (Martin 1986). Present results with tilapia did not confirm earlier finding in terrestrial animals that phosphorous reduces Al uptake (Table 5). The excretion of faecal Al and Fe was decreased as dietary P increased. However, dietary P level led to a higher excretion in total ash and other minerals such as P, Ca and S (Table 5). A further study should be conducted to investigate the interaction between minerals as organic and inorganic forms are metabolized differently in gastro-intestinal tract.

In the studies on Fe requirement in tilapia, the highest weight gain and feed utilization efficiency

were observed at the concentration of 85 mg kg^{-1} of ferrous sulphate (II) or 150 mg kg^{-1} as ferric citrate (III) (Shiau & Su 2003). Therefore, in our studies algae provided highly excessive amount of Fe. Furthermore, Baker, Martin and Davies (1997) reported that an increase in ferrous iron to 6345 mg Fe kg^{-1} diet fed to African catfish resulted in liver Fe level of 754 μg g^{-1} in comparison with fish fed a diet unsupplemented with Fe (523 μg g^{-1}). The effect of dietary iron toxicity and lipid oxidation in fish diets is further elaborated by studies of Desjardins, Hicks and Hilton (1987) in rainbow trout. The authors observed no detrimental effect of dietary Fe level of up to 1380 mg Fe kg^{-1} when antioxidant, ethoxiquin was added to dietary lipids. The Fe concentrations in liver and kidney increased as dietary Fe levels increased and amounted to 536 and 493 μg g^{-1} dry tissues in comparison to control fish 69 and 138 μg g^{-1} respectively. Present study with tilapia demonstrated that Fe is excreted in faeces and resulting accumulation in the body is disproportionally small in comparison to dietary levels (Fig. 1).

Mineral compositions of the faeces in the present study showed high levels of Al and Fe (Table 5) suggesting that Al and Fe were excreted and were not accumulated in the fish body. These results are in agreement with those found by Santos, Paulino, Tambourgi and Almeida (2011) showing that Nile tilapia juveniles (13.2 ± 0.9 g) when fed with Al^{+3}-enriched diets containing up to 100 mg of Al^{+3} (Al $(NO_3)_3$) kg^{-1} feed excreted this metal in the faeces (448 mg kg^{-1}). It indicated that Al was eliminated in the faeces and was not absorbed by tilapia organs.

Mineral concentrations of diets and algae extracted by different methods (Table 6) showed higher concentrations of Al and Fe in HCl soluble fraction that represented both inorganic and organic association. This result was in agreement with an earlier study (Hargrove & Thomas 1982) and suggests that the greater amounts of Al are bond to inorganic portion of the minerals in algae. Also, Al from soils was extracted by $LaCl_3$ and KCl (Bloom 1979) suggesting that Al extracted from Al-organic matter by $LaCl_3$ was higher than KCl. However, in our experiment with high organic matter content in feeds and algae, results of extraction with $LaCl_3$ were equivocal in comparison to soil samples (data not shown). This may suggest that the high level of Al and Fe found in the algae used in this experiment can probably be related to mineral fraction in the algae (ash content in algae was 16–19%). However, we found in histological observation that gastro-intestinal tract of fish fed algae containing Al and Fe showed the formation of folds fusion of the intestine, and the numbers of goblet cells were elevated in this group (Fig. 3). Interestingly, the addition of phosphorus into this diet did not adversely affect the digestive functions in Nile tilapia. Penn *et al.* (2011) reported histological changes in the distal intestine with respect to several variables including mucosal fold height, fold fusion, lamina propria width, lamina propria infiltration, enterocyte vacuolization and displacement of enterocyte nuclei when Atlantic salmon were fed high level (35%) of plant protein (pea protein concentrate) for 8-weeks. The number of goblet cells may differ due to the feeding level (De Silva & Anderson 1995). Thus, even though the level of dietary algae did not affect fish growth in the current study, dietary P must be included in algae-containing diet to eliminate a potential risk of decreased digestibility of proteins and growth depression for long-term feeding until marketable size.

In conclusion, our findings suggest that algae meal protein can replace approximately 50% of gluten protein concentrate in diets for juvenile Nile tilapia without any adverse growth effects. Diets containing algae should be supplemented with phosphorus, particularly di-calcium phosphate to reduce Al impact on absorption of other minerals. Further research will be required to determine the feasibility of using algae with lower ash content and examine algae supplements (replacement) when fed *ad libitum*.

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