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RESEARCH ARTICLE

Toward environmentally sustainable aquafeeds: Managing phosphorus discharge from Nile tilapia (*Oreochromis niloticus*) aquaculture with microalgae-supplemented diets

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Aquaculture is the fastest growing food production sector and currently supplies almost 50% of fish for human consumption worldwide. There are significant barriers to the continued growth of industrial aquaculture, including high production costs and harmful environmental impacts associated with the production of aquaculture feed. Most commercial aquaculture feeds are based on fish meal, fish oil, and terrestrial plant ingredients, which contain indigestible forms of phosphorus. Phosphorus loading from aquaculture effluent can lead to eutrophication in aquatic ecosystems. Formulating fish feeds using ingredients that contain highly bioavailable forms of phosphorus in nutritionally appropriate quantities will reduce phosphorus loading. Using both in vivo and in vitro experiments, we examined the digestibility of phosphorus in three experimental tilapia feeds supplemented with two freshwater microalgae (*Spirulina* sp., *Chlorella* sp.) and one marine microalga, *Schizochytrium* sp., relative to a reference diet containing fish meal and fish oil. We also calculated a phosphorus budget to quantify metabolic phosphorus waste outputs. The marine *Schizochytrium*-supplemented diet had the highest phosphorus digestibility and the lowest solid phosphorus discharge compared to the reference diet and the other experimental diets. The *Schizochytrium* ingredient also had the highest phosphorus digestibility among the three microalgae tested in vitro experiments. These results suggest that *Schizochytrium* sp. is a highly digestible source of phosphorus and findings on metabolic phosphorus waste outputs have implications for the formulation of sustainable diets for tilapia. Further research must examine the economic feasibility and environmental impacts of producing *Schizochytrium* sp. as an aquafeed ingredient.

Keywords: Aquaculture, Phosphorus digestibility, Tilapia, Microalgae, Eutrophication

Introduction

Aquaculture is the fastest growing food sector globally, having grown almost 600% between 1990 and 2018, while capture fisheries around the world are either at or beyond their sustainable limits and have plateaued at around 90 million tons/year (Food and Agriculture Organization of the United Nations [FAO], 2020). Aquaculture has increasingly filled the growing demand for seafood, now producing over half of all fish and seafood for human consumption. Aquaculture is projected to continue its remarkable rise and to produce nearly 109 million metric tons in 2030 (FAO, 2020), and a majority of this production will be in the form of industrial aquaculture (Delgado et al., 2003; Sarker et al., 2013).

Current industrial aquaculture practices can have harmful ecological consequences. One such consequence is the eutrophication of surface waters from aquaculture effluents containing phosphorus excreted by cultured species (Bureau and Hua, 2010; Sarker et al., 2011; Sarker et al., 2014). Previous work suggested that anthropogenic alteration of the phosphorus cycle could lead to nonlinear environmental degradation (Rockström et al., 2009). In spite of this, as much as 70%–80% of phosphorus contained in fish feed in intensive aquaculture systems is excreted into the environment rather than retained by the fish (Soto et al., 2008). Because phosphorus is a limiting nutrient in all freshwater ecosystems and certain marine ecosystems, release of dissolved phosphorus from

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aquaculture wastewater into aquatic environments can result in severe eutrophication and anoxia (Ricklefs and Miller, 1999; Bureau and Hua, 2010; Sarker et al., 2011). This problem is especially important to solve, given that 60% of global aquaculture production is carried out in freshwater (Hall et al., 2011). In some countries, regulations have been put in place restricting the amount of phosphorus that aquaculture operations may discharge (Sarker et al., 2011). Altering feed composition may be an efficient way to alter the amount and composition of fish waste (Bureau and Hua, 2010; Sarker et al., 2016a; Sarker et al., 2016b; Sarker et al., 2018). In order to ensure the sustainability of aquaculture as a solution to food security concerns, we must develop aquaculture feeds that are both nutritionally adequate and result in minimal phosphorus excretion.

Most commercial fish feeds currently use fish meal as their primary protein source. One challenge with fish meal–based feeds is that they rely on wild-caught forage fish, which are overharvested and in decline (Delgado et al., 2003). The second challenge is that the majority of phosphorus in fish meal–based feeds is in the form of hydroxyapatite from bone content, which is not digestible for monogastric animals such as fish (Bureau and Hua, 2010; Sarker et al., 2011). As a result, phosphorus retention rates are low and excretion rates are high (Sarker et al., 2011; Sarker et al., 2014).

Due to the increasing costs of fish meal and fish oil, the aquaculture feed industry has recently focused on substituting plant proteins such as soybean meal for fish protein (Naylor et al., 2009; Troell et al., 2014). Although this substitution may address the problem of relying on wild forage fish populations, it does not solve the problem of phosphorus digestibility. Phosphorus in soybean products and many other terrestrial plants comes in the form of phytic acid, which also cannot be digested by fish (Naylor et al., 2009). Previous studies have shown that the bioavailability of phosphorus in fish feed is more important than the absolute phosphorus content. For example, Sarker et al. (2011) have shown that rainbow trout retain more phosphorus from an experimental diet with lower phosphorus content in less digestible forms than from a commercial fish meal–based diet with higher phosphorus content. Thus, it is essential that feeds be developed with appropriate amounts of bioavailable phosphorus, so that fish nutritional needs are met and phosphorus excretion levels are low. The ideal aquaculture feed would result in high phosphorus retention with minimal phosphorus loading, as discharge of soluble phosphorus contributes to eutrophication and discharge of solid phosphorus (fecal phosphorus) indicates low phosphorus digestibility.

Aquatic microalgae present a promising alternative protein and oil source for fish feed. Currently, the environmental sustainability of algae culture is limited by high production costs related to fossil fuel use for energy and mined or manufactured nutrients for growth media (Wijffels and Barbosa, 2010). Microalgae production has the potential to be more sustainable than the production of fish meal and fish oil made from industrial reduction of wild forage fisheries, especially if it can be produced using renewable energy and fish effluent or an optimal mixture of fish effluent and inorganic nutrients as a growth medium.

Tilapia are an ideal species for aquaculture because they are low trophic level feeders and can tolerate low dissolved oxygen levels (Shelton and Popma, 2006). Nile tilapia (*Oreochromis niloticus*) is currently the world's third most farmed fish; its production has grown 7%–10%/year since the 1990s (Wang and Lu, 2016; FAO, 2020). Tilapia will also be one of the two fastest growing aquaculture products in the next decade (Kobayashi et al., 2015) and has been a key driver of the U.S. and global consumer demand for farmed fish (FAO, 2018).

Industrially produced tilapia feeds are currently based on a mixture of fish meal and plant proteins. However, a relatively high proportion of fish meal is still included in tilapia feeds, and a high percentage of the phosphorus content (40%–75%) is nondigestible (Schneider et al., 2004; Köprücü and Özdemir, 2005; Sugiura, 2018). Although a few studies have shown that partial substitution of certain microalgae species (*Scenedesmus* spp., *Chlorella* spp., and *Spirulina* spp.) for fish meal in tilapia feed results in satisfactory growth (Olvera-Novoa et al., 1998; Badwy et al., 2008; Hussein et al., 2013), no research has been done on the digestibility of phosphorus from microalgae-based diets in tilapia. Understanding ways to optimize phosphorus digestibility in tilapia feed by manipulating feed formulation is imperative to ensure the minimization of harmful waste from tilapia aquaculture.

We recently evaluated the digestibility of protein, amino acids, lipid, and all unsaturated fatty acid fractions in two freshwater microalgae (*Chlorella* spp. and *Spirulina* spp.) and one marine microalga (*Schizochytrium* spp.) for Nile tilapia. Results suggested that *Spirulina* sp. is a good alternative protein and *Schizochytrium* sp. is a quality fish oil substitute or omega-3 polyunsaturated fatty acids, including docosahexaenoic acid (DHA) supplement for tilapia diets (Sarker et al., 2016a). We also detected significantly improved weight gain, feed conversion ratio, and protein efficiency ratio when *Schizochytrium* fully replaced dietary fish oil compared to reference feed containing fish oil (Sarker et al., 2016b). Understanding phosphorus digestibility, retention, and budget (metabolic waste outputs) is an important step toward low pollution feed design for sustainable aquaculture.

In this study, we fed Nile Tilapia four different diets: a reference diet based on commercial aquafeed, and three experimental diets which we supplemented with two freshwater microalgae (*Chlorella* sp. and *Spirulina* sp.) and one marine microalga (*Schizochytrium* sp.). We assessed the digestibility of phosphorus in each diet and also assessed phosphorus digestibility in each of the experimental ingredients both in vivo and in vitro to develop a generalizable digestibility assay for other feeds and fish species. Specifically, our objectives were to (1) determine and compare the amount of phosphorus in each of the four treatment diets and each of the three experimental microalgae ingredients alone, (2) determine and compare the digestibility of phosphorus in each of the treatment diets in vivo in Nile tilapia, (3) determine and compare the

Table 1. Composition of the four test diets. DOI: https://doi.org/10.1525/elementa.2020.00170.t1

Ingredient (g/kg)	Reference Diet (A)	*Spirulina* Diet (B)	*Chlorella* Diet (C)	*Schizochytrium* Diet (D)
Fish meal	300	210	210	210
Soybean meal	170	119	119	119
Corn gluten meal	130	91	91	91
Fish oil	100	70	70	70
Wheat flour	280	196	196	196
Vitamin/ mineral[a]	10	7	7	7
Sipernat 50™[b]	10	7	7	7
Test ingredient	0	300	300	300
Total	1,000	1,000	1,000	1,000

Test ingredient refers to the dried microalgae species being tested in each diet as indicated in the diet name. Proximate composition of each diet is reported in Sarker et al. (2016a).

[a]Vitamin/mineral premix (mg kg^{-1} dry diet unless otherwise stated): vitamin A (as acetate), 7,500 IU kg^{-1} dry diet; vitamin D3 (as cholecalciferol), 6,000 IU kg^{-1} dry diet; vitamin E (as DL-a-tocopherylacetate), 150 IU kg^{-1} dry diet; vitamin K (as menadione Na-bisulphate), 3; vitamin B12 (as cyanocobalamin), 0.06; ascorbic acid (as ascorbyl polyphosphate), 150; D-biotin, 42; choline (as chloride), 3,000; folic acid, 3; niacin (as nicotinic acid), 30; pantothenic acid, 60; pyridoxine, 15; riboflavin, 18; thiamin, 3; NaCl, 6.15; ferrous sulphate, 0.13; copper sulphate, 0.06; manganese sulphate, 0.18; potassium iodide, 0.02; zinc sulphate, 0.3; carrier (wheat middling or starch).

[b]Sipernat 50: Source of AIA comprises 98.50% SiO_2 with an average particle size of 50 μm.

digestibility of phosphorus in each algal ingredient alone (*Spirulina*, *Chlorella*, and *Schizochytrium*) in vivo and in vitro, and (4) calculate a phosphorus budget for each of the diets in relation to their use in Nile tilapia aquaculture.

Materials and methods

Feed formulation

To determine phosphorus digestibility of whole cells of *Spirulina sp.*, *Chlorella sp.*, and *Schizochytrium sp.*, four feeds were formulated: One is a nutritionally complete reference feed and three test diets with 30% of each microalgal species replacing the reference feed. For this, we manufactured a high-quality reference diet containing fish meal and fish oil (**Table 1**; Sarker et al., 2016a; Sarker et al., 2018) and combined it with each test microalgal species (pure algae) at a 7:3 ratio to produce three test diets (one for each microalgal species) following the apparent digestibility protocol of Cho et al. (1982). The test diets included an acid-insoluble marker (acid-insoluble ash [AIA]) that assisted with later nutrient digestibility analysis. The ingredients and AIA were mixed with 33% deionized water in a KitchenAid mixer, then put through a Panasonic meat grinder and dried at room temperature for 12 h. Feed was stored at –20°C throughout the experiment.

In vivo digestibility experimental design

The in vivo experiment consisted of four treatments (three microalgae-supplemented diets and a reference diet) and three replicate tanks per treatment for a total of 12 tanks of Nile tilapia. Each tank was randomly assigned to be fed one of the four diets (**Table 1**).

Nile tilapia were held in conical 30-gallon tanks with removable feces collectors. Each tank had an internal sponge-type biological filter with a central air stone. Fresh, dechlorinated tap water was added to each tank as needed to make up for water lost during the removal of feces from the collector. Tanks were held at 27°C with a 10 h light/14 h dark photoperiod. To acclimate the tilapia, all fish were fed the control diet for 1 week after their initial placement in the tanks, then fed their assigned diet for 1 week prior to the first fecal collection. Average biomass in each tank ranged from 20.5 to 22.8 g at the beginning of the experiment and from 31.07 to 40.77 g at the end of the experiment (7 days acclimation on same feed + 7 days acclimation on assigned diet + 55 days on assigned diet = 69 days total). Treatments did not differ in their starting biomass.

All tanks were fed equal amounts twice daily Monday through Friday and once on Sunday afternoons. After feeding, we removed any uneaten feed pellets found in the feces collectors, recorded their weight, and subtracted that weight from our record of the total weight of feed given to that tank on that day. To account for fish growth, each tank was fed to apparent satiation every Monday morning. The smallest amount of feed fed to any tank on Monday morning was set as the mass of feed to be given to each tank during every feeding for the rest of the week. To ensure every tank received the same amount of feed for each 1-week period, the Tuesday morning feed weights for each tank were adjusted by subtracting the difference between the tank's Monday morning feed mass and the set feed mass for the week. Feces were collected from each tank before feeding and stored at –20°C. Feces were collected over 55 days.

To determine phosphorus retention, we euthanized 10 fish with clove oil prior to the start of the experiment. These fish were ground into a homogenous slurry, freeze-dried for 48 h, reground, and stored at –20°C until analyzed for phosphorus content. We repeated this procedure on the final day of the experiment using three fish from each tank. These final whole-body fish samples were pooled by tank. Feces were also pooled by tank, freeze-dried, and homogenized prior to phosphorus analysis.

Three samples of feces and whole-body fish per tank were analyzed for phosphorus content. We analyzed three samples of each of the four diets and each of the three experimental protein source ingredients (dried microalgae) for phosphorus content. The mean phosphorus content for each diet and ingredient was used in the digestion analysis and for the phosphorus budget calculation.

In vivo phosphorus digestibility analysis and calculations

We analyzed the three types of samples (pure microalgae, diets, and feces) for phosphorus in the EMS lab at the Environmental Studies Program at Dartmouth College. To determine the phosphorus content, each dried sample was heated in a porcelain crucible at 550°C for 12 h in a muffle furnace, then weighed. Samples were then boiled for 2 min in 18 mL of 50% HCl and three drops of HNO_3, filtered through "ash less" #40 filter paper into a 250-mL volumetric flask, and diluted to a final volume of 250 mL; 8 mL of each digested sample were run by Flow Injection Analysis by a Lachat QuickChem 8500 Auto Analyzer, in which the phosphorus in each sample reacts with ammonium molybdate, antimony potassium tartrate, and ascorbic acid to form a blue complex. The absorbance of each sample at 880 nm is then directly proportional to the phosphorus concentration. This method is capable of detecting phosphorus concentrations as small as 3 μgP/L (Hach Company 2004). Following this procedure, the filter paper was returned to its porcelain crucible and incinerated at 600°C overnight. The mass of this AIA was recorded once the sample had cooled.

To measure the bioavailability of phosphorus, apparent digestibility coefficients (ADC) for each diet were calculated according to Cho et al. (1982):

$$\sqrt{\mathrm{ADC}_{\mathrm{Diet}} = 1 - \left(\frac{F}{D} \times \frac{D_i}{F_i}\right)},$$

where $F = \%$ phosphorus of feces, $D = \%$ phosphorus of diet, $D_i = \%$ digestion indicator (AIA) of diet, and $F_i = \%$ digestion indicator (AIA) of feces. ADC of each protein-source ingredient (fish meal and each of the three microalgae) was calculated according to the National Research Council (2011):

$$\sqrt{\mathrm{ADC}_{\text{test ingredient}} = \frac{(0.7 \times D_{\mathrm{ref}} + 0.3 \times D_{\mathrm{ingredient}}) \times \mathrm{ADC}_{\text{test diet}} - (0.7 \times D_{\mathrm{ref}} \times \mathrm{ADC}_{\mathrm{ref}})}{0.3 \times D_{\mathrm{ingredient}}}},$$

where D_{ref} is the % phosphorus of the reference diet and $D_{\mathrm{ingredient}}$ is the % phosphorus of the ingredient.

The amount of phosphorus digested (g/kg) was also calculated for each diet and microalgae ingredient by multiplying the ADC by the amount of phosphorus in the diet or ingredient. The phosphorus budget was estimated following the methods of Cho and Bureau (1998) and Koko et al. (2010) using the following equations:

$$\sqrt{\text{Retained } P(g) = \text{feed consumed (g)} \times \mathrm{ADC}_{\mathrm{Diet}}},$$
$$\sqrt{\text{Solid } P \text{ waste } (g) = \text{feed consumed (g)} \times (1 - \mathrm{ADC}_{\mathrm{Diet}}) \times \text{phosphorus content}_{\mathrm{diet}}},$$
$$\sqrt{\text{Dissolved } P \text{ waste } (g) = \text{digestible } P \text{ intake} - \text{retained } P},$$
$$\sqrt{\text{Total } P \text{ Load } (g) = \text{Solid } P \text{ waste } + \text{ Dissolved } P \text{ waste}}.$$

In vitro digestibility experimental design

We ran the three microalgae ingredients (*Spirulina* sp., *Chlorella* sp., and *Schizochytrium* sp.) through a two-step in vitro digestion process, called the pH stat method, to simulate digestion in the Tilapia stomach and small intestine. We introduced 80 mg by protein basis of each microalga with 25 mL D.I. water in a 50-mL Pyrex beaker that was held at 25°C via water bath. The reaction mixture was adjusted to 2.0 pH with 0.1 M HCl using a Hannah instrument HI-901C1 potentiometric autotitrator to dose 0.3 mL HCl per 2-min interval until pH equilibrium was met (approximately 30 min). After equilibrium, we introduced 200 μL stomach crude enzyme extract prepared according to Yasumaru and Lemos (2014) with slight modifications sourced from Chaijaroen and Thongruang (2016; Sarker et al., 2020b).

We made minor pH changes using 0.1 M HCl or 0.01 M NaOH if necessary. Once we introduced the crude enzyme extract, we initiated a set program on the autotitrator to dose 0.025–0.075 mL per 3 min intervals to keep the pH at 2.0 for 1 h. After the 1-h stomach digestion period, we recorded the total volume dosed. Then, we adjusted the pH to 8.0 using 0.1 M NaOH and allowed the autrotitrator to dose 0.025 mL 0.1 M NaOH for approximately 1 h to allow the mixture to reach equilibrium. Once pH equilibrium had been reached, we introduced 250 μL intestinal crude enzyme extract, prepared in the same way as the stomach crude enzyme extract (Yasumaru and Lemos, 2014; Sarker et al., 2020b). Then, we initiated the autotitrator method to dose 0.01–0.025 mL 0.1 m NaOH to hold the pH at 8.0 for 1 h and recorded the total volume dosed. All samples were run in triplicate on different machines and frozen immediately at –20°C after the final step (Yasumaru and Lemos, 2014; Tibbetts et al., 2017).

In vitro phosphorus digestibility analysis and calculations

All in vitro digestion samples were defrosted at room temperature then centrifuged at 4,000 rpm for 30 min to settle any solids. The supernatant was separated into designated beakers for acid digestion (see below). The solids were rinsed using 15 mL D.I. water and centrifuged two additional times, then transferred into crucibles where we processed them using the ash-free dry weight method described above. We then digested the ash

content in 10 mL 50% HNO_3 for 15 min, filtered each sample through #40 ashless filters, and brought the final volume to 45 mL. The liquid samples were processed using the Environmental Protection Agency method 3050b: acid digestion for sediments, sludges, and soils to improve clarity. Each sample was brought up to 45 mL final volume. All samples (liquid and solid) were run through the same inductively coupled plasma mass spectrometry analysis for Phosphorus as the in vivo samples to get Total P (mg/L). The in vitro ADC percentages were calculated as:

$$\sqrt{\text{Liquid } P(\text{mg/L}) = \text{Soluble Phosphorus in the liquid sample,}}$$
$$\sqrt{\text{Solid } P(\text{mg/L}) = \text{Insoluble or not solubilized Phosphorus in solid sample,}}$$
$$\sqrt{\text{Total } P(\text{mg/L}) = \text{Solid} + \text{Liquid } P,}$$
$$\sqrt{\text{In vitro ADC } (\%) = \frac{\text{Liquid } P}{\text{Total } P} \times 100.}$$

Statistical analysis

We conducted a one-way analysis of variance of phosphorus content (g/kg) in each diet and microalgae ingredients, ADC of P in diet and ingredients, amounts of phosphorus digested, phosphorus budget fractions (retention, solid, dissolved, and total), and in vitro phosphorus digestibility data. When significant differences were found, the means among treatments were compared using a Tukey's test of multiple comparison, and differences were considered statistically significant at $P < 0.05$. Digested phosphorus and percent AIA values were excluded from analysis as outliers if they were outside the 95% confidence interval for the diet, ingredient, or tank being analyzed. Based on these criteria, one digested phosphorus value from the *Chlorella* diet and five whole-body fish digested phosphorus values (two from tank 10 [*Spirulina* diet], two from tank 3 [*Chlorella* diet], and one from tank 8 [*Spirulina* diet]) were excluded. One percent AIA value from the *Chlorella* diet and one from the *Schizochytrium* diet were also excluded as outliers, along with 2% AIA values from tank 7 feces (reference diet) and 1% AIA value from tank 8 (*Spirulina* diet).

Results

In vivo experiment

Throughout our results, we present means ± *SE*s. Fish appeared healthy and gained weight throughout the experiment (Table S1). The total phosphorus content of the reference diet (8.50 g P/kg feed) was higher than the three microalgae-supplemented diets (6.03–7.49 g P/kg feed; $F_{3,10} = 3.87$, $P = 0.064$, **Figure 1A**, Table S3). However, the ADC of phosphorus in all three microalgae-supplemented diets (74.02%–81.53%) were higher than the ADC for phosphorus in the reference diet (71.70%; $F_{3,11} = 6.39$, $P = 0.016$, **Figure 1B**, Tables S3 and S4). The amount of phosphorus digested also differed significantly among diets and ranged from 4.60 (Chorella) to 6.11 g P/kg feed (Spirulina; $F_{3,11} = 37.24$, $P < 0.0001$). A Tukey HSD (honestly significant difference) showed that the amounts of phosphorus digested in the *Schizochytrium* and reference diets were significantly higher than those of both the *Chlorella* and *Spirulina* diets (**Figure 1C**, Tables S3 and S4).

The three microalgae ingredients did not differ in their amount of phosphorus ($F_{2,8} = 2.23$, $P = 0.19$, **Figure 2A**, Table S5). The ADC of phosphorus differed significantly among microalgae species ($F_{2,8} = 6.18$, $P = 0.03$, **Figure 2B** and Tables S4 and S5). The ADC of phosphorus in *Schizochytrium* was 12% higher than that in Spirulina and 19% higher than in Chlorella. *Schizochytrium* also had a higher amount of digestible P (6.15 ± 0.49 g/kg) than *Spirulina* (4.55 ± 0.49 g/kg) and *Chlorella* (4.77 ± 0.49 g/kg; $F_{2,8} = 4.29$, $P = 0.07$, **Figure 2C** and Tables S4 and S5).

Phosphorus budget

Diets differed significantly in retained phosphorus percentage ($F_{3,11} = 5.72$, $P = 0.02$), total phosphorus load ($F_{3,11} = 4.11$, $P = 0.049$), solid phosphorus discharge ($F_{3,11} = 4.67$, $P = 0.04$; Tables S6 and S7), and dissolved phosphorus discharge ($F_{3,11} = 4.11$, $P = 0.049$). The percentage of retained phosphorus was highest in Tilapia fed the *Spirulina* diet (86.80% ± 4.05%) and lowest in those fed the *Schizochytrium* diet (38.90 ± 10.64; **Figure 3A**). The total phosphorus load was highest for the *Schizochytrium* diet (18.60 g P/kg feed) and lowest for the *Spirulina* diet (4.05 ± 1.72 g P/kg feed; **Figure 3B**). Solid phosphorus discharge was highest for the reference diet (11.27 ± 1.42 g P/kg feed) and lowest for the *Schizochytrium* diet (5.56 ± 0.69; **Figure 3C**). The *Schizochytrium* diet discharged the highest amount of dissolved phosphorus (13.04 ± 4.18 g P/kg feed) and the *Spirulina* diet discharged the least (–2.72 ± 1.29; **Figure 3D**).

In vitro experiment

In vitro phosphorus digestibility differed significantly among the experimental microalgae ingredients ($F_{2,8} = 104.96$, $P < 0.0001$). The *Schizochtrium* ingredient had the highest phosphorus digestibility (93.9% ± 0.8%) followed by *Spirulina* (81.9% ± 0.6%) and *Chlorella* (75.2% ± 1.2%). A Tukey HSD test showed that the ADC of phosphorus from each species was significantly different from each of the other two species.

Discussion

Currently, aquaculture feeds use unsustainably sourced ingredients that supply largely indigestible forms of phosphorus, producing both solid and dissolved phosphorus waste that leads to eutrophication in aquatic ecosystems

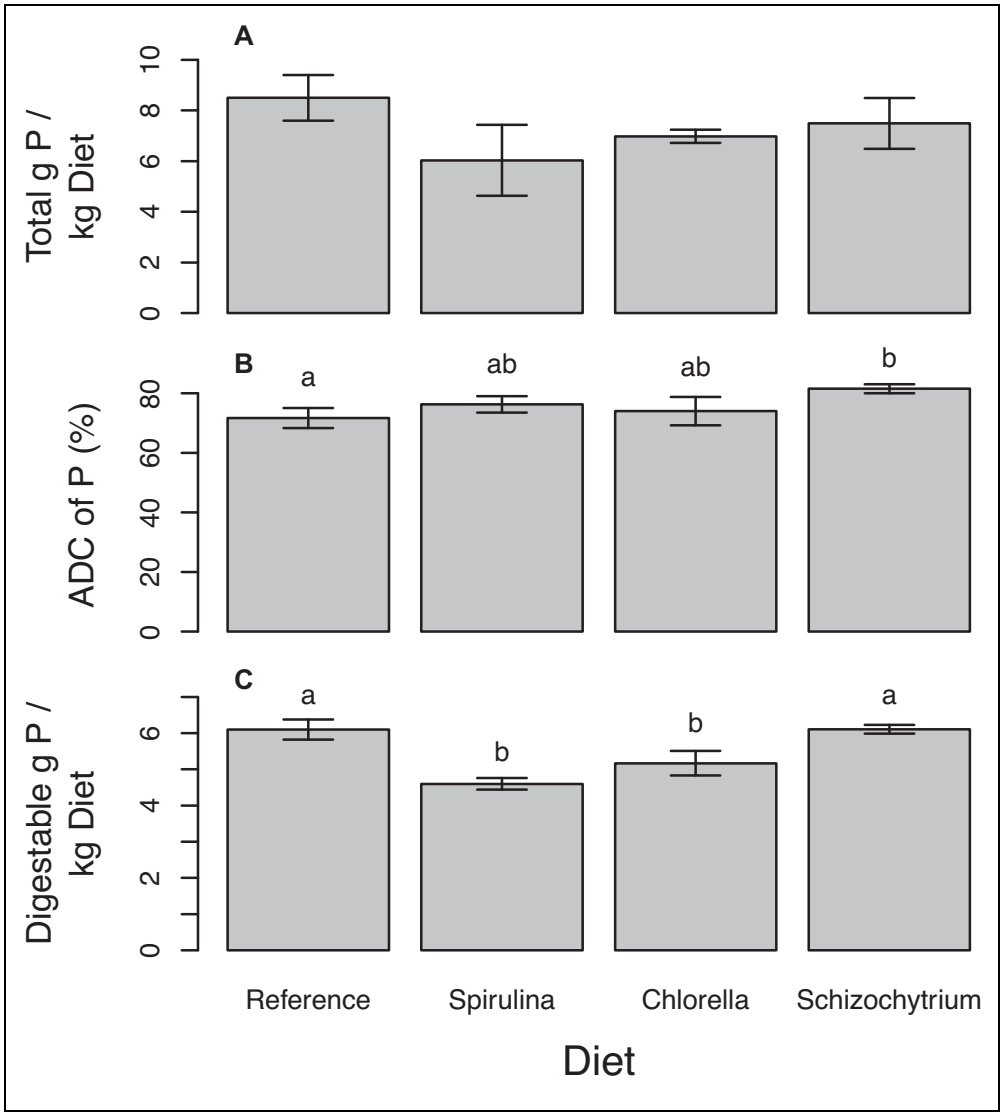

Figure 1. Quantity and digestibility of phosphorus in the control (Reference) and experimental diets (*Spirulina*, *Chlorella*, and *Schizochytrium*). (A) Total amounts of phosphorus in the four diets tested ($n = 2$ replicates for the Chlorella diet; $n = 3$ for all other diets). (B) The apparent digestibility coefficient of phosphorus in the four diets tested ($n = 3$ replicates for each diet). (C) The amount of digestible phosphorus per kg feed in the four diets tested ($n = 3$ replicates for each diet). Error bars indicate $\pm$ 2 *SE*. DOI: https://doi.org/10.1525/elementa.2020.00170.f1

(Ricklefs and Miller, 1999; Soto et al., 2008; Bureau and Hua, 2010; Sarker et al., 2011; Sarker et al., 2014). Our results show that substituting *Schizochytrium* into a traditional diet based on fish meal and fish oil can improve phosphorus digestibility and thus reduce phosphorus waste and discharge in Nile Tilapia aquaculture. These results, combined with those from other studies on the nutritional characteristics and digestibility of *Schizochytrium* spp. in general, suggest a path forward in the development of more sustainable aquaculture.

Among the three microalgae ingredients tested, *Schizochytrium* spp. had the highest ADC of phosphorus. This was supported by both the in vivo and in vitro experiments. The *Schizochytrium* diet also had the highest ADC of phosphorus among the four diets tested and contained an amount of digestible phosphorus comparable to the reference diet. The phosphorus budget supports these results—the *Schizochytrium* diet had the lowest P retention, the highest dissolved phosphorus discharge, and the lowest solid phosphorus discharge. A low phosphorus retention coupled with a high dissolved phosphorus discharge indicates that the fish are receiving a surplus of digestible phosphorus from their feed, while the low solid phosphorus discharge suggests that most of the total phosphorus contained in the *Schizochytrium* diet can be digested. This suggests that the fish fed the *Schizochytrium* diet were able to digest more phosphorus than they needed, and subsequently excreted the superfluous digested phosphorus through their gills or kidneys, leading to high soluble phosphorus. The excretion of soluble phosphorus for each diet should be studied further in order to formulate feeds that contain just enough phosphorus for Nile tilapia, since excreted soluble phosphorus is readily available to other organisms in aquatic ecosystems and thus contributes directly to eutrophication.

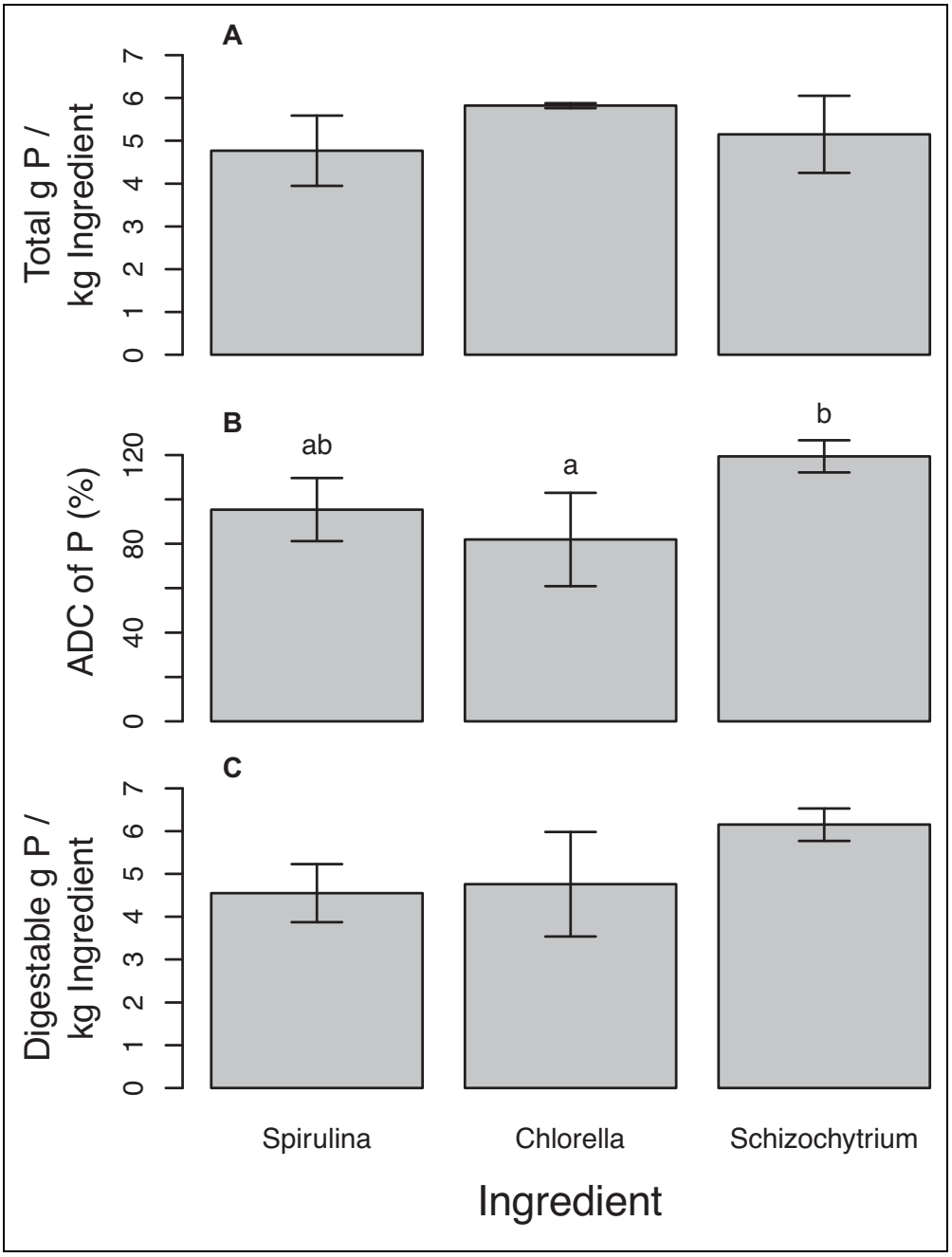

Figure 2. Quantity and digestibility of phosphorus in three experimental algal ingredients (*Spirulina*, *Chlorella*, and *Schizochytrium*). (A) Total amounts of phosphorus in each experimental algal ingredient ($n = 3$ replicates per ingredient). (B) Apparent digestibility coefficient of phosphorus for each algal ingredient ($n = 3$ replicates per ingredient). (C) Digestible phosphorus per kg in each algal ingredient ($n = 3$ replicates per ingredient). Error bars indicate $\pm$ 2 *SE*. DOI: https://doi.org/10.1525/elementa.2020.00170.f1

The high phosphorus retention and negative dissolved phosphorus discharge associated with the *Spirulina*-supplemented diet indicates that the diet is phosphorus-deficient, despite the fact that the total phosphorus content of the spirulina diet did not differ from that of the *Schizochytrium* diet. Tilapia fed the *Spirulina* diet were retaining as much digestible phosphorus as possible, and even absorbing dissolved phosphorus from their surroundings to make up for this deficit (Sugiura et al., 2003; Sarker et al., 2014). Our results show this is likely due to the low digestibility of phosphorus in the *Spirulina* diet. Although this diet resulted in the lowest phosphorus loading into the tanks, the low digestibility of phosphorus suggests that a *Spirulina* diet would not be healthy for Tilapia.

The lower digestibility of the *Chlorella* diet and ingredient is consistent with qualitative observations noted throughout the feeding trial. Tilapia fed the *Chlorella* diet often spat out the feed pellets after eating them, suggesting low palatability. There was also a qualitatively higher amount of fecal matter from tanks fed the *Chlorella* diet and the *Spirulina* diet compared to the other two diets.

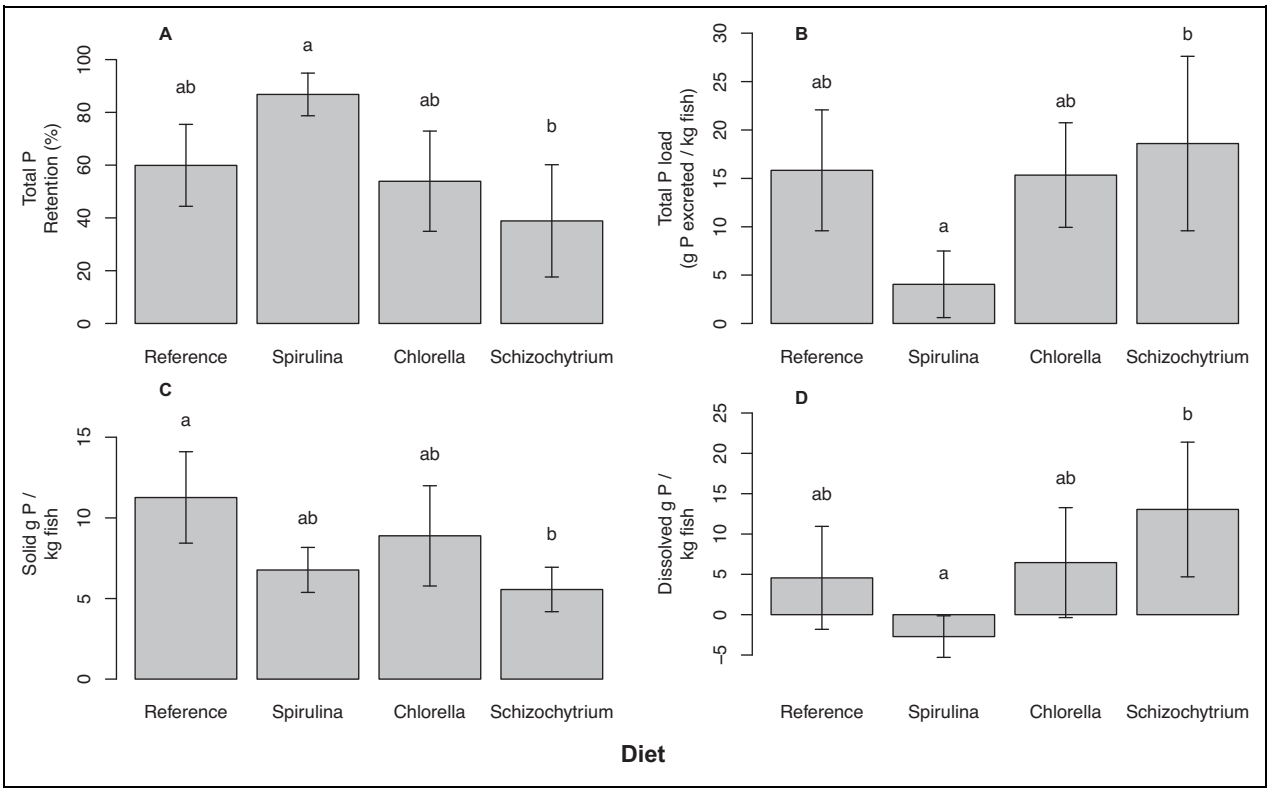

Figure 3. Phosphorus budget for control (reference) and experimental diets (*Spirulina*, *Chlorella*, and *Schizochytrium*). (A) Retained phosphorus (%) in each diet ($n = 3$ replicates per diet). (B) Total P load in each diet ($n = 3$ replicates per diet). (C) Solid phosphorus discharge for each diet ($n = 3$ replicates per diet). (D) Dissolved phosphorus discharge for each diet ($n = 3$ replicates per diet). Error bars indicate $\pm$ 2 *SE*. DOI: https://doi.org/10.1525/elementa.2020.00170.f1

This could be due to differences in the cell structure of these organisms, antinutritional factors, or the biochemical forms of the phosphorus in each microalga (Sarker et al., 2016a). Microalgae-based diets are currently in the infant stage of development for rainbow trout. The goal of this article is not related to cell disruption or processing of microalgae to improve the quality of ingredients for aquafeeds. However, further processing of the *Chlorella* diet and the *Spirulina* ingredients into concentrates, disruption of cell walls, exogenous enzymes, and extrusion pelleting could be useful to enhance nutrient digestibility of certain microalgal species.

The apparent overestimation of the in vivo ADC of phosphorus in the *Schizochytrium* spp. ingredient, 119.3% (**Figure 2**, Table S6), could be due to several factors. Phosphorus contamination of the lab equipment is possible, although all precautions were taken against it. More likely, this overestimation is a mathematical artifact, since the equation used to calculate the ADC of phosphorus in the test ingredients includes outputs from the equation for the ADC of phosphorus in the diets as inputs. More derived equations will magnify any small measurement errors present in the calculated values it uses as inputs and thus may result in high values such as this one. Calculated nutrient ADCs above 100 can occur in experiments, and feed manufacturers should round to 100 when they pursue diet formulation on a digestible nutrient basis (Glencross et al., 2007).

The documented digestibility of phosphorus in reference diets is between 17% and 81% in rainbow trout (Hua et al., 2005) and approximately 60% in tilapia (Schneider et al., 2004). The value reported for tilapia is slightly lower than the ADC of phosphorus in the reference diet used in this experiment. This may be due to the extensive sieving and processing of the plant meals that occurred during the formulation of our experimental diets, which increases the digestibility of the whole diet by reducing fiber content. A study examining the effects of replacing fish meal with various plant protein sources (wheat gluten, soybean extract, soybean meal, duckweed, and single-cell protein [yeast and bacteria mixture]) found that the ADCs of phosphorus in plant-based tilapia diets ranged from 62% to 67% (Schneider et al., 2004). These ADCs of phosphorus are lower than all three microalgae-supplemented diets tested in this experiment, indicating that microalgae may offer more bioavailable forms of phosphorus for tilapia. The same study also reported that solid phosphorus discharge was highest for the fish meal–based diet and that the fish meal diet resulted in one of the highest phosphorus retention percentages (Schneider et al., 2004), which is consistent with our results.

Our experiments together with previous studies suggest that *Schizochytrium sp.* is the most likely microalgae that could be used to replace fish meal ingredients and may even be nutritionally superior for Nile tilapia. We recently evaluated the digestibility of other nutrients from

Schizochytrium sp. in Nile tilapia and found high digestibility of lipid and all unsaturated fatty acid fractions (Sarker et al., 2016a). We then conducted a growth experiment with *Schizochytrium sp.* and found significantly improved weight gain, feed conversion ratio, and protein efficiency ratio when *Schizochytrium sp.* fully replaced dietary fish oil compared to control feed containing fish oil—the first report of complete replacement of fish oil with whole cells of *Schizochytrium sp.* in a tilapia diet (Sarker et al., 2016b).

Currently, farmed fish including tilapia are fed diets derived from overharvested wild-caught forage fish and crops like corn and soy, which result in chemical and nutrient pollution that impairs waterways. Additionally, plant-based feeds have nutritional deficiencies such as unbalanced essential amino acids and lack the most health-promoting omega-3 fatty acids DHA and EPA. Terrestrial plant ingredients also contain high levels of anti-nutritional factors that limit nutrient uptake for omnivorous species like tilapia (Shiau et al., 1990; Thompson et al., 2012; Sarker et al., 2013; Sarker et al., 2018). These ingredients are the major constituent of commercial feeds that contain adequate amounts of phosphorus for tilapia nutrition, but much of this phosphorus is not digestible. Because phosphorus is a limiting nutrient in freshwater (and some marine) ecosystems, the excretion of this undigested phosphorus into aquatic environments can result in eutrophication and ultimately anoxic conditions (Ricklefs and Miller, 1999). Manipulation of feed composition is one way to manage the composition of aquaculture waste (Bureau and Hua, 2010; Sarker et al., 2014). This, along with the fact that fish meal and fish oil are increasingly more costly and less sustainable protein source ingredients, puts a high priority on research regarding substitutes for fish meal and terrestrial plant proteins.

Marine and freshwater microalgae offer a potentially sustainable substitute for fish meal and terrestrial plant proteins in aquaculture feeds. Microalgae store phosphorus as polyphosphate granules/phospholipid form rather than phytic acid (Mukherjee et al., 2015; Sarker et al., 2016a) and thus may contain more digestible forms of phosphorus. We previously reported the improved digestibility of lipids and all unsaturated fatty acid fractions from *Schizochytrium* in tilapia (Sarker et al., 2016a), and the current results also show the highest digestibility of phosphorus from its phospholipid sources.

Although multiple studies have examined tilapia growth when fed microalgae-supplemented diets (Olvera-Novoa et al., 1998; Lu et al., 2004; Schneider et al., 2004; Badwy et al., 2008; Hussein et al., 2013; Stadtlander et al., 2013; Sarker et al., 2016b; Sarker et al., 2018), to our knowledge, this is the first study to investigate total phosphorus content, phosphorus digestibility, retention, and loading in microalgae-supplemented diets for tilapia. Our phosphorus digestibility results suggest that *Schizochytrium* sp is a good candidate for developing tilapia feeds that minimize phosphorus loading. Partial or complete substitution of conventional ingredients with certain microalgae species could result in a feed with greater phosphorus digestibility, reducing the phosphorus load in the aquatic environment into which the aquaculture waste is discharged.

One current challenge is the cost to produce microalgae for aquaculture feed. To this end, Naylor et al. (2009) have suggested that the biofuels industry could spearhead research and development surrounding microalgae culture in order to lower feed costs. We have also shown that aquaculture feeds can successfully incorporate microalgal co-products from omega-3 fatty acid extraction used in producing human nutritional supplements (Sarker et al., 2018; Sarker et al., 2020b). Economic analyses should take a full cost accounting approach that would include the relative digestibility (and thus the efficiency of resource use) of essential nutrients, such as phosphorus, in protein and oil ingredients such as fish meal, terrestrial plant proteins, and microalgae. Furthermore, economic analyses should consider the cost of eutrophication and pollution that arises from the entire life cycle of each ingredient, from production to excretion.

Aquatic microalgae present a promising alternative protein and oil source for fish feed. Currently, the environmental sustainability of algae culture is limited by high production costs related to fossil fuel use for energy and mined or manufactured nutrients for growth media (Wijffels and Barbosa, 2010). Microalgae production has the potential to be more sustainable than the production of fish meal and fish oil made from industrial reduction of wild forage fisheries, especially if it can be produced using renewable energy and fish effluent or an optimal mixture of fish effluent and inorganic nutrients as a growth medium.

Conclusions

Aquaculture has definitive environmental advantages over the production of terrestrial animal protein sources for human consumption, but biological, environmental, and economical constraints on feed production pose significant obstacles to continued development of aquaculture as a major producer of fish for human consumption (Duarte et al., 2009; Hall et al., 2011; Pikitch, 2015; Froehlich et al., 2018). Fish meal and fish oil for aquafeeds are currently obtained from overharvested wild forage fish populations. Terrestrial plant protein sources are also grown using environmentally unsustainable methods, including high use of fossil fuels, inefficient use of manufactured fertilizers, and water-intensive methods (Foley et al., 2011). Additionally, fish meal and plant meals contain phosphorus in forms that are largely indigestible to fish. This results in high phosphorus loading, which can cause eutrophication in the aquatic environments into which aquaculture waste is discharged. Because feeds are the biggest contributor to the cost of aquaculture, it is essential to formulate feeds that use minerals and nutrients efficiently. Thus, finding feed ingredients that can be produced sustainably and contain highly bioavailable phosphorus is essential to the sustainability of industrial aquaculture.

In general, marine microalgae are considered among the most promising sustainable replacements or supplements for fish oil in aquaculture feeds. Mainly DHA-rich

Schizochytrium sp. have been examined to replace fish oil in aquafeeds for shrimp and other finfish including tilapia (Sarker et al., 2016b; Wang et al., 2017; Bélanger-Lamonde et al., 2018; Sarker et al., 2020a; Sarker et al., 2020b). In our earlier study, we reported the full replacement of fish oil using Schizochytrium sp. in juvenile Nile tilapia (Sarker et al., 2016b). Our current study shows that *Schizochytrium* sp. is a highly digestible source of phosphorus and the data will guide for fine-tuning feed formulations for sustainable tilapia aquaculture. However, data on life cycle environmental impacts (energy use, greenhouse gas emissions, etc.) and the economic viability of growing *Schizochytrium* for aquaculture diet are very limited. Thus, environmental impacts and economic analysis of this marine microalga in aquaculture feed should be fully studied. Toward this end, we are now focusing the research on life cycle environmental analysis and technoeconomic analysis of marine microalgae-based feed for aquaculture.

Data accessibility statement

All raw data have been included in the supplementary materials.

Supplementary files

The supplemental files for this article can be found as follows:

Text S1.Docx
Phosphorus Data and Calculations.xlsx
InVitro Results.xlsx
Fish Biomass by tank.xlsx

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Competing interests

No competing interests. Our coauthor Dr Kapuscinski is an editor in chief at *Elementa* Sustainability knowledge domain. However, Dr Kapuscinski was not involved at all in handling or reviewing this article.

Author contributions

Conceived and designed the experiments: PKS, ARK, MMG.

Performed the experiments: MMG, PKS, ARK, SK, DSF, BS, AVBDS, TT.

Analyzed the data: MMG.

Contributed reagents/materials/analysis tools: MMG, DSF.

Wrote—original draft: MMG.

Wrote—review and editing: PKS, MMG, ARK, SK, DSF.

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