

Original Research

Metabolomics and Nutritional Profiling of *Azolla microphylla*: Insights from Amino Acid Quantification and GC-MS Analysis

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Abstract

Azolla microphylla, a free-floating nitrogen-fixing aquatic fern, has emerged as a promising sustainable bioresource owing to its rapid biomass accumulation, high protein content, and rich phytochemical composition. The present study investigated the seasonal growth dynamics, biomass productivity, nutritional composition, amino acid profile, mineral content, and phytochemical constituents of *A. microphylla* cultivated under controlled pond conditions across three climatic quarters over one annual cycle. Species authentication through molecular barcoding confirmed 99% homology with *Azolla microphylla*. Biomass productivity exhibited significant seasonal variation ($p < 0.05$), with maximum wet biomass yield ($40,584.25 \pm 2,093.37 \text{ kg ha}^{-1}$) and dry matter yield ($8,116.85 \pm 418.60 \text{ kg ha}^{-1}$) recorded during Q1 (May-August), characterized by elevated temperature, humidity, and rainfall, whereas reduced yields were observed during Q2 (September-December) under comparatively cooler and drier environmental conditions. Despite fluctuations in biomass production, proximate analysis demonstrated substantial nutritional stability throughout the year, with crude protein content consistently maintained at approximately 30%, indicating resilience of nitrogen assimilation and protein biosynthesis under varying climatic conditions. Amino acid profiling revealed significant seasonal modulation ($p < 0.05$), with elevated concentrations of leucine, lysine, glutamic acid, glycine, threonine, and isoleucine during Q2, while arginine and methionine reached maximum levels during Q1. Mineral analysis confirmed the presence of appreciable concentrations of calcium, phosphorus, potassium, magnesium, sodium, and zinc, supporting the nutritional suitability of the biomass for feed applications. GC-MS analysis of the n-hexane extract identified twelve major phytochemical constituents, predominantly fatty acids, phytosterols, and long-chain hydrocarbons, including stigmasterol (42.30%), phytol (23.79%), n-hexadecanoic acid (12.35%), oleic acid, campesterol,

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and gamma-sitosterol, compounds previously associated with antimicrobial, antioxidant, anti-inflammatory, and pharmacological activities. Collectively, the findings indicate that optimum cultivation of *A. microphylla* is achieved at temperatures ranging from 29 to 33°C and pH 6.3-6.8, while its stable nutritional quality and diverse bioactive profile establish this species as a sustainable alternative feed resource and a potential reservoir of industrially and pharmacologically valuable metabolites.

Keywords: *Azolla microphylla*, biomass, seasonal variation, nutritional profile

Introduction

Food security is the greatest challenge and an important component of sustainable developmental goals. This scarcity and threat compel researchers around the globe to explore new and unconventional feed and food resources. Aquatic plants cover almost 50% of total fresh water cover. Currently, the feed industry is facing the challenges of availability and cost. So, in order to bridge this huge gap between demand and supply and reduce costs for small-scale producers, it is highly recommended that non-conventional feed sources be explored. In the last 5 years, *Azolla* spp. have gained much attention due to their high protein content.

Azolla utilization was documented as early as 1969 by Moore in rice fields to increase yield and production, but its use remained limited to inclusion in feed for backyard poultry and ruminants. Animal feed available on the market is expensive and can account for 60% of the variable cost, especially in fish, poultry and livestock, and this has forced scientists to consider alternative sources of plant-based protein such as soybeans, groundnut cake and others at low cost for fish, poultry and livestock products. The use of *Azolla* spp. in animal feed will reduce the present dependence on these competitive agricultural crops used in compounding feeds. Few studies reported that 10% replacement of the concentrate with an *Azolla* mixture improves the digestibility of dry matter, crude fiber and total digestible proteins [1]. *Azolla microphylla*, a fast-growing aquatic fern, is increasingly recognized as a sustainable bio resource with significant potential for commercialization due to its high nutritional value, nitrogen-fixing ability, and minimal environmental footprint. It forms a symbiotic association with the cyanobacterium *Anabaena azollae*, which enables biological nitrogen fixation, reducing dependency on synthetic fertilizers. The plant's rapid biomass accumulation, rich protein content (ranging between 25 and 30%), and adaptability to various aquatic environments make it a promising candidate for applications in livestock feed, aquaculture, organic fertilizers, and even bioremediation. Establishing a sustainable commercial supply chain for *A. microphylla* involves the systematic development of interconnected processes including large-scale cultivation, harvesting, processing, storage, distribution, and market linkages. Each stage requires optimization to maintain quality,

reduce losses, and ensure the environmental and economic sustainability of the enterprise [2].

The yield pattern of *Azolla microphylla* is highly influenced by seasonal variations. During warm and humid months, especially in tropical and subtropical regions, the plant exhibits its highest growth rates due to favorable temperatures, high light intensity, and sufficient nutrient availability in the water. Under these optimal conditions, doubling times can be as short as 2-3 days, allowing for frequent harvesting and high biomass turnover. However, in cooler or dry seasons, growth slows considerably because of low temperatures, shorter daylight hours, and increased water evaporation. Water stress, salinity buildup, or nutrient deficiency can further suppress productivity during these periods. Consequently, the commercial supply chain must anticipate and manage these seasonal fluctuations to maintain consistent output and fulfill market demand throughout the year.

To stabilize supply, several sustainability strategies can be adopted. Controlled-environment cultivation using lined ponds, raceways, or greenhouse systems helps regulate temperature, humidity, and light exposure, ensuring continuous growth even in adverse weather. Integration with aquaculture or livestock farms allows efficient nutrient recycling, where wastewater rich in organic matter sustains *Azolla* growth while improving water quality [3]. Selecting locally adapted or genetically improved strains can enhance tolerance to temperature extremes and salinity. In addition, effective harvesting and post-harvest management, such as sun-drying, low-temperature dehydration, or pelletization, extend shelf-life and reduce post-harvest losses. Developing cooperative models or community-based clusters of small producers ensures equitable participation, shared infrastructure, and economies of scale in production and marketing.

A well-structured sustainable supply chain for *Azolla microphylla* not only mitigates seasonal yield variability but also aligns with circular bio economy principles by turning aquatic biomass into renewable feed and fertilizer resources. It supports rural livelihoods, promotes environmentally friendly agricultural practices, and reduces reliance on chemical fertilizers and imported protein feed sources [4]. Furthermore, its cultivation contributes to carbon sequestration, improved water quality, and overall ecosystem resilience. Therefore, building a sustainable commercial model

for *A. microphylla* requires an integrated approach that combines ecological sustainability, economic viability, and social inclusion, transforming this small aquatic fern into a reliable component of future green supply chains.

A commercially viable, sustainable supply chain for *Azolla microphylla* hinges on pairing ecological strengths (fast growth, nitrogen fixation) with industrial-scale thinking: standardized propagation, seasonal risk management, value-added processing and robust market linkages. Important gaps for practitioners and researchers include local data on seasonal yield curves under scalable systems, cost-benefit analyses of controlled vs open cultivation, and consumer acceptance/quality standard development for different product forms. Addressing those will make *A. microphylla* a reliable, climate-smart input for feed, fertilizer and other circular bio-economy uses.

Materials and Methods

Study Area

Azolla spp. were collected from the pond prepared at the University of Veterinary and Animal Sciences (UVAS), Pattoki Campus.

Collection And Preservation of Samples

Whole plant roots, shoots and leaves were dried, ground at -4°C into powder with a particle size of 2 mm, and stored at 4°C in a refrigerator.

Environmental Variables

The environmental variables studied for the growth of *Azolla* spp. included temperature, pH of water, humidity in the air and rainfall.

Identification

The plant species was identified by the plant DNA barcoding method. RUBISCO Sanger sequence analysis revealed that the tested sample had a 99% evolutionary relationship with *Azolla microphylla*, as shown in Table 1.

Sanger sequence analysis (Fig. 1) shows that the tested sample has a 99% evolutionary relationship with *Azolla microphylla*.

Cultivation of *Azolla microphylla*

Three areas of land with even bottoms, each measuring one acre (43,560 square feet), were selected for the study at the University of Veterinary and Animal Sciences, Pattoki, Pakistan. All the stones, stubble and unwanted particles were removed from the place. A pit 18 inches deep was made and the natural pond containing the bed of soil from the pond area was filled with water up to 10-12 inches and 10 kg of dry cow dung mixed in 20 L of water was added. After the preparation of the pond, 5 kg of azolla culture was spread in the pond, and 900 g of single super phosphate (SSP) was added to the pond by dissolving it in water for the availability of phosphorus. The pH of the water was maintained at 6.3-6.8. During

Table 1. Subject score identities for the *Azolla* spp.

Subject				Score			Identities		
Accession	Description	Length	Start	End	Coverage	Bit	E-Value	Match/Total	Pct. (%)
MF177089.1	<i>Azolla microphylla</i>	617	1	617	100	1099	0	610/617	99

Note: Embryophyta; Tracheophyta; Polypodiopsida; Polypodiidae; Salviniiales; Salviniaceae; *Azolla*.

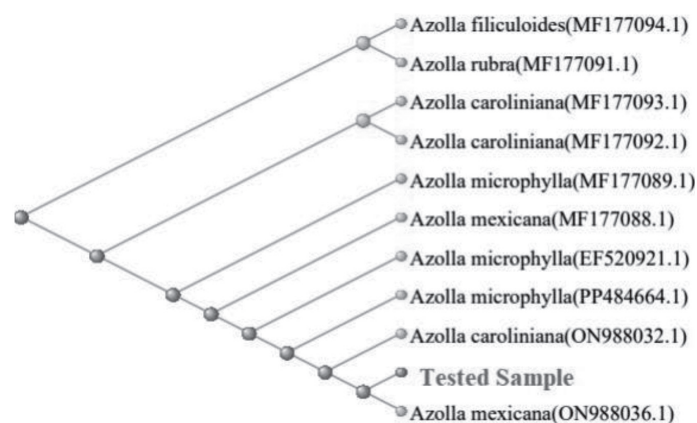


Fig. 1. Phylogenetic tree of *Azolla* spp.

the daytime, the temperature of the surroundings of the pond was approximately 30-31°C and 21-23°C at night. Optimized conditions were favorable for the cultivation of azolla. Once every 40 days, 10 kg of dry cow dung and 800-900 g of SSP were added to obtain continuous growth of azolla and to avoid nutrient deficiency. In case of pond contamination with insects, a fresh pure culture was added. Various environmental factors influenced *Azolla microphylla* growth as the pond was prepared in an open land area.

Determination of pH

pH of the pond water was determined at intervals of 15 days by using the Digital pH meter Jenway UK in all three quarters of the year and was maintained between 6.3 and 6.8.

Determination of Temperature and Humidity

Temperature and humidity of the pond area were measured using a ZEAL Digital thermo-hygrometer P-1001, which displays humidity up to 95% and a temperature range between 0°C and 50°C. Rainfall data and climatic temperature were collected from the National Weather Forecasting Centre (NWFC), Pakistan Meteorological Department.

Collection and Storage of *Azolla*

Azolla multiplied at high rates and covered the area in 25 days. Fully grown *Azolla* was harvested every 40 days and three times in a quarter from the water ponds. Approximately 5 kg of *Azolla* was left in the pond for the next growth. Harvested *Azolla* was cleaned, thoroughly washed, sun-dried for 2-3 days, dried to 10-15% moisture, and stored in airtight bags.

Proximate Analysis

The experimental aquatic plant *Azolla microphylla* was analyzed for moisture, crude proteins, crude lipids, crude ash, and crude fiber following AOAC (2005) methods.

GC-MS Analysis

GC-MS analysis of the n-hexane extract of *Azolla microphylla* was conducted at the UVAS Pattoki campus and key components were identified.

Determination of Mineral Composition

Mineral composition of the *Azolla microphylla* sample was determined by using the wet digestion method with perchloric acid as a solvent. The digested plant samples were filtered through glass filters and diluted to a final volume of 100 mL with deionized water. The samples were then subjected to atomic absorption

spectrometry (AAS) (Shimadzu AA 6701F, Atomic Absorption Spectrometer) to determine the percentage chemical composition. A triplicate experimental procedure was followed for internal control and to assess analytical precision using the method explained by Richard Koplik.

$$\text{Mineral (mg/kg)} w = fd (\rho - \rho_0) \frac{v}{ms} \left[\frac{\text{mg}}{\text{kg}} \right]$$

where ρ is the mass concentration of the element in the dilute sample ($\mu\text{g/mL}$), fd is the dilution ratio for the dilution applied before AAS measurement, ρ_0 is the average mass concentration of the element in the series of corresponding blank samples ($\mu\text{g/mL}$), v is the final volume of sample solution = (total volume of the ash solution or the extract) (mL), ms is the sample mass in grams.

Amino Acid Profiling

Amino acid profiling was determined using a BIOCHROM 30+ series amino acid analyzer in which amino acids are separated by IEC with a visible wavelength detector (IEC-VIS) and determined by reaction with ninhydrin using a spectrophotometric detector at 570 nm (440 nm for proline).

Statistical Analysis

The acceptable limit of error for measurements of chemical and nutritional analysis was 5%. All tests were performed in triplicate. Data values were expressed as means $\pm$ standard deviations. One-way ANOVA was performed using SPSS Statistics 26.0 (IBM Corporation, Armonk, New York, USA). p-value <0.05 was regarded as significant in the experiment.

Results and Discussion

Influence of Temperature and Rainfall

The quantitative parameters of rainfall and temperature were studied in three quarters of the year to describe the phenological analysis of the pond located at the University of Veterinary and Animal Sciences, Pattoki campus. With the help of the three-quarter study, the phytomass amount was recorded according to variations in climatic conditions. The observed physicochemical parameters, temperature and rainfall, influenced the growth of *Azolla microphylla* species, as shown in Table 2. The environmental parameter data show that Q1 (May to August) is characterized by significantly higher temperatures ($35.85 \pm 1.87^\circ\text{C}$) and rainfall (32.7375 mm) compared with the other quarters. This climate directly impacts the productivity of *Azolla microphylla*. The wet yield in Q1 ($40584.25 \pm 2093.37 \text{ kg ha}^{-1}$) is marked with

the superscript “c”, indicating it is statistically superior ($p < 0.05$) to the yields in Q2 and Q3, which are both marked “a” and do not differ significantly from each other. Interestingly, while the wet yield is much higher in Q1, the pH remains relatively stable across all quarters, ranging from 6.417 to 6.667, suggesting that soil or medium acidity is not a primary driver of the seasonal yield variance. Rainfall observed in the months of July and August was high, ranging from 42.87 mm to 48.97 mm, respectively, as shown in Fig. 2, and high yields were observed in these two months. In the second quarter, Q2, from September to December, the average rainfall decreased and the temperature also decreased, which caused a decrease in humidity in the environment and a lower yield of *Azolla microphylla*. The yield observed in the second quarter was 31948.75 ± 3033.52 kg per hectare, and the dry mass was 6389.60 ± 606.62 kg per hectare, as shown in Fig. 3. The temperature favored *Azolla microphylla* growth in the months of September and October. In the months of November and December, the average temperature was 26°C and 19.2°C , which reduced the growth yield of *Azolla microphylla*. The third quarter, Q3, was again potentially good for

Azolla microphylla growth compared with the second because the humidity and rainfall increased again, while the mean temperature of Q3 was $22.125 \pm 6.75^\circ\text{C}$ and humidity was more than $60.1 \pm 14.53\%$, as shown in Table 2. The lowest yield was obtained in the month of January, which was 26092 kg per hectare, with a dry mass of 5218.4 kg per hectare, because of cloudy conditions and fog concentration. The temperature was very low, at 13.9°C , with poor humidity of 38.6%, as shown in Fig. 4. The first quarter, Q1, gave the maximum yield of 40584.25 kg per hectare compared with the second and third quarters, with $p < 0.05$, because the climatic conditions favored the production of *Azolla microphylla*.

The rainfall data (mm) in Pattoki for three quarters of the year. Precipitation was significantly high from May to August in the first quarter (Q1), which influenced the high production of *Azolla microphylla*.

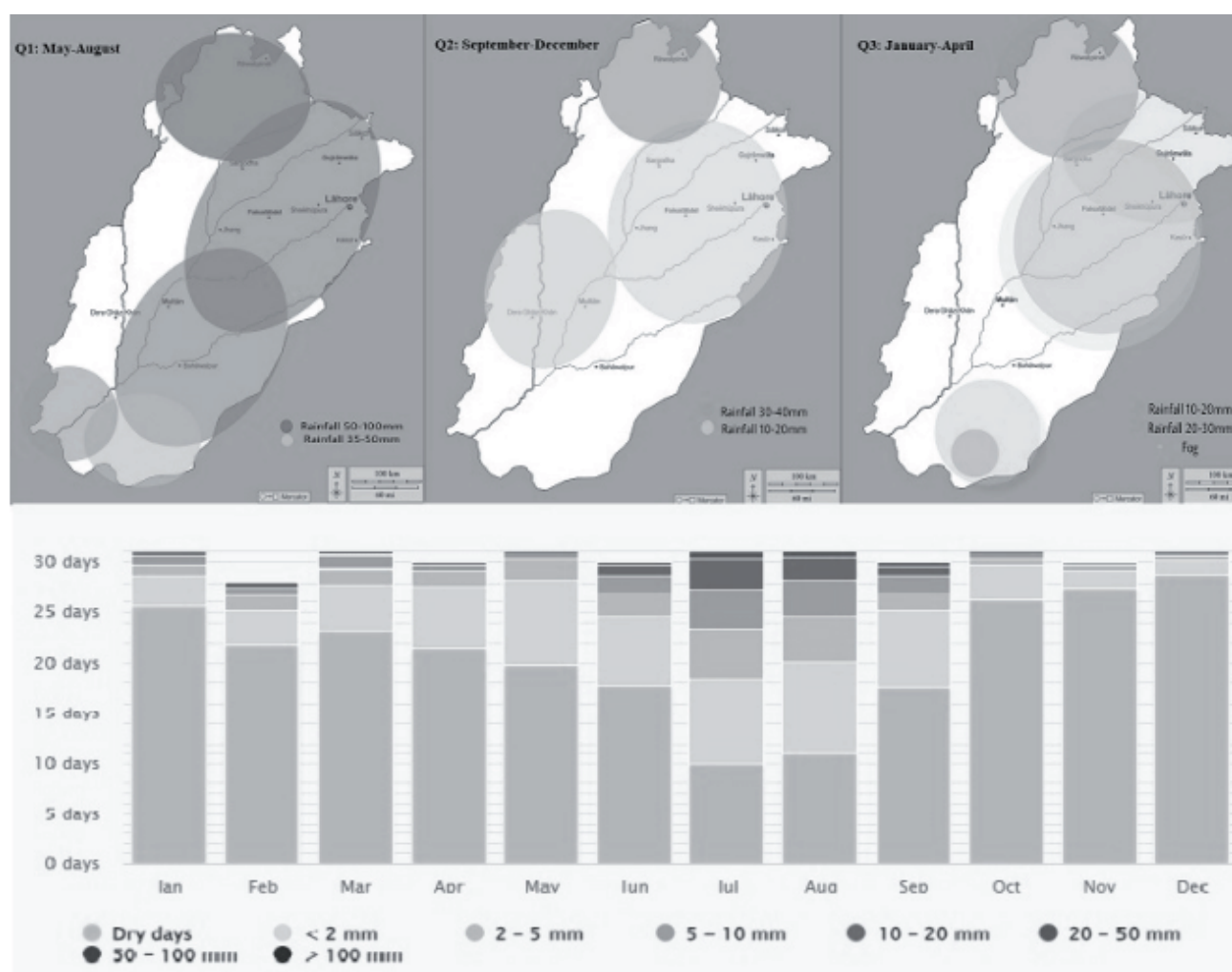
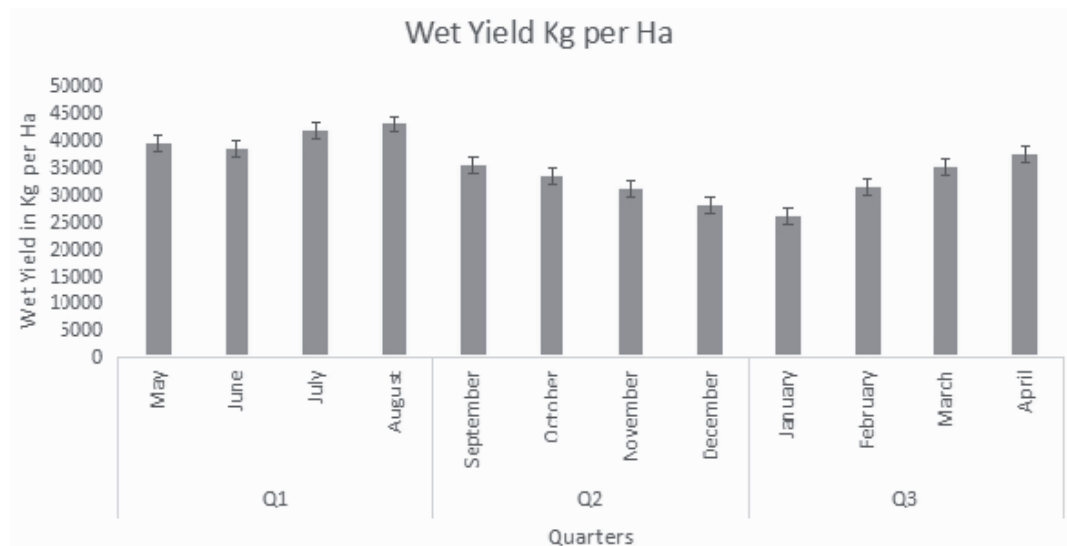
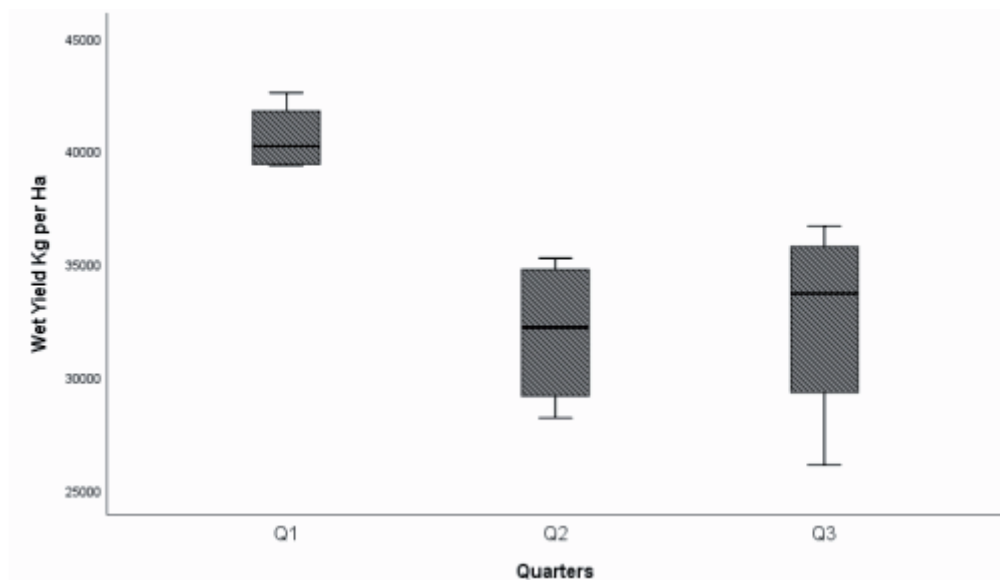


Fig. 2. Rainfall data of Punjab in three quarters of year.

Table 2. Environmental factors affecting *Azolla microphylla* yield production.

Quarters	Temperature °C	Humidity %	pH	Rainfall (mm)	Yield (Wet) kg per hectare	Yield (Dry) kg per hectare
Q1	35.85±1.87	70.225±9.02	6.667±0.23	32.7375	40584.25±2093.37 ^c	8116.85±418.6
Q2	27.825±6.88	55.775±23.56	6.457±0.22	15.855	31948.75±3033.52 ^a	6389.60±606.62
Q3	22.125±6.75	60.1±14.53	6.417±0.11	28.95	32536.01±4970.00 ^a	6507.20±992.64

Note: Biomass production (wet yield) was significantly high in Q1 ($p < 0.05$).

Fig. 3. *Azolla microphylla* fresh Biomass kg per ha in three quarters of year.Fig. 4. Box plot mean of fresh biomass of *Azolla microphylla*.

The yield of *Azolla microphylla* significantly increases in Q1, the first quarter, with $p < 0.05$, and no significant change is observed in Q2 and Q3, with $p > 0.05$.

Proximate Analysis

Proximate analysis of *Azolla microphylla* in the three quarters of the year indicated that total protein % remained remarkably stable ($p > 0.05$) across the year,

with values of 30.11%, 30.17%, and 30.40% for Q1, Q2, and Q3, respectively. This indicates that the nitrogen-fixing or protein-synthesis capacity of the species is resilient to seasonal changes. However, moisture % does show variance, peaking in Q3 at $91.02 \pm 1.21\%$. Other components like crude fats, fiber, and ash show minor fluctuations, but the consistency in protein suggests that the core nutritional value (in terms of bulk protein) is maintained year-round.

In the second quarter, Q2, from September to December, the mean crude fat content was $3.93 \pm 0.279\%$. The maximum crude fat content of 4.28% was observed in the month of September, and the minimum of 3.69% was observed in the month of December. In the third quarter, Q3, from January to April, the crude fat content observed was $4.44 \pm 0.429\%$. Results show that Q1 and Q3 had greater crude fat contents compared with Q2. The environmental factors favored the growth of *Azolla microphylla*, and the months from May to August, as well as March and April, provided the best environmental conditions for growth.

Environmental factors influenced the production of *Azolla microphylla* and indicated the favorable conditions for the production of *Azolla microphylla*, but these factors could not affect the percentage of crude protein contents and other nutritional components present in *Azolla microphylla*, as shown in Table 3 and Fig. 5.

Amino Acid Profile of *Azolla microphylla*

Both essential and non-essential amino acids were present in *Azolla microphylla* in all three quarters of the year. Results indicated that the specific amino acid concentrations varied significantly ($p < 0.05$), with Q2 (September to December) emerging as the period of peak protein quality. Peak values were observed in Q2. Major essential amino acids such as Leucine ($10.16 \pm 0.029\%$), Lysine ($2.57 \pm 0.026\%$), Phenylalanine ($5.18 \pm 0.038\%$), and Threonine ($7.81 \pm 0.126\%$) all carry the “c” superscript in Q2, meaning they are significantly higher than in Q1 and Q3. Regarding Q1 and Q3 variations, arginine and methionine are at their statistical maximum in Q1 (superscript “c”) and decrease significantly as the year progresses. Conversely, Histidine shows a significant increase over time, reaching its peak in Q3 ($1.79 \pm 0.017\%$), which is statistically different from both Q1 and Q2. Cysteine was not detected (ND) in any of the three quarters of the year.

Valine ($7.28 \pm 0.145\%$) and serine ($10.01 \pm 0.062\%$) were observed in the first quarter, where the yield was maximum. In the second quarter, Q2, the amino acids Isoleucine $5.75 \pm 0.049\%$, Threonine $7.81 \pm 0.126\%$, Tryptophan $0.85 \pm 0.026\%$, Aspartic acid $11.57 \pm 0.157\%$, Glutamic acid $14.32 \pm 0.028\%$, and glycine $8.59 \pm 0.016\%$ were obtained, indicating that under harsh conditions *Azolla microphylla* significantly improved the nutritional

Table 3. Proximate analysis of *Azolla microphylla* in three quarters of the year.

Quarters	Moisture %	Total protein %	Crude fats %	Crude fiber %	Crude ash %
Q1	90.317 ± 3.05	30.111 ± 0.697	4.43 ± 0.414	12.28 ± 0.258	15.34 ± 0.147
Q2	83.95 ± 0.90	30.17 ± 0.328	3.93 ± 0.279	11.72 ± 0.413	15.63 ± 0.244
Q3	91.02 ± 1.21	30.40 ± 1.011	4.44 ± 0.429	12.09 ± 0.435	15.45 ± 0.337

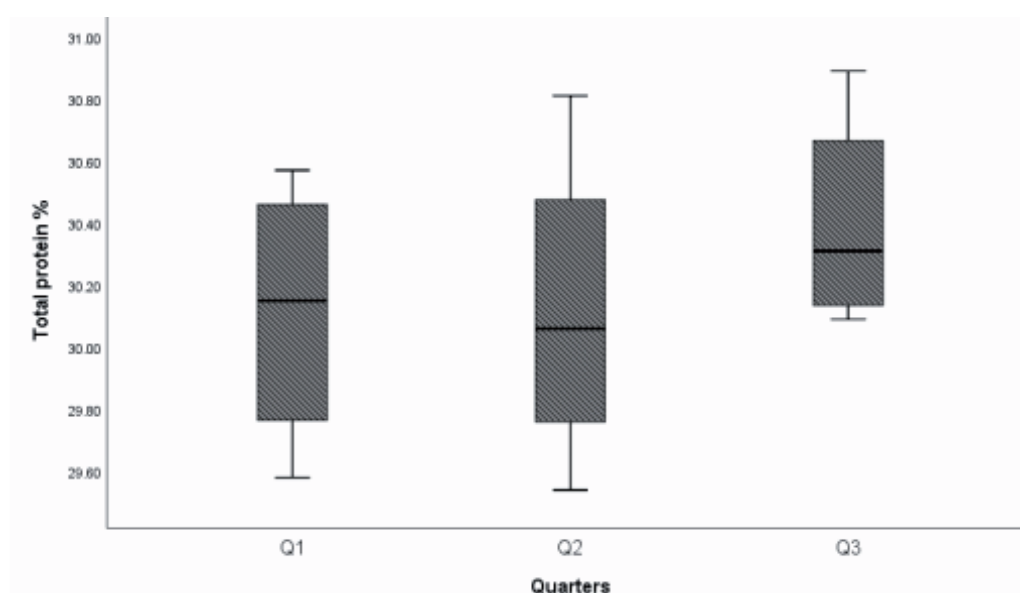


Fig. 5. Mean box plot of total protein contents.

profiling, especially acidic amino acids. Overall, *Azolla microphylla* was a rich source of amino acids and had a better nutritional profile and was utilized as an alternate source of protein for livestock and poultry feed.

Fig. 6 and Table 4 show that environmental factors affect the amino acid profile, with significant changes in

amino acid concentration observed at $p < 0.05$ in the first quarter, Q1, and the second quarter, Q2, of the year.

GC-MS Analysis

The GC-MS analysis of *Azolla microphylla* identified the presence of compounds with greater percentage

Table 4. Amino Acid profiling of *Azolla microphylla*.

Amino acids	Q1	Q2	Q3
Arginine	2.24±0.035 ^c	2.04±0.298 ^b	1.81±0.022 ^a
Histidine	0.86±0.0244 ^a	1.02±0.015 ^b	1.79±0.017 ^c
Isoleucine	4.12±0.020 ^a	5.75±0.049 ^c	4.52±0.112 ^b
Leucine	9.91±0.005 ^b	10.16±0.029 ^c	9.67±0.015 ^a
Lysine	1.95±0.031 ^a	2.57±0.026 ^c	2.22±0.116 ^b
Methionine	0.65±0.020 ^c	0.46±0.013 ^b	0.39±0.012 ^a
Phenylalanine	4.33±0.021 ^a	5.18±0.038 ^c	5.07±0.034 ^b
Threonine	6.10±0.049 ^a	7.81±0.126 ^c	6.41±0.032 ^b
Tryptophan	0.80±0.022 ^{ab}	0.85±0.026 ^b	0.77±0.033 ^a
Valine	7.28±0.145 ^a	7.60±0.036 ^b	7.16±0.057 ^a
Aspartic acid	9.40±0.052 ^a	11.57±0.157 ^b	9.27±0.061 ^a
Glutamic acid	10.20±0.051 ^a	14.32±0.028 ^c	11.62±0.045 ^b
Cystine	0.20±0.017 ^a	0.51±0.0216 ^c	0.29±0.002 ^b
Glycine	7.70±0.018 ^b	8.59±0.016 ^c	7.18±0.014 ^a
Serine	10.01±0.062 ^c	9.36±0.029 ^b	9.08±0.038 ^a
Cysteine	ND	ND	ND

Note: Table shows the percentages of various amino acids, with significant differences at $p < 0.05$.

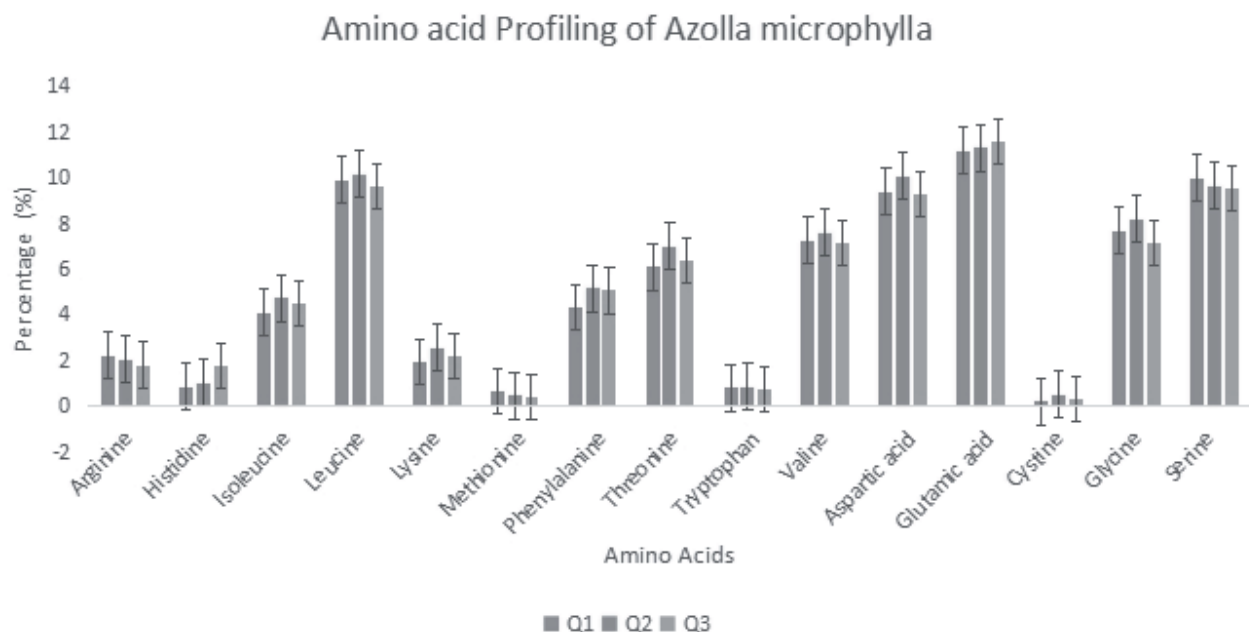


Fig. 6. Amino acid profile of *Azolla microphylla* in the three quarters of the year.

areas obtained for stigmasterol, phytol, n-hexadecanoic acid, oleic acid and campesterol (Table 5, Fig. 7).

Mineral Composition

The mineral-rich profile of *Azolla microphylla* makes it a valuable biofertilizer, livestock feed and sustainable resource in agricultural systems. Its ability to thrive in nutrient-deficient waters while fixing nitrogen adds to its utility in enhancing soil fertility. Mineral composition can vary based on the growing conditions, such as water quality and nutrient availability. Common minerals found in *Azolla microphylla* in three quarters of the year under various conditions, as shown in Table 6, were, in the first quarter, calcium $1.02 \pm 0.02\%$, phosphorus $1.88 \pm 0.05\%$, magnesium $0.36 \pm 0.04\%$, potassium $1.65 \pm 0.05\%$, sodium $2.12 \pm 0.08\%$ and a trace amount of zinc $0.008 \pm 0.0002\%$, while in the second quarter, the calcium concentration of $1.13 \pm 0.05\%$ was higher than in the first and third quarters, phosphorus was $1.95 \pm 0.02\%$, while potassium, at $1.68 \pm 0.01\%$, was greater than in the first quarter, and the highest concentrations of phosphorus, potassium, and magnesium, at $1.98 \pm 0.04\%$, $1.70 \pm 0.04\%$, and $0.35 \pm 0.05\%$, respectively, were observed in the third quarter.

In the third quarter, the climatic conditions became more moderate and were best in the months of March and April. The mean total protein content of Q3 was

$30.40 \pm 1.011\%$. The protein percentage was much higher than the $25.9 \pm 0.72\%$ [16] and 20.02% [1] reported previously. [17] suggested that the crude protein percentage was 24.8% and that it was a good alternate source of protein for ruminants. [18] discussed that the percentage of crude protein of *A. microphylla* was 25.32% , which was higher than that of *A. pinnata*, which was 24.14% , due to the nitrogen-fixing bacterium *Anabaena azollae*, which lives symbiotically. More appropriate conditions for the bacterium enhanced the protein percentage [19]. The reason for the variation of total crude protein contents may be due to temperature, soil nutritional contents, pH of pond water, pest growth and the mineral mixture obtained from an outside source that may have affected its growth rate and composition [20]. The crude fiber contents ranged from $11.72 \pm 0.413\%$ to $12.28 \pm 0.258\%$, with no significant difference at $p > 0.05$. [18] suggested that *Azolla microphylla* contained 13.29% crude fiber. [21] reported 13.64% , which was quite close to the results shown for Q2 and Q1 in Table 3.

GC-MS analysis shown in Fig. 6. identified a total of 12 peaks. The bioactive components detected were mainly fatty acids and long-chain hydrocarbons such as alkenes and alkanes. Key components shown by the chromatogram in Table 5. include eicosane, heptane, phytol, cyclohexane, oleic acid, octadecane, n-hexadecanoic acid, campesterol and gamma-sitosterol,

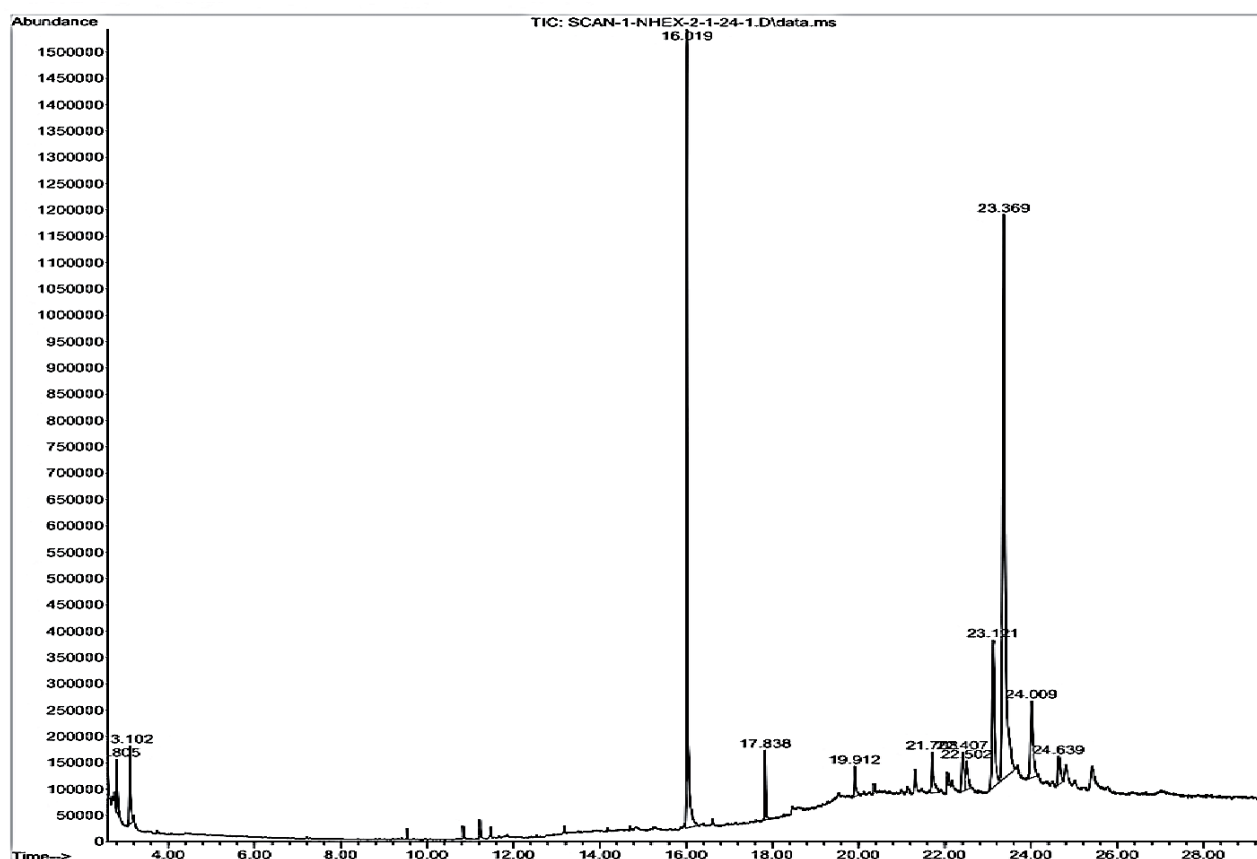


Fig. 7. GC-MS chromatogram of the n-hexane extract of *Azolla microphylla*.

Table 5. Components identified by GC-MS of n-hexane extract of *Azolla microphylla*.

Compound name	Area (%)	RT	M.F	M.W (g/mol)	Medical Importance
Heptanoic acid	1.78	2.85	C ₇ H ₁₆	100.21	Antiallergic, antimalarial, antiviral, and antimicrobial [5]
Cyclohexane, methyl-	3.12	2.8	C ₆ H ₁₂	84.16	Antimicrobial and anti-inflammatory [6]
Phytol	23.79	16.019	C ₂₀ H ₄₀ O	296.53	Antiarthritic, anti-inflammatory and fungicide [7]
Stigmasterol	42.30	23.369	C ₂₉ H ₄₈ O	412.69	Anticancer, anti-osteoarthritis, anti-inflammatory, anti-diabetic, antibacterial [8]
Eicosane	2.74	21.708	C ₂₀ H ₄₂	282.54	Antimicrobial, anti-inflammatory, antitumor, analgesic and antipyretic [8]
Oleic acid	23.12	10.48	C ₁₈ H ₃₄ O ₂	282.46	Anti-inflammatory, immunomodulatory, anti-cancer [9]
Tetrasiloxane, decamethyl-	2.15	22.50	C ₁₀ H ₃₀ O ₃ Si ₄	310.69	Antimicrobial, lubricant, skin and hair care [10]
n-hexadecanoic	12.35	22.407	C ₁₆ H ₃₂ O ₂	256.43	Antioxidant, hypocholesterolemic, pesticide, hepatoprotective and anti-inflammatory [11]
Gamma-sitosterol	6.35	24.009	C ₂₉ H ₅₀ O	414.70	Antimicrobial and antioxidant [12]
Cyclotrisiloxane, hexamethyl	2.09	24.639	C ₆ H ₁₈ O ₃ Si ₃	222.46	Antimicrobial, antifungal, antioxidant [13]
Octadecane	2.74	21.708	C ₁₈ H ₃₈	254.49	Antimicrobial [12]
Campesterol	10.48	23.12	C ₂₈ H ₄₈ O	400.69	Anti-inflammatory [14], anti-atherosclerotic, neuroprotective and antidiabetic [15]

Table 6. Chemical analysis of *Azolla microphylla*.

Components	Q1	Q2	Q3
Calcium (%)	1.02±0.02 ^a	1.13±0.05 ^c	1.08±0.07 ^b
Phosphorus (%)	1.88±0.05 ^a	1.95±0.02 ^b	1.98±0.04 ^b
Magnesium (%)	0.36±0.04 ^a	0.31±0.07 ^a	0.35±0.05 ^a
Potassium (%)	1.65±0.05 ^a	1.68±0.01 ^a	1.70±0.04 ^b
Sodium (%)	2.12±0.08 ^b	2.09±0.06 ^a	2.11±0.03 ^b
Zinc (%)	0.008±0.0002 ^b	0.007±0.0006 ^{ab}	0.004±0.0004 ^a

Note: the mineral contents of Ca, P, Mg, K, Na, and zinc in three quarters of the year shows significant changes at $p < 0.05$, indicating that environmental variables could affect mineral concentrations in *Azolla microphylla*.

with greater percentage areas obtained for stigmasterol 42.30%, n-hexadecanoic acid 12.35%, phytol 23.79% and campesterol 10.48%, which are reported to possess antimicrobial and anti-inflammatory properties [12]. [22] concluded that *Azolla microphylla* methanolic extract contained 2-butanone, 3-methoxy-3-methyl 0.865%, '2,2-dimethyl propionic acid, cyclopentyl ester' 0.670%, 'butane, 1-bromo-2-methyl' 0.398%, '2-hexen-1-ol, 2-ethyl' 0.269%, '5-hydroxy-2,2-dimethyl hexan-3-one' 0.212%, 'phthalic acid, ethyl pentyl ester' 0.21%, and 'pentanoic acid, 2-methyl' 0.182% in minor quantities, which do not cause any cellular and nuclear toxicity.

Due to environmental stress, the construction of NADPH-producing glycolytic pathways can enhance the production of important intermediates like oxaloacetate (OAA) and pyruvate, ultimately leading to increased

lysine synthesis [23]. The glutamic acid percentage increased in the second quarter because of calcium (Ca²⁺) signaling mediated by glutamate receptor-like proteins (GLRs) in plants. For aquatic plant growth and development, GLR-mediated Ca²⁺ signaling triggered by glutamate potentially enhances seed germination by suppressing the effect of abscisic acid (ABA) and/or its receptor ABA-insensitive 4 (ABI4). In addition, the glutamic acid response to environmental stress involves GLR-mediated Ca²⁺ signaling, which triggers salt tolerance by nitric oxide (NO) and activates the salt overly sensitive (SOS) signaling pathway; induces heat tolerance; governs drought tolerance via interaction with ABA to regulate stomatal movement; and hence adapts the plant to improve cold tolerance by the ROS-glutathione (GSH) cascade [24]. Extracellular glutamate,

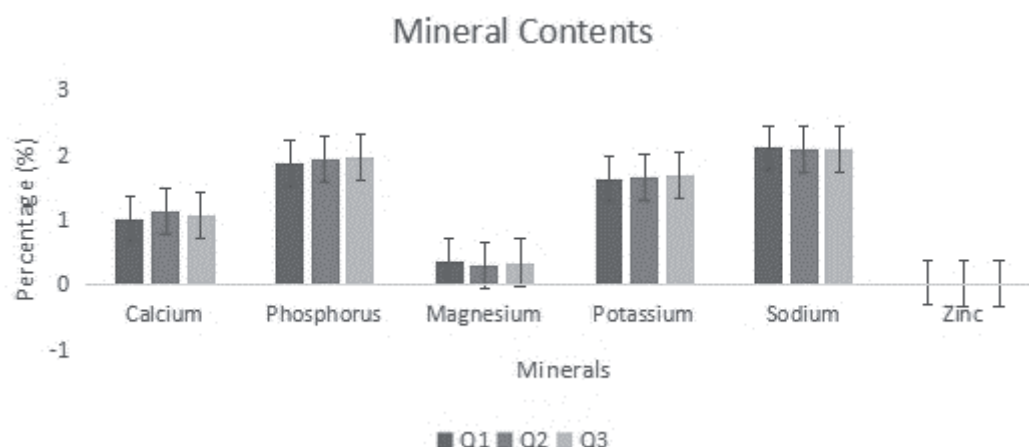


Fig. 8. Mineral composition of *Azolla microphylla*.

which is produced by plants, can be imported into cells and act as a mediator to synthesize proteins, glutamine, glutathione, arginine, proline, gamma-aminobutyric acid (GABA), heme, chlorophyll, and other components, which in turn regulate plant growth, development, and response to environmental stress [25].

Isoleucine percentage increased in Q2 to $5.75 \pm 0.049\%$, compared with $4.12 \pm 0.020\%$ in Q1 and $4.52 \pm 0.112\%$ in Q3 [21]. Methionine percentage was higher in Q1 at $0.65 \pm 0.020\%$ and gradually decreased in Q2 and Q3, with concentrations of $0.46 \pm 0.013\%$ and $0.39 \pm 0.012\%$. Cystine concentration increased in Q2 to $0.51 \pm 0.0216\%$. Histidine concentration was significantly high in Q3 at $1.79 \pm 0.017\%$, compared with the observed values in Q1 and Q2 of $0.86 \pm 0.0244\%$ and $1.02 \pm 0.015\%$. [25] reported a histidine concentration of 0.856% , which closely resembled the concentration obtained in Q1. Amino acid percentages discussed by [26] show similarities with the results of this study: alanine 1.52% and arginine $4.6\text{--}7\%$. Amino acid profile data reported that lysine, isoleucine, leucine, arginine, phenylalanine, glycine, aspartic acid and glutamic acid existed in higher concentrations compared with methionine and cystine, which contain sulphur.

Azolla microphylla contains various essential minerals which are important for plant growth. The results obtained in the study of three quarters of the year, with the percentages of Ca, Mg, P, K, Na and Zn given in Fig. 8, show that with changes in environmental variables, there were significant changes at $p < 0.05$ in the mineral contents observed. The Ca percentage ranged from $1.02 \pm 0.02\%$ to $1.13 \pm 0.05\%$, and [19] studied $0.3 \pm 0.02\%$, which was lower than that observed in this study. The minimum concentration of Ca was observed in the first quarter, Q1, with a mean value of $1.02 \pm 0.02\%$, which may be due to poor rainfall and high temperature. The percentage of phosphorus ranged from $1.88 \pm 0.05\%$ to $1.98 \pm 0.04\%$. [27] discussed $0.5\text{--}0.9\%$ in his study and [28] studied $0.2\text{--}0.3\%$. The results show that *Azolla microphylla* contains phosphorus for the development of chlorophyll and roots in various

environmental conditions. The maximum P percentage was observed in the third quarter, Q3, due to extreme winter conditions in the month of January.

Magnesium (Mg) production in plants is influenced by several environmental factors, primarily because magnesium is a crucial nutrient for photosynthesis and overall plant health. Increased availability of magnesium can enhance chlorophyll production, leading to improved photosynthetic efficiency. Additionally, proper magnesium levels help activate specific enzymes that aid in growth and cellular functions, resulting in healthier and more productive plants. It's important to ensure that soil conditions and nutrient supply are conducive to magnesium uptake for optimal plant growth. In this study, the percentage of Mg obtained ranged from $0.31 \pm 0.07\%$ to $0.36 \pm 0.04\%$, and [29] suggested 0.43% , while a higher value of 2.27% was observed by [30]. The results of the study were quite dissimilar due to environmental stress conditions. Potassium (K) concentration ranged from $1.65 \pm 0.05\%$ to $1.70 \pm 0.04\%$. [19] explained a potassium concentration of $1.22 \pm 0.62\%$, which was dissimilar to the obtained results. Sodium percentage ranged from $2.09 \pm 0.06\%$ to $2.12 \pm 0.08\%$. No significant effect on Na concentration was observed in three quarters at $p > 0.05$, as shown in Fig. 8. Zinc ranged from $0.004 \pm 0.0004\%$ to $0.008 \pm 0.0002\%$. [16] documented that *Azolla microphylla* contained 0.0054% . These results indicated that zinc percentage varied with fluctuations in environmental factors. In Q3, the minimum concentration of Zn obtained was $0.004 \pm 0.0004\%$.

Conclusions

The present study establishes *Azolla microphylla* as a highly productive and nutritionally resilient aquatic bioresource with substantial potential for sustainable feed, agricultural, and biotechnological applications. Seasonal variation significantly influenced biomass productivity, with maximum yield recorded under

warm and humid environmental conditions, confirming temperature, humidity, and rainfall as critical determinants of growth performance. Nevertheless, the nutritional composition, particularly crude protein content, remained remarkably stable throughout the year, demonstrating the species' adaptability and consistent nutritional quality under fluctuating climatic conditions. Comprehensive amino acid profiling further revealed the presence of essential amino acids in appreciable concentrations, while mineral analysis confirmed a rich supply of macro- and micronutrients important for livestock and poultry nutrition. GC–MS characterization identified diverse bioactive metabolites, predominantly phytosterols, fatty acids, and long-chain hydrocarbons, including stigmaterol, phytol, n-hexadecanoic acid, and campesterol, compounds associated with antimicrobial, antioxidant, and anti-inflammatory activities. Seasonal environmental variations, particularly fluctuations in temperature, humidity, and rainfall, were identified as the major factors influencing biomass productivity, with optimum growth of *Azolla microphylla* observed within a temperature range of 29–33°C and pH 6.3–6.8. Collectively, these findings demonstrate that *Azolla microphylla* can serve as a sustainable, low-cost, and environmentally compatible alternative protein source with additional pharmacological potential, thereby supporting its integration into circular bioeconomy systems, climate-smart agriculture, livestock feed formulations, biofertilizer development, and future value-added nutraceutical applications.

Author Contributions

Muhammad Ahmar Ali performed the experiments and wrote the manuscript. Rahat Naseer (Project Manager, PARB Research Project 22-42) analyzed the data and wrote the manuscript. Shagufta Saeed, Munazza Raza Mirza and Ovais Omer contributed to the experimental design and manuscript editing. All authors reviewed and approved the final manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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