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Evaluation of Nitrogen and Phosphorus Wastes Produced by Nile Tilapia (*Oreochromis niloticus* L.) Fed *Azolla*-Diets in Earthen Ponds*

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ABSTRACT

Nitrogen (N) and phosphorus (P) wastes produced by Nile tilapia *Oreochromis niloticus* L. fed *Azolla*, an aquatic atmospheric nitrogen fixing fern, was evaluated for 90 days in pond experiment. Six isonitrogenous (29.2% crude protein) and isoenergetic (16.9 $Kj \cdot g^{-1}$) diets A_0, A_{10}, A_{20}, A_{30}, A_{40} and A_{50}, containing 0%, 10%, 20%, 30%, 40% and 50% of *Azolla* meal (AM) respectively, as partial fishmeal (FM) substitutes, was provided to experimental fish. The *Azolla*-free diet A_0 served as a control. Fish specific growth rate (SGR) was higher with the control diet, the lower values being obtained in A_{50}-fed fish ($P < 0.05$). Crude protein and P content in experimental fish showed similar values. Evaluation of the nutrient wasted show identical values (84.8% - 87.8% of supplied) for total P (TP); while total N (TN) discharged into ponds by fish increased significantly when AM level greater than 30% in diets ($P < 0.05$), amounting 63.9% - 74.2% of that supplied. From these findings, the fern *Azolla* could be used in diet to sustain Nile tilapia growth and as "environmentally-friendly" ingredient to limit P loss, while providing N to the field, beneficially in tropical marshland pond where this nutrient is already limiting.

Keywords: *Azolla*; Aquaculture; Environment; Fish Meal Replacement; *Oreochromis niloticus*; Nitrogen; Phosphorus; Nutrient Balance

1. Introduction

One of the major problems currently facing aquaculture industry is the projected increase in production in order to meet the worldwide demand for fish. Beside from this challenge, aquaculture productions are unfortunately faced with the need to resolve another problem that constrains its sustainability. Indeed, the intensification of production leads to the release of organic wastes and inorganic nutrients, such as phosphorus (P) and nitrogen (N), which are known to enrich and promote eutrophication in aquatic ecosystems [1]. Thereby, environmental pollution associated with aquaculture becomes another critical issue for sustainability and future expansion of this activity [2]. In aquaculture, both P and N originate mainly from fish feeds [3] due to their high amounts in fishmeal (FM) that is rich in P [4,5]. Several studies have reported that the high P of fish meal-based diets is not well utilized by many fish species, such as common carp *Cyprinus carpio* L., gibel carp *Carassius auratus gibelio* or rainbow trout *Onchorhynchus mykiss* Walbaum [6-8]. As a consequence, P loading into the water by fish is generally high, and thus fishmeal-based-diets are considered as primary pollutants of aquatic ecosystems. In Benin, this situation causes generalized eutrophication that has compromised the use of many stagnant earthen ponds in small-scale farms. Therefore, one should appropriately balance this nutrient in feeds for farmed species. One of the numerous ways to reduce P waste produced by aquaculture is the use of FM substitutes that contain lower P with high availability [9]. Among the most popular alternative ingredients usable as FM substitutes, plants proteins appear to be suitable in practice to achieve this goal [10]. *Azolla*, a small floating freshwater fern that has been successfully used recently in fish farming [11-15] could be a good candidate. P content in that species is less than 0.77% dry matter [16], which is by far lower

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than the levels of 2% - 4% generally found in FM. This may encourage testing *Azolla* in low-polluting feeds. In particular, *Azolla* species develop a symbiotic relationship with a cyanobacteria named *Anabaena azolla* Strasburger, an atmospheric nitrogen fixing organism. Due to its presence in *Azolla* fronds cavity, *Anabaena azollae* make the fern naturally rich in nitrogen. This property is exploited in rice-field culture where *Azolla* is known as biofertilizer [17].

The main purpose of this study was to investigate P and N wastes discharged into water by Nile tilapia *O. niloticus* fed with diets containing gradual levels of AM in earthen ponds.

2. Material and Methods

2.1. Experimental Design and Set-Up

Eighteen small earthen ponds of 30 m^2 (10 m × 3 m × 1 m) filled naturally from the water table were newly constructed for a 90-days experiment at Louho village, in Porto-Novo suburb, Benin (West Africa).

Male Nile tilapia *O. niloticus* (initial mean weight = 16.3 ± 0.1 g) from a same cohort was stocked at a density of 2 m^{-2} (60 fish·pond^{-1}).

These ponds were randomly assigned to 6 triplicate (6 × 3) groups, each set attributed one of the experimental diets. Diets are isonitrogenous (29.2% crude protein) and isoenergetic (16.9 kJ·g^{-1}), formulated using locally available ingredients and the freshwater fern *Azolla filiculoides* Lamarck. Diets were formulated to contain 0% (A_0), 10% (A_{10}), 20% (A_{20}), 30% (A_{30}), 40% (A_{40}) and 50% (A_{50}) of *Azolla* meal (AM). The *Azolla*-diets were compared with a control (A_0) without AM.

Formulation and proximate composition of experimental diets are given in **Table 1**. They were prepared according to the procedure described in Abou *et al*. [14] and preserved in the refrigerator (+4˚C) until used for feeding fish.

Fish were hand-fed daily according to Melard [19]. Daily rations were divided into two parts, each distributed at 8:00 h and 16:00 h, respectively. They were adjusted every two weeks according to the fish biomass in each tank.

Once every fortnight, at least 40% of the fish in stock were sampled with a dip net, without entering the pond, and weighed. The daily ration was adjusted according to the actual body weight, and used for the next fortnight.

2.2. Analysis

The diets used were reduced in meal and preserved at –20˚C for biochemical analysis. 30 fishes were randomly sampled at the beginning from the initial batch, and at the end of the experiment 6 fish·pond^{-1}, and stored at –20˚C for analysis of whole-body composition. Whole fish were mashed, using a Robot coupe food processor. Diets and whole fish were analyzed for dry matter (drying samples in an oven at 105˚C, [20]) and crude protein (N × 6.25, Kjeldahl method). Body P concentrations in fish were determined by persulphate digestion [21] with boric acid and sodium hydroxide.

Table 1. Formulation and proximate composition of experimental diets.

Diets	A_0	A_{10}	A_{20}	A_{30}	A_{40}	A_{50}
Ingredients (g/100g diet)						
Fish meal	30	25	20	15	10	5
Azolla meal	0	10	20	30	40	50
Cottonseed meal	30	30	30	30	30	30
Maize bran	18	17	16	15	14	10
Brewery draff	20	16	12	8	4	3
Binder	1	1	1	1	1	1
Salt (NaCl)	1	1	1	1	1	1
Proximate composition[¥]						
Dry matter (%)	90.3	90.2	91.1	89.4	90.5	90.3
Crude protein (% DM)	29.3	29.3	29.2	29.1	29.2	29.0
Crude lipid (% DM)	10.8	10.0	9.4	9.0	8.4	8.0
Crude ash (% DM)	14.3	13.8	12.5	12.2	10.9	10.0
Crude fibre[§] (% DM)	9.9	10.0	10.2	10.4	10.5	10.8
NFE[†] (% DM)	35.7	36.8	38.7	39.4	41.0	42.2
Gross energy[‡] (kJ/g)	17.0	16.9	16.9	16.9	17.0	17.0
Phosphorus (% DM)	1.13	1.08	1.06	1.00	0.95	0.76

[¥]Means of two analyses values; [§]Calculated according to Ovograin Feeds Depot, Abomey-Calavi, Bénin and Leonard [16] for ingredients and *A. filiculoides*, respectively; [†]Nitrogen-Free Extract, calculated as: 100 – (%protein + %lipid + %ash + %crude fibre); [‡]According to [18]; DM: dry matter.

2.3. Calculations

Growth and feed performances parameters were calculated as follow:

$$\text{Final mean weight } \left(W_f\text{, in g}\right) = \text{FB }(\text{g})/\text{number of fish harvested}$$

$$\text{SGR}\left(\%\text{day}^{-1}\right) = 100 \times \left[\ln\left(W_f\right) - \ln\left(W_i\right)\right]\text{day}^{-1}$$

$$\text{Food conversion ratio }(\text{FCR}) = \text{TFI} \times (\text{FB} - \text{IB})^{-1}$$

$$\text{Apparent protein utilization }(\text{APU, }\%) = 100 \times \text{TPG} \times \text{DPI}^{-1}$$

where W_i and W_f are initial and final mean wet weight in g; TFI the total feed intake; FB and IB are final and initial fish biomass in g; DPI the total dietary protein intake;

TPG the total body protein gain.

Apparent retention rate (%) of P and N, and the amount of P and N retained and that is discharged into water as wastes (as faecal + urinary + gill) by fish were evaluated according to Cho *et al.* [22] as follow:

$$\text{Apparent retention rate } (\text{ARR, \%}) \text{ of P and N} = 100 \times (\text{TN}_f - \text{TN}_i)\,\text{N}_{\text{supplied}}^{-1}\,(\text{g}),$$

$$\text{Nutrient}_{\text{supplied}}\,(\text{NS, g}) = \text{N}_{\text{feed}} \times \text{TFI}$$

$$\text{Nutrient}_{\text{retained}}\,(\text{NR, g}) = \text{NS} \times \text{ARR} \times 100^{-1}$$

$$\text{Total nutrient waste}(\text{TN or TP, g}) = \text{NS} - \text{NR}$$

where: TN_f and TN_i are the final and initial nutrient (P or N) body content in (g); $N_{supplied}$ the quantity in (g) of nutrient (P or N) content in the total food supplied; N_{feed} is the quantity of feed supplied.

During the experiment, very limited feed loss was noticed. As this amount was unknown, we assume for calculations that the amount lost is 0.

2.4. Statistical Analysis

Means for growth and feed performances, body composition, and nutrient balance were analyzed using one-way ANOVA, after verifying the homogeneity of their variance [23]. Values for percentage data and ratios were log-transformed prior to analyses. When the effect was significant, comparisons between treatment means was run using Duncan's multiple range test [24] at P = 0.05.

3. Results

3.1. Growth Rate and Feed Utilization

As shown in **Table 2**, survival rate did not show any significant differences and values were higher in each treatments (P > 0.05). Fish specific growth rate decreased from fish fed the control diet to those fed A_{50} ($P < 0.05$), values ranging from 2.35 ± 0.04 %day^{-1} to 1.87 ± 0.06 %day^{-1}. Significant differences appear at AM levels higher than 20% in diets. FCR increased from 1.26 for A_0-fish to 1.68 for A_{50}-fish (P < 0.05). Apparent net protein utilization decreased significantly at AM levels higher than 30% in diets (P < 0.05).

3.2. Body Composition and Nutrient Retention

No significant differences were found in body crude protein (range: 12.1% - 12.9% fresh matter) among all the experimental diets (P > 0.05). Body P (0.73% - 0.85% dry matter) content in fish showed unchanged values in all experimental fish (P > 0.05).

Total P or N supplied to fish and nutrient balance at the end of the experiment are presented in **Table 3**. Total P or N supplied decreased as AM increased in the diets, from 86.1 ± 0.7 to 47.9 ± 1.2 for TP and from 357.4 ± 3.0 to 292.3 ± 7.0 for TN (P < 0.05). No differences were found in P apparent retention rate (P > 0.05) whereas a decrease in N retention rate was only significant in fish fed A_{40} and A_{50}. P waste discharged into the water showed significant decreasing values, from fish fed the control diets to those fed diets containing 50% AM (P < 0.05). Conversely, a similar quantity of N was wasted by all experimental fish (P > 0.05). The evaluation as percentage of the amount supplied show no difference for TP (84.8% - 87.8%), whereas TN increased from 63.9% to 74.2% when dietary AM increased from 0% to 50%, difference being significant when AM was more than 30% in diet (P < 0.05).

4. Discussion

In this study, all the diets used contain sufficient amounts of phosphorus to meet this need. Although a low level of methionine has been reported in *Azolla filiculoides* [25], the required aminoacids and minerals needed to balance the dietary deficiency at high content in AM could be

Table 2. Growth, feed performance and body composition (% fresh matter basis) of Nile tilapia fed in ponds with diets containing increasing level of *Azolla filiculoides* for 90 days. (A_0 to A_{50}: diet with 0% to 50% *Azolla*).

Diets	A_0	A_{10}	A_{20}	A_{30}	A_{40}	A_{50}	
Survival rate (%)	86.7 ± 6.7	87.8 ± 4.2	90.0 ± 2.9	88.3 ± 2.9	85.0 ± 4.4	89.4 ± 4.2	
SGR (% day^{-1})	2.35 ± 0.04^a	2.31 ± 0.06^a	2.27 ± 0.06^{ab}	2.19 ± 0.04^b	1.96 ± 0.01^c	1.87 ± 0.06^c	
FCR	1.26 ± 0.11^b	1.26 ± 0.10^b	1.28 ± 0.08^b	1.35 ± 0.01^b	1.67 ± 0.12^a	1.68 ± 0.15^a	
Protein efficiency ratio	2.78 ± 0.22^a	2.78 ± 0.21^a	2.73 ± 0.23^a	2.60 ± 0.01^a	2.14 ± 0.13^b	2.13 ± 0.18^b	
Apparent net protein utilization (%)	36.1 ± 3.1^a	35.6 ± 5.2^a	34.0 ± 4.0^a	31.9 ± 0.9^a	25.8 ± 1.8^b	26.0 ± 3.6^b	
Whole body composition							Initial
Crude protein (% fresh matter)	12.9 ± 0.7	12.6 ± 0.8	12.3 ± 0.5	12.2 ± 0.2	12.1 ± 0.1	12.1 ± 0.7	10.2 ± 0.0
Phosphorus (% dry matter)	0.83 ± 0.10	0.85 ± 0.10	0.76 ± 0.06	0.73 ± 0.07	0.77 ± 0.02	0.75 ± 0.08	0.40 ± 0.01

[a,b,c]In each line, means with no letters or with the same letters as superscripts are not significantly different (P > 0.05). Data are means of three replicates.

Table 3. Phosphorus and nitrogen budget from Nile tilapia fed *Azolla* meal diets in earthen ponds for 90 days. (A_0 to A_{50}: diet with 0% to 50% *Azolla*).

Diets	A_0	A_{10}	A_{20}	A_{30}	A_{40}	A_{50}
Feed supplied (g/pond)	7623 ± 64^a	7420 ± 303^a	7450 ± 118^a	6950 ± 105^b	6460 ± 203^c	6300 ± 151^c
P supplied (g/pond)	86.1 ± 0.7^a	80.1 ± 3.3^b	79.0 ± 1.2^b	69.5 ± 1.1^c	61.4 ± 1.9^d	47.9 ± 1.2^e
N supplied (g/pond)	357.4 ± 3.0^a	347.8 ± 14.2^a	348.1 ± 5.5^a	323.6 ± 4.9^b	301.8 ± 9.5^c	292.3 ± 7.0^c
P retention rate (%)	14.3 ± 2.1	15.2 ± 1.9	13.7 ± 2.4	13.1 ± 1.4	12.2 ± 1.1	14.3 ± 1.5
N retention rate (%)	36.1 ± 3.1^a	35.6 ± 5.2^a	34.0 ± 4.0^a	31.9 ± 0.9^a	25.8 ± 1.8^b	26.0 ± 3.6^b
P retained (g/pond)	12.4 ± 1.9^a	12.2 ± 1.3^a	10.8 ± 2.0^{ab}	9.09 ± 0.92^{bc}	7.49 ± 0.52^c	6.86 ± 0.72^c
N retained (g/pond)	129.2 ± 11.8^a	123.5 ± 13.5^{ab}	118.4 ± 15.1^{ab}	103.1 ± 1.7^b	77.7 ± 3.5^c	76.1 ± 12.6^c
Total P waste (g/pond)	73.8 ± 1.3^a	67.9 ± 3.8^b	68.1 ± 1.0^b	60.4 ± 1.5^c	53.9 ± 2.3^d	41.0 ± 1.2^e
Total N waste (g/pond)	228.2 ± 9.3	224.4 ± 27.2	229.7 ± 12.2	220.5 ± 6.1	224.1 ± 12.1	216.2 ± 5.6
Total P waste (% supplied)	85.7 ± 2.1	84.8 ± 1.9	86.3 ± 2.4	86.9 ± 1.4	87.8 ± 1.1	85.7 ± 1.5
Total N waste (% supplied)	63.9 ± 3.1^b	64.4 ± 5.2^b	66.0 ± 4.0^b	68.1 ± 0.9^b	74.2 ± 1.8^a	74.0 ± 3.6^a

[a,b,c,d,e] In each line, means with no letters or with the same letters as superscripts are not significantly different ($P > 0.05$). Data are means of three replicates.

likely met from pond ecosystem through natural productivity and water [26]. Thus, since *A. filiculoides* showed relatively low fibre content, and no antinutrient factors have been detected in that fern, the reduction observed in growth could probably be due to the lower protein digestibility of this fern, as mentioned by Leonard *et al.* [11] and Micha and Leonard [27] in *Oreochromis aureus* Steindachner and *O. niloticus*, respectively. The improved protein efficiency and protein utilization in group of fish fed with diets A_0, A_{10}, A_{20} and A_{30} could implies that the potentiality of natural food failed to balance *Azolla* deficiency in A_{40} and A_{50}. As a consequence, nitrogen retention in fish fed the last two diets was lower than that in fish fed the other diets. Although P content in diet decreased obviously with increasing AM level, a stable P retention was obtained in all experimental fish. According to Hernández *et al.* [28], P provided by FM-based diets generally surpasses the minimal requirements needed for optimal fish growth and in fact are less utilized by some cultivated species [5]. Therefore, it was suggested to provide fish with just the quantity required in order to keep the diets environmentally friendly [5]. Since tilapia P requirements vary from 0.90% [29] to 0.46% [30] depending on the species, fish size and diet composition, only P content in diet A_{50} comply with this suggestion. However, because the quantity of P or N supplied was adjusted fortnightly based on fish biomass, the amount provided in ponds receiving diets containing high AM was lower, and so the amount retained in fish decreased with increasing AM in diets.

Conversely, N waste discharged into ponds was similar in all groups of fish whereas lower P loaded decreased from ponds A_0 to ponds A_{50}. These findings confirm the lower utilization of nitrogen from *Azolla* at high levels of the fern in diets and its efficiency in reducing P output, and in turn to limit eutrophication. This result agreed with Jahan *et al.* [3], and Kaushik *et al.* [31] who found a reduction in P discharge when feeding fish with diets containing lower P, in which FM was replaced by corn gluten meal. These results could be compared to studies relating dietary P (or N) to P (or N) discharged. In rainbow trout fed 2.2%, 1.4% and 1.0% dietary P, 1.24%, 0.64% and 0.54% P was excreted [32], respectively. Investigating salmonid cage culture, Phillips *et al.* [33] found 79% loss of N and 85% loss of P supplied with feed, which amounted to 104 $kg \cdot N \cdot ton^{-1}$ fish production. Values in our study were similar for N and slightly higher for P, ranging from 69.5% to 80.7% and from 89.6% to 91.2%, respectively. In contrary, values obtained in this study are lower to those of Abou *et al.* [34] from the same experiment conducted in concrete tanks, who found that 90% and 80% of total phosphorus and total nitrogen supplied was wasted by the experimental fish; thus confirming the beneficial role of natural food in improving the nutrient utilization, and then in reduction in the amount wasted. When compared to that in control diet, the values of nutrient wasted by fish fed *Azolla*-diets were reduced, and the percentage of reduction increased with increasing AM level, reaching 8.7% to 46% for P and 2.9% to 8.8% for N at 50% *Azolla*-diet. The corresponding nutrient biomass ranged from 4.90 g to 25.9 g for P and 5.27 g to 15.8 g for N, which could not be negligible in preventing eutrophication in the field.

5. Conclusions

These results further contribute toward the comprehension of the role of *Azolla* in economical and environmental management of semi-intensive tilapia cultures in the tropics, where feed costs are a major factor limiting fish production and where, unfortunately, it is imperative to protect the ponds used, as many of them are stagnant earthen ponds. From the present study, the following conclusions could be drawn in low-polluting feed field:

1) Because of its low P content, *Azolla* meal could be used in diets for Nile tilapia to develop low-polluting feeds. However, more investigations are needed in that area;

2) High levels of *Azolla* meal in diets reduce growth, but positively, its non well-utilized nitrogen could enrich the fish ponds; which will be quite good in tropical marshland ponds where nitrogen is already limiting;

3) Hence, *Azolla* could be considered as "environmentally-friendly" ingredient that could limit the eutrophication, as well as preventing oligotrophication.

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