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Biotechnology of Stepwise Acclimatization of *Azolla Caroliniana* Willd. to Fishpond Wastewater and Its Bioconversion Under Field Conditions

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Abstract: In this study, a simple and reproducible biotechnological scheme was developed for the stepwise acclimatization of *Azolla caroliniana* Willd. to wastewater discharged from fishponds, its controlled clonal propagation under field conditions, and the preparation of the resulting biomass for subsequent nutritional bioconversion. Initially, 0.1×, 0.4× and 1.0× concentrations of a nitrogen-free modified Hoagland medium were tested. Each experiment used 4.0 g of fresh biomass; growth was evaluated in terms of final biomass, covered area and relative growth rate (RGR). Across three independent temporal replicates, the 0.4× medium gave the highest performance: 47.04 ± 0.28 g biomass, 320.0 ± 2.82 cm² area and an RGR of 0.246 ± 0.001 day⁻¹. Thereafter, three parallel clonal lines were sequentially transferred to 25, 50, 75 and 100% fishpond wastewater. As the wastewater proportion increased, day-10 biomass rose consistently from 48.20 ± 0.63 g to 53.88 ± 0.24 g, and RGR from 0.24890 ± 0.00130 to 0.26005 ± 0.00045 day⁻¹. The lines acclimatized to 100% wastewater were transferred to floating field units with a surface area of 0.49 m². In the 10-day field validation, biomass reached 68.12 ± 0.28 g, covered area 1055.67 ± 4.25 cm² and RGR 0.28349 ± 0.00040 day⁻¹; net fresh biomass productivity amounted to 13.09 ± 0.06 g FW·m⁻²·day⁻¹. Drying 50.0 g samples of field biomass at 45°C for 6 h yielded 3.57 ± 0.13 g of dried mass, corresponding to a yield of $7.13 \pm 0.25\%$. The results demonstrate the feasibility of clonal propagation and biomass production of *Azolla caroliniana* Willd. in fishpond wastewater without the addition of external mineral nitrogen. This approach integrates the conversion of wastewater biogenic elements into plant biomass, the reuse of water resources and the production of an alternative feed raw material for local aquaculture within a single technological chain.

Keywords: *Azolla caroliniana* Willd.; nitrogen-free Hoagland; acclimatization; fishpond wastewater; clonal propagation; bioconversion; phytoremediation; RGR; biomass productivity; field

validation; aquaculture; protein raw material; drying; circular bioeconomy.

Introduction

With the rapid development of aquaculture, the environmentally safe management of nutrient-rich wastewater discharged from ponds and the reduction of feed costs are becoming increasingly pressing issues. When nitrogen, phosphorus and other mineral components of wastewater are discharged without control, they increase the risk of eutrophication; however, they can be reincorporated into biomass by fast-growing aquatic macrophytes [1]. Floating ferns of the genus *Azolla* are of particular interest, since, together with their cyanobacterial symbiont, they fix atmospheric nitrogen and are able to grow actively even in media with relatively low mineral nitrogen [2]. Moreover, their high content of protein, amino acids, minerals and bioactive compounds strengthens the prospects for processing the biomass as a feed raw material [3].

The growth physiology of *Azolla caroliniana* Willd., its development in nitrogen-free Hoagland medium, its responses to temperature and light, and its tolerance to certain stress factors have been studied previously. Studies on wastewater treatment with *Azolla* have reported reductions in phosphorus, nitrogen, organic load and certain heavy metals. Work in the field of aquaculture has shown that *Azolla* biomass can be used as a partial source of plant protein in fish feed [4]. However, the available literature has most often examined individual stages – laboratory growth, wastewater phytoremediation or application in feed – independently. Data on an integrated scheme comprising the sequential adaptation of *Azolla caroliniana*, starting from a nitrogen-free nutrient medium, to increasing proportions of fishpond wastewater, the transfer of the same clonal lines to 100% wastewater, followed by validation in a field drainage channel and low-temperature drying of the biomass, remain limited [5].

The aim of this study was to generate practical, uncomplicated and reproducible biotechnological data for *Azolla caroliniana*. To this end, the following tasks were defined: 1) to select the effective concentration of nitrogen-free Hoagland medium; 2) to standardize the initial inoculum; 3) to acclimatize three parallel clonal lines to 25→50→75→100% fishpond wastewater; 4) to validate the laboratory results under field conditions; 5) to evaluate biomass productivity and RGR; and 6) to dry the resulting biomass at low temperature and prepare it for subsequent biochemical and feed-technology studies. This approach is based on the principle of bioconverting wastewater biogenic elements into biomass and returning resources to the aquaculture system [6].

The practical novelty of the study lies in the fact that the plant is not transferred directly to 100% wastewater; instead, the proportion of mineral nutrient medium is reduced stepwise while the proportion of wastewater is increased, and after each stage the biomass of the same clonal line is transferred to the next medium [7]. As a result, a continuous experimental chain is established between laboratory acclimatization and field production. This approach is of practical importance, particularly in arid regions, for water reuse and the cultivation of a local, low-cost protein raw material.

One of the key methodological studies on the controlled growth of *Azolla caroliniana* is that of Kösesakal and Yıldız, who evaluated the plant in nitrogen-free Hoagland solution under day/night conditions of 28±2/21±2°C on days 3, 5, 10 and 15 and recorded a mean RGR of approximately 0.120 g·g⁻¹·day⁻¹. Mostafa and Tammam, using a modified Hoagland medium at 2/5 strength, analysed the 10-day stress responses of *A. caroliniana*; this identifies the 0.4× medium as a species-appropriate experimental reference. Peters et al. evaluated the effects of pH, temperature, photoperiod and light intensity on growth and N₂ fixation in various *Azolla*–*Anabaena* associations and showed that under optimal conditions biomass can double within two days or less [8].

Temperature is an important limiting factor in *Azolla* physiology. Mostafa and Hassan, returning *A. caroliniana* to nitrogen-free and nitrate-containing Hoagland media after low-temperature stress, demonstrated a substantial effect of chilling on N₂ fixation, nitrate/nitrite reductase activity and protein metabolism. Da Silva et al., in a comprehensive test of Hoagland strength, light, humidity, pH control and mineral nitrogen availability in *A. pinната*, found that growth rate depends strongly on medium

strength and pH control; the highest RGR reached 0.321 day⁻¹. These results indicate that, rather than relying on a “single standard medium”, it is necessary to test concentrations appropriate to the species and local conditions for *Azolla* [9].

With regard to biomass production and nutritional value, Brouwer et al. cultivated *A. pinnata* and *A. filiculoides* in a semi-continuous system for more than 100 days and achieved a productivity of 90.0-97.2 kg DW·ha⁻¹·day⁻¹, with a protein content of 176-208 g·kg⁻¹ in the dry biomass [10]. Winstead et al. cultivated *A. caroliniana* under controlled conditions at 173 g FW·m⁻²·day⁻¹ and 5.53 g DW·m⁻²·day⁻¹, with a mean water content of the fresh biomass of 96.8%. Abdel Rahman et al. characterized the phenolic compounds, fatty acids and other bioactive components of *A. caroliniana*, demonstrating the potential of the biomass not only as a protein source but also as a functional phytochemical raw material.

In the FAO technical review on the use of aquatic macrophytes as feed in small-scale aquaculture, *Azolla* was considered as a separate group, and its cultivation, chemical composition and application in fish feed were summarized. Camargo et al. cultivated *A. pinnata* biomass in fishpond effluent and linked its processing and conversion into feed with the concept of the circular bioeconomy. The 2023 review by Yohana et al. analysed the protein, amino acid, vitamin and mineral value of *Azolla* meal and showed that there are prospects for partial replacement of fishmeal in various fish species. Refaey et al. reported that replacing 20% of the diet of Nile tilapia (*Oreochromis niloticus*) with fresh *A. pinnata* gave one of the best results in terms of growth and feed efficiency [11].

The practical value of *Azolla* in wastewater bioremediation has been confirmed in several directions. Forni et al. evaluated growth, nitrogenase activity and nitrogen and phosphorus removal during the treatment of aquaculture facility wastewater with *A. filiculoides* and presented this system as a low-cost tertiary treatment method. Costa et al. compared the growth of the *Azolla*–*Anabaena* system in urban wastewater with that in mineral media and reported an RGR of approximately 0.122-0.126 day⁻¹ in wastewater and a potential to reduce phosphate by 40-65%. Amare et al. treated mixed wastewater with *A. filiculoides* and *Lemna minor* in a 28-day batch system under semi-arid tropical conditions and demonstrated the treatment potential of both macrophytes.

Taghilou, Peyda and Mehrasbi modelled wastewater treatment by *A. filiculoides* using response surface methodology and described the effects of biomass quantity and exposure time on the reduction of TP, TN and COD. Khodadad Hosseini et al., using live *A. filiculoides* in batch and continuous bioreactors with dairy wastewater, demonstrated reductions in phosphorus, COD, BOD and TDS. Goala et al. tested *A. pinnata* in dairy wastewater at various dilutions, reported high phytoremediation efficiency at 75% dilution and monitored growth by image processing. These studies show that the stepwise optimization of wastewater concentration is practically justified [12].

Azolla caroliniana may also have a high capacity to accumulate heavy metals. Bennicelli et al. cultivated *A. caroliniana* for 12 days in water contaminated with Hg(II), Cr(III) and Cr(VI) and recorded a 74-100% decrease in metal concentrations, although metal stress reduced growth by 20-31%. Therefore, biomass grown in wastewater must be tested for heavy metals and microbiological safety before being directed to fish feed [13]. The 2023 review by Taghilou et al. summarized the phytoremediation potential of *Azolla* with respect to ammonium, phosphorus, COD, dyes and metals.

Recent reviews emphasize the need to scale *Azolla*-based systems up from the laboratory to pilot and integrated systems. In 2025, Goala, Bachheti and Kumar summarized recent findings on wastewater phytoremediation by *Azolla* species and identified scaling up, determination of species-specific efficiency and safe management of the resulting biomass as the main research gaps. In the 2026 integrated aquaculture study by Saif et al., *A. filiculoides* reduced NH₄-N, NO₃-N and phosphate and was applied within the same system as tilapia culture; at the same time, the accumulation of heavy metals in the biomass confirmed the importance of safety control [14].

Post-harvest processing determines the subsequent nutritional value of the biomass. Chandrababu et al. compared sun, cabinet tray and hot-air drying methods for *A. pinnata* and *A. caroliniana* and reported that cabinet drying at 40-45°C for 4-6 h was distinguished by high nutrient retention. Rahmah et al. used *Azolla* biomass grown in aquaculture wastewater as a partial protein source in the diet of *Pangasius* catfish and showed improved growth and feed efficiency at low inclusion

levels. Tammam and Mostafa demonstrated the molecular and amino acid responses of *A. caroliniana* to nitrate and salinity, confirming that medium chemistry directly affects plant metabolism [15].

Thus, although the nitrogen fixation, growth physiology, wastewater treatment capacity and feed raw material potential of *Azolla* are sufficiently covered in the international literature, an integrated approach involving the transfer of *A. caroliniana* from nitrogen-free 0.4× Hoagland medium through a 25→50→75→100% sequence of fishpond wastewater, the monitoring of the same clonal lines up to the field drainage channel, and the calculation of the dried biomass yield within the same experimental chain is rarely encountered. The present study was aimed precisely at filling this practical gap [16].

Materials and Methods

Biomass of *Azolla caroliniana* Willd. growing under natural conditions was collected. After removal from the water, free surface moisture was removed with absorbent paper following a uniform procedure. In each replicate, 4.0 g of fresh biomass was weighed (n=5), the fronds were counted, and the area freely covered in a rectangular water-filled vessel was determined planimetrically. The initial inoculum was standardized at 4.0 g for subsequent experiments (Table 1).

Table 1. Measurement parameters of the initial *Azolla caroliniana* inoculum (n=5)

Replicate	Biomass, g	Number of fronds	Occupied area, cm ²
R-1	4.0	255	62.3
R-2	4.0	251	61.5
R-3	4.0	257	62.6
R-4	4.0	253	61.7
R-5	4.0	254	62.0
Mean ± SD	4.0	254.0 ± 2.15	62.0 ± 0.44

The mean mass of a single frond was calculated as $4.0/254 = 0.01575$ g (15.75 mg), and the mean area occupied by a single frond as $62.0/254 = 0.2441$ cm² (24.41 mm²).

The nutrient medium was based on studies in which *A. caroliniana* was cultivated in nitrogen-free Hoagland medium. The mineral nitrogen sources KNO₃, Ca(NO₃)₂ and NH₄⁺ salts were not added. Potassium was supplied as KCl and KH₂PO₄, and calcium as CaCl₂·2H₂O. The target pH range was set at 5.6-6.0, with a working setpoint of 5.8. When necessary, 0.1 M HCl or 0.1 M NaOH was added dropwise (Table 2).

Table 2. Composition of the 1× nitrogen-free modified Hoagland medium

Group	Compound	Formula	1×, mg/L	Per 1 L	Per 15 L
Macronutrients	Potassium chloride	KCl	74.55	74.55 mg	1.1182 g
	Potassium dihydrogen phosphate	KH ₂ PO ₄	136.08	136.08 mg	2.0412 g
	Calcium chloride dihydrate	CaCl ₂ ·2H ₂ O	147.02	147.02 mg	2.2053 g
	Magnesium sulfate heptahydrate	MgSO ₄ ·7H ₂ O	246.08	246.08 mg	3.6912 g
	Boric acid	H ₃ BO ₃	2.86	2.86 mg	42.90 mg
Micronutrients	Manganese(II) chloride	MnCl ₂ ·4H ₂ O	1.82	1.82 mg	27.30 mg
	Zinc sulfate	ZnSO ₄ ·7H ₂ O	0.22	0.220 mg	3.30 mg
	Sodium molybdate	Na ₂ MoO ₄ ·2H ₂ O	0.09	0.090 mg	1.35 mg
	Copper sulfate	CuSO ₄ ·5H ₂ O	0.09	0.090 mg	1.35 mg
Iron	Iron(III) chloride	FeCl ₃ ·6H ₂ O	4.84	4.84 mg	72.60 mg
Chelating agent	Disodium EDTA	Na ₂ EDTA	15.00	15.00 mg	225.00 mg

To prepare 15 L of working solution, approximately 12 L of distilled water was first placed in the vessel, the stock solutions A-S1→A-S6 were added sequentially, and the solution was gently mixed after each addition. The final volume was then brought to 15.0 L. Calcium, phosphate and sulfate components were not stored together in a single concentrated stock. To prepare the Fe-EDTA stock, Na₂EDTA was dissolved in warm distilled water, the FeCl₃·6H₂O solution was added, and the stock was stored in a light-protected container (Table 3).

Table 3. 100× stock solutions and working volumes for nitrogen-free Hoagland medium

Stock	Composition	100× stock, 1 L	15 L for 0.4×	15 L for 1×	
A-S1	KCl	7.455 g/L	60 mL	150 mL	
A-S2	KH ₂ PO ₄	13.608 g/L	60 mL	150 mL	
A-S3	CaCl ₂ ·2H ₂ O	14.702 g/L	60 mL	150 mL	
A-S4	MgSO ₄ ·7H ₂ O	24.608 g/L	60 mL	150 mL	
A-S5	H ₃ BO ₃ ;	0.286; 0.182;	60 mL	150 mL	
	MnCl ₂ ·4H ₂ O;				
	ZnSO ₄ ·7H ₂ O;	0.022; 0.009; 0.009			
	Na ₂ MoO ₄ ·2H ₂ O;				
A-S6	CuSO ₄ ·5H ₂ O	0.484 + 1.500 g/L	60 mL	150 mL	
	FeCl ₃ ·6H ₂ O +				
	Na ₂ EDTA				

The 0.1×, 0.4× and 1.0× variants of nitrogen-free Hoagland medium were tested in a 15 L working volume. Each variant received 4.0 g of fresh inoculum. The experiment lasted 10 days. Three independent 10-day temporal replicates were performed, with an interval of 10 days between replicates. At the end, fresh biomass, covered area and RGR were determined. Differences in biomass between concentrations were evaluated exploratively by one-way ANOVA (Table 4).

Table 4. Growth of *A. caroliniana* at different nitrogen-free Hoagland concentrations (n=3)

Replicate	0.1× biomass, g	0.1× area, cm ²	0.4× biomass, g	0.4× area, cm ²	1× biomass, g	1× area, cm ²
1	12.44	130.2	46.72	317.0	36.48	256.6
2	12.76	132.5	47.16	320.7	37.08	260.0
3	12.96	134.0	47.24	322.5	37.32	261.7
Mean ± SD	12.72 ± 0.26	132.2 ± 1.92	47.04 ± 0.28	320.0 ± 2.82	36.96 ± 0.43	259.4 ± 2.58
RGR, day ⁻¹	0.116 ± 0.002	—	0.246 ± 0.001	—	0.222 ± 0.001	—

After 0.4× nitrogen-free Hoagland medium was selected as the most effective variant, three parallel clonal lines were maintained: vessel 1→vessel 1, vessel 2→vessel 2 and vessel 3→vessel 3. At each stage, the initial biomass was 4.0 g, the working volume 15 L and the observation period 10 days. The sequence of media was as follows: I – 25% wastewater (WW) + 75% 0.4× Hoagland; II – 50% wastewater + 50% 0.4× Hoagland; III – 75% wastewater + 25% 0.4× Hoagland; IV – 100% wastewater without added Hoagland medium (Tables 5 and 6).

Table 5. Laboratory acclimatization of *A. caroliniana* to 25→50→75→100% wastewater

Stage	Medium	Vessel	W ₀ , g	W ₁₀ , g	Net growth, g	Area, cm ²	RGR, day ⁻¹	Fold increase
I	25% WW	1	4.0	47.76	43.76	740.0	0.24799	11.94
	+ 75%	2	4.0	48.92	44.92	758.0	0.25039	12.23
	0.4×	3	4.0	47.92	43.92	742.8	0.24832	11.98
	Mean ± SD	—	4.0	48.20 ± 0.63	—	746.93 ± 9.69	0.24890 ± 0.00130	12.05 ± 0.16
	50% WW	1	4.0	51.32	47.32	795.2	0.25518	12.83
II	+ 50%	2	4.0	51.84	47.84	803.2	0.25619	12.96
	0.4×	3	4.0	51.12	47.12	792.0	0.25479	12.78
	Mean ± SD	—	4.0	51.43 ± 0.37	—	796.80 ± 5.77	0.25538 ± 0.00072	12.86 ± 0.09
	75% WW	1	4.0	52.68	48.68	816.4	0.25779	13.17
	+ 25%	2	4.0	52.16	48.16	808.4	0.25680	13.04
III	0.4×	3	4.0	52.04	48.04	806.4	0.25657	13.01
	Mean ± SD	—	4.0	52.29 ± 0.34	—	810.40 ± 5.29	0.25706 ± 0.00065	13.07 ± 0.09
	100% WW	1	4.0	53.64	49.64	831.2	0.25960	13.41
		2	4.0	53.88	49.88	834.8	0.26005	13.47
		3	4.0	54.12	50.12	838.8	0.26049	13.53
IV	Mean ± SD	—	4.0	53.88 ± 0.24	—	834.93 ± 3.80	0.26005 ± 0.00045	13.47 ± 0.06

Table 6. Dynamics of growth parameters with increasing wastewater proportion

Stage	Medium	Day-10 biomass, g	Area, cm ²	RGR, day ⁻¹	ΔB relative to the preceding stage, %
I	25% WW + 75% 0.4×	48.20 ± 0.63	746.93 ± 9.69	0.24890 ± 0.00130	—
II	50% WW + 50% 0.4×	51.43 ± 0.37	796.80 ± 5.77	0.25538 ± 0.00072	+6.70
III	75% WW + 25% 0.4×	52.29 ± 0.34	810.40 ± 5.29	0.25706 ± 0.00065	+1.69
IV	100% WW	53.88 ± 0.24	834.93 ± 3.80	0.26005 ± 0.00045	+3.03

From each of the three clonal lines acclimatized to 100% wastewater in the laboratory, a 4.0 g inoculum was taken. The inocula were stocked separately into three buoyant wooden units measuring 70×70 cm (0.49 m²), placed 2 m apart in the drainage channel carrying fish-farm wastewater. No supplementary mineral nutrients were provided. The experiment lasted 10 days. The initial stocking density was $4.0/0.49 = 8.16$ g FW·m⁻² (Table 7).

Table 7. 10-day field validation in wastewater discharged from the fishpond (n=3)

Unit	W ₀ , g	W ₁₀ , g	Covered area, cm ²	RGR, day ⁻¹	Net FW productivity, g·m ⁻² ·day ⁻¹	Surface coverage, %
1	4.00	68.10	1055.5	0.28347	13.08	21.54
2	4.00	68.40	1060.0	0.28391	13.14	21.63
3	4.00	67.85	1051.5	0.28310	13.03	21.46
Mean ± SD	4.00	68.12 ± 0.28	1055.67 ± 4.25	0.28349 ± 0.00040	13.09 ± 0.06	21.54 ± 0.09

The biomass from each field unit was harvested separately, rinsed in clean water, and the free water remaining on the surface and roots was removed with absorbent paper following a uniform procedure. From each replicate, 50.0 g of fresh biomass was weighed separately and dried in a drying oven at 45°C for 6 h. This regime is consistent with the low-temperature drying at 40-45°C for 4-6 h reported for *Azolla* species (Table 8).

Table 8. *A. caroliniana*: dried mass yield from 50 g of fresh biomass

Replicate	Fresh biomass, g	Mass after 45°C/6 h, g	Mass yield, %	Mass loss, g	Mass loss, %
1	50.00	3.55	7.10	46.45	92.9
2	50.00	3.45	6.90	46.55	93.1
3	50.00	3.70	7.40	46.30	92.6
Mean ± SD	50.00	3.57 ± 0.13	7.13 ± 0.25	46.43 ± 0.13	92.87 ± 0.25

The relative growth rate was calculated using the formula $RGR = [\ln(W_t) - \ln(W_0)]/t$, where W_0 is the initial fresh biomass (4.0 g), W_t is the fresh biomass on day 10, and t is 10 days. Net fresh biomass productivity was expressed by the formula $PFW = (FW_t - FW_0)/(A \times t)$, where A is the area of the field unit (0.49 m²). The inter-stage change in biomass was calculated using the formula $\Delta B(\%) = [(B_{\text{current}} - B_{\text{previous}})/B_{\text{previous}}] \times 100$. Results are presented as mean ± sample SD. The concentration screening was evaluated by one-way ANOVA, and the sequential acclimatization stages by an exploratory Friedman test across the three parallel lines; the field stage and the 100% laboratory stage were treated as a paired comparison. Owing to the small n , statistical inferences were interpreted with caution.

Results

Among the three concentrations, 0.4× nitrogen-free Hoagland medium yielded the highest biomass and surface coverage. On day 10, biomass was 47.04 ± 0.28 g, area 320.0 ± 2.82 cm² and RGR 0.246 ± 0.001 day⁻¹. In the 1.0× variant, biomass was 36.96 ± 0.43 g, and in the 0.1× variant 12.72 ± 0.26 g. The effect of concentration on biomass was significant in one-way ANOVA ($F_{2,6}=8374.91$; $p<0.003$); a pronounced difference was also observed for area ($F_{2,6}=4526.90$; $p<0.003$). Accordingly, 0.4× nitrogen-free Hoagland medium was adopted as the base medium for the subsequent acclimatization experiments [17].

No decline in growth parameters was observed when the wastewater proportion was increased from 25% to 100%. Whereas mean biomass at stage I was 48.20 ± 0.63 g, at stages II, III and IV it reached 51.43 ± 0.37 , 52.29 ± 0.34 and 53.88 ± 0.24 g, respectively. RGR increased from 0.24890 ± 0.00130 to 0.26005 ± 0.00045 day⁻¹. The dynamics of biomass and area across stages differed in the exploratory Friedman test ($\chi^2=9.00$; $df=3$; $p=0.029$). Despite the absence of Hoagland supplementation at the 100% wastewater stage, the highest laboratory values of the experiment were obtained at this stage [18].

When the three clonal lines acclimatized to 100% wastewater were grown in the field drainage channel for 10 days, mean fresh biomass amounted to 68.12 ± 0.28 g, covered area to 1055.67 ± 4.25 cm² and RGR to 0.28349 ± 0.00040 day⁻¹. Net fresh biomass productivity was 13.09 ± 0.06 g FW·m⁻²·day⁻¹. Field biomass was 26.42% higher than that of the laboratory 100% wastewater stage, and the differences among the three units were very small. The paired exploratory comparison indicated higher field biomass ($t_2=56.07$; $p<0.003$) [19].

From each 50.0 g of fresh biomass, 3.45-3.70 g of dried mass was obtained. The mean yield was 3.57 ± 0.13 g, or $7.13 \pm 0.25\%$; the mass loss amounted to $92.87 \pm 0.25\%$. Across the three replicates, a total of 10.70 g of dried biomass was obtained from 150 g of fresh biomass. The 45°C/6 h regime was chosen as a low-temperature treatment aimed at minimizing heat-induced nutrient losses (Table 9).

Table 9. Final biotechnological parameters of the study

Parameter	Result
Effective nitrogen-free Hoagland concentration	0.4×
Day-10 biomass in 0.4× Hoagland	47.04 ± 0.28 g; RGR 0.246 ± 0.001 day ⁻¹
25% wastewater	48.20 ± 0.63 g; RGR 0.24890 ± 0.00130 day ⁻¹
50% wastewater	51.43 ± 0.37 g; RGR 0.25538 ± 0.00072 day ⁻¹
75% wastewater	52.29 ± 0.34 g; RGR 0.25706 ± 0.00065 day ⁻¹
100% wastewater	53.88 ± 0.24 g; RGR 0.26005 ± 0.00045 day ⁻¹
Field drainage channel, 10 days	68.12 ± 0.28 g; RGR 0.28349 ± 0.00040 day ⁻¹
Net field productivity	13.09 ± 0.06 g FW·m ⁻² ·day ⁻¹
Dried mass from 50 g of fresh biomass	3.57 ± 0.13 g (45°C, 6 h)
Dried mass yield	$7.13 \pm 0.25\%$

Discussion

The superiority of 0.4× nitrogen-free Hoagland medium over the 0.1× and 1.0× variants indicates that a physiologically balanced, rather than an absolute maximal, nutrient medium strength is important for *Azolla* growth. Kösesakal and Yıldız successfully cultivated *A. caroliniana* in nitrogen-free Hoagland medium while Mostafa and Tammam applied a medium at 2/5 strength. The experiment by Da Silva et al. on another *Azolla* species also showed that interactions between nutrient solution strength, pH, light and humidity markedly alter growth rate. From this perspective, it is appropriate to interpret the 0.4× variant as the “most effective tested variant” specifically for the conditions of the present experiment [20].

The consistent increase in biomass and RGR with increasing wastewater proportion along the acclimatization chain indicates that the plant was able to utilize the biogenic elements present in the fishpond effluent. Costa et al. reported a specific growth rate of the *Azolla*–*Anabaena* system in urban wastewater close to that in mineral medium while Forni et al. demonstrated *Azolla* growth and N-P removal in aquaculture wastewater. The role of wastewater as a nutrient source has also been observed in other studies on *A. pinnata* and *A. filiculoides*. In the present experiment, the absence of growth decline

in 100% effluent indicated that additional Hoagland medium may not be required at subsequent stages [21].

In the field validation, RGR was higher than in the laboratory. This result may be associated with a combination of natural light, gas exchange, a larger surface area and nutrient flux in the water in the outdoor environment; however, these factors were not experimentally separated. Therefore, the superiority of the field stage should be regarded as a descriptive rather than a causal conclusion. Brouwer et al. specifically emphasized the need to distinguish RGR from areal productivity; in production systems with limited surface area, the $\text{g DW}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$ parameter is considered more practical. Winstead et al. reported high FW and DW areal productivity for *A. caroliniana*. Hence, optimization of the semi-continuous harvesting regime is required at the next stage [22].

The use of the biomass as a feed raw material is promising, but any macrophyte grown in wastewater must undergo safety control. *Azolla* can efficiently accumulate heavy metals and recent integrated aquaculture studies also confirm this property. Therefore, in addition to the protein, fat, ash, fibre and amino acid profile, Pb, Cd, Hg, Cr, As and microbiological parameters should be monitored. This requirement is also consistent with reviews on the application of *Azolla* meal in fish feed [23].

The drying regime also determines the quality of the feed raw material. The yield at $45^{\circ}\text{C}/6\text{ h}$ was $7.13 \pm 0.25\%$; this value was interpreted not as absolute dry matter but as the dried mass yield after this low-temperature regime. Chandrababu et al. showed that cabinet drying at $40\text{--}45^{\circ}\text{C}$ for 4–6 h is effective in terms of nutrient retention. In the study by Winstead et al., the mean water content of fresh *A. caroliniana* biomass was 96.8% hence, the dry matter fraction depends strongly on the cultivation and drying processes. Prior to subsequent feed formulation, the true dry matter content should be determined separately by standard proximate analysis.

Overall, the study makes it possible to regard wastewater not merely as waste but as a controllable bioconversion medium. The sequence laboratory $0.4\times$ nitrogen-free Hoagland $\rightarrow 25\rightarrow 50\rightarrow 75\rightarrow 100\%$ effluent $\rightarrow$ field drainage channel $\rightarrow$ low-temperature drying established a simple and reproducible technological platform for *A. caroliniana* [24]. This integration is consistent with the FAO concept of linking aquatic macrophytes to aquaculture feed with approaches to cultivating *Azolla* biomass on aquaculture effluent and with the circular bioeconomy principles of wastewater phytoremediation.

Conclusion

Among the tested nitrogen-free Hoagland concentrations, the $0.4\times$ variant gave the highest biomass, surface coverage and RGR for *Azolla caroliniana*. When the culture prepared in $0.4\times$ nitrogen-free Hoagland medium was sequentially transferred to $25\rightarrow 50\rightarrow 75\rightarrow 100\%$ fishpond wastewater, growth did not decline in any of the three parallel clonal lines; laboratory values were maximal at the 100% wastewater stage. In the 10-day validation in the field drainage channel, biomass reached $68.12 \pm 0.28\text{ g}$ and RGR $0.28349 \pm 0.00040\text{ day}^{-1}$, with a net fresh biomass productivity of $13.09 \pm 0.06\text{ g FW}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$. Drying 50 g of fresh field biomass at 45°C for 6 h yielded $3.57 \pm 0.13\text{ g}$, i.e., $7.13 \pm 0.25\%$, of dried mass. The results demonstrated the practical feasibility of acclimatizing *A. caroliniana* from a nitrogen-free starter culture to fishpond wastewater, subsequently propagating it clonally under field conditions, and bioconverting wastewater biogenic elements into biomass. To optimize separately the initial stocking density and semi-continuous harvesting intervals of 3, 5 and 7 days at the field production stage; to evaluate productivity in $\text{g DW}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$ alongside FW and to validate it with seasonal/spatial replicates; to test the biomass grown in wastewater for protein, lipid, ash, crude fibre, amino acid and mineral composition, heavy metals and microbiological safety prior to its inclusion in fish feed; and to test dried *A. caroliniana* biomass in feeding trials with stepwise inclusion levels in diets appropriate to the fish species and age.

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