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Enhancing sustainable agriculture and mitigating environmental impacts: Azolla cultivation to treat fattener pig farm effluent wastewater

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Abstract

The number of pig farms in Thailand is increasing to meet domestic and worldwide consumer demand as society moves from agricultural to industrial operations. Waste generation, especially wastewater, has increased due to this development, with pig farm owners struggling to meet environmental regulations before wastewater release. Azolla, a floating aquatic fern, is a promising sustainable agricultural resource. This study proposed the growth of high-quality Azolla using a pig fattener farm effluent. Microorganisms at 1% (v/v) were added to wastewater from the 3rd stabilization pond of a fattener pig farm to improve water quality. Compared to the non-treated group, Azolla crops grew faster with doubling time (DT) 5.92 days and relative growth rate (RGR) 10.00 $g.day^{-1}$. The Azolla contained 9.43% dry matter, 24.30% ash, 16.22% total Kjeldahl nitrogen (TKN), 0.78% fat, 12.62% dietary fiber, 0.14% calcium, 0.26% potassium, and 1.75% phosphorus that were within conventional ranges, suggesting agricultural applications. Azolla cultivation reduced Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), TKN, phosphorus, and potassium contents by 75.45% (498.00 mg/L), 73.44% (940.00 mg/L), 70.74% (20.61 mg/L), 63.11% (179.41 mg/L), and 83.67% (747.27 mg/L), respectively. The findings highlighted the capability of Azolla to effectively improve wastewater quality.

Keywords: agricultural, *Azolla microphylla*, phytoremediation, plant treatment, wastewater

1. Introduction

Pig farming in Thailand is expanding with the transformation of society from agriculture to industry. This transition, coupled with advanced knowledge and technology in pig farming, has led to larger farms to meet the dietary needs of both domestic and international populations. The wastewater generated during various stages of the pig farming process from cleaning the pens contains manure and serves as a source of nutrients. However, uncontrolled release into the environment can cause pollution of public water sources and negatively impact the ecosystem by promoting the rapid growth of aquatic plants, leading to eutrophication [1].

Azolla, "Red Stem Floating-heart" is a small water plant belonging to the floating-heart family. It typically grows on the water surface in stagnant environments such as ponds, marshes, waterlogged areas, or locations with year-round standing water. This plant is commonly found in warm and tropical regions and comprises seven species: *Azolla nilotica*, *A. pinnata*, *A. caroliniana*, *A. filiculoides*, *A. mexicana*, *A. rubra*, and *A. microphylla*. In Thailand, two common varieties of Azolla are *A. pinnata* and *A. microphylla*. Large hollow structures within the leaves of Azolla serve as the habitat for Anabaena filamentous cyanobacteria which aid in nitrogen fixation from the surrounding atmosphere at 1.1 tons per hectare, significantly higher than the 0.4 tons per hectare achieved by legumes alone [2-3]. Azolla has accelerated biomass growth, with a doubling time (DT) ranging from 2 to 6 days [4-6]. Azolla can generate biomass of 15 tons per hectare within 3 months through an initial inoculation of 1.3 kg/m^2 [7]. Wastewater treatment is required to control nutrient levels and meet environmental standards. The nutrients found in pig farming wastewater such as total Kjeldahl nitrogen (TKN) and phosphorus do not meet the discharge standards [8]. Recent research studies have identified the potential utility of Azolla for biological wastewater treatment. This plant has shown high efficiency in removing contaminants such as cadmium (Cd), chromium (Cr), copper (Cu), and zinc (Zn) and can also reduce the emission of greenhouse gases from agriculture [9]. Azolla grows rapidly and can effectively reduce organic matter content in wastewater. Biological wastewater treatments are becoming increasingly popular due to their efficiency, lack of persistent pollutants, and safety for users and the environment. Azolla offers multiple benefits including efficient reduction of organic matter, biological nitrogen fixation [10], enhancement of organic matter in soil and improved efficiency of chemical fertilizer in crop production [11-13], use in organic farming, weed control in rice fields, reduced chemical herbicide usage [14-15], and application as animal feed [16]. Azolla can also be used for biological wastewater treatment.

2. Materials and methods

2.1 Material

A. microphylla Kaulf were obtained from The Research and Development Center for Agriculture in Nakhon Ratchasima located at 196 Moo 3, Lad Bua Khao Sub-district, Sikhio District, Nakhon Ratchasima Province, 30340.

The wastewater samples used in the experiments were collected from the 3rd stabilization pond in a fattener farm that raised type B pigs. The farm owner was Mrs. Benjamat Intayawong and the farm was located at 1 Moo 18, Banlang Sub-district, Non-Thai District, Nakhon Ratchasima Province. The farm standard code was 02-6403-30-030084 GAP.

2.2 Preparation of wastewater

Wastewater samples (150 L) were collected from the 3rd stabilization pond of the fattener farm.

Experimental Set 1 (FS):

Wastewater (100%) (FS). A volume of 75 liters of wastewater was divided into three test containers, each containing 25 liters.

Experimental Set 2 (FSEM):

The wastewater was mixed with 1% v/v Effective Microorganisms (EM) and incubated for 3-7 days (FSEM). A volume of 75 liters of wastewater was distributed equally between three containers, each holding 25 liters. (Figure 1).

Figure 1 Preparation of wastewater from the 3rd stabilization pond of the fattener farm. (A) Un-treated EM (B) Treated EM.

2.3 Wastewater analysis

Samples of wastewater before and after the experiment were collected to analyze their characteristics. The samples were tested at the Faculty of Environmental and Resource Studies, Mahasarakham University. Water quality indices were assessed based on the standards set by the Ministry of Natural Resources and Environment in the Royal Gazette on April 4, 2021 [17] including pH, Biochemical Oxygen Demand (BOD), Chemical Oxygen

Demand (COD), Suspended Solids (SS), Total Kjeldahl Nitrogen (TKN), Phosphorus (P), and Potassium (K). These indices were used to evaluate the characteristics of the wastewater based on standard methods for the examination of Water and Wastewater [18]. The results were used to calculate the efficiency of improving wastewater quality according to Equation (1).

$$\%Removal = \left[\frac{concentration\ before - concentration\ after}{Concentration\ before}\right] x\ 100 \qquad (1)$$

2.4 Inoculation of substrate seeding and spawning

The Azolla was weighed, with cultivation in each experiment set at 100 grams (fresh weight). The temperature and light intensity were controlled to mimic natural conditions with light levels ranging from 300,000 to 400,000 lux. The temperature during daylight hours was maintained between 37 and 42 °C. The growth capabilities of Azolla were observed and their characteristics were documented, with photographs taken every 7 days for 21 days. After 21 days, the water was drained and the Azolla was weighed to calculate the DT and RGR. The Azolla was then dried at 70 to 80 °C for 48 hours, as presented in Figure 4

2.4.1 Doubling time: DT

The doubling time (DT) is the time taken by the biomass of Azolla to double in weight, and was calculated using Equation (2) [19]:

$$DT\ (day) = t/(log\ (W1/W0))\ x\ 0.031 \qquad (2)$$

where:

It is the duration of the experiment (in days).
W_1 is the weight of Azolla after t days.
W_0 is the initial weight of Azolla used in the experiment.

The term log is the natural logarithm (log base e) and dividing division by 0.301 are presents the conversion factor from the natural logarithm to base 10.

2.4.2 Relative growth rate: RGR

The relative growth rate (RGR) of Azolla was expressed as the daily growth rate and calculated by comparing the biomass before and after the experiment. The RGR was computed using Equation (3) [20]

$$RGR\ (\frac{g}{day}) = (ln(W2) - ln(W1))/t \qquad (3)$$

where:

W1 and W2 are the initial and final fresh weights of Azolla, respectively.
It is the duration of the experiment in days.

2.4.3 Dry matter content: % DM

The samples were dried using a hot air oven at 100-105 °C for 8 hours (AOAC, 2004) [21]. The moisture content was calculated using Equation (4).

$$\%humidity = \frac{(W1-W2)\ x\ 100}{Weight\ of\ Sample} \qquad (4)$$

where:

W1 is the combined weight of the sample and the drying dish before drying.
W2 is the combined weight of the sample and the drying dish after drying.

The dry matter (% Dry Matter, DM) can be was determined by subtracting the moisture content from 100.

2.4.4 Ash

The sample was incinerated in a furnace at 550 to 570 °C until the ash turned white or light gray. This process took approximately 2 hours (counting from the time when the furnace reached the specified temperature) (AOAC, 2004) [21]. The percentage of total ash (% Total Ash) was calculated using Equation (5).

$$\%Total\ Ash = \left(\frac{Weight\ of\ total\ ash}{Weight\ of\ Sample}\right) x\ 100 \qquad (5)$$

2.4.5 T Total Kjeldahl nitrogen: TKN

The Kjeldahl method is a technique used for nitrogen determination (AOAC, 2004) [21]. The percentage of nitrogen was calculated using Equation (6).

$$\%Nitrogen = (S - B)\ x\ 0.014\ x\ N\ x \frac{100}{W} \qquad (6)$$

where:

S is the volume of NaOH used to titrate the sample.
B is the volume of NaOH used to titrate the blank.
N is the normality of the standard NaOH.
W is the weight of the sample.

$$\%Crude\ Protein = \%Nitrogen\ x\ 6.25 \qquad (7)$$

A protein factor of 6.25 is commonly used in these calculations.

2.4.6 Fiber

Crude fiber analysis is a technique used for fiber determination (AOAC, 2004) [21]. The percentage of fiber was calculated using Equation (8).

$$\%Total\ Fiber = \left(\frac{Weight\ of\ Residue\ after\ drying - Weight\ of\ residue\ after\ ignitation}{Weight\ of\ sample}\right) x\ 100 \qquad (8)$$

2.4.7 Calcium: Ca

The sample was prepared by the dry ashing method for decomposing plant samples (Hoenig) [22]. The quantity of calcium was determined using an Atomic Absorption Spectrophotometer (AAS) (AOAC, 2004) [21] and calculated as follows using Equation (9) .

$$\%\text{Ca} = \frac{\text{ppm Reading x dilution factor x volume (subdued with acid) x 100}}{\text{10 to the power of 6 x Weight of sample}} \qquad (9)$$

2.4.8 Potassium: K

The sample was prepared by the dry ashing method for decomposing plant samples (Hoenig) [22]. The quantity of potassium was determined using an Atomic Absorption Spectrophotometer (AAS) (AOAC, 2004) [21] and calculated using Equation (10).

$$\%\text{K} = \frac{\text{ppm Reading x dilution factor x volume (subdued with acid) x 100}}{\text{10 to the power of 6 x Weight of sample}} \qquad (10)$$

2.4.9 Phosphorus: P

The sample was prepared by the dry ashing method for decomposing plant samples [22]. The quantity of phosphorus was determined using a Spectrophotometer and calculated using Equation (11).

$$\%Total\ P = (E\ x\ D\ x\ 250\ x\ 100)/(C\ x\ 1000\ x\ W) \qquad (11)$$

where:

E is the concentration of phosphorus (P) in the sample determined from the standard graph of phosphorus, reading the concentration of P from the absorbance (OD) values of the sample.
D is the adjusted volume before measurement (50 mL)
C is the measured volume (10 mL)
W is the weight of the sample used for incineration (g)

2.5 Experimental design

The experiment followed a Completely Randomized Design (CRD) with each stage having three replications. After completing the experimental procedures, a statistical analysis was performed to evaluate the results including percentage, mean (average), standard deviation, analysis of variance (ANOVA), and comparison of means at a 95% confidence level ($p < 0.05$). The analysis was performed using SPSS software version 29.0 for Windows.

3. Results and discussions

3.1 Characteristics of wastewater samples from the fattener farm before culturing

Analysis parameters of wastewater from the 3rd fattener farm stabilization pond are shown in Table 1. BOD, COD, and TP exceeded the prescribed standards. Specifically, the BOD should not exceed 80 mg/L, the COD should not exceed 350 mg/L and the phosphorus content should not exceed 5 mg/L. These standards were set by the Ministry of Natural Resources and Environment to regulate wastewater discharge from sources related to pig farming in 2021. When efficient microorganisms (EM) were introduced into the wastewater the COD increased (Table 1). Microorganisms play a vital role in the breakdown and decomposition of complex organic compounds into forms more readily usable by plants, highlighting the potential suitability of employing biological processes for wastewater treatment.

Table 1 Characteristics of wastewater samples from the 3rd stabilization pond of the fattener farm before the experiment.

Treatment	pH	BOD (mg/L)	COD (mg/L)	SS (mg/L)	TKN (mg/L)	TP (mg/L)	TK (mg/L)
FS	8.10 (±0.00)	1950.00 (±0.00)	920.00 (±0.00)	0.30 (±0.04)	54.34 (±0.04)	212.19 (±0.74)	452.12 (±1.98)
FSEM	7.30 (±0.00)	1660.00 (±0.00)	1280.00 (±0.00)	0.50 (±0.02)	29.14 (±0.15)	284.30 (±11.62)	833.36 (±0.86)

Note: FS = Wastewater from the 3rd stabilization pond of the fattener farm
FSEM = Wastewater from the 3rd stabilization pond of the fattener farm treated with EM (Effective Microorganisms)

3.2 The ability of A. microphylla to grow in wastewater.

After 7 days of cultivation, the Azolla plants exhibited growth and uniform distribution. At 14 days of cultivation, there was noticeable growth and an increase in the number of Azolla, filling the surface of the container. In the group cultured in wastewater with the addition of EM, the Azolla had thicker, greener, and healthier-looking leaves. At the 21-day mark of cultivation, the growth rate remained consistent. Notably, the group cultured in wastewater containing effective microorganisms (EM) exhibited a fresher and greener appearance compared to the group without EM. Therefore, adding EM to the wastewater increased the decomposition of organic substances into nutrients that promoted the growth and proliferation of Azolla [23]. The Azolla continued to grow, with a shift in the color of the wastewater from the FS group toward yellowish. The group with EM addition maintained a vibrant green color (Figures 2 and 3).

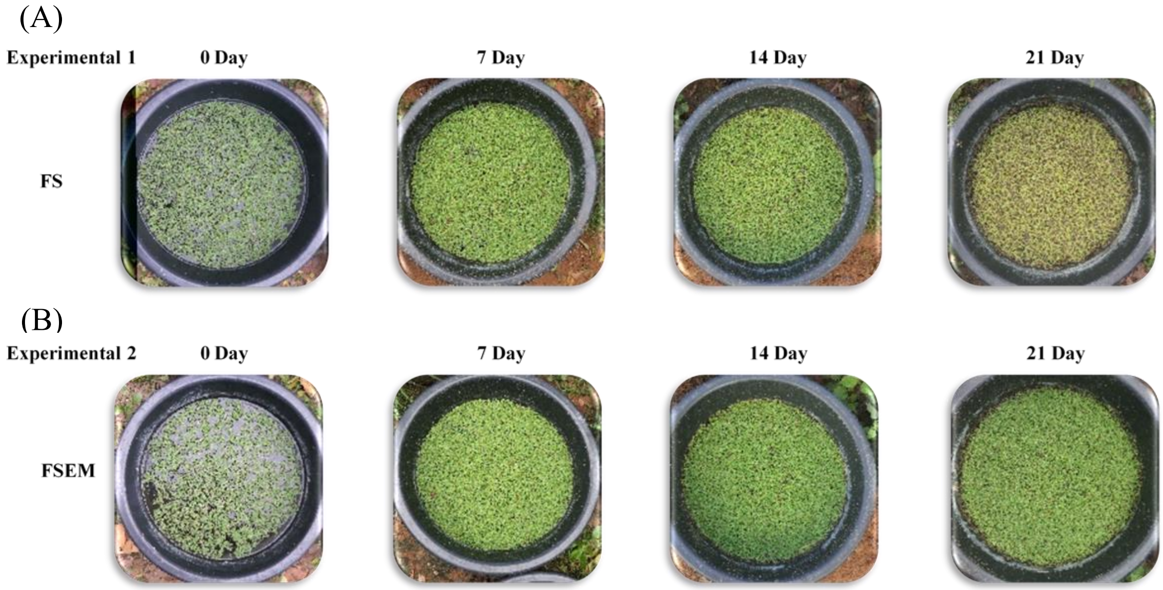


Figure 2 Growth of *A. microphylla* in wastewater from the fattener farm at 0, 7, 14, and 21 days. (A)Un-treated EM (B) Treated EM.

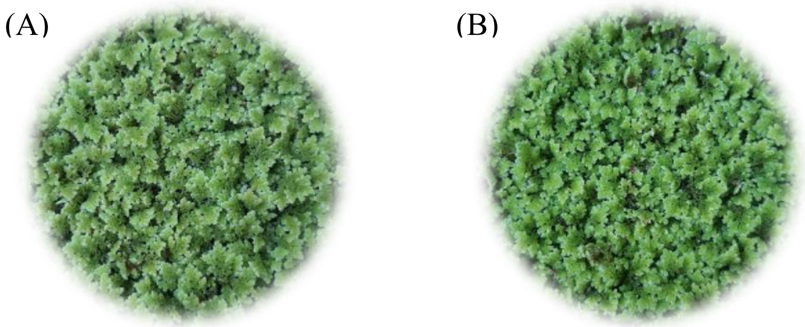
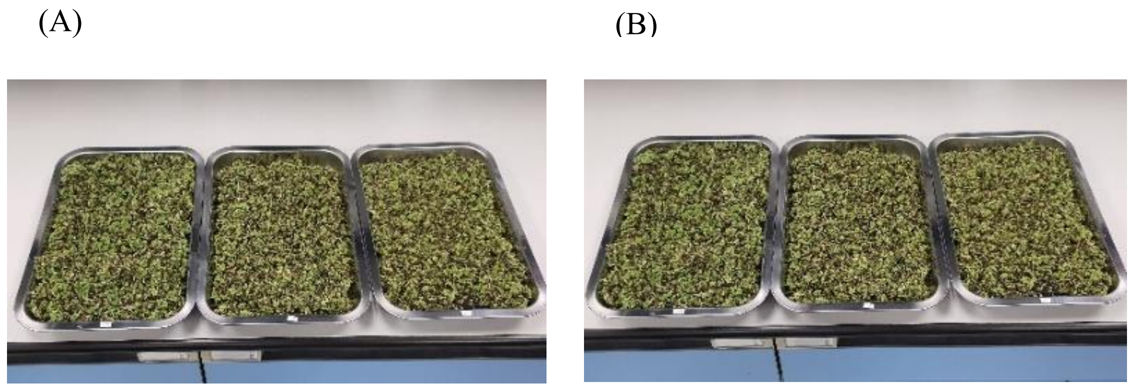


Figure 3 Characteristics of *A. microphylla* after 21 days of culture. (A) Un-treated EM (B) Treated EM.



4**Figure 4** Azolla after drying at 70-80 °C for 48 hours (A) Un-treated EM (B) Treated EM.

3.3 Growth rate of A. microphylla (DT, RGR, DM and Ash)

Fresh biomass of Azolla was harvested and weighed after 21 days after cultivation. The DT and RGR were calculated using fresh biomass values as 7.08, 5.92 days and 7.54, 10.00 g/day respectively (Figure 5). There was a significant difference between the groups ($p \leq 0.05$). Azolla grown in wastewater with the addition of EM exhibited a significantly superior growth rate and, consequently, lower dry matter and ash content compared to the group that did not receive EM treatment. The doubling time observed in this research aligned with Sawadee and Taengcham [24] and Chuenban and Chuenban [25] who recorded the doubling time of Azolla as 3 to 6 days.

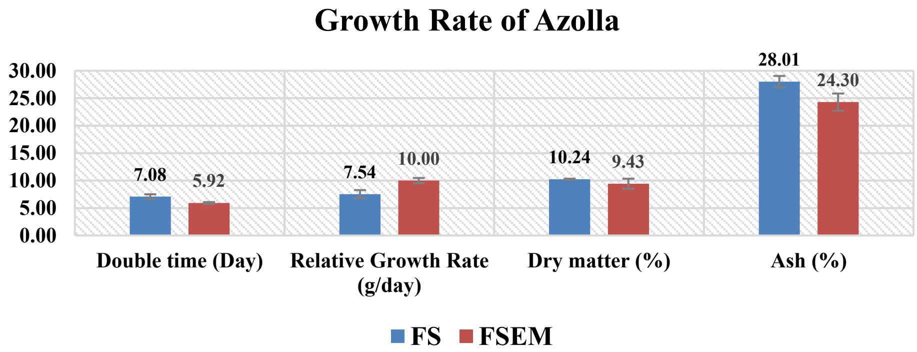


Figure 5 Growth rate parameters of *A. microphylla* cultured in wastewater from the fattener farm after 21 days.

3.4 Nutritional values of A. microphylla (TKN, Fat, Fiber, Ca, P, and K)

The nutrient analysis results for *A. microphylla* following cultivation in wastewater for nitrogen (TKN), calcium (Ca), phosphorus (TP), and potassium (K) (Figure 6) were 14.55, 16.22 %N, 0.13, 0.14 %Ca, 1.50, 1.75 %P, and 0.23, 0.26 %K, respectively. These results concurred with Lartdavong et al. [26] who presented the nitrogen value from Water Lettuce grown in wastewater from a pig farm at 17.64%. Azolla grown in wastewater from a pig farm was effective under certain conditions. Alalade & Lyayi [27] reported Ca and P contents of 0.16% and 1.29%, similar to the calcium content found in this experiment. The accumulation of minerals in Azolla is influenced by the concentration of mineral elements in the water, pH levels, temperature, and the availability of nutrient elements. The results also revealed fat and fiber contents of 1.88, 0.78%, and 12.12, 12.62%. Azolla demonstrated low fat levels, and fat accumulation was not correlated with efficient EM inoculation and growth rates. Azolla cultivated in nutrient-rich wastewater exhibited enhanced growth, resulting in thickened stems and increased water content. Alalade & Lyayi [27], Basak et al. [28], and Tamang & Samanta [29] reported fat contents in Azolla at 2.70%, 3.47%, and 2.70%, respectively. However, despite substantial growth, the nutrient fiber was relatively low. Dietary fiber values in this study concurred with Alalade & Lyayi [27], 12.70%, Basak et al. [28], 15.71%, Khatun et al. [30], 12.30%, and Tamang & Samanta [29], 12.70%.

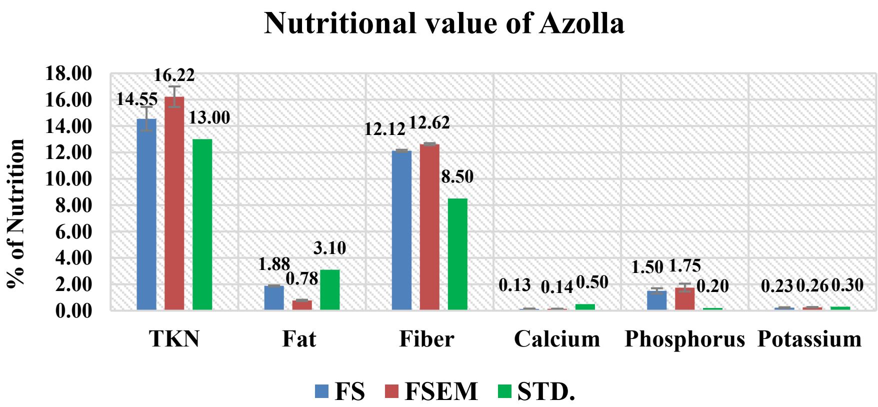


Figure 6 Nutritional values of *A. microphylla* in wastewater from the fattener farm after 21 days.

The nutrient analysis results for *A. microphylla* following cultivation in wastewater for nitrogen (TKN), calcium (Ca), phosphorus (TP), and potassium (K) (Figure 6) were 14.55, 16.22 %N, 0.13, 0.14 %Ca, 1.50, 1.75 %P, and 0.23, 0.26 %K, respectively. These results concurred with Lartdavong et al. [26] who presented the nitrogen value from Water Lettuce grown in wastewater from a pig farm at 17.64%. Azolla grown in wastewater from a pig farm was effective under certain conditions. Alalade & Lyayi [27] reported Ca and P contents of 0.16% and 1.29%, similar to the calcium content found in this experiment. The accumulation of minerals in Azolla is influenced by the concentration of mineral elements in the water, pH levels, temperature, and the availability of nutrient elements. The results also revealed fat and fiber contents of 1.88, 0.78%, and 12.12, 12.62%. Azolla demonstrated low fat levels, and fat accumulation was not correlated with efficient EM inoculation and growth rates. Azolla cultivated in nutrient-rich wastewater exhibited enhanced growth, resulting in thickened stems and increased water content. Alalade & Lyayi [27], Basak et al. [28], and Tamang & Samanta [29] reported fat contents in Azolla at 2.70%, 3.47%, and 2.70%, respectively. However, despite substantial growth, the nutrient fiber was relatively low. Dietary fiber values in this study concurred with Alalade & Lyayi [27], 12.70%, Basak et al. [28], 15.71%, Khatun et al. [30], 12.30%, and Tamang & Samanta [29], 12.70%.

3.5 The efficiencies of A. microphylla for water quality improvement

Introduction of *A. microphylla* resulted in substantial improvements in BOD, COD, TKN, P, and K levels under both experimental conditions. As shown in Table 2, BOD value decreased by 86.26% (1682.00 mg/L) and 75.45% (498.00 mg/L). COD value decreased by 70.58% (649.33 mg/L) and 73.44% (940.00 mg/L). TKN value decreased by 84.09% (45.69 mg/L) and 70.74% (20.61 mg/L). TP value decreased by 79.99% (137.20 mg/L) and 63.11% (179.41 mg/L), and TK value decreased by 65.01% (293.92 mg/L) and 83.67% (747.27 mg/L). The experimental results closely aligned with Chatchamni et al. (2013) [31] who used Wolffia for wastewater g et al.[26] used Water Lettuce and reported a reduction at 94.70%. Our experimental results indicated s more effective COD decrease than found by Klom Jek et al. in 2015 [32], who reported 35.40%. Chuenban and Chuenban [25] used Azolla for wastewater treatment and reported an efficiency of 50.00%, similar to the findings of Lartdavong et al. in 2021 [26] who used Water Lettuce and reported an efficiency of 88.92%. In 2013, Chatchamni et al. [31], used Wolffia for wastewater treatment and demonstrated an efficiency of nearly 100.00% for N value. Khom jek et al. (2022) [32], used *microphylla* for treatment and reported an efficiency of 51.50%, while Chuenban and Chuenban (2019) [25] reported an efficiency range of 70.00-76.00% using *A. microphylla*. A 2021 study by Lartdavong et al. [26] employed Water Lettuce for treatment and reported efficiency at 77.70%. Khom jek et al. (2022) [32] used *A. microphylla* and reported phosphate removal of 52.00%. Chuenban and Chuenban [25] used *A. microphylla* for treatment and reported an efficiency range of 65.00-74.80%. In the case of pH, Azolla absorbs organic matter from wastewater and utilizes CO_2 in the water for photosynthesis. Azolla takes in CO_2, HCO_2^-, CO_3^{2-}, $H^{+,}$ and OH from various chemical processes [33], resulting in an increase in pH [32] and suspended solids. Azolla exhibited the maximum growth rate between 7 and 14 days. After this period, the Azolla started to age, with mature roots and leaf petioles breaking off, contributing to increased suspended solids in the cultivation water. Overall, *A. microphylla* demonstrated significant potential for improving wastewater quality by reducing pollutants and enhancing water characteristics.

Table 2 Evaluation of the ability to improve the quality of wastewater from the fattener farm after 21 days of Azolla cultivation (% removal).

Variable	Wastewater/Day								
	FS					FSEM			
	STD	Day 0	Day 21	Removal	%	Day 0	Day 21	Removal	%
pH	5.5-9.0	8.10 (±0.00)	8.77 (±0.06)	+0.67	8.23	7.30 (±0.00)	7.93 (±0.06)	+0.63	8.68
BOD (mg/L)	≤ 80	1950.00 (±0.00)	268.00 (±19.00)	-1682.00	-86.26	660.00 (±0.00)	162.00 (±4.50)	-498.00	-75.45
COD (mg/L)	≤ 350	920.00 (±0.00)	270.67 (±6.43)	-649.33	-70.58	1280.00 (±0.00)	340.00 (±10.00)	-940.00	-73.44
SS (mg/L)	≤ 200	0.30 (±0.04)	1.40 (±0.02)	+1.10	373.03	0.50 (±0.04)	2.05 (±0.05)	+1.55	307.28
TKN (mg/L)	≤ 200	54.34 (±0.04)	8.65 (±0.18)	-45.69	-84.09	29.14 (±0.15)	8.53 (±0.27)	-20.61	-70.74
TP (mg/L)	≤ 5	212.19 (±0.74)	74.80 (±4.93)	-137.20	-79.99	284.30 (±11.62)	104.89 (±1.74)	-179.41	-63.11
TK (mg/L)	-	452.12 (±2.98)	158.20 (±2.38)	-293.92	-65.01	833.36 (±0.86)	136.09 (±2.18)	-747.27	-83.67

(–) – Performance of *A. microphylla* that can improve wastewater quality.
(+) – Performance of *A. microphylla* that cannot improve wastewater quality.

4. Conclusions

Results demonstrated that *Azolla microphylla* cultivation, utilizing effluent from fattener pig farms was effective for wastewater treatment. Treatment with *A. microphylla*, both with and without EM, significantly reduced BOD, COD, TKN, TP, and TK levels compared to the control group. Nutrient analysis of *A. microphylla* cultured in wastewater treated with EM showed enhanced N, P, K, and Ca content compared to the non-EM group. These findings suggest that *A. microphylla* has considerable potential as a means of wastewater treatment and a biofertilizer to promote sustainable agriculture while simultaneously mitigating environmental impacts.

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