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Comparative Effects of Vermicompost and NPK Fertilization on Growth, Heterocyst Formation, and Nutritional Quality of *Azolla microphylla*

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ABSTRACT. *Azolla* sp. is a rapidly growing aquatic fern valued as a nitrogen-rich biofertilizer and sustainable feed source. Its nutritional value is attributed to its high protein content, essential lipids, vitamins, and minerals, which contribute to improved animal nutrition. Ecologically, the plant maintains a permanent symbiotic association with nitrogen-fixing cyanobateria, enabling its adaptation to diverse and extreme environments. This study compared the effects of vermicompost (VC) and NPK (15:15:15) fertilizers at five concentrations (0–1000 ppm) on biomass accumulation, heterocyst frequency, and protein–lipid content of *A. microphylla* under greenhouse conditions. Relative growth rate (RGR) was calculated every three days, heterocyst frequency was quantified microscopically, and protein/lipid contents were determined using AOAC (2016) methods. Results showed that VC treatment significantly enhanced biomass yield (RGR = 0.23 at 750 ppm, $p < 0.05$), sustained higher heterocyst frequency across concentrations, and produced greater protein (24.31 ± 0.60%) and lipid (13.71 ± 0.08%) contents compared to NPK (17.62 ± 0.80%, 8.53 ± 0.05%, respectively). In contrast, NPK promoted growth only at 250 ppm and inhibited heterocyst formation and nutrient accumulation at higher concentrations. These findings indicate that VC provides a balanced, slow-release nutrient supply that supports both growth and symbiotic nitrogen fixation, highlighting its potential to enhance *Azolla* production for biofertilizer and feed applications.

Key words: Azolla microphylla, *Vermicompost*, *NPK fertilizer*, *Heterocyst frequency, Protein-lipid accumulation*

1. INTRODUCTION

The global agricultural sector faces the dual challenge of reducing dependence on chemical fertilizers while ensuring sustainable sources of protein-rich feed. *Azolla* sp., a fast-growing aquatic fern with a permanent symbiotic association with cyanobacteria such as *Anabaena azollae*, has gained recognition as a dual-purpose bioresource, functioning as both as biofertilizer and a high protein feed supplement (>30% crude protein) (Ahirwar & Leela, 2012). Dietary inclusion of *Azolla* can replace 10–25% of conventional feed, while simultaneously improving animal growth performance and immunity (Caruso et al., 2023). Native to Americas, Azolla has now become widely distributed across tropical, subtropical, and temperate regions, including Southern Africa and Asia (Hamdan & Houri, 2021). To date, it remains the only known plant to host a permanent nitrogen-fixing cyanobacterial symbiont, underscoring its ecological and agronomic significance.

Cyanobacteria, often mischaracterized as blue green algae, are prokaryotic phototrophs that have existed for

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approximately 3.5 billion years. They play a central role in global nitrogen and carbon cycles, with the ability to fix atmospheric nitrogen under diverse ecological conditions (Chittora et al., 2020; Nawaz et al., 2025). They are widely distributed across freshwater, terrestrial and marine environments, including extreme conditions, such as geothermal springs, frozen habitats, and hypersaline waters (Allaf & Peerhossaini, 2022; Waditee-Sirisattha & Kageyama, 2022). This adaptability highlights their ecological importance in mitigating nitrogen pollution derived from anthropogenic sources.

Within this context, the nutritional quality of *Azolla* is closely linked to its growth conditions and to the activity of heterocysts, the specialized cyanobacterial cells responsible for nitrogen fixation. Heterocyst differentiation is highly sensitive to nutrient availability, particularly nitrogen status (Zulkefli & Hwang, 2020; Waditee-Sirisattha & Kageyama, 2025). While chemical fertilizers such as NPK may enhance biomass accumulation, they often compromise protein quality and essential amino acid content, e.g. lysine and methionine, due to nutrient imbalances (Brouwer et al., 2018). Conversely, vermicompost (VC), a nutrient-dense organic amendment, has been reported to improve protein biosynthesis in *Azolla* by providing slow-release micronutrients, essential for cyanobacterial activity (Li et al., 2020), as well as phytohormones, e.g. auxins, that regulate protein metabolism (Zhang et al., 2015). Moreover, VC minimizes the risk of ammonia toxicity, which can inhibit amino acid production.

Produced through earthworm-mediated decomposition of organic matter, VC also improves soil health and stimulates beneficial microbial communities (Mulatu & Bayata, 2024). Unlike chemical fertilizers that may suppress nitrogen fixation in cyanobionts (Kalika-Singh et al., 2022), VC offers gradual nutrient release, potentially supporting both fern growth and symbiotic nitrogen fixation. However, comparative studies assessing the influence of VC and NPK on *Azolla* remain scarce. In particular, the relationship between fertilization regime, heterocyst development, and nutritional composition (protein–lipid content) has yet to be systematically examined.

The present study addresses this gap by comparing the effects of VC and NPK fertilizers on the growth performance, heterocyst frequency, and protein-lipid composition of *A. microphylla*. By elucidating the differential impacts of organic and chemical fertilizers, this work contributes to a deeper understanding of how fertilization strategies influence *Azolla's p*otential as a sustainable biofertilizer and protein-rich feed resource.

1. METHODOLOGY

2.1. Maintenance of *A. microphylla*

This study utilized *A microphylla* propagation under a controlled environment, in a greenhouse at UiTM Puncak Alam, Selangor (3°11'55"N, 101°26'43"E), providing more than 80% sunlight exposure. Cultivation was conducted in rectangular canvas containers (1.2m width×1.8m length×0.45m depth) containing tap water maintained at 0.25±0.05m depth, with initial inoculation of 50±1.0g of fresh *A. microphylla* fronds into each container, covering approximately 10% of the water surface area.

2.2. Fertilization regime for *A microphylla*

The fertilization protocol was designed to evaluate the growth responses of *A. microphylla* under varying nutrient conditions using both organic and chemical fertilizers. Five concentration levels (0, 250, 500, 750, and 1000 ppm) were systematically prepared for each fertilizer type: vermicompost (VC) and NPK (15:15:15) (Tayeb et al., 2020). VC was sourced from three different local suppliers, and each treatment was conducted in triplicate. The fertilizers were allowed to equilibrate in the cultivation water for 48 hours prior to inoculation. Growth performance of *A. microphylla* under various treatments was evaluated by monitoring and calculating its biomass every third day. Morphological parameters, including frond length, frond width and root length, were also measured. The fully grown plant from each treatment container was harvested, thoroughly washed, gently blotted dry and weighed to determine the relative growth rate (RGR), which was calculated using a formula reported by Hechler and Dawson (1995).

$$\text{RGR (g/g/d)} = (\ln W_2 - \ln W_1) / (t_2 - t_1)$$

where, W2 = the mean final biomass,
W1 = the mean initial biomass,
t2 – t1 = number of days between W2 and W1

2.3. Observation of indigenous cyanobacterial cells

A plant tissue squash technique was employed to observe *A. microphylla* frond samples. A single fresh frond was placed in a drop of distilled water on a clean glass slide, followed by gently placing a cover slip over it, which was then lightly tapped to squash the tissue and spread the cells. The prepared slide was examined under a light microscope. The cell arrangement, the presence of heterocyst and the numbers of vegetative cells between heterocyst were noted. The numbers of heterocyst within every 100 vegetative cells were quantified for each treatment concentration, and the percentage frequency was calculated accordingly.

2.4. Determination of total protein and lipid content

Samples of *A. microphylla* were initially dried under greenhouse sunlight conditions for 2-3 days until complete desiccation, followed by oven drying at 60 °C for 3 h to ensure uniform moisture removal. The dried material was cooled to room temperature, ground into a fine powder, and stored in airtight containers at 4 °C until further analysis. Proximate composition, specifically crude protein and lipid content, was quantified using the standard protocols of the Association of Official Analytical Chemists (AOAC, 2016).

2.5. Statistical analysis

Data were analyzed using one-way ANOVA followed by Tukey's HSD post-hoc test to compare means between treatments ($\alpha = 0.05$). Statistical analyses were performed using SPSS v25.0 (IBM Corp., USA). Results are presented as mean ± standard deviation ($n = 3$).

RESULTS AND DISCUSSION

3.1. Cultivation of *A. microphylla*

The successful propagation of *A. microphylla* under controlled greenhouse conditions (Figure 1) provided a consistent and reproducible biomass source for downstream analysis. The environmental parameters, particularly the provision of over 80% natural sunlight exposure, mimicked near-natural conditions while ensuring sufficient light intensity for optimal growth. Light is one of the most critical factors influencing *Azolla*'s photosynthetic rate and symbiotic nitrogen fixation (Brouwer et al., 2018).

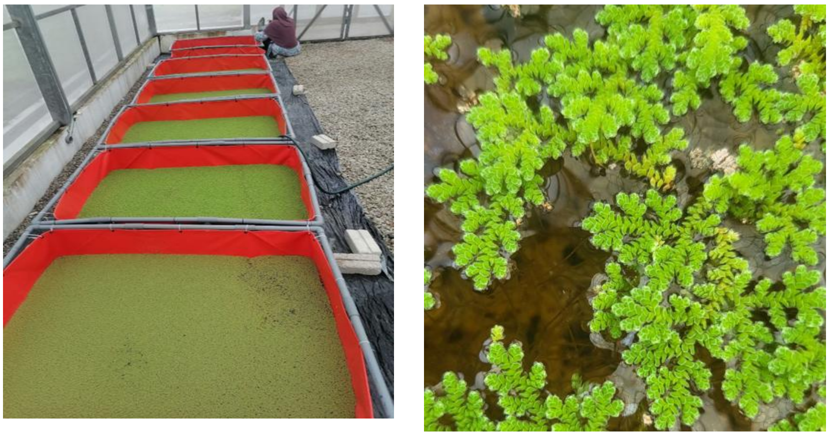

Figure 1. Cultivation of *A. microphylla* in ponds under greenhouse conditions at UiTM Puncak Alam Selangor (left), and morphology of *A. microphylla* fronds (right).

3.2. Fertilization regime for *A. microphylla* propagation

The RGR of *A. microphylla* responded strongly to fertilizer type and concentration (Figure 2). VC treatments showed sustained growth across concentrations, with maximum RGR (0.23 ± 0.02) recorded at 750 ppm ($p < 0.05$), which agrees with Adzman et al. (2022). In contrast, NPK treatments peaked at 250 ppm and declined sharply thereafter, suggesting possible nutrient stress or osmotic inhibition at higher levels. This finding supports the hypothesis that slow-release nutrients in VC create a more stable growth environment for *Azolla*.

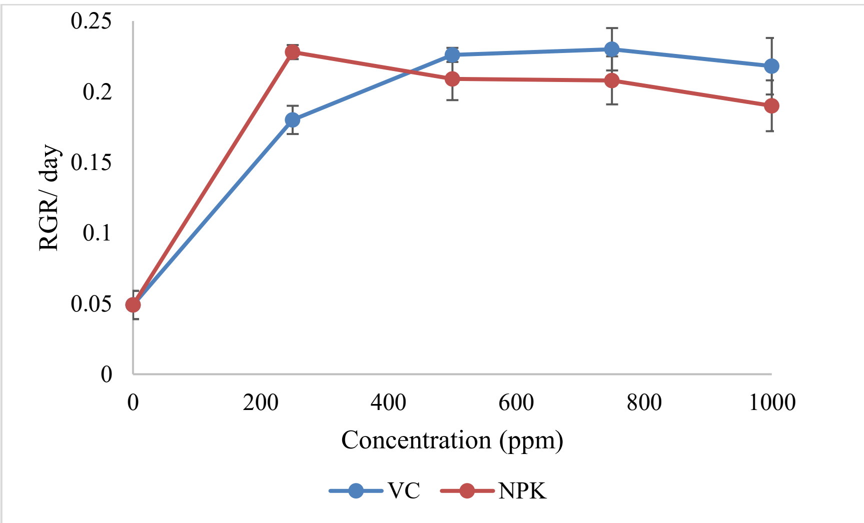

Figure 2. RGR of *A. microphylla* against fertilizers concentration.

This trend is likely attributable to the high phosphorus content in NPK formulations, as phosphorus is a critical driver of biomass accumulation in *Azolla*. A similar observation was made by Hossain et al. (2021), who demonstrated that phosphorus supplementation in aquatic systems enhanced *Azolla* growth. However, at higher NPK concentrations, the growth-promoting effect appears to diminish, possibly due to nutrient imbalance or stress induced by excessive inorganic inputs.

The frond width of *A. microphylla* responded positively, reaching a maximum value of 0.42 ± 0.02cm at a VC concentration of 750 ppm (p <0.05), after which a slight decline was observed (Figure 3). This trend suggests that moderate levels of VC provide sufficient macro- and micronutrients essential for cellular metabolism, tissue expansion, and overall plant vigor, while excessive concentrations may create nutrient imbalances or induce mild physiological stress, thereby limiting further growth. In contrast, frond width in NPK-treated plants peaked at 250 ppm, followed by a sharp decline at higher concentrations. This pattern mirrors the RGR results, indicating that although low levels of NPK may enhance frond development through rapid nutrient availability, excessive application likely disrupts nutrient homeostasis or imposes osmotic stress, ultimately constraining growth performance.

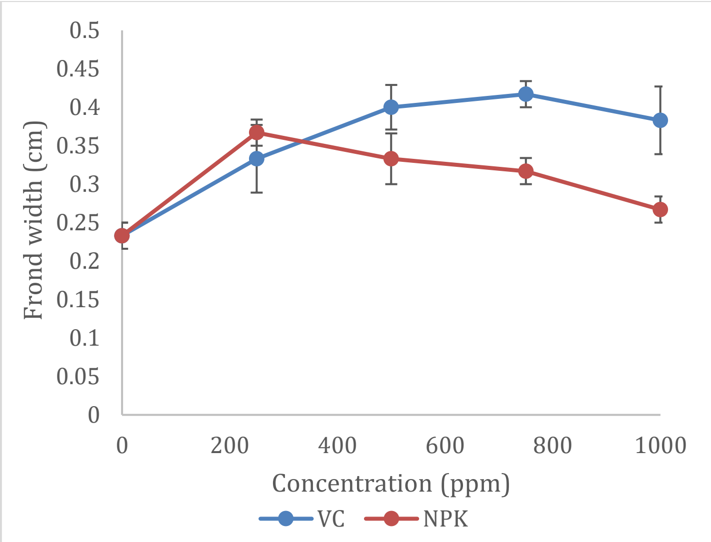

Figure 3. Effect of fertilizer concentration on the frond width of *A. microphylla.*

The average roots length of *A. microphylla* in both fertilized treatments was inversely proportional to the treatment concentration (Figure 4). Under nutrient-deprived conditions, the plant has elongated roots to enhance their surface area for the purpose of nutrient absorption. This is a survival mechanism to maximize the uptake of scarce resources. When there is an abundance of nutrients present in the environment, the need for extensive root development decreases as the plant can efficiently obtain the necessary nutrients with a smaller network of roots (Lopez et al., 2023).

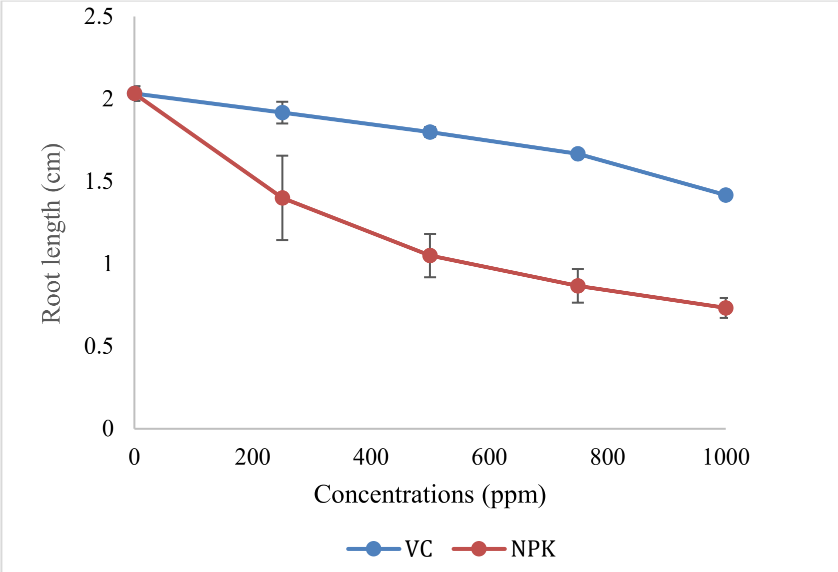

Figure 4. Effect of fertilizer concentration on the root length of *A. microphylla.*

Notably, the decline in root length was more pronounced under NPK fertilizer treatment compared to VC reduction. Roots exposed to NPK fertilizer showed a sharper reduction, 63.9%, suggesting possible osmotic or phytotoxic stress that constrained growth. By contrast, VC treatment exhibited a relatively moderate decline (30.3%), indicating a less inhibitory effect on root morphology. This differential response may be attributed to the gradual nutrient release and the presence of organic matter and growth-promoting substances in VC, which buffer against nutrient shock and mitigate toxicity (Kalika-Singh et al., 2022).

These findings highlight that while nutrient sufficiency reduces the necessity for extensive root systems, the form of fertilizer plays a critical role in modulating root morphology. VC not only sustains root growth at higher concentrations but also appears to provide a more balanced nutrient environment compared to NPK fertilizers. This observation supports the growing evidence that organic amendments promote plant resilience under variable nutrient regimes, making them a more sustainable option for long-term cultivation systems.

3.3. Observation of indigenous cyanobacterial cells

Heterocysts are specialized nitrogen-fixing cells that lack functional photosystem II, thereby creating a microoxic environment favorable for nitrogenase activity (Waditee-Sirisattha & Kageyama, 2022). Their differentiation is strongly regulated by nitrogen availability, with heterocyst frequency typically increasing under nitrogen-limiting conditions. In the present study, chains of cyanobiont cells associated with *A. microphylla* were clearly observed under light microscopy (Figure 5), with arrows indicating the presence of heterocyst. The occurrence of these specialized cells confirms the active role of the cyanobacterial symbionts in sustaining nitrogen fixation within the *A. microphylla* system.

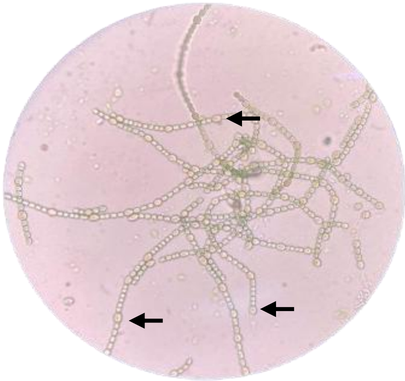

Figure 5. Chains of cyanobiont cells of *A. microphylla* observed under light microscope (40x10). Arrows indicate the presence of heterocyst.

In the present study, heterocyst frequency in *Azolla* cyanobiont displayed a concentration-dependent response to fertilizer application (Figure 6). Both VC and NPK fertilizer treatments exhibited the highest heterocyst frequency at 250 ppm, followed by a marked decline as fertilizer concentrations increased. Interestingly, the control (0 ppm) did not exhibit the maximum heterocyst formation, suggesting that a moderate nutrient input may act as a physiological trigger, promoting cyanobiont proliferation and heterocyst differentiation before inhibitory effects of higher nutrient levels set in.

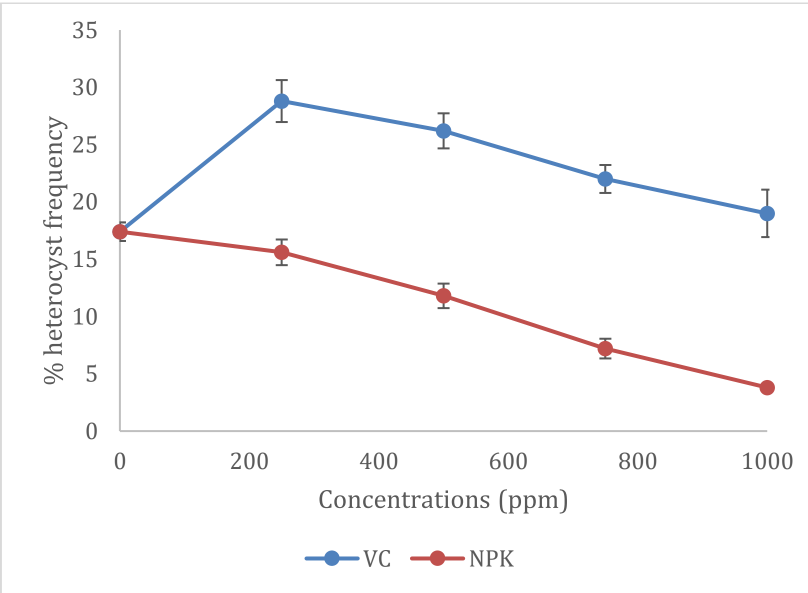

Figure 6. Percentage of heterocyst frequency of cyanobiont against fertilizers concentration.

The contrasting responses between fertilizer types are noteworthy. VC consistently sustained higher heterocyst frequencies compared to chemical fertilizer across all concentrations. This suggests that vermicompost may provide a more balanced nutrient profile and gradual nutrient release, minimizing osmotic stress and creating a favorable environment for symbiotic cyanobacteria. In contrast, chemical fertilizer appeared to suppress heterocyst formation more rapidly, which may be attributed to excessive inorganic nitrogen availability, reducing the need for biological nitrogen fixation, or to potential inhibitory effects on cyanobacterial physiology. Environmental stressors, such as salinity, have also been reported to significantly influence heterocyst differentiation and abundance (Yadav et al., 2016).

These findings align with previous reports that heterocyst frequency is not solely a function of absolute nitrogen scarcity but also depends on the quality, form, and release dynamics of nutrient sources (Gunawardana, 2020; Roumezi et al., 2020). Thus, the current results reinforce the ecological advantage of VC as a nutrient amendment that supports the functional activity of *Azolla*–cyanobiont associations, with potential implications for sustainable biofertilizer applications.

3.4. Total protein and lipid analysis

Protein and lipid contents are critical indicators of plant nutritional quality and growth performance. In the present study, the effects of VC and NPK fertilizer on the biochemical composition of *A. microphylla* were compared. While *Azolla* is capable of generating its own assimilates via photosynthesis and benefits from its cyanobionts as biological nitrogen fixers (Brouwer et al., 2018), the macronutrient composition of the plant can vary considerably depending on environmental conditions, species type, and cultivation practices. Previous studies have reported protein contents in *Azolla* sp. ranging from 17.6-20.8% and lipid contents between 7.9-10.0% (Brouwer et al., 2018). Under stress conditions, such as salinity, protein levels have been shown to decline to 12.9-19.3% (Yadav et al., 2016). Conversely, nutrient supplementation has been demonstrated to enhance protein accumulation to 27.1%, although in some cases lipid content declined to as low as 6.37% (Adzman et al., 2022). Such variability underscores the sensitivity of *Azolla* biochemical composition to both abiotic stressors and nutrient management strategies. It is also important to recognize that differences in analytical methodologies may contribute to variation in reported values across studies.

In this study, VC supplementation resulted in significantly higher protein (24.31%) and lipid (13.71%) contents compared to NPK (17.62% and 8.53%, respectively) (Table 1). This enhancement under VC treatment may be attributed to the gradual nutrient release and presence of organic matter that supports cyanobacterial proliferation and heterocyst differentiation, thereby sustaining symbiotic nitrogen fixation. By contrast, NPK-treated plants exhibited reduced protein and lipid levels, which is consistent with the observed decline in heterocyst frequency under chemical fertilizer application. Such reductions suggest that excessive inorganic nitrogen may suppress symbiotic nitrogen fixation and induce physiological stress, ultimately lowering the accumulation of nitrogen-rich compounds such as proteins and lipids.

Table 1. Comparative analysis of protein content (%) in dried *A. microphylla*. Note: different letters indicate statistically significant differences (p<0.05).

Fertilizers	Protein content (%)	Lipid content (%)
NPK	17.62 ± 0.80^{a}	8.53 ± 0.05^{a}
VC	24.31 ± 0.60^{b}	13.71 ± 0.08^{b}

The macronutrient composition of *Azolla* is known to vary considerably depending on species and external growing conditions (Yadav et al., 2016; Brouwer et al., 2018). The higher protein–lipid content observed in VC-treated *A.*

microphylla may also reflect an increased density of cyanobiont cells, which themselves are rich in proteins and lipids, further contributing to the host's biochemical composition. These findings highlight the dual role of fertilizer source in modulating not only plant growth but also the functional activity of symbiotic microorganisms. Consequently, organic fertilizers such as VC appear to confer both nutritional and ecological advantages over synthetic fertilizers by enhancing the biofertilizer potential of *Azolla*.

4. CONCLUSION

This study demonstrates that vermicompost (VC) application significantly improves biomass accumulation, heterocyst frequency, and protein–lipid content in *A. microphylla* compared to NPK fertilizer under greenhouse conditions. VC provided a balanced nutrient supply that supported sustained symbiotic nitrogen fixation, whereas higher NPK concentrations suppressed heterocyst formation and reduced nutritional quality. These findings suggest that VC is a more sustainable fertilization strategy for optimizing *Azolla* production as both a biofertilizer and a protein-rich feed source. Future studies should validate these results under field conditions and assess economic feasibility for large-scale cultivation systems.

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AUTHOR CONTRIBUTIONS

Rohana Mat Nor is responsible for writing the manuscript. **Nur Aina Zulaikha Arbain** and **Dinah Aidileyna Mohamad Shukry Fairuz** are responsible for additional data collection. **Farizan Aris**, **Sharifah Raina Manaf** and **Alawi Sulaiman** are responsible for supervising and managing the project.

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The project did not receive any funding.

DECLARATION OF GENERATIVE AI IN THE WRITING PROCESS

During the preparation of this manuscript, the author(s) used ChatGPT (OpenAI) to assist with language editing, including grammar and clarity improvement. The author(s) carefully reviewed and revised the content as necessary and take full responsibility for the final version of the publication.

DATA AVAILABILITY

All data supporting the findings of this study are included in the article.

COMPETING INTEREST

The authors declare that there are no competing interests.

COMPLIANCE OF ETHICAL STANDARDS

The authors declare that this research did not involve human or animal subjects and this research does not include any ethical issue. All experimental procedures were conducted in accordance with the institutional Safety, Health, and Environmental (HSE) protocols of Universiti Teknologi MARA (UiTM).

SUPPLEMENTARY MATERIAL

No supplementary material is associated with this article.

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