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Phytoremediation capability of *Azolla pinnata* for the removal of malachite green from aqueous solution

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ORIGINAL RESEARCH ARTICLE

ABSTRACT

Phytoremediation potential of *Azolla pinnata* (AP) was investigated for the remediation of malachite green (MG) dye from aqueous solution. This study involved the use of artificial neural network (ANN) as one of the mathematical tools for modelling the experimental data which showed good accuracy (R=0.867). The parameters of this study included the pH, initial dye concentration and plant weight. The optimum pH for phytoremediation of MG was in the range of 4-5, with reasonably high removal efficiencies of 81 - 84%. Reusability studies of spent AP showed good removal efficiency of 75% in the second cycle indicating its usefulness in repeated batch treatment. The growth estimation as indicated by relative frond number (RFN) showed that AP can tolerate a concentration of up to 15 mg/L MG.

KEYWORDS
artificial neural network; *Azolla pinnata*; biotechnology; malachite green; phytoremediation

1. INTRODUCTION

Industrialisation and urbanisation are associated with an increased level of pollutants (Kaur et al., 2016). Among the different types of environmental pollution, water pollution is one of the major problems for most countries. Pollutants may enter water bodies as leachates or through the improper disposal of industrial wastes which may include pesticides, heavy metals, textile wastes, inorganic anions and radioactive compounds. Textile industry is one of the most water intensive industries where a textile mill with a production rate of 8 tonnes per day uses 1.6 million liters of freshwater and its dyeing department can generate 15% of the total wastes (Kant, 2012). Many methods are available for the remediation of textile wastes which include the use of oxidising/reducing agents, ozonation, adsorption, membrane filtration, electrodegradation, photodegradation and phytoremediation (Crini, 2006; Dahri et al., 2014; Vijayaraghavan et al., 2015; Kooh et al., 2016a; Vijayaraghavan, 2016).

In recent years, phytoremediation has emerged as an alternative approach for wastewater remediation (Dhir, 2013). Phytoremediation is defined as the use of plants to remediate contaminated soil, sediments and water. This method involves the use of plant root systems which have the ability to translocation, bio-accumulate and degrade pollutants (Dhir, 2013). Phytoremediation is considered as a green technology since the whole remediation process is driven by solar energy and it is also carbon neutral as it does not involve electricity or fossil fuel (Dietz and Schnoor, 2001).

In this study, *Azolla pinnata* (AP) was investigated as a potential plant species for the phytoremediation of malachite green (MG) wastewater. AP is a water fern with fast growth and proliferative ability, and can thrive in water with little nutrient (Brouwer et al., 2014). AP forms a symbiotic

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relationship with *Anabaena azollae*, a nitrogen-fixing bacteria which can convert nitrogen gas from the air into nitrogen source that can be assimilated by the plant. In addition, AP is also more tolerant to environmental stress. In general, at the same level of salinity, *Azolla* has a better survival rate than other common floating aquatic plants such as *Lemna minor* (duckweed), *Pistia stratiotes* (water lettuce) and *Eichornia crassipes* (water hyacinth) (Haller et al., 1974).

There are numerous studies on the phytoremediation of heavy metals such as Hg, Cd, Zn and Pb using AP (Jain et al., 1990; Rai, 2008). However there are currently no studies involving the use of AP in the phytoremediation of dyes. In our previous studies, AP was investigated as a potential low-cost material for remediation of triphenylmethane dye (Kooh et al., 2016b), xanthene dye (Kooh et al., 2016d) and acid dye (Kooh et al., 2015) by adsorption, which is different from phytoremediation.

MG is chosen as a focus of this study due to its vast application as colouring agent and it is widely used in the dyeing of wool, leather, silk and cotton (Mittal, 2006). Apart from its colouring purposes, MG is also used as antiparasitic agents in aquaculture industry for the control of parasites residing in the guts of aquatic fauna (Schmahl et al., 1991). The antifungal property of MG is useful for preserving animal feed (Kleter et al., 2009; Schmahl et al., 1991). Ironically, MG is also highly toxic to aquatic fauna where toxicity testing on fish reported LC50 for *Ictalurus punctatus* and rainbow trout at 0.1 and 1.4 mg/L, respectively (Srivastava et al., 2004). MG can also be converted into metabolic form known as leucomalachite green which can be easily bioaccumulated in human through food chain. Animal testing on rat revealed an affinity of MG to liver, thyroid gland and bladder and it has been shown to cause damages to DNA which indicates a risk of cancer (Sudova et al., 2007).

Artificial neural network (ANN) is a powerful mathematical tool and its development was inspired by the concept of a biological neuron. Its usefulness extends from financial and weather forecasting to medical research and environmental monitoring. Some of the studies involving the use of ANN modelling in water remediation are the removal of Cr (IV) using fly ash (Asl et al., 2013) and phytoremediation of acid blue 92 using duckweed (Khataee et al., 2012).

2. MATERIALS AND METHODS

2.1. Preparation of materials

Malachite green oxalate ($C_{23}H_{25}N_2.C_2HO_4.0.5C_2H_2O_4$, Mr 463.50, 90% dye content) was obtained from Sigma-Aldrich Corporation. MG stock solution of concentration 1000 mg/L was prepared, and serial dilutions were carried out to obtain dye solutions of lower concentrations. All the dye solutions contained 2 mmol/L $CaCl_2$, 1 mmol/L KNO_3, 3.3 mmol/L $Mg(NO_3)_2$, 1.3 mmol/L K_3PO_4, 5.4 μmol/L $FeCl_3$, 0.038 μmol/L $Zn(NO_3)_2$, 0.016 μmol/L $Cu(NO_3)_2$, 0.004 μmol/L $(NH_4)_6Mo_7O_{24}.4H_2O$, following an optimum method for *Azolla* growth as described by Wagner, but with some modifications (Wagner, 1997).

AP was obtained from the Brunei Agriculture Research Center, Brunei Darussalam. The AP was washed with distilled water, and gently patted dry with paper towels before use.

2.2. Phytoremediation procedures

The effects of initial dye concentration, pH and plant weight on phytoremediation of MG using AP were studied, with the change of one variable at a time while the rest of the variables remain constant. The effect of dye concentrations (5-20 mg/L) was studied, at intervals of 5 mg/L, with 0.4 g AP (fresh weight of whole plant) and unadjusted pH of 4. AP was patted dry using paper towel, and measured using an analytical mass balance. The effect of pH experiments were carried out at pH between 3-6, at AP weight of 0.4 g and a dye concentration of 10 mg/L (dye volume 100 mL). Lastly, the effect of plant weight was studied at an amount ranged from 0.2 to 0.8 g, at initial dye concentration of 10 mg/L with unadjusted pH of 4. Glass containers with volume capacity of 200 mL were used in all experiments. To account for water lost through evaporation from the water surface and transpiration by AP, the total volume of dye in the glass container was measured using a measuring cylinder and an accurate volume of distilled water was added to replace the water lost. The phytoremediation setup was placed on a balcony outside the laboratory to avoid direct sunlight and was subjected to a photoperiod of 13 h of natural light and 11 h of darkness. The remediated dye solutions were analysed daily at 12 noon using a Shimadzu UV-1601PC UV–visible spectrophotometer at λ_{max} of 618 nm.

The removal efficiency was calculated by the following equation:

$$\text{Removal efficiency} = \frac{(C_i - C_f) \times 100\,\%}{C_i} \quad (1)$$

where C_i is the initial dye concentration and C_f is the recorded dye concentration at n^{th} day.

2.3. Artificial neural network (ANN) modelling

The ANN modelling was built using a data mining software, Weka Version 3.6 (Hall et al., 2009) using the classifier, "Multilayer Perceptron". The ANN is a supervised learning method based on the generalisation of the least mean square error algorithm using a gradient descent method to minimise the cost function which is the mean square difference between the target and actual net output. The ANN model consists of three main layers: the input layer (independent variables), output layer (dependent variable) and usually one or more hidden layers. The input layer consists of 4 input neurons (plant weight, pH, initial dye concentration and duration of phytoremediation process) while the output layer has only a single neuron (removal efficiency). The ANN model was built using two-third of the experimental data for training and one-third for testing and validation. The training and validation sets for phytoremediation of MG consist of 140 sets of experimental data in total. The logsig activation function was used in the hidden layer. The hidden layer was optimised by testing with a different number of neurons and the number that yielded the highest correlation coefficient and lowest error was chosen as the optimum number of neurons in the hidden layer.
All the input data was normalised to values between 0 and 1 by the equation as follow:

$$X_N = \frac{(X_i - X_{min})}{(X_{max} - X_{min})} \quad (2)$$

where X_i is the experimental data and X_N is the normalised value, while X_{max} and X_{min} are the maximum and minimum experimental data, respectively. The ANN predicted values were converted back to their original scale for comparing with the experimental data.

The performance of the ANN model was evaluated using the correlation coefficient (R), mean absolute error (MAE) and root mean square error (RMSE). The equations are listed as follows,

$$R = \frac{\sum_{i=1}^{n}(x_i - x_m)(y_i - y_m)}{\sqrt{\sum_{i=1}^{n}(x_i - x_m)^2 \sum_{i=1}^{n}(y_i - y_m)^2}} \quad (3)$$

$$MAE = \frac{1}{n}\sum_{i=1}^{n}|y_i - x_i| \quad (4)$$

$$RMSE = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(y_i - x_i)^2} \quad (5)$$

where n is the number of data, x is the experimental data and y is the ANN predicted data, while x_m and y_m are the mean experimental data and mean predicted data, respectively.

The R and the error measurements were generated by the WEKA software. Correlation indicates the closeness between the experimental data and predicted data. MAE and RMSE measure average differences between the experimental and predicted data. One unit of MAE or RMSE represents a difference of 1 unit between the experimental and predicted data.

2.4 Growth rate estimations

Estimation of the plant growth rate was determined by the relative frond number (RFN) (White, 1936) using the following equation:

RFN = (frond number at n^{th} day – frond number at 0 day) / frond number at 0 day (6)

The fronds were counted over a duration of 7 days for AP submerged in MG of initial concentrations of 0, 5, 10, 15 and 20 mg/L.

3. RESULTS AND DISCUSSION

3.1. Visual analysis

AP bears small leaves which are alternatively arranged and each leaf consists of two lobes. The aerial dorsal lobe contains chlorophyll pigments while the ventral lobe is colourless, cup-shaped and partially submerged in water which provides the buoyancy for the plant (Wagner, 1997). As seen in Figure 1, the MG dye was concentrated on the ventral lobes and the roots of AP, while the dorsal lobe was not affected. This behaviour is due to the absence of cuticle on the ventral lobes and root tissue which allow direct contact with the dye solution, while the cutinised dorsal lobes are waterproof and hence prevent their contact with the dye solution.

3.2. ANN modelling

Prior to the building of the ANN model, the number of neurons in the hidden layer need to be optimised to yield a model with minimal error. The optimum number of neurons in the hidden layer for phytoremediation of MG is 3, as shown in Figure 2A, with good correlation coefficient R at 0.867, and low error with MAE at 0.093 and RMSE at 0.117. R with the value close to 1 indicates strong linear relation between the experimental and the ANN predicted data which is illustrated by the plot of experimental data versus the ANN predicted data. In general, most of the predicted ANN was close to the experimental data, with the exception of a few data, such as at pH 3 and 6 and at plant weight of 0.2 g, where the predicted data was slightly deviated. This behaviour is due to the complexity of biological processes involved in the phytoremediation processes. The correlation between the experimental data and the ANN predicted data can be visualised in Figure 2B. The framework of the ANN model which was used for the phytoremediation of dyes using AP is shown in Figure 3.

Figure 1. A photograph showing the accumulation of MG on the ventral lobes and the roots of AP after the phytoremediation process

3.3. Effect of pH

The favourable pH range for optimum AP growth was reported to be between 4.5 and 7.0, but it was found to tolerate pH ranges of 3.5 to 10.0 (Wagner, 1997). In the present study, the effect of pH on MG phytoremediation was studied at a pH range from 3.0 to 6.0. Experiments beyond pH 6 was not conducted as pH beyond this value leads to a change of colour intensity of the MG dye (Dahri et al., 2014).

The effect of pH on the phytoremediation of MG using AP is summarised in Figure 4. It was observed that the rate of MG uptake comprises of two phases: rapid sorption (1st to 3rd day) and slower gradual removal phase (4th to 7th day). This behaviour can be explained with the Fick's diffusion law, where the concentration gradient is the driving force for the sorption of dye (Frijlink et al., 2015). The rapid phase is the result of higher driving force due to higher dye concentration at the initial phase of the experiment, while the slower removal phase is due to the lower driving force because of the reduction in dye molecules in the liquid phase over time.

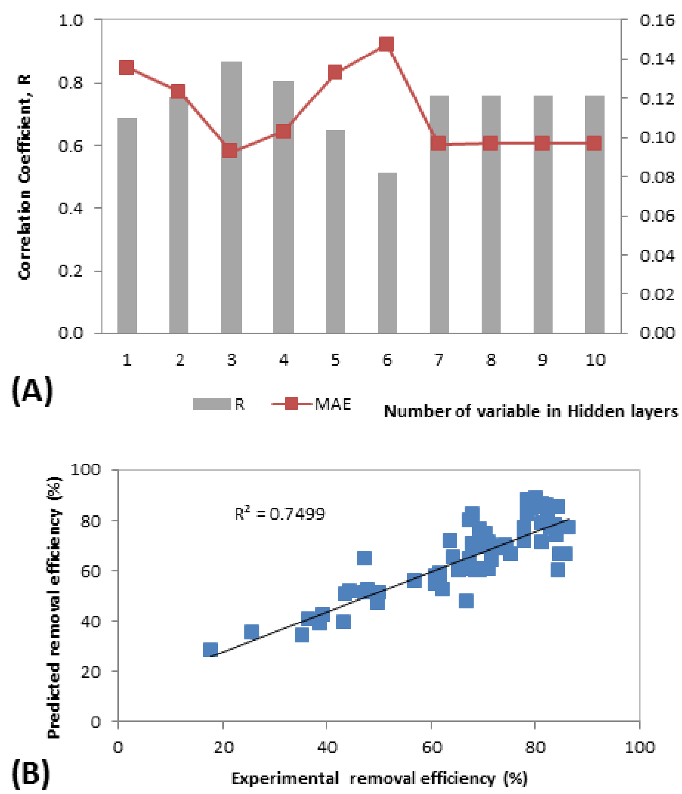

Figure 2. Plots showing (A) the effect of the number of neurons in the hidden layer on the correlation coefficient and MAE, and (B) the closeness between the experimental and the ANN predicted data for the phytoremediation of MG using AP (R^2 = 0.7499)

The solution pH affected the MG-phytoremediation potential of AP in order of pH 3.0 <

6.0 < 5.0 < 4.0 with removal efficiency (7th day) at 65.9%, 71.7%, 81.3% and 84.4%, respectively. When referring to our previous work regarding the adsorption of MG using AP powder, where the optimum adsorption of MG dye was at pH 5.0, while a lower adsorption occurred at pH 3.0 and 6.0 which matched the phytoremediation pattern of the same dye (Kooh et al., 2016c). All these results showed that pH is one of the major factors that influence the phytoremediation process, and it also hints that adsorption is also involved in the removal of dye. Similar behaviour on the pH influencing ability of phytoremediation ability was also observed in the phytoremediation of MG using duckweed (Torbati, 2016).

It was also observed that AP has the capacity to alter the pH of the dye solution. In Table 1, it can be seen that pH outside the optimum growth of AP at pH 3.2 (0.4 g AP) was adjusted to favourable pH at 4.8. The reason for such a behaviour is not known, however other aquatic plants such as water lettuce (Oladejo et al., 2015) and water hyacinth (Gopal, 1987) were also able to alter the pH of the water in which they grow.

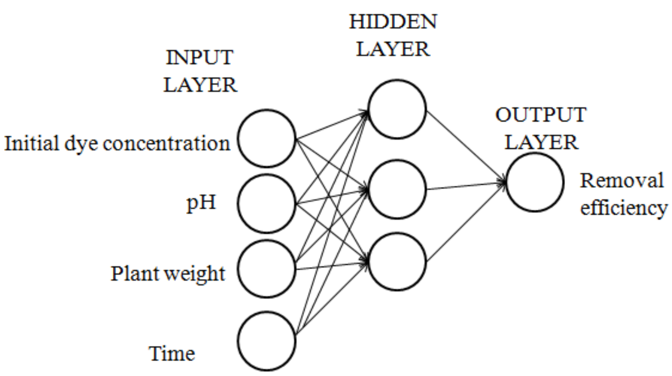

Figure 3. Framework of the ANN-model built for the phytoremediation of MG using AP.

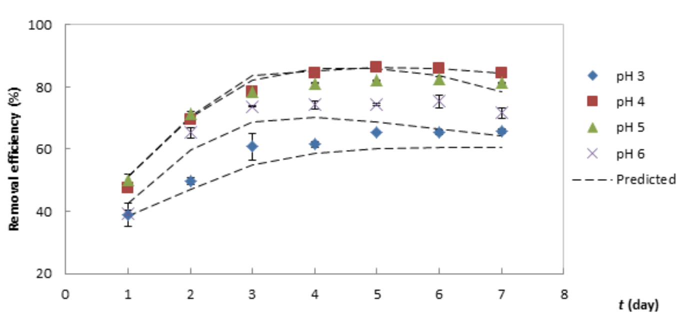

Figure 4. Comparison of the experimental data and the ANN predicted data obtained during investigation on the effect of pH on the phytoremediation of 10 mg/L MG using 0.4 g AP

3.4. Effects of plant weight

The effect of plant weight is summarised in Figure 5, where removal efficiency of MG for plant weight of 0.2 g, 0.4 g, 0.6 g and 0.8 g on the 7th day was 69.5%, 84.4%, 78.0% and 81.4%, respectively. The increase in removal efficiency with increasing AP weight may be due to a higher plant weight which provides higher root surface area that promotes better plant-dye interaction and hence a higher sorption of dye. However it is important to note that the glass container provided a fixed and limited water surface area for the AP to grow where over time it leads to an overcrowding and overlapping of the AP for plant weight of 0.6 g and 0.8 g. This resulted in reduced photosynthetic activities, which explained the slightly lower removal efficiency when compared to the plant weight of 0.4 g.

Table 1. The pH of MG dye before and after phytoremediation process using AP.

	Effect of plant weight (g)				Effect of pH			
	0.2	0.4	0.6	0.8	3	4	5	6
(Day 0) pH_i	4.2	4.2	4.2	4.2	3.2	4.2	5.0	5.9
(Day 7) pH_f	4.9	4.8	4.8	5.2	4.8	4.8	5.1	5.8

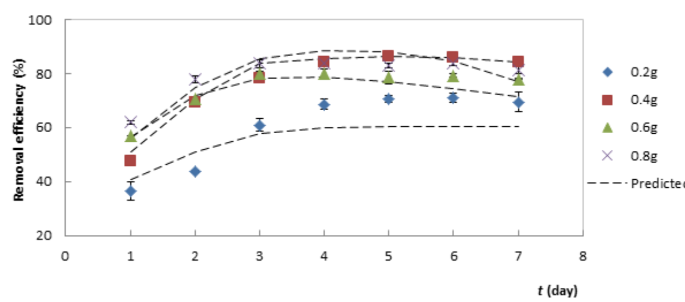

Figure 5. Comparison of the experimental data and the ANN predicted data in the investigation of the effect of AP weight on the phytoremediation of 10 mg/L MG dye at pH 4.2.

3.5. Effects of initial dye concentration

The effect of initial dye concentration was investigated for four concentrations (5 to 20 mg/L) and the results are summarised in Figure 6. It was observed that a higher concentration of dye resulted in a higher dye removal due to the Fick's diffusion law where the concentration gradient acts as the driving force for the sorption of dye (Frijlink et al., 2015). A similar trend was reported in the removal of acid blue 29 (Khataee et al., 2012) and MG (Torbati, 2015) using duckweed.

The phytoremediation capability of AP was compared with those of other plants as shown in Table 2. Even though AP displayed high effectiveness in the phytoremediation of MG, duckweeds such as *Lemna minor* and *Spirodela polyrhiza* exhibited slightly higher removal efficiency than the AP at similar experimental conditions. The main advantage of AP over the duckweeds and other aquatic plants such as water lettuce and water hyacinth, is its higher environmental stress tolerance and hence a better survival rate (Haller et al., 1974).

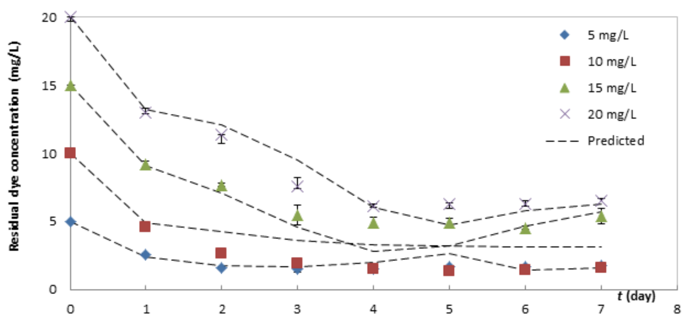

Figure 6. Comparison of the experimental data and the ANN predicted data in the investigation of the effect of dye concentration on the phytoremediation of MG using AP.

3.6. Reusability of AP

To investigate if AP can be reused after the phytoremediation process, the spent AP was introduced to a new batch of 10 mg/L MG dye and the experiment was repeated for the same period of time. The results are summarised in Figure 7, where at the end of the second cycle, the AP showed a good removal efficiency of 75%, which was slightly lower than the first cycle at 84%. This result indicates added value of AP to be utilized for phytoremediation of MG dye. The possibility of being reusable for multiple cycles decreases the dependency of the process on the plant biomass.

3.7. Estimation of AP growth by RFN

Relative frond number (RFN) is one of the useful tools commonly used to estimate the plant growth and it can be used to gauge the effect of dye concentration on plant growth (White, 1936). If the RFN value is 1.0, it indicates that the plant growth has doubled, while a value of -1.0 indicates that all the plants have died.

The measurement of plant growth by RFN is summarised in Figure 8. In the controlled experiment where no dye was added (Figure 8A), a constant growth was observed during the first 3 days, and the growth remained almost stagnant thereafter at a RFN value of 0.26. The slowing down of growth was due to the exhaustion of micronutrients, especially phosphate which is the main growth limiting nutrient of AP (Wagner, 1997).

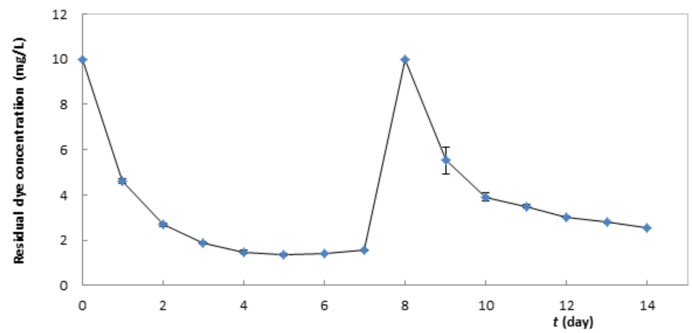

Figure 7. Repeated batch removal of 10 mg/L MG up to two cycles at pH 4.2 and using 0.4 g AP.

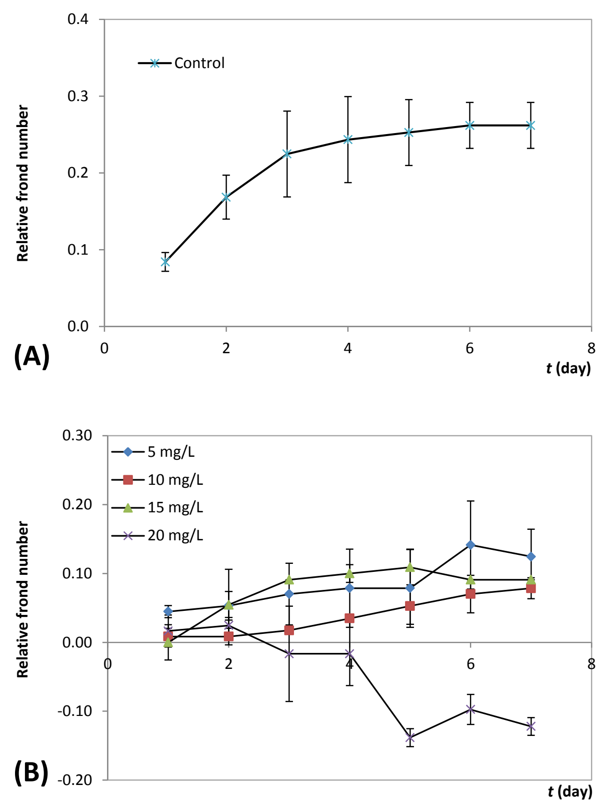

Figure 8. Estimation of plant growth by relative frond number for (A) control experiment of AP growing under optimum conditions without MG dye, and (B) phytoremediation of MG using 0.04 g of AP at pH 4.2.

Table 2. Comparison of phytoremediation potential of various plants for remediation of dyes

Plant	Dye concentration (mg/L)	Duration of phytoremediation (day)	Removal efficiency (%)	References
AP	10	7	84.4	This work
Lemna minor	10	7	88.0	(Torbati, 2015)
Spirodela polyrhiza	10	7	95.0	(Torbati, 2016)

For the phytoremediation of MG as shown in Figure 8B, dye concentrations from 5 to 15 mg/L resulted in little AP growth which was much lower than that of the control. At 20 mg/L MG, the AP could not grow and dead fronds were observed from day 3 onward which resulted in a negative RFN value. Similar study on the removal of MG using *Lemna minor* reported that there was no negative effect on the RFN at 10 mg/L, while a negative effect on plant growth was observed at concentrations of dye at 20 mg/L and above (Torbati, 2015).

4. CONCLUSIONS

Phytoremediation potential of AP was investigated for the treatment of MG dye wastewater. ANN modelling was built using experimental data which showed good accuracy (R=0.867). The solution pH strongly influenced the phytoremediation process, and therefore affects the remediation of dyes. The optimum pH for phytoremediation of MG was in the range of 4-5, with reasonably high removal efficiencies at 81% to 84%. Higher AP weight allows higher removal efficiency due to the exposure of higher surface area of AP to MG. The removal efficiency was also higher at higher initial dye concentration due to the driving force exerted by the concentration gradient that overcome all mass transfer resistances of the dyes between the aqueous and solid phase. Reusability studies of spent AP indicated good removal efficiency of 75% during the second cycle which indicates its usefulness in repeated batch treatment. The growth estimation by RFN indicates that AP can tolerate a MG concentration of up to 15 mg/L MG.

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