Egyptian Journal of Aquatic Biology and Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 29(6): 17 – 38 (2025)
www.ejabf.journals.ekb.eg

Green Remediation Strategy Using *Azolla pinnata*: Heavy Metals Removal and Waste Water Ultra-Purification

Noha Abdelwahab[1]*, Amira Faysal[2], Marzouka Shaban[2], Asmaa A. Amin[2], Seham M. Hamed[3]

[1]Department of Microbiology, Soil, Water and Environment Research Institute (SWERI), Agricultural Research Center (ARC), Giza 12619, Egypt

[2] Beni-Suef Water and Sanitation Company, a subsidiary of the holding company of water and sanitation, Egypt

[3]Department of Biology, College of Science, Imam Mohammad Ibn Saud Islamic University (IMSIU), P. O. Box: 90950, Riyadh 11623, Saudi Arabia

*** Corresponding Author : abdelwahab.noha@yahoo.com**

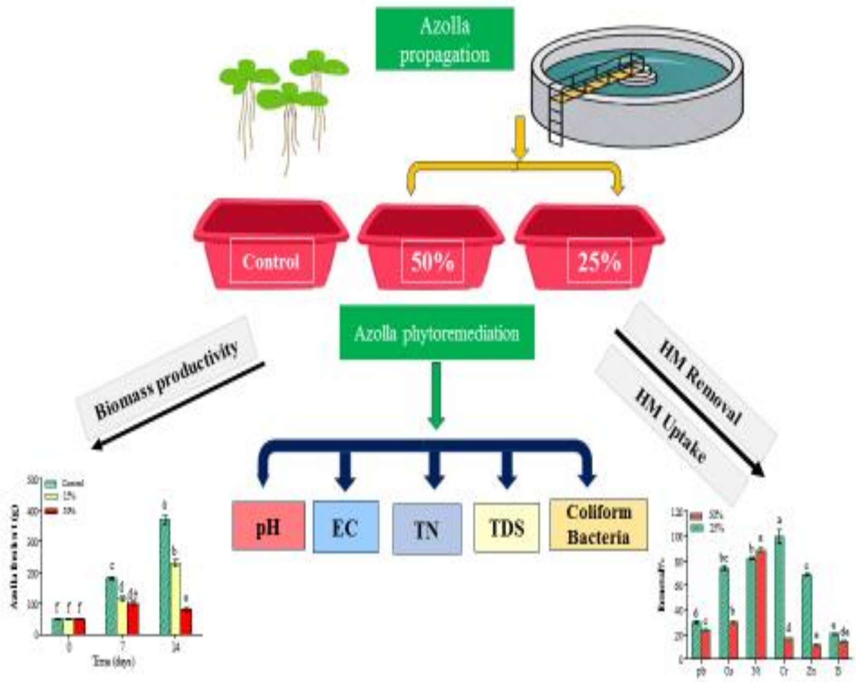
ARTICLE INFO

Article History:
Received: Aug. 30, 2025
Accepted: Oct. 14, 2025
Online: Nov. 3, 2025

Keywords:
Azolla pinnata,
Phytoremediation,
Heavy metal,
Municipal wastewater,
Water quality,
Biofuel production

ABSTRACT

Phytoremediation using aquatic macrophytes is a scalable and affordable technique. Hereby, this study aims to determine the potential of *Azolla pinnata* to enhance the chemical and biological properties of treated municipal wastewater from the Beni-Suef treatment plant, Dandil, as well as its capacity for heavy metal removal at two dilution ratios (25% and 50%). The propagation of *A. pinnata* for 14 days significantly increased pH, electrical conductivity, total dissolved solids, and total Kjeldahl's nitrogen in two tested concentrations, particularly at 50% by 8.5, 632.9, 627.7 and 1620%, respectively. However, the count of coliform bacteria was significantly decreased to less than 180 CFU/100 ml at 25 and 50% treatments. Water analysis showed that *A. pinnata* significantly decreased the concentration of Pb, Cu, Ni, Cr, Zn, and B. Biomass analysis implied differential affinity of heavy metals uptake and removal efficiency by *A. pinnata* biomass which is typically higher at 25% concentration. A 50% dilution ratio resulted in removal efficiencies of Ni (88.7%), Cu (30%), Pb (24%), Cr (17.2%), B (14.5%), and Zn (11.4%), whereas a 25% dilution ratio showed removal efficiencies of Cr (100%), Ni (82%), Cu (74%), Zn (68.5%), Pb (30%), and B (20.8%). Compared to 50% dilution, the use of 25% significantly increased fresh weight (230 g) and relative growth rate (and 0.043 g/day), and achieved the shortest doubling ~ 6 days after 14 days of growth. *A. pinnata* may serve as an effective and reliable phytoremediator for municipal wastewater, and its biomass could be a sustainable biorefinery platform for various biofuel industries.



Graphical abstract

INTRODUCTION

Anthropogenic activities in home, workplace, and agriculture produce a variety of organic and inorganic compounds that are suspended or dissolved in water. Today, over 1 billion people globally are impacted by freshwater shortages **(Muradov *et al.*, 2014)**. According to the results of the simulation model, Egypt's overall water demands are expected to increase from 78.40 billion cubic meters (BCM) in 2023 to 81.02 BCM in 2037. It is anticipated that the agricultural sector's water demand will drop from 61.30 BCM in 2023 to 60.14 BCM in 2037. However, the municipal sector will grow from 12.58 BCM in 2023 to 14.88 BCM in 2037 **(Esraa *et al*., 2023)**. Therefore, wastewater treatment is required to keep the water supply available to meet demand while lowering water pollution.

Several aquatic floating macrophytes have been proposed as bioagents for phytoremediation due to their rapid growth rates and ease of management, their ability to accumulate nutrients, heavy metals and harmful substances while enhancing water quality by controlling oxygen balance were studied **(Echiegu *et al*., 2021; Jayasundara, 2022; Nyein & Iwai, 2025)**. Among these macrophytes is *Azolla*, a rapidly growing, free-floating water fern. It can thrive without nitrogen (N) in the water due to a symbiotic relationship with the N_2-fixing cyanobacterium *Anabaena azollae* Strasburger (*A. azollae*) **(Mishra, 2023)**.

The phytoremediation potential of *A. pinnata* has been previously demonstrated in numerous studies which recommend its use for treating various types of wastewaters, including paint wastewater **(Echiegu *et al*., 2021)**, petroleum-polluted freshwater **(Mostafa *et al*., 2021)**, agricultural water **(Saad *et al*., 2022)**, sewage wastewater (Singh *et al.*, 2024) and textile dye wastewater **(Tasnim *et al*., 2024)**. In this context, *A. pinnata* R.Br. grown for 14 days in a 75% dilution of dairy wastewater, significantly reduced total phosphorus (65.37%), total Kjeldahl's nitrogen (73.25%), total dissolved solids (TDS) (71.56%), electrical conductivity (61.42%), and pH (9.41%) **(Goala *et al*., 2021; Singh *et al*., 2024)**. The application *A. pinnata* in aquaculture wastewater considerably decreased microbial counts by 50 -77% and heavy metal by 2.98 and 82.4% **(Adabembe *et al*., 2022)**.

A. pinnata successfully removed Cr (VI) from chromium-polluted water at concentration range (0.05 to 90 ppm) using a ratio of 25:1 (volume (mL): fresh weight of *Azolla* (g)) after 2 days incubation **(Soliman *et al*., 2025)**. The application of fresh *A. pinnata* covering 75% of the surface area had the maximum iron removal efficiency by 98.10% from sand mining waters **(Hasani *et al*., 2021)**. *A. pinnata* has been demonstrated to accumulate considerable amounts of Pb, Fe, Zn under greenhouse conditions after 20 days of incubation **(Mohamed & Elshahat, 2021)**. Additionally, the use of *A. filiculoides* as dry biomass showed high biosorption capacity of Pb, Cd, Cu and Zn by 228, 86, 62 and 48 mg/g, respectively **(Taghi Ganji *et al*., 2005)**.

Azolla spp. has been demonstrated to reduce eutrophication in streams and lakes along with the production of biomasses. *Azolla* has a doubling time of 2-4 days, indicating a high rate of growth. The pectin that makes up *Azolla*'s cell wall has a strong affinity for organic material adsorption. Thus, *Azolla* acts as a "biofilter" while wastewater is being treated **(Muradov *et al*., 2014)**.

Azolla spp. are rich source of growth promoting substances, minerals (e.g. Calcium, nitrogen, phosphorus, potassium, magnesium, copper and iron), beta-carotene, vitamins A, B12, essential amino acids, and proteins **(Riaz *et al*., 2022)**. Their low lignin content and high nutritional contents make them an excellent source of nutrients for animals. Also, *Azolla* with high phosphorus and nitrogen contents have been effectively used as a feed additive for both aquatic and terrestrial animals **(Nasir *et al*., 2022)** as well as a green manure in rice fields in Asia and Africa **(Kimani *et al*., 2025)**.

The heavy metal concentrations in *Azolla* biomass used for phytoremediation process make the extraction of these metabolites unsuitable for use as feed additive from an economic standpoint. Consequently, it is more reliable to convert this biomass into different bio-based energy carriers **(Christy *et al*., 2024; Sambasivam *et al*., 2024)**. In spite of extensive studies on the phytoremediation capability of *Azolla* species in various agricultural and industrial effluents, the relationship between wastewater concentration, pollutant removal efficiency, and biomass productivity of *A. pinnata* remains poorly understood. Previous studies often highlighted contaminant removal alone**,** without combining the dual objective of water quality improvement and biomass valorization for potential bioenergy applications. This study addresses these gaps by evaluating *A. pinnata* under local Egyptian wastewater conditions (Beni-Suef, Dandil) to determine the optimal conditions for both phytoremediation and biomass yield.

Accordingly, the main objectives of this study are (1) to assess the phytoremediation potential of *Azolla pinnata* cultivated in treated wastewater samples collected from the Beni-Suef wastewater treatment plant (Dandil), aiming to minimize the negative environmental impacts of nutrients and residual heavy metals in treated wastewater discharged into waterways, and (2) determine the optimum concentration of treated wastewater at which *A. pinnata* achieves its highest efficiency in both phytoremediation and biomass production.

MATERIALS AND METHODS

1. Experimental location

Phytoremediation experiment was conducted at the Wastewater Treatment Plant (WWTP), Beni-Suef governorate, Egypt. Beni-Suef Governorate is situated along the River Nile, about 120 kilometers south of Cairo, the capital city of Egypt, between latitudes 28˚45' and 29˚25' N and longitudes 30˚45' and 31˚15' E. The experiment was conducted in September 2024.

2. *Azolla* propagation

A healthy aquatic plant of *A. pinnata* was collected from the artificial pond located at Sids Agricultural Research Station (SARS), Beni-suef governorate, Egypt (Fig. 1) The research station is located at 29°3′58.06″ N latitude and 31°5′57.79″ E longitude, with an elevation of 32.2 meters above sea level, situated in a semi-arid region.

Fig. 1. *Azolla* propagation ponds at Sids Agricultural Research Station (SARS), ARC

3. Experimental design

The treated wastewater samples were collected from Beni-Suef wastewater treatment plant (Dandil). Samples were subsequently subjected to chemical analysis following standard methods for water and wastewater examination **(APHA, 2017)**. Ten litres of treated wastewater with concentrations of 50 and 25%, were placed in 15-liter plastic tubs (37.5cm in diameter, 45cm in length and 18.0cm in depth), filled with one kg soil. Conventional irrigation water was used for control conditions propagation and for adjusting dilution ratios, each concentration was conducted in three replicates throughout the study.

The phytoremediation experiment was conducted by introducing 50g of fresh, healthy *Azolla pinnata* at the early growth stage into the experimental media (standard control medium, 25 and 50% of treated municipal wastewater for fourteen days. Tubs were propagated under natural outdoor conditions of light and temperature and were placed in randomized design (Fig. 2).

Fig. 2. Fresh *A. pinnata* cultured in the plastic tubs using treated municipal wastewater at dilution ratio of 50% and 25%

4. Estimation of *Azolla* growth characteristics

The growth characteristics of *A. pinnata*, including biomass production, relative growth rate (RGR), and doubling time (Td), were assessed on the seventh and fourteenth days of its cultivation. The biomass production of *A. pinnata* was calculated based on fresh weight at certain time (t_n) and initial fresh weight at time zero (t_0). RGR was calculated as set in Eq. (1) **(Pabby *et al.*, 2001)**.

$$RGR = \frac{\log W_1 - W_0}{(t_1 - t_0)} \quad (1)$$

Doubling time (Td) was calculated using the following Eq. (2) **(Kannaiyan & Kumar, 2005)**:

$$Td = \frac{(t_1 - t_0) \times \log 2}{\mathrm{Log}\,(W_1/W_0)} \quad (2)$$

In both equations, t_0 = time initial (0 day); t_1 = time of harvest (days); W_0 = fresh *Azolla* biomass at initiation of experiment (grams); W_1 = fresh *Azolla* biomass at harvest time (grams).

5. Characterization of treated wastewater effluent

5.1. Chemical analysis

Water samples were collected from each plastic tub at time zero and after 14 days of phytoremediation experiments, and analyzed for a range of chemical, biological, and heavy metal analyses. The pH was measured using an electrometric pH meter (ORION 2

STAR). Electrical conductivity (EC) and total dissolved solids (TDS) were assessed using a conductivity meter (Jenway 4320).

5.2. Heavy metals analysis

Lead (Pb), copper (Cu), nickel (Ni), chromium (Cr), zinc (Zn) and boron (B) were determined in both treated water and in *Azolla* biomass at time zero and at the end of the experiment according to the method described by the **APHA (2017)**. 10mL of treated effluent water and 1.0g of air-dried *A. pinnata* were placed in separate digestion tubes. Then, 2– 3mL of concentrated HNO_3 was added to each tube, which was subsequently digested in an electrically heated block for 1 hour at 150°C. The mixture was cooled and was then filtered using Whatman No. 42 filter paper. The volume was adjusted to 50mL by adding 1% HNO_3 and was prepared for heavy metals analysis. Heavy metals were analysed using inductively coupled plasma (ICP), Perkin Elmer Model: ICP – OES Optima 7300 DV.

5.3. Biological analysis

Total coliform bacteria were counted in the treated water samples before and after remediation process using the membrane filter method, procedure number 9222B **(APHA, 2011)**.

6. Heavy metal removal efficiency (%)

The efficiency (%) of *A. pinnata* for removal of heavy metal was calculated using the Equation (1) **(Kumar *et al*., 2017; Kooh *et al*., 2018)**.

$$\text{Removal efficiency \%} = \frac{C_a - C_z \times 100}{C_a} \qquad \text{Eq.(1)}$$

Where, C_a represents the concentration of the parameter before phytoremediation, and C_z denotes the concentration of the parameter after phytoremediation.

7. Heavy metals uptake capacity

It quantifies the ability of *Azolla* plant to accumulate heavy metals from the surrounding environment, focusing on the change over time in the concentration of the metal in the plant biomass using equation (2) **(Arora *et al*., 2006; Kumar *et al*., 2018)**.

$$\text{Heavy metal uptake} = C_{after} - C_{before} \qquad \text{Eq. (2)}$$

Where, C_{after} and C_{before} represent concentration of metal in the plant after and before phytoremediation process

8. Statistical analysis

One-way ANOVA was used for statistical analysis using SPSS 20 software (IBM, USA). Dunkin's multiple range tests were used to statistically compare sample means at a significance level of $P < 0.05$. Data were expressed as the average of three replicates± S.E.

RESULTS

1. Water analysis

The phytoremediation potential of *A. pinnata* was investigated under control conditions (using irrigated water) and two different concentrations (25% and 50%) of treated municipal wastewater. The water quality was assessed using various chemical and biological measurements, including pH, EC, TDS, total nitrogen and total coliform bacteria count before introducing *A. pinnata* and after 14 days of cultivation with *A. pinnata* (Fig. 3). A significant reduction in pH was observed in both wastewater treatments compared with the control, declining by 5% and 8% in 25% and 50% treatments, respectively. In contrast, pH changes in the control medium were minimal (<3%) after 14 days of cultivation. Under control conditions, electrical conductivity (EC) and total dissolved solids (TDS) decreased by ~47% and ~38%, respectively, while total nitrogen content (TN) increased by 185%. In contrast, wastewater dilutions resulted in sharp increases. At 25% dilution, EC, TDS and TN significantly increased by 2940%, 2237%, and 6257%, respectively, relative to the control, and further by 3642%, 2802% and 9657%, at 50 % ($P < 0.05$). Biological analysis indicated that no coliform bacteria were detected under control conditions. Meanwhile, the concentration of coliform bacteria significantly decreased ($P < 0.05$). It dropped to below 180 CFU/100 ml in both the 25% and 50% diluted water. At the beginning of the experiment, the corresponding values were 210,000 and 380,000 CFU/100 ml, respectively.

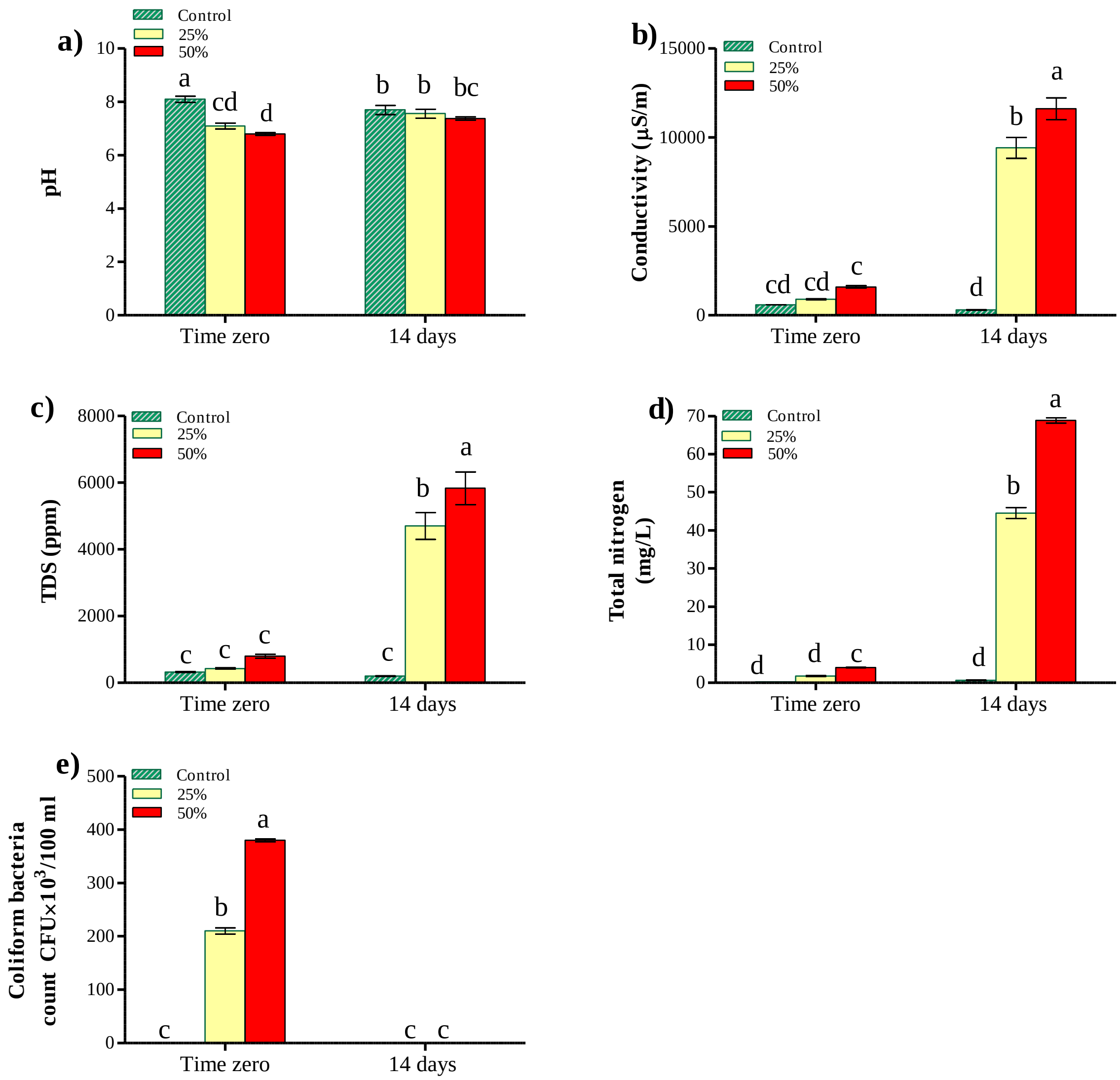


Fig. 3. Chemical and biological analysis of the control and treated municipal wastewater at dilution ratio of 50% and 25% at time zero and after 14 days of *A. pinnata* cultivation. Significant differences between treatments are denoted by lowercase letters ($P < 0.05$).

Water chemical analysis results in Fig. (4) indicate that heavy metals were below detection levels in the conventional irrigation water at control conditions. They occurred only in trace quantities, if present, throughout the duration of the experiment. In contrast, significant decreases in the concentration of the heavy metals Pb, Cu, Ni, Cr, Zn, and B were detected in the treated water over 14 days of phytoremediation, relative to their initial concentrations. For instance, Pb, Cu, and Ni decreased by 22.2%, 74%, and 82% at 25% concentration, and by 24%, 23.2%, and 88.7% at 50%, respectively (Fig. 4a–c).

Furthermore, Cr, Zn, and B showed significant reductions ($P < 0.05$). Their concentrations declined by 100%, 68.5%, and 20.8% at 25%, and by 17.4%, 11.7%, and 14.5% at 50%, respectively (Fig. 4d–f). These results suggest a differential affinity for heavy metal uptake by *A. pinnata,* which appears to be higher at 25% concentration.

2. Heavy metals uptake and removal by *A. pinnata*

The removal efficiency and intracellular heavy metals uptake in *A. pinnata* biomass are illustrated in Fig. (5). *A. pinnata* was found to effectively accumulate significant amounts of Pb, Cu, Ni, Cr, Zn, and B in their cells with varying concentration at the two tested dilution ratios. For example, B was the highest metal content (26.67 mg/kg), followed by Ni (15.29 mg/kg), Zn (13.74 mg/kg) and Cu (7.12 mg/kg) at 50% dilution ratio. At 25% concentration, B was also the most absorbed element (11.08 mg/kg), followed by Zn (7.5 mg/kg), Cu (2.79 mg/kg) and Ni (1.72 mg/kg). On contrary, Pb recorded the lowest ~~metal~~ uptake by 0.006 and 0.018 mg/kg at 25% and 50%, respectively.

Phytoremediation results revealed that *A. pinnata* showed differential removal efficiencies of heavy metals accordingly, Ni (88.7%) followed by Cu (30%), Pb (24%) Cr (17.2%) B (14.5%), and Zn (11.4%) at 50% dilution ratio. Meanwhile, at 25% dilution ratio, the removal efficiencies were varied as follows: Cr (100%), Ni (82%), followed by Cu (74%), Zn (68.5%), pb (30%) and B (20.8%). Obviously, the removal efficiency of Cr, Cu, Zn, Pb and B was higher at 25% dilution compared to 50% dilution.

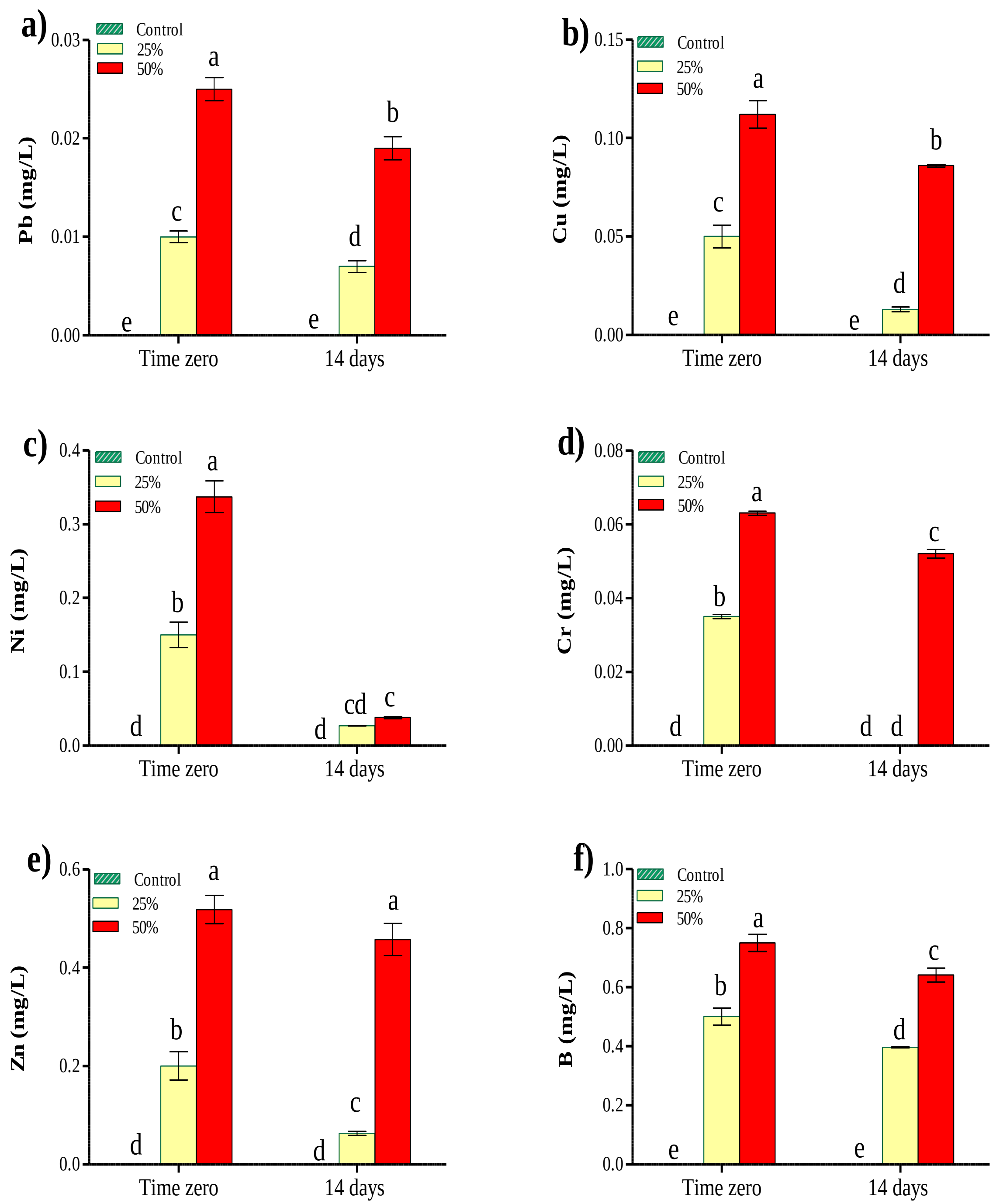


Fig. 4. Heavy metal analysis of treated wastewater at time zero and after 14 days of growing *A. pinnata* under control conditions and under dilution ratio of 50% and 25% of treated waste water. Significant differences between treatments are denoted by lowercase letters ($P< 0.05$).

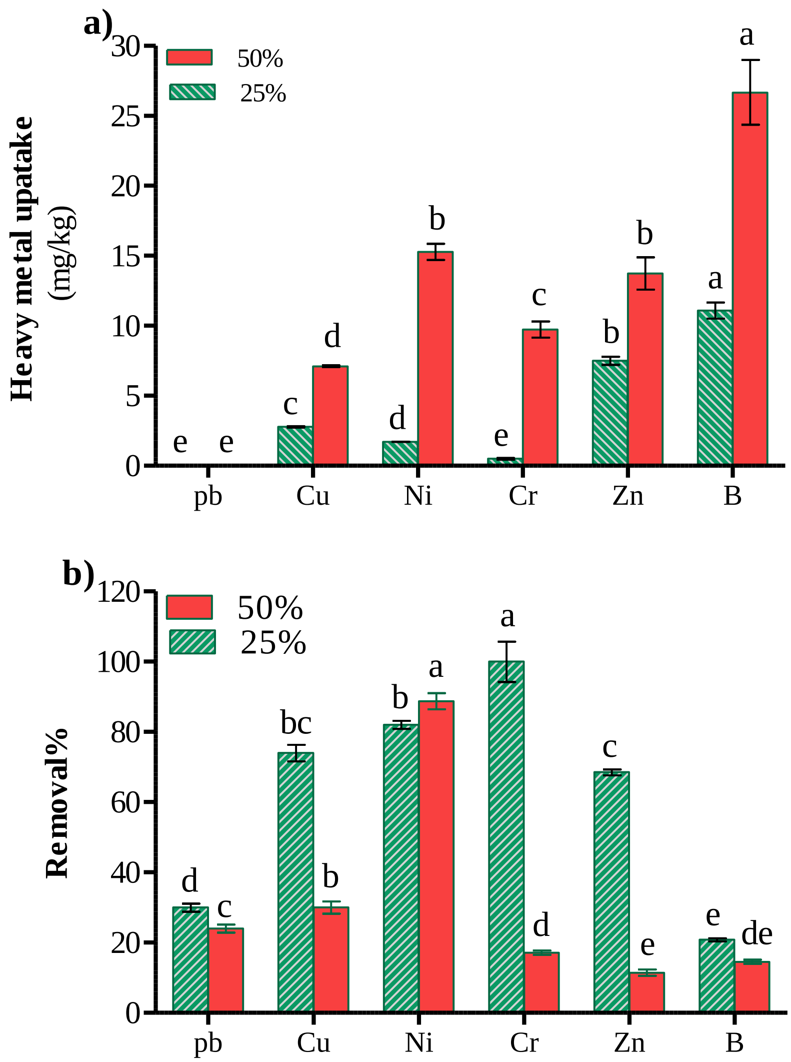


Fig. 5. Influence of dilution ratio of treated municipal wastewater on heavy metal uptake (mg/kg) and heavy metal removal (%) by *A. pinnata* biomass. Significant differences between treatments are denoted by lowercase letters ($P< 0.05$).

3. Impact of treated wastewater on *Azolla* growth parameters

The effects of treated wastewater (25% and 50%) on the growth parameters of *A. pinnata* compared to standard control conditions were evaluated at 7 and 14 days (Fig. 6). Results indicated that fresh weight of *A. pinnata* increased under all treatments during the 14-day period; however, wastewater dilutions caused pronounced growth inhibition compared with the control. At day 7, the control reached 180g, compared to

115 and 100g at 25% and 50% of wastewater, showing significant decreases by 36% and 44, respectively. By day 14, the control achieved the highest biomass (370g), whereas the 25% and 50% wastewater treatment achieved 230 and 80g, showing significant decreases by 38% and 78%, with respect to control conditions.

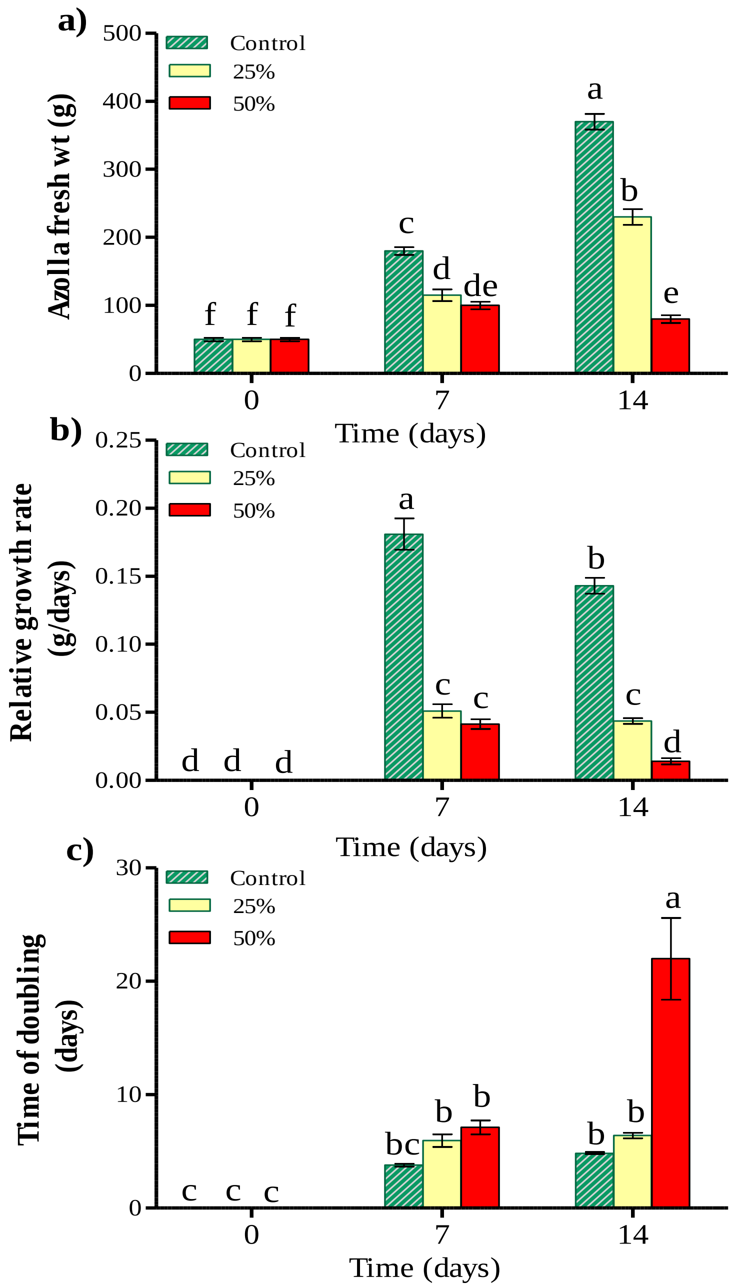


Fig. 6. Growth rate and biomass productivity of *A. pinnata* cultivated in standard control conditions or under dilution ratio of 50% and 25% of treated wastewater at time zero and after 14 days. Significant differences between treatments are denoted by lowercase letters (P< 0.05).

The relative growth rate (RGR) of *A. pinnata* markedly decreased under wastewater dilutions compared with the control. At day 7, the control had RGR of 0.18, whereas the 25% and 50% wastewater treatments recorded 0.05 and 0.04, corresponding to decreases of 72% and 76%, respectively. By day 14, the control maintained RGR of 0.14, while the 25% and 50% wastewater treatments dropped to 0.04 and 0.01, representing decreases of 69% and 90%, respectively, relative to the control. Overall, increasing the proportion of municipal wastewater resulted in progressive decreases in biomass and RGR values, particularly high at 50% dilution.

Results indicated that the doubling time of *A. pinnata* increased under wastewater dilutions compared to the control. At day 7, the control showed the shortest doubling time (3.79 days), while the 25% and 50% wastewater treatments recorded 5.96 and 7.13 days, respectively, indicating slower growth under wastewater conditions. By day 14, the control reached 4.85 days, compared to 6.40 days at 25% wastewater and a much longer doubling time of 21.98 days at 50% wastewater. Overall, higher wastewater concentrations substantially delayed biomass doubling, with the 50% dilution showing the most pronounced inhibitory effect.

The relative growth rate (RGR) of *A. pinnata* is significantly affected by wastewater dilution ratio and incubation period. For example, at the 7th day, RGR was 0.05g/ day at 25% compared to 0.04g/ day at 50%; this reduction was further increased at the 14th day when RGR recorded 0.014g/ day at 50% compared to 0.043g/ day at 25%. The shortest doubling time was approximately six days observed at 25% concentration compared to 22 days at the 50% concentration.

DISCUSSION

1. *A. pinnata* improved wastewater quality

The reuse of treated wastewater is increasingly recognized as a sustainable strategy for saving water resource, particularly in arid and semi-arid regions. It offers considerable economic benefits by reducing the pressure on freshwater resources and lowering irrigation costs **(Costa *et al*., 2025)**. Thus, the irrigation of trees and non-food crops could minimize the health risks and provides long-term sustainability **(Meiramkulova *et al.*, 2024)**. However, treated wastewater may still contain residual nutrients, pathogens, and trace levels of heavy metals. These substances, if not adequately managed, can accumulate in the soil over time or be taken up by plants, potentially posing environmental and health risks **(Shomar & Rovira, 2024)**. Therefore, additional purification steps or careful monitoring are essential to ensure that reused water meets safety standards and supports sustainable agricultural practices.

In the present study, we investigated the potential of *A. pinnata* in ultra-purification of treated municipal wastewater of Beni-Suef treatment plant, Dandil at two dilution levels compared to recommended control conditions. Our results indicated a positive impact of *A. pinnata* on decreasing density of coliform bacteria. The efficacy of *Azolla* sp. in the phytoremediation of wastewater, particularly in reducing coliform bacterial counts has been previously investigated. This reduction is primarily attributed to the antibacterial and antioxidant properties of *Azolla* **(Nayak *et al*., 2015)**, which inhibit microbial growth and contribute to improved water quality. For example, *A. caroliniana* achieved a 100% removal rate of *Escherichia coli* and total coliforms in treated wastewater, highlighting its potential as a natural disinfectant agent **(León *et al.*, 2018)**.

Our results indicated significant increases in pH, total dissolved solids (TDS), electrical conductivity (EC), and total nitrogen content after 14 days of *A. pinnata* growth, which contrasts with previous reports **(Echiegu *et al*., 2021; Goala *et al*., 2021; Tasnim *et al*., 2024)**. These increases can be attributed to the release of ammonium, phosphate, and potassium ions from *A. pinnata* biomass during decomposition, as well as the enhanced microbial activity. These processes lead to higher concentrations of dissolved salts in the water, which in turn increase the EC levels. Additionally, the metabolic byproducts of microbial communities associated with *A. pinnata* may contribute additional ionic species to the aquatic phase which further influencing conductivity **(Mahdavi *et al*., 2021)**.

Moreover, the innate symbiotic association between *A. pinnata* and the nitrogen-fixing cyanobacterium *Anabaena azollae* significantly increases the total nitrogen content in aquatic systems. This in turn enriches the surrounding environment with bioavailable nitrogen compounds, thereby enhancing the fertility of treated wastewater and supporting plant growth in reuse applications **(Akhtar *et al*., 2021; Abd El-Aal, 2022)**. Additionally, the type and condition of the wastewater significantly influence its chemical and physical properties, thereby affecting the efficiency and outcomes of phytoremediation processes. Though previous studies have primarily utilized dairy wastewater **(Goala *et al*., 2021)**, paint wastewater **(Echiegu *et al*., 2021)**, or textile **(Tasnim *et al*., 2024)** characterized by high organic loads and variable pollutant profiles, in the current study we employed treated municipal wastewater, which tends to have more consistent and regulated parameters.

2. *A. pinnata* decreased heavy metal content

The water analysis after 14 days of propagation in 25 and 50% diluted wastewater indicated significant decreases in the concentration of the heavy metals Pb, Cu, Ni, Cr, Zn, and B compared to their initial concentrations. Consistently, the application of *A. pinnata* in untreated and treated water from the constructed wetland for 5 days achieved heavy metal removal efficiencies within a range of 2.98 - 82.4% **(Adabembe *et al*., 2022)**. The phytoremediation potential of *A. pinnata* using emulsion paint wastewater indicated heavy metals reduction range from 11.0 to 92.5% **(Echiegu *et al*., 2021)**. *A. pinnata* significantly decreased Fe concentration in water by 98.10%, depending on cover area **(Hasani *et al*., 2021)**. In this context, *A. pinnata* achieved maximum removal efficiency of Fe (87%) and Zn (81.14%) after 20 days of incubation and had greater efficiency than *Lemina minor* in removing Pb with a considerable biomass productivity **(Mostafa & Hegazy, 2021)**. *A. pinnata* significantly decreased Cr (VI) from chromium-polluted water by 70% at a concentration of 0.1ppm **(Soliman *et al*., 2025)**.

The biomass analyses were consistent with those of water analyses where *A. pinnata* was found to effectively accumulate significant amounts of Pb, Cu, Ni, Cr, Zn, and B with varying concentration at the two tested concentrations, showing heavy removal efficiencies of 20.8-100% at 25% and 11.4%- 88.7 at 50% dilution ratios. This demonstrates that *A. pinnata* is capable of metal biosorption and bioaccumulation via complexation with thiol-rich compounds, intracellular sequestration, and surface binding sites **(Goala, *et al*., 2025)**.

Results also denoted significant metal uptake and removal efficiency at low concentration where, the removal efficiency of Cr, Cu, Zn, Pb and B was higher at 25% dilution compared to 50% dilution. This can be explained by the fact that moderate pollution loads induce ideal physiological stress that stimulates metal absorption mechanisms, whereas greater pollutant concentrations may hinder growth and reduce biosorption effeciency **(Zeid *et al*., 2024)**. In fact, variable factors such as metal valency, the number of active binding sites, and the types of functional groups present on the cell surface could affect the differential affinities of heavy metal uptake by aquatic macrophytes **(Rahman & Hasegawa, 2011)**. Moreover, the rates at which the metal ion forms complexes with the thiol compounds in cell such as glutathione and phytochlatins **(Hamed *et al*., 2024)**. Metal oxidation state, levels of metal chelators and antioxidant enzymes influence metabolism and detoxification pathway in aquatic macrophytes **(Talebi *et al*., 2019)**. Aquatic plant and macrophytes have been proposed to accumulate heavy metals by passive uptake mechanisms through aquaporins/aquaglyceroporins **(Rahman *et al*., 2011; Hamed *et al.*, 2022)**. Although certain heavy metals like As (V) inter cell via active uptake using phosphate uptake carriers of plasma membrane. Thereby, increasing phosphate concentrations in the solution are expected to enhance the desorption of analogous heavy metal ions **(Rahman *et al*., 2007)**.

3. Dilution factor of wastewater influences *A. pinnata* growth attributes

Treated wastewater may serve as rich source of residual organic matter nutrient and considerably low level of heavy metals. In this regard, the 25% dilution ration achieved greater fresh weight and relative growth rate along with the shortest doubling time compared to 50%. High concentrations of pollutants and heavy metals negatively affected the growth of *Azolla* at the 50% dilution ratio. Similar finding was reported by **Khosravi et al. (2005)**, who observed growth inhibition and limited biomass production of *Azolla filiculoides* exposed to elevated levels of Pb, Cd, Ni and Znsu. Plant phytotoxicity and low biomass production were observed in *A. japonica* and *A. pinnata* exposed to combined heavy metal stress **(Akhtar *et al*., 2019)**. They added that the excessive uptake of heavy metal impairs physiological functions of cell and hinder the proliferation of aquatic macrophytes due to over intracellular accumulation of heavy metals by *A. japonica* and *A. pinnata* **(Akhtar *et al*., 2019)**.

4. Fate of *A. pinnata* biomass after phytoremediation

Based on results of biomass analysis, produced biomass is no longer suitable for animal fodder or nutraceutical industries due to high heavy metal concentration. The use of aquatic macrophytes as a feedstock for biofuel production has primarily focused on the saccharification of their cell walls to produce fermentable sugars, which can then be converted into value-added products or ethanol **(Christy *et al*., 2024)**. In addition, technologies such as hydrothermal carbonization and pyrolysis could be a good strategy for production of phenolic compounds and aromatic hydrocarbons (e.g. pyrrole derivatives, pyridine, benzene, and phenol) **(Babinszki *et al.*, 2020**) or *in-situ* transesterification with acid catalyst for biodiesel production **(Sambasivam *et al*., 2024)** may attract researcher for future work. For the first time, this study clearly demonstrates the potential of *A. pinnata* for heavy metal removal and water purification in treated effluent from the Beni-Suef wastewater treatment plant in Dandil. Moreover, its biomass could serve as a sustainable and cost-effective bioresource for future biofuel production.

CONCLUSION

This study identifies *A. pinnata* as a promising bioagent for advanced wastewater treatment, owing to its fast growth and substantial biomass yield. It effectively reduces coliform bacteria and accumulates various heavy metals, including Pb, Cu, Ni, Cr, Zn, and B. Its symbiosis with nitrogen-fixing cyanobacteria further enhances nutrient absorption, making it ideal for large-scale use in contaminated water systems. Moreover, the resulting biomass holds potential as an eco-friendly and economical resource for biofuel production. The findings highlight the importance of further research into safe and sustainable utilization of metal-enriched biomass, alongside improving its remediation performance.

ACKNOWLEDGEMENTS

The authors wish to thank Soils, Water and Environment Research Institute (SWERI), Sids Agricultural Research Station (SARS), Agriculture Research Centre

(ARC), Giza, Egypt and Wastewater Treatment Plant (WWTP), Beni-Suef Governorate, Egypt, for facilities provided to conduct this research work.

REFERENCES

Abd El-Aal, A.A. (2022). *Anabaena-azollae*, significance and agriculture application: A case study for symbiotic cyanobacterium. In: *Microbial Syntrophy-Mediated Eco-Enterprising*. Academic Press, 1-14.

Adabembe, B.A.; Fasinmirin, J.T.; Olanrewaju, O.O.; Dada, A.A. and Faloye, O.T. (2022). Phytoremediation of aquaculture wastewater using *Azolla pinnata* and evaluation of its suitability for irrigation purpose. *Sustainable Water Resources Management*, *8*(5), 166.

Akhtar, M.S.; Oki, Y.; Bich, B.T.N. and Nakashima, Y. (2019). Estimation of phytofiltration potential for Cu and Zn and relative growth response of *Azolla japonica* and *Azolla pinnata*. *Journal of Agricultural Science and Technology*, *21*(4), 895-909.

Akhtar, M.; Sarwar, N.; Ashraf, A.; Ejaz, A.; Ali, S. and Rizwan, M. (2021). Beneficial role of *Azolla* sp. in paddy soils and their use as bioremediators in polluted aqueous environments: implications and future perspectives. *Archives of Agronomy and Soil Science*, *67*(9), 1242-1255.

APHA. (2011). *Standard Methods for the Examination of Water and Wastewater* (21st ed.). American Public Health Association.

APHA. (2017). *Standard Methods for the Examination of Water and Wastewater* (23rd ed.). American Public Health Association.

Arora, A.; Saxena, S. and Sharma, D.K. (2006). Tolerance and phytoaccumulation of chromium by three *Azolla* species. *World Journal of Microbiology and Biotechnology*, *22*(2), 97–100.

Babinszki, B.; Jakab, E.; Sebestyén, Z.; Blazsó, M.; Berényi, B.; Kumar, J. and Czégény, Z. (2020). Comparison of hydrothermal carbonization and torrefaction of azolla biomass: Analysis of the solid products. *Journal of Analytical and Applied Pyrolysis*, *149*, 104844.

Christy, E.J.S.B.A.; Wickramasinghe, I.; Kapilan, R. and Wijesekara, I. (2024). Enhancing bioethanol production from *Azolla filiculoides* through optimization of pretreatment and culture conditions with *Saccharomyces cerevisiae*. *Journal of Agricultural Sciences–Sri Lanka*, *19*(1).

Costa, C.S.; Carlos, C.; Oliveira, A.A. and Barros, A.N. (2025). Reuse of Treated Wastewater to Address Water Scarcity in Viticulture: A Comprehensive Review. *Agronomy*, *15*(4), 941.

Echiegu, E.A.; Ezimah, C.O.; Okechukwu, M.E. and Nwoke, O.A. (2021). Phytoremediation of emulsion paint wastewater using *Azolla pinnata*, *Eichhornia crassipes* and *Lemna minor*. *Nigerian Journal of Technology*, *40*(3), 550-557.

Esraa, E.; Mohamed, N.; Fatma, S. and Ahmed, B. (2023). WEAP analysis for enhancing water resource sustainability in Egypt: a dynamic modeling approach for long-term planning and management. *HBRC Journal*, *19*(1), 253-274.

Goala, M.; Bachheti, A. and Kumar, V. (2025). A comprehensive review of recent advances in phytoremediation of wastewaters using *Azolla* species. *3 Biotech*, *15*(8), 238.

Goala, M.; Yadav, K.K.; Alam, J.; Adelodun, B.; Choi, K.S.; Cabral-Pinto, M.M. and Shukla, A.K. (2021). Phytoremediation of dairy wastewater using *Azolla pinnata*: Application of image processing technique for leaflet growth simulation. *Journal of Water Process Engineering*, *42*, 102152.

Hamed, S.M.; Al-Nuaemi, I.J.; Korany, S.M.; Alsherif, E.A.; Mohamed, H.S. and AbdElgawad, H. (2022). Hazard assessment and environmental fate of propiconazole degradation by microalgae: differential tolerance, antioxidant and detoxification pathway. *Journal of Environmental Chemical Engineering*, *10*(4), 108170.

Hamed, S.M.; Ali, H.S.; Mohamed, M.Y.A.; Alammari, B.S. and AbdElgawad, H. (2024). Toxicity of mercuric oxide nanoparticles on freshwater microalgae: Comprehensive analysis on their interactive effects and detoxification pathways. *Journal of Water Process Engineering*, *57*, 104583.

Hasani, Q.; Pratiwi, N.T.M.; Effendi, H.; Wardiatno, Y.; Raja Guk Guk, J.A.; Maharani, H.W. and Rahman, M. (2021). *Azolla pinnata* as phytoremediation agent of iron (Fe) in ex sand mining waters. *CMU Journal of Natural Sciences*, *20*(1), e2021017.

Jayasundara, P. (2022). Wastewater treatment by Azolla: a review. *Diyala Agricultural Sciences Journal*, *14*(1), 40-46.

Kannaiyan, S. and Kumar, K. (2005). *Azolla Biofertilizer for Sustainable Rice Production*. Daya Publishing House.

Khosravi, M.; Taghi Ganji, M. and Rakhshaee, R. (2005). Toxic effect of Pb, Cd, Ni and Zn on *Azolla filiculoides* in the international Anzali wetland. *International Journal of Environmental Science & Technology*, *2*, 35-40.

Kimani, S.M.; Sekishita, N.; Hosogoe, Y.; Kaustar, V.; Tawaraya, K.; Tokida, T. and Cheng, W. (2025). Successive incorporation of *Azolla* (*A. filiculoides* Lam.) as green manure increases rice yield in lowland paddy soil. *Soil Science and Plant Nutrition*, 1-8.

Kooh, M.R.R.; Lim, L.B.; Lim, L.H. and Malik, O.A. (2018). Phytoextraction potential of water fern (*Azolla pinnata*) in the removal of a hazardous dye, methyl violet 2B: artificial neural network modelling. *International Journal of Phytoremediation*, *20*(5), 424–431.

Kumar, V.; Othman, N. and Asharuddin, S. (2017). Applications of natural coagulants to treat wastewater − a review. *MATEC Web of Conferences*, *103*, 06013.

Kumar, V.; Singh, J. and Chopra, A.K. (2018). Assessment of plant growth attributes, bioaccumulation, enrichment, and translocation of heavy metals in water lettuce (*Pistia stratiotes* L.) grown in sugar mill effluent. *International Journal of Phytoremediation*, *20*(5), 507–521.

León, R.; Pernía Santos, B.M.; Siguencia, R.; Franco, S.; Noboa, A. and Cornejo, X. (2018). Potential of aquatic plants for the removal of total coliforms and *Escherichia coli* in wastewaters. *Enfoque UTE*, *9*(4).

Mahdavi, M.; Naseri, S.; Yunesian, M.; Mahvi, A. and Alimohaadi, M. (2021). Modeling of wastewater treatment by *Azolla filiculoides* using response surface methodology. *Environmental Science and Pollution Research*, *28*(4), 4567–4578.

Meiramkulova, K.; Kydyrbekova, A.; Kydyrbekova, A.; Daldabayeva, G.; Bauyrzhan, O.; Shegenbayev, A. and Mkilima, T. (2024). Evaluating the Long-Term Effects of Recycled Wastewater Irrigation on Soil Health, Crop Yield, and Ecological Sustainability in Arid Regions. *Journal of Ecological Engineering*, *25*(12).

Mishra, N. (2023). Cyanobacterial symbiotic interaction in pteridophytes. In: *Microbial Symbionts*. Academic Press, 3-13.

Mohamed, S. and Elshahat, R. (2021). Accumulation of Iron, Zinc and Lead by *Azolla pinnata* and *Lemna minor* and activity in contaminated water. *Egyptian Journal of Chemistry*, *64*(9), 5017-5030.

Mostafa, A.A.; Hegazy, A.K.; Mohamed, N.H.; Hafez, R.M.; Azab, E.; Gobouri, A.A. and Mustafa, Y.M. (2021). Potentiality of *Azolla pinnata* R. Br. for phytoremediation of polluted freshwater with crude petroleum oil. *Separations*, *8*(4), 39.

Muradov, N.; Taha, M.; Miranda, A.F.; Kadali, K.; Gujar, A.; Rochfort, S. and Mouradov, A. (2014). Dual application of duckweed and azolla plants for wastewater treatment and renewable fuels and petrochemicals production. *Biotechnology for Biofuels*, *7*, 1-17.

Nasir, N.A.N.M.; Kamaruddin, S.A.; Zakarya, I.A. and Islam, A.K.M.A. (2022). Sustainable alternative animal feeds: Recent advances and future perspective of using azolla as animal feed in livestock, poultry and fish nutrition. *Sustainable Chemistry and Pharmacy*, *25*, 100581.

Nayak, N.; Padhy, R.N. and Singh, P.K. (2015). Evaluation of antibacterial and antioxidant efficacy of the fern *Azolla caroliniana* symbiotic with the cyanobacterium *Anabaena azollae*. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*, *85*, 555-569.

Nouri, T.; Bouyahia, H.; Amari, Z.; Arbaoui, D.; Mekhloufi, M.B. and Badji, A. (2020). Growth and biochemical response of *Azolla Caroliniana* wild to soluble

NPK fertilizers. *Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii*, *30*(4), 193–199.

Nyein, N. and Iwai, C.B. (2025). Using *Azolla microphylla* in investigating different agro-wastewaters treatment and its biomass growth for carbon sequestration. *Results in Engineering*, *25*, 103865.

Pabby, A.; Dua, S. and Ahluwalia, A.S. (2001). Changes in ammonia-assimilation enzymes in response to different nitrate levels in *Azolla pinnata* and *A. microphylla*. *Journal of Plant Physiology*, *158*(7), 899–903.

Rahman, M.A. and Hasegawa, H. (2011). Aquatic arsenic: phytoremediation using floating macrophytes. *Chemosphere*, *83*(5), 633-646.

Rahman, M.A.; Hasegawa, H.; Ueda, K.; Maki, T.; Okumura, C. and Rahman, M.M. (2007). Arsenic accumulation in duckweed (*Spirodela polyrhiza* L.): a good option for phytoremediation. *Chemosphere*, *69*(3), 493-499.

Rahman, M.A.; Kadohashi, K.; Maki, T. and Hasegawa, H. (2011). Transport of DMAA and MMAA into rice (*Oryza sativa* L.) roots. *Environmental and Experimental Botany*, *72*(1), 41-46.

Riaz, A.; Khan, M.S.; Saeed, M.; Kamboh, A.A.; Khan, R.U.; Farooq, Z. and Farid, M.U. (2022). Importance of Azolla plant in poultry production. *World's Poultry Science Journal*, *78*(3), 789-802.

Saad, F.N.M.; Remlee, N.N.; Zakarya, I.A.; Kamaruddin, M.A.; Izhar, T.N.T.; Ibrahim, M.H. and Syafiuddin, A. (2022). Phytoremediation potential of *Azolla pinnata* on water quality and nutrients from agricultural water. *Desalination and Water Treatment*, *271*, 38-47.

Sambasivam, K.; Kuppan, P.; Laila, L.S.; Rajamohan, N. and Abinandan, S. (2024). Valorizing *Azolla pinnata* biomass for sustainable bioenergy production: a novel catalyst transesterification approach. *Waste and Biomass Valorization*, 1-10.

Shomar, B. and Rovira, J. (2024). Human health risk assessment associated with the reuse of treated wastewater in arid areas. *Environmental Pollution*, *345*, 123478.

Singh, J.; Soni, S.K. and Yadav, P. (2024). Potential of *Azolla pinnata* for phytoremediation of sewage waste water. *Sustainability, Agri, Food and Environmental Research*, *12*(2).

Soliman, E.R.; Moustafa, K.; Khamis, M. and Shedeed, Z.A. (2025). Chromium"(VI)" phytoremediation using *Azolla pinnata*: effects on *Vicia faba* growth, physiology, cytogenetics, and gene expression profiling. *BMC Plant Biology*, *25*(1), 160.

Taghi Ganji, M.; Khosravi, M. and Rakhshaee, R. (2005). Biosorption of Pb, Cd, Cu and Zn from the wastewater by treated *Azolla filiculoides* with H_2O_2/$MgCl_2$. *International Journal of Environmental Science & Technology*, *1*, 265-271.

Talebi, M.; Tabatabaei, B.E.S. and Akbarzadeh, H. (2019). Hyperaccumulation of Cu, Zn, Ni, and Cd in *Azolla* species inducing expression of methallothionein and phytochelatin synthase genes. *Chemosphere,* *230*, 488-497.

Tasnim, N.; Das, E.J.; Tania, K.A.; Bhuiyan, M.A.R. and Swarna, F.A. (2024). Assessment of the efficacy of *Azolla pinnata* in improving textile dye wastewater quality at varying concentrations. *Bioremediation Journal*, 1-14.

Zeid, I.; Ghaly, E.K. and Shedeed, Z.A. (2024). *Azolla pinnata* as a phytoremediator: improves germination, growth and yield of maize irrigated with Ni-polluted water. *Scientific Reports*, *14*, 22284.