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Nutritional Composition of Duckweed (*Lemna minor*) Cultured with Inorganic Fertilizer and Organic Manure in Earthen Ponds

Mary OPIYO[1*], Kevin MBOGO[2], Kevin OBIERO[3], Paul ORINA[4], Patricia MUENDO[5]

[1] Kenya Marine and Fisheries Research Institute, National Aquaculture Research Development and Training Centre, Sagana, Kenya.

[2] Department of Biochemistry, Jomo Kenyatta University of Agriculture and Technology,Nairobi, Kenya

[3] Kenya Marine and Fisheries Research Institute, Sangoro Aquaculture Station, Pap-Onditi, Kenya

[4] Kenya Marine and Fisheries Research Institute, Kegati Aquaculture Center, Kisii, Kenya

[5] Department of Biological Sciences, Machakos University, Machakos, Kenya.

ABSTRACT

Duckweed (*Lemna minor*) have been used for several years to recover nutrients from wastewater and as feed ingredient for livestock, including fish and poultry. This study aimed at assessing the effect of different manure sources; inorganic fertilizer (a combination of DAP and Urea), and organic sources (chicken manure and cow dung manure) on the proximate composition, amino acid and fatty acid profile of *L. minor* under culture conditions. Results indicated that *L. minor* cultured with chicken manure had significantly higher crude protein level (36.8%) ($P < 0.05$) and lower crude fat content (8.10%) compared to the ones cultured with inorganic fertilizer and cow dung manure. Essential amino acids proportion was 50% in *L. minor* cultured with inorganic fertilizer, 45.4% in chicken manure and 44.8% in cow dung manure with lysine and phenylalanine being the most abundant amino acids. The total polyunsaturated fatty acids (PUFA) were significantly higher ($P < 0.05$) in *L. minor* cultured using inorganic fertilizer (43.05 mg/100 g) with linoleic acid being the most dominant PUFA. The presence of high levels of amino acids and PUFA in the *L. minor* cultured with organic and inorganic fertilizer respectively indicates that it can provide quality protein and PUFA required for fish growth and well-being.

Keywords: Organic manure, inorganic fertilizer, nutrients, *Lemna minor*, composition

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*** CORRESPONDING AUTHOR**

marybede@gmail.com
Phone: +254 721 782 665

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Introduction

Duckweed (*Lemna minor*) is a free-floating freshwater macrophyte belonging to the family *Lemnacea* and is found in freshwater ponds, lagoons, ditches and streams in both tropical and subtropical climates (Culley et al. 1981; Hassan and Edwards 1992; Young et al. 2006). In most cases, duckweed has been used for wastewater treatment, as food for humans, fish and terrestrial animals (Culley et al. 1981; Chakrabarti et al. 2018; Nesan et al. 2020). Previous studies have shown that *L. minor* is a source of protein, minerals and vitamins important for cultured fish (Yılmaz et al. 2004). The application of this aquatic macrophyte in fish nutrition requires continuous and sustainable production which is not achievable through collection from natural water bodies and wastewaters. Furthermore, macrophytes collected from natural/wastewater systems may contain contaminants that may affect fish or animal production and/or the quality of these products for human consumption.

Duckweed is readily consumed by Nile tilapia (*Oreochromis niloticus*), Common carp (*Cyprinus carpio*) and other herbivorous fish

(Hassan and Edwards 1992; Yılmaz et al. 2004). It is reported that it contains up to 45% crude protein (CP) of the plant's dry weight and can be easily cultured in nutrient-rich waters in tropical and subtropical countries (Culley et al. 1981; Hassan and Edwards 1992; Hasan and Chakrabarti 2009; Chakrabarti et al. 2018). However, conditions for the growth of duckweed are not generally possible in semi-intensive and intensive aquaculture systems which are used for fish, hence, it is important to culture them separately and incorporate them in fish feeds for fish to get the benefits of their nutrients (Bag et al. 2011).

The nutritional composition of cultured aquatic plants depends on the culture medium and can differ from the natural water bodies to the controlled environments with known fertilization rates. Inorganic and organic manures have been used in the production of duckweed in Bangladesh (Chakrabarti et al. 2018; Sharma et al. 2019). However, their nutritional composition normally varies depending on the type of manure used and the nitrogen content of the water used which arises from fertilization (Chakrabarti et al. 2018). The present study analyzed the proximate, amino acid and fatty acid composition of *L. minor* cultured with locally available organic and inorganic fertilizers, to evaluate their suitability for utilization in fish feeds.

Materials and Methods

Culture Unit Preparation

The study was carried out in earthen ponds at Kenya Marine and Fisheries Research Institute (KMFRI) Sangoro Aquaculture Station, Kisumu County, Kenya. Nine ponds measuring 169 m^2 each were drained off, dried and limed using agricultural lime at 100 g/m^2 to minimize unwanted macrophytes growth and nutrient load management. The ponds were filled with water to a depth of 40 cm (0.4 m) and were assigned fertilizer treatments randomly with T1 (inorganic fertilizer), T2 (chicken manure), and T3 (Cow dung manure) according to the procedures documented by Chakrabarti et al. (2018). Initial fertilization with organic manure was done at 1052 g/m^2 using chicken manure and cow dung, respectively. After every 10 days, fertilization was done at ¼ dose of the initial treatment. The manures were mixed with water and allowed to decompose for 3 days before applying to the culture units. Fertilization by a combination of DAP and urea was done at the rate of 1 g/m^2 for DAP and 1.5 g/m^2 for urea, respectively. After every 10 days, fertilization was repeated at a ¼ dose of the initial treatment. The nutritional content of the fertilizers used is summarized in Table 1. Nitrogen (N) and phosphorus (P) analyses of inorganic and organic fertilizers used in *L. minor* culture were performed according to standard methods. Available N was determined by extracting 5 g (dry weight equivalent) of each fertilizer with 25 mL of 2 M KCl (Mulvaney 1996). Available P was determined by extracting 2.5 g (dry weight equivalent) of each fertilizer with 25 mL of Mehlich-3 extracting solution (Frank et al. 1998).

Table 1. Nutritional composition of inorganic and organic fertilizers used in *L. minor* culture

Fertilizer	Nitrogen (%)	Phosphorus (%)
Diammonium phosphate (DAP)	18	46
Urea	46	-
Cow dung	2.03	1.54
Chicken manure	2.57	1.58

L. minor inoculation and culture

L. minor was collected from the Ahero Irrigation Scheme, Kisumu, County, Kenya and transported to KMFRI, Sangoro aquaculture station. The macrophytes were introduced to the culture system at a rate of 5 g (wet weight) per m^2 for the 3 treatments; T1 (inorganic fertilizer (DAP + Urea)), T2 (chicken manure), and T3 (cow dung manure) in triplicates (Chakrabarti et al. 2018). The duckweed was harvested after 60 days and washed with clean water to remove soil particles and any other impurities before drying under a shed.

Nutritional composition of *L. minor*

Biochemical (proximate) compositions of *L. minor* cultured in ponds using different fertilizers were determined by standard methods (AOAC 1990). Crude protein (CP) was analyzed by the copper catalyst Kjeldahl method. Analysis was done by taking 1g of each sample and 2 tablets of catalyst (Kjeldahl tablets) which were digested in 15 ml concentrated sulfuric acid (H_2SO_4) at 420 °C. The samples were cooled and automatically distilled in Kjeldahl equipment with 40% NaOH and ammonia gas trapped in 4% boric acid and reverse titrated using 0.2N HCl. The nitrogen content was determined and converted to crude protein content using a nitrogen factor for the crude protein calculation of 6.25. Ash was determined by expressing the weight of 2 g of the ground sample burnt at 600°C for 3 hours in a muffle furnace as a percentage of the un-burnt sample weight. Crude fat (CF) was extracted by heating 3 g of the sample in diethyl ether under reflux at 105 °C for 30 minutes in a VELP Solvent Extraction unit. The ether extract was calculated as the difference between the original sample and the ether extract residue. Crude fibre (CF) was determined gravimetrically by chemical digestion and solubilization, and quantified by: CF (%) = dried sample (g) – ashed sample (g)/initial sample weight × 100). Carbohydrate content was

determined by subtraction of protein, lipid and ash values.

Amino acid analysis

Amino acids were analysed from the *L. minor* using standard methods (Otter 2012). Analysis was done on 1 g of pooled finely ground *L. minor* from each treatment using ion exchange liquid chromatography via continuous flow chromatography. The compounds were identified and quantified using an authentic sample mixture (amino acid standard solution (AAS 18) from Sigma-Aldrich (Chemie GmbH, Munich, Germany).

Extraction of lipids and fatty acid analysis

Lipid extraction was performed according to the procedure of Bligh and Dyer (1959). Total lipid was extracted from 0.5 g samples of pooled finely ground *L. minor* by homogenization in 10 ml chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene (BHT) as an antioxidant and 2 ml cold isotonic saline, 0.9% Sodium chloride (NaCl). Homogenates were mixed vigorously and allowed to stand for 20 minutes before being centrifuged at 3000 rpm for 10 minutes and the upper aqueous layer aspirated before the lower organic/chloroform layer was transferred to a 100 ml reflux flask and evaporated to dryness under a vacuum. Fatty acid methyl esters (FAME) were prepared from the extracted total lipid and fatty acid standards by acid-catalyzed transmethylation. Briefly, 5 ml of 1% H_2SO_4 (v/v) in methanol was mixed with 1 ml of extracted total lipid in a 50 ml reflux flask and refluxed at 70°C for 3 hours. FAME was extracted into 750 ml of distilled water and 10 ml of hexane and then dehydrated using anhydrous Sodium sulphate (Na_2SO_4). The extracted FAME was concentrated at 0.5 ml in a vacuum evaporator and transferred into gas chromatography (GC) vials before GC analysis.

Gas chromatography analysis

FAME was separated and quantified by gas chromatography (GC) (Shimadzu Model GC14B, Japan) fitted with on-column injection and equipped with a fused silica capillary column (SUPELCO Column Omega wax^{tm} 530, 30 m x 0.5 mm x 0.5 µm) with nitrogen as a carrier gas. Temperature was increased from 170 °C to 220 °C at 1.8 °C min^{-1}, then 220 °C for 47 minutes and a total run time of 75 minutes. Injection and detection temperatures were 240 °C and 260 °C, respectively. All GC analyses were performed under the same conditions. Individual methyl esters were identified by comparing the retention times of FAME with known FAME standard (Supelco 37 Component FAME Mix standard (Sigma-Aldrich, Munich, Germany) obtained from Kobian© Kenya Chemicals. Fame analysis and identification were done according to Indarti et al. (2005).

Statistical analysis

All data were presented as mean ± SE. One-way analysis of variance (ANOVA) was used for mean comparisons and Tukey post hoc analysis for multiple comparisons of means (Bhujel, 2011). The significance level was set at $P < 0.05$. All statistical analyses were carried out using Statistical Package and Service Solutions (SPSS version 23).

Results

Proximate composition of *L. minor*

The proximate composition analysis indicated there were differences in the composition of *L. minor* cultured with inorganic and organic fertilizers. *L.minor* cultured with chicken manure had significantly higher crude protein levels (36.8 ± 0.05%), and lower fat content (8.10 ± 0.05%) compared to the ones cultured with inorganic manure which had (31.4 ± 0.02%) and (8.7 ± 0.53%) for protein and fat respectively ($P < 0.05$) (Table 2). Among the organic manure used, cow dung manure produced *L. minor* with a relatively higher percentage of ash (13.67%) ($P < 0.05$). Carbohydrates were also significantly higher in *L. minor* cultured with inorganic fertilizer ($P < 0.05$).

Table 2. Proximate composition of *Lemna minor* cultured with inorganic and organic fertilizer

Parameter (% dry matter)	Inorganic fertilizer (DAP+Urea)	Chicken manure	Cow dung manure
Crude protein	31.4 ± 0.02^a	36.8 ± 0.05^b	35.34 ± 0.03^c
Crude fat	8.7 ± 0.30^a	8.1 ± 0.05^b	8.36 ± 0.01^c
Crude fibre	5.3 ± 0.04^a	4.6 ± 0.02^b	5.35 ± 0.01^a
Ash	11.6 ± 0.24^a	11.8 ± 0.01^a	13.67 ± 0.01^b
Carbohydrates	43.1 ± 0.6^a	38.5 ± 0.13^b	37.25 ± 0.06^b

*Values are reported as mean ± SE. Values followed by different superscript letters across one row are significantly different (one-way ANOVA, Tukey post hoc test, P<0.05)

Amino acid profile of *L. minor*

The amino acid profile of *L. minor* cultured with different fertilizers is shown in Table 3. The essential amino acids and non-essential amino acids were present in *L. minor* cultured with both inorganic and organic manure. Essential amino acids were present in the following proportions: inorganic fertilizer (50%), chicken manure (45.4%) and cow dung manure (44.8%). The proportion of essential amino acids was significantly higher in *L. minor* cultured with inorganic fertilizer ($P < 0.05$). Most of the essential amino acids apart from methionine, isoleucine, threonine and tryptophan were significantly higher in *L. minor* cultured with chicken manure and cow dung manure but were significantly lower in *L. minor* cultured with inorganic fertilizer ($P < 0.05$). Lysine levels were not significantly different

in the *L. minor* cultured with inorganic fertilizer and cow dung manure (P > 0.05). The proportion of non-essential amino acids was significantly lower in *L. minor* cultured with inorganic fertilizer (50%) compared to chicken and cow dung manure.

Table 3. Amino acid profile (µg/100 mg) of *Lemna minor* cultured with inorganic and organic fertilizer
*Values are reported as means. Values followed by different superscript letters across one row are significantly different

Amino acid (µg/100 mg)	Inorganic fertilizer (DAP +Urea)	Chicken manure	Cow dung manure
Essential			
Lysine	1.6[a]	2.08[b]	1.8[a]
Histidine	0.3[a]	0.39[b]	0.35[c]
Leucine	3.3[a]	0.39[b]	0.35[c]
Valine	2.1[a]	2.3[b]	2.3[b]
Methionine	0.1[a]	0.13[a]	0.11[a]
Isoleucine	2.2[a]	2.26[a]	2.2[a]
Phenylalanine	2.5[a]	3.25[b]	3.2[b]
Threonine	0.1[a]	0.13[a]	0.12[a]
Tryptophan	0.1[a]	0.13[a]	0.12[a]
Non-essential			
Glycine	0.2[a]	0.26[a]	0.26[a]
Alanine	1.2[a]	1.56[b]	1.46[c]
Glutamic acid	5.2[a]	5.26[a]	5.16[a]
Proline	1.2[a]	1.56[b]	1.5[b]
Tyrosine	1.3[a]	1.39[b]	1.35[b]
Cysteine	0.1[a]	0.13[a]	0.13[a]
Arginine	3.1[a]	3.13[a]	3.13[a]
***Essential* (%)**	**50.0[a]**	**45.4[b]**	**44.8[b]**
***Non-Essential* (%)**	**50.0[a]**	**54.6[b]**	**55.2[b]**

(one-way ANOVA, Tukey post hoc test, $P < 0.05$)

Fatty acid profile of *L. minor*

In the current study, 21 fatty acids were identified in the dried *L. minor* 14 of them were saturated fatty acids. Palmitic acid (C16.0) was the dominant saturated fatty acid. The total saturated fatty acids (∑SFAs) were significantly higher (P < 0.05) in the *L. minor* cultured with chicken manure (35.90 mg/100 g). *L. minor* cultured with inorganic fertilizer had significantly higher palmitic acid values compared to the *L. minor* cultured with chicken manure and cow dung manure (P < 0.05). Among the monounsaturated fatty acids (MUFAs), Oleic acid (C18.1) was significantly higher in the *L. minor* cultured in inorganic fertilizer (19.33 mg/100 g). In addition, Oleic acid, (C18:1) was the dominantnounsaturated fatty acid across all treatments. Two polyunsaturated fatty acids were detected namely linoleic acid (C18:2) and linolenic acid (C18:3). Linoleic acid (C18:2) was significantly higher (P < 0.05) in the *L. minor* cultured with inorganic fertilizer (41.12 mg/100 g) and was the dominant polyunsaturated fatty acid across the treatments. Eicosapentaenoic acid, (C20:5) was the only highly polyunsaturated fatty acid (HUFA) detected in the cultured *L. minor* and was significantly lower (P < 0.05) in *L. minor* cultured in cow dung manure (Table 4). The total polyunsaturated fatty acids were significantly higher (P < 0.05) in *L. minor* cultured with inorganic manure (43.05 mg/100 g).

Table 4. Composition of fatty acids (mg/100 g) of *Lemna minor* cultured with inorganic and organic fertilizers

Fatty acid (mg/100 g)	Chemical structure	Inorganic fertilizer (DAP +Urea)	Chicken manure	Cow dung manure
Butanoic acid	C4:0	1.03 ± 0.02[a]	1.43 ± 0.01[b]	1.43± 0.02[b]
Caprylic acid	C8:0	1.77 ± 0.01[b]	3.01 ± 0.02[b]	2.96 ± 0.02[b]
Capric acid	C10:0	0.93 ± 0.11[b]	1.90 ± 0.3[b]	1.77 ± 0.31[b]
Undecanoic acid	C11:0	0.07 ± 0.02[a]	0.13 ± 0.02[b]	0.13 ± 0.02[b]
Lauric acid	C12:0	0.74 ± 0.19[a]	0.93 ± 0.24[b]	0.93 ± 0.24[b]

Table 4. Continues

Myristic acid	C14:0	1.03 ± 0.45[a]	1.98 ± 0.47[b]	1.82 ± 0.50[c]
Pentadecanoic acid	C15:0	1.74 ± 0.09[c]	2.26 ± 0.23[b]	2.25 ± 0.23[b]
Palmitic acid	C16:0	21.12 ±0.59[a]	15.03 ± 0.60[b]	14.83 ± 0.60[b]
Margaric acid	C17:0	1.26 ± 0.04[a]	2.25 ± 0.35[b]	1.19 ± 0.35[c]
Stearic acid	C18:0	0.55 ± 0.23[a]	2.85 ± 0.14[b]	3.48 ± 0.13[c]
Nonadecanoic acid	C19:0	0.52 ± 0.09[a]	0.73 ± 0.13[b]	0.67 ± 0.12[b]
Heneicosanoic acid	C21:0	0.25± 0.01[a]	1.68 ± 0.06[b]	1.69 ± 0.03[b]
Dodecenoic acid	C12:1	0.13 ± 0.13[a]	1.10 ± 0.13[a]	0.93 ± 0.11[b]
Myristoleic acid	C14:1	1.21 ± 0.06[a]	0.63 ± 0.04[b]	0.45 ± 0.05[c]
∑SFAs		**32.35 ± 0.01[a]**	**35.90 ± 0.02[b]**	**34.54 ± 0.01[c]**
Palmitoleic acid	C16:1	0.83 ± 0.56[a]	5.95 ± 2.01[b]	6.12 ± 1.60[c]
Palmitelaidic acid	Trans C16:1	2.31 ± 0.20[a]	1.06 ± 0.50[b]	1.31 ± 0.45[c]
Oleic acid	C18:1	19.33± 0.37[a]	15.43 ± 0.31[b]	14.97 ± 0.21[c]
Eicosenoic acid	C20:1	2.01± 1.15[c]	2.05 ± 0.39[b]	1.96 ± 0.29[a]
∑MUFAs		**24.48 ± 0.11[a]**	**24.50 ± 0.27[a]**	**24.36 ± 0.37[a]**
Linoleic acid	C18:2	41.12 ± 1.83[a]	37.13 ± 1.47[b]	38.42 ± 1.32[b]
Linolenic acid	C18:3	1.64 ± 0.93[a]	1.68 ± 0.32[a]	1.51 ± 0.22[b]
Eicosapentaenoic acid	C20.5	0.29 ± 0.14[a]	0.28 ± 0.01[a]	0.26 ± 0.02[b]
∑PUFAs		**43.05 ± 0.96[a]**	**39.08 ± 0.96[b]**	**40.19 ± 0.22[c]**

*∑SFA, total saturated fatty acids; ∑MUFA, total monounsaturated fatty acids; ∑PUFA, total polyunsaturated fatty acids. Values are reported as mean ± SE. Values followed by different superscript letters across one row are significantly different (one-way ANOVA, Tukey post hoc test, $P < 0.05$)

Discussion

The protein content of *L. minor* cultured in both organic and inorganic manures was higher than earlier reported values of 18% for *L. minor* collected from raceways, and other aquatic macrophytes like *Salvinia molesta* which had 17.3% crude protein (Yılmaz et al. 2004; Moozhiyil and Pallauf 2016). The protein content for *L. minor* cultured with chicken manure was similar to the results obtained by Chakrabarti et al. (2018) when organic manure containing chicken manure was used in the culture of *L. minor* in concrete ponds. The high crude protein level could be due to the culture environment which contained the required levels of nutrients as a result of fertilization, which is similar to the environment found in natural eutrophic waters where *L. minor* has traditionally been collected for fish feeds (Yılmaz et al. 2004). In addition, the high protein content of *L. minor* cultured with chicken manure could be related to the ability of the macrophyte to accumulate nitrogen for a longer period. The crude protein and crude fibre reported in this study are lower than the values reported for duckweed (*Lemna gibba*) grown in wastewater (Landesman et al. 2002). The carbohydrates were higher in *L. minor* cultured with inorganic fertilizer. Previous studies have reported higher carbohydrate levels (51.2%) in *L. minor* cultured in nutrient-enriched waters (Al-snafi 2019). This is higher than the results obtained from the present study in which 43.1% was recorded in *L. minor* cultured with inorganic manure. Similarly, organic manure (Chicken manure) resulted in lower carbohydrates in *L. minor* compared to inorganic fertilizer (Chakrabarti et al. 2018).

The amino acid levels in *L. minor* cultured with different manures were comparable to the ones collected from the wild and used for fish feeds (Yılmaz et al. 2004). The presence of all the essential amino acids was also reported by Landesman et al. (2002) in *L. gibba* cultured in wastewater even though the levels reported were higher than the levels in the present study. This is consistent with the reports that *L. minor* from natural water bodies and nutrient-enriched waters contains all the essential amino acids which make it suitable as a fish feed ingredient (Culley et al. 1981). Lysine and phenylalanine were the most abundant essential amino acid in this study contradicting the finding of Landesman et al. (2002) which had Leucine and valine as the abundant amino acids in *L. gibba*.

The low values of methionine in the present study are coherent with a previous study which had low

methionine of clones of duckweed in comparison with the requirement of 2.2 for fish feeds by FAO (Culley et al. 1981). *L. minor* has been previously reported to be having low levels of methionine, threonine, tryptophan and cysteine (Hammouda et al. 1995). Landesman et al. (2002) also reported the same deficiencies for *L. gibba* cultured in wastewater. *L. minor* cultured with chicken manure and cow dung manure were richer in the essential amino acid. Since fish requires the presence of 10 essential amino acids in their diets, *L. minor* cultured with organic manures can promote good growth, survival of fish and antibody production as they have high levels of essential and non-essential amino acids (Moyo et al. 2011). Additionally, previous studies have reported that the protein of duckweed is rich in certain amino acids that were often low in other plant proteins making it suitable for fish feed (Guha 1997).

The fatty acid profile of *L. minor* was significantly affected by the different manures. *L. minor* grown with inorganic fertilizers had higher levels of oleic acid, linoleic acid and a higher proportion of total polyunsaturated fatty acids (PUFA). The PUFAs were significantly higher compared to the saturated and monosaturated fatty acids across all the treatments. This is similar to previous studies which documented higher levels of polyunsaturated fatty acids in *L. minor* (Chakrabarti et al. 2018; Al-snafi 2019). Similar trends were reported for duckweed (*Spirodela polyrhiza*) cultured with inorganic manure (Sharma et al. 2019). Linoleic acid was the most predominant proportion of unsaturated fatty acids and has been reported to be essential for the growth and proper performance of fish (Mukherjee et al. 2010). Previous studies have documented that *L. minor* contains a larger proportion of polyunsaturated fatty acids mainly linolenic acid (41 to 47%) and linoleic acid (17–18%) (Chakrabarti et al. 2018). In this study, the saturated fatty acids were predominately palmitic and stearic acids in the *L. minor* cultured in chicken manure and cow dung manure. This agrees with the findings of Yan et al. (2013) which documented more than 70% PUFA and that palmitic acid and stearic acid were the predominant fatty acids in *L. minor*. The high level of PUFA indicates the enhanced nutritional value of the duckweeds cultured in inorganic manure.

The study indicates that duckweed cultured with chicken manure, contains high protein and can be suitable for use as fish feed. Large-scale production of duckweed using chicken manure should be encouraged for its sustainable use in aquaculture. The presence of high levels of essential amino acids and polyunsaturated fatty acids in the *L. minor* cultured with inorganic fertilizer indicates that it can provide the quality protein and polyunsaturated fatty acids required for fish growth and well-being. However, due to the high prices of inorganic fertilizers, organic manures are recommended for the production of *L. minor* for cost-effective fish-feed formulation.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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