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Full Length Article

Microcosm Investigation on Differential Potential of Free Floating Azolla Macrophytes for Phytoremediation of P-controlled Water Eutrophication

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Abstract

Eutrophication is caused by an over enrichment of aquatic ecosystems with nutrients, principally with chronic release of phosphorus (P) from point and non-point sources, leading to nuisance algal blooms, anoxic events, impaired water quality and stubborn environmental issues. Aquatic macrophytes display an efficient phytosequestration of inorganics in plant parts due to their non-degradable nature. Serial microcosm experiments were conducted to estimate differential phytoremdiation ability of *A. japonica, A. pinnata, and A. hybrid* to remove P from different P-eutrophicated solutions under different incubation periods. Azolla plants showed substantial P-removal efficiency from P-eutrophicated solutions, and removed P-amounts were significantly correlated with P-accumulated in plant biomass. About 1-fold decrease in solution pH might be ascribed to H^+-efflux. Plants without P-hunger showed lower P-removal rates compared to P-hunger plants. *A. japonica* displayed highest P-removal efficiency in different experiments. From these kinetic experiments, it is plausible to conclude that phytoaccumulation was the possible mechanism for P-removal, and due to fast growth, high tolerance and accumulation ability, free floating Azolla might be the best candidate among macrophytes to combat P-driven eutrophication. Results obtained will not only provide information to environmental managers to mitigate P-eutrophication but will also provide data base to scientists for their future ventures.

Keywords: Azolla; Phosphorus; Phytoaccumulation; Phytoremediation; Eutrophication

Introduction

Phosphorus (P) ('bearer of light' in Greek mythology and 'conveyor of light' in plant biology'; Ticconi and Abel, 2004) enters the biosphere by natural weathering of rocks or mining and other land disturbances by anthropogenic activities. Presently, annual global mining flux of P (≈18.5 Tg y^{-1}) is almost similar in magnitude to weathering flux (15–20 Tg y^{-1}), and global P flux to biosphere increased from ≈18.5 Tg y^{-1} in preindustrial times to 33–39 Tg y^{-1} at the beginning of 21th century (Bennett *et al.*, 2001; Carpenter, 2005). Phosphorus display an equivocal paradox due to its elusive chemistry and density in two extreme environments (P-loaded/enriched and P-starved) prevailing in synchrony.

From the context of plant biology, although total P is abundant in the lithosphere, however, the elusive soil chemistry of orthophosphate (Pi) renders the element the most immobile, inaccessible and unavailable nutrient in many natural and agricultural ecosystems (Vance *et al.*, 2003; Lambers *et al.*, 2006; Schachtman and Shin, 2007; Lynch, 2007). Negligible Pi-transport by mass flow (1–5% of plant's P-demand; Lambers *et al.*, 2006), low diffusion coefficient (10^{-12}–10^{-15} m^2 s^{-1}; Rausch and Bucher, 2002), high P-sorption (e.g., to Fe/Al oxides) in soil, salt P-complexation (e.g., Ca-P as apatite), and organic fixation results in P-starvation. A great disparity in Pi-distribution between plant cells (mM) and soil solution (µM; well below the K_m for plant uptake) affecting 5.7 billion ha (> 30% of world's arable land) is one of the major reasons for poverty and malnutrition in tropics and sub-tropics (Matar *et al.*, 1992; Hinsinger, 2001; Vance *et al.*, 2003). Hence, traditional approach of heavy fertilization for high crop yield is leading to a P-store/P-bank in soils.

From the context of environmental ecology, chronic release of P from these over enriched soils is the major cause for persistent eutrophication. Water run-off, soil erosion and leakage may cause environmental problems such as toxic algal blooms, eutrophication and hypoxia of lakes, rivers and marine estuaries (Tilman *et al.*, 2001;

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Withers *et al.*, 2001). Some of the P washed into lakes dissolves and stimulates the growth of phytoplankton and aquatic plants. P-concentrations ([P]s) in lake water above 0.02 ppm generally accelerate eutrophication that is lower an order of magnitude than soil solution [P]s (0.2 to 0.3 ppm) (Sharpley *et al.*, 2003). Excess P may come from different point and non-point sources including industrial and municipal discharges, sewage, wind eroded sediments, septic systems, construction sites, stream bank erosions, and run off from urban and agricultural fields. P-eutrophication results in excess plant biomass, harmful algal blooms and anoxic events along with economic losses such as water purification cost, losses of fish and recreational amenities (Carpenter, 2005). As an example, Kojima Lake (biggest artificial lake in Japan) situated in Okayama, Japan has been converted into entropic lake due to this persistent eutrophication problem. An average [Pi] in this lake is 0.107 ppm measured annually between 1996 and 2014 that is much higher than USEAP acceptable limit of P (0.025 ppm) in lakes/reservoirs (USEAP, 1986). This problem will be further intensified in coming decades unless otherwise there would be substantial P-management in the surrounding soil and/or aquatic environments. Rates of recovery from non-point sources of nutrients (mostly run off from agriculture and urban lands) are highly variable and recovery is often slow to combat this problem. Therefore, there is an urgent need to explore and deploy cheap eco-friendly strategies to combat P-driven eutrophication of aquatic systems. 'Tailoring the plant to fit the environment instead of tailoring the environment to fit the plant' is an effective, alternative eco-friendly strategy ensuring sustainable P-management.

Phytoremediation is a cost effective, eco-friendly noninvasive alternative or complementary green technology for engineering based remediation methods. Due to non-degradable nature, inorganics released into environment can be phytoremediated by higher plants either by phytostabilization or phytosequestration in harvestable plant parts (Pilon-Smits, 2005). Recently, aquatic macrophytes attain the greatest interest in phytoremediation due to their undeniable function in balancing the environment. P is a limiting factor for the growth of Azolla. Due to fast growth, high biomass, high tolerance and accumulation ability, Azolla are best phytoremditaor plants for heavy metals (Sood *et al.*, 2012). However, research about the remediation ability of Azolla species under P-enriched environment been scarcely documented in pertinent literature. Therefore, to estimate the phytoremediation ability of three types of free floating Azolla plants for P-removal from P-eutrophicated solutions with various [P]s, microcosm experiments were conducted under climatically controlled environmental conditions along with control treatments. The effectiveness in removing P along with the potential of plants to accumulate P in biomass was investigated. Plant biomass and pH in each solution were also measured.

Materials and Methods

Experimental Protocol and Plant Culture Environment

Plant material and growth characteristics: Photographic representations of plant material and culture conditions are depicted in Fig. 1. Azolla is a small free floating aquatic fern belong to Phylum-Pteridophyta, Class Polypodiopsida, Order Salviniales, Family Azollaceae with a monotypic genus (Wagnar, 1997). Three Azolla species (*A. japonica, A. pinnata* and *A. hybrid)* were tested in present experiments (Figs. 1B-D). *A. japonica* is an indigenous Japanese endemic fern with thickly pinnately–branched stems and slight leaves like lined tiles. The form is triangle, length is about 1.5~7 cm, and slightly green in summer but becomes showy pink in winter. It is widely distributed in Asian tropics and subtropics. The length is about 1.5~2.5 cm and leaves are 1~2 mm long with pinnately arranged side branches, progressively longer towards the base and triangular in shape. *A. japonica* was collected form Toyooka city, Hyogo prefecture, Japan, while *A. pinnata* is used from lab stock. *A. hybrid* (*A. cristata x A. filiculoides*) was collected from a river near Kojima Lake, Okayama, where it is frequently distributed in lake and nearby water bodies.

Culture conditions and experimental set-up: Fern plants were re-cultured in a glass house of Tsushima campus, Okayama University, Japan (Fig. 1E). Plants were washed twice with tap water and cultured in 50-L containers having 20-L water and 10-kg soil to get reasonable biomass. Plants were then re-cultured again in nutrition free distilled water for 7-days in a climatically controlled growth chamber for acclimatization prior to their actual exposure to different P-treatments in study 1 and 2 (Figs. 1F-G). Considering that plants at this stage had already extensive roots and well acclimatized to an ambient environment, kinetic studies were conducted to estimate P-depletion form solutions.

Earlier, in order to estimate phytoremediation potential of these fern plants, we did experiments with 0, 0.03, 0.3 and 1 ppm of P along with 0 and 2 ppm N treatment combinations for 1, 2 and 3 weeks, respectively under controlled environment. However, when solutions were analyzed for P, P-concentrations were not detected in samples significantly during any incubation period. This leads to the hypothesis that at these [P]s and incubation periods, almost all P was depleted in solutions due to uptake by fern plants. Therefore, phytoremediation potential of Azolla plants was estimated by using higher [P]s and shorter incubation periods in the present experiments.

4 g fronds of Azolla with approximately the uniform size and age were used to estimate P-removal efficiency and P-uptake by plants (Fig. 1H). Plants were transferred into a 250-mL capacity rounded plastic pots (Ø=10.40 cm) containing 200 mL of 1% modified Hoagland P-free medium having initial pH of 6.5 in an environmentally controlled growth chamber and culture conditions were as follows: light/dark 16/8 h; temperature 25/20^{o}C,

respectively; light intensity 60 µmol m^{-2} s^{-1}; relative humidity 65%. Elemental composition along with salts of P-free medium was; [in mg/L]: N = [2]-NH_4NO_3, K = [2]-KCl, Ca = [2.06]-$CaCl_2.2H_2O$, Mg = [0.48]-$MgSO_4.7H_2O$, Fe = [0.062]-EDTA-Na-Fe.H_2O, Mn = [0.005]-$MnSO_4.5H_2O$, B = [0.005]-H_3BO_3, Mo = [0.005]-$H_2MoO_4.H_2O$, Cu = [0.006]-$CuSO_4.5H_2O$ and Zn = [0.007]-$Zn.SO_4{\cdot}6H_2O$. Solutions in the pots were modified by adding 5 and 10 ppm P by using $Na_2H_2PO_4.12H_2O$ as a P-source in study 1 and 2, respectively. Pots containing modified Hogland solutions with P but without fern plants were used as control treatments for valid comparisons. All treatments were replicated at least thrice by using completely randomized design. The incubation periods were ranging from 0 to 144 h after transferring of fronds for different experiments, and all the pots were covered with pierced nylon cover having 75 holes per cover. Study 1 and 2: Azolla plants (with one week P-hunger period i.e. plants were cultured in nutrition free distilled water) were exposed to 5 and 10 ppm P, respectively, and treatment solutions (with plants) and control solutions (without plants) were sampled at 24 h intervals for 144 h incubation period followed by immediate filtration of solution samples. Plants were harvested for different plant measurements. Study 3: Azolla plants (without P-hunger period i.e. plants were cultured in complete nutrition for one week) were exposed to 5 ppm P along with control treatments, and P-depletion study was conducted by solution sampling at 0, 1.5, 3, 4.5, 6, 16, 24, 48 and 96 h intervals followed by immediate filtration of samples. In study 3, Azolla plants without P-hunger period were used in order to compare the P-removal ability of these fern plants with the Azolla plants used in study 1 with P-hunger period. Study 4: water form Kojima Lake, Okayama, Japan having initial [P] of 0.095 ppm was collected and plants (with P-hunger period) were exposed to the lake water along with control (without plants), and P-depletion study was conducted by sampling at 0, 2, 6 and 8 h intervals followed by immediate filtration of samples.

Phosphorus Assay and Plant Measurements

Phosphorus assay and related parameters: P-concentrations (mg/L) in solution samples were analyzed by molybdenum blue method using spectrophotometry technique and UV-visible spectrophotometer (Hitachi, U-1100; Hitachi, Tokyo, Japan).

P-removed amount (mg/m^2) in solutions was calculated by the following expression.

P-removed amount (mg/m^2) = (mass of initial water x initial [P] - mass of water at the termination of experiment x final [P])/surface area (1)

P-removal efficiency was calculated by using the following expression.

P-removal efficiency (%) = (Ci-Cf/Ci) x 100 (2)

Where Ci is the initial P-amount and Cf is the remaining P-amount in solutions.

P-removal rate was calculated by using the following expression.

P-removal rate= (initial P amount-final P amount)/(surface area x treatment time) (3)

pH of each sample solution was also recorded at the time of sampling after filtration.

Biomass assay and plant P-concentrations and contents: Whole plants were harvested at 24 h intervals during 144 h incubation in study 1 and 2. Plants collected at the start of experiment were used as control plants. Plants were washed twice with distilled water, excess water was allowed to drain off and the plants were stored in craft paper bags. Subsequently, the samples were dried in a forced air-driven oven for 24 h at 80°C, and dry mass (g pot^{-1}) was recorded. Oven dried plants were grounded into fine powder and 0.3 g powdered samples were taken into porcelain crucibles. Crucibles were placed into a cool muffle furnace, and temperature was increased gradually to 550^0C and continued ashing for 12 h after attaining 550^0C temperature of furnace. Then furnace was shut off and opened it after cooling. Cooled ash was dissolved in 5 mL of 2 N HCl and thoroughly mixed. For complete digestion, solutions containing 5 mL of 2N HCl were evaporated at 80^0C until we get pellets. Pellets were dissolved again in 5 mL of 2 N HCl and mixed well. Solutions were filtered into 100 mL flask by washing crucibles with demineralized water thrice and made the volume up to the mark. These plant samples were subsequently used for P analysis. Plant P-content (mg pot^{-1}) was estimated by multiplying plant-[P]s in plant samples with the dry biomass of plants.

P-uptake (mg pot^{-1}) = P concentration ([P]) (mg g^{-1}) X dry biomass (g pot^{-1}) (4)

Statistical Analysis

Data were analyzed according to standard procedures. Means of three estimations were presented with standard errors and correlations were estimated by using treatment means. $P < 0.05$ was considered statistically significant.

Results

Biomass Accumulation and Solution pH Changes

Fig. 2 depicted solution pH changes and dry biomass accumulation of Azolla plants exposed to 5 and 10 ppm P in study 1 and 2, respectively during 144 h incubation period. Biomass recorded at 0 h is the biomass of control plants. At 5 ppm P, plant dry biomass was differentially increased with an increase in the exposure time and the same trend was observed at 10 ppm P except for *A. japonica* at 2^{nd} and 6^{th} day and *A. hybrid* at 2^{nd} day of exposure (Figs. 2A-B). More than 1-fold average decrease in solution pH during incubation was depicted in Fig. 2C-D.

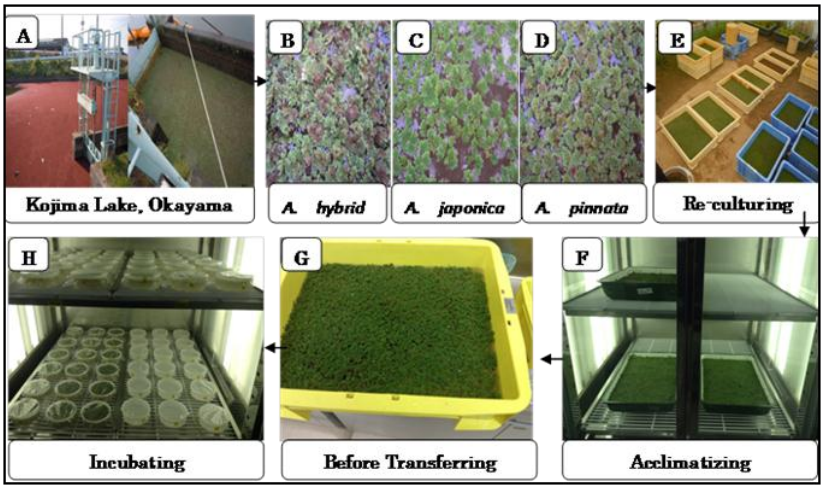

Fig. 1: Photographic representations of plant culture technique

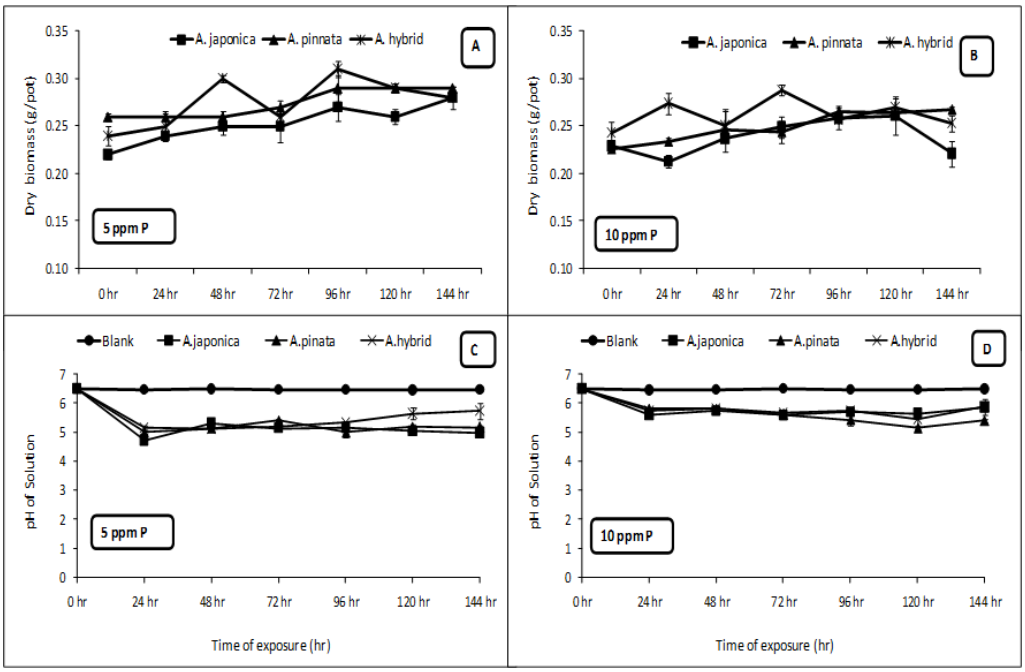

Fig. 2: (A-B) Dry biomass of Azolla species exposed to 5 ppm P (study 1) and 10 ppm P (study 2), and (C-D) solution pH changes during incubation period; error bars show ± SE (n = 3)

P-concentrations and Amounts in Solutions

Fig. 3 showed [P]s and P-amounts remained in solutions exposed to P-hunger plants in study 1 and 2. Solution [P]s were significantly decreased after the start of experiment. In study 1, [P]s were completely depleted within 24 h of exposure to *A. japonica and A. pinnata* compared to control, whereas, in case of *A. hybrid*, [P] was depleted within 48 h (Fig. 3A). Fig. 3C depicted P-amounts remained in solutions in study 1. In study 2, [P]s were depleted from 10 to 2 ppm within 96 h when exposed to *A. japonica and A. pinnata* and remained less than 1 ppm after 144 h, whereas in case of *A. hybrid*, [P] was depleted from 10 to 4 ppm within 96 h and remained about 3 ppm after 144 h of P-exposure (Fig. 3B). P-amounts remained in study 2 are depicted in Fig. 3D. In study 3, [P]s and amounts remained in solutions during 96 h exposure of plants without P-hunger period are depicted in Fig. 4A-B. P-concentrations were almost completely depleted within 96 h and Azolla plants showed similar trend in P-removal from solutions (Fig. 4A). P-amounts remained in solutions are depicted in Fig. 4B. In study 4, [P]s and percent P-removal from lake water during 8 h exposure to Azolla are depicted in Fig. 4C-D. P-concentration was depleted from 0.095 to 0.01 ppm

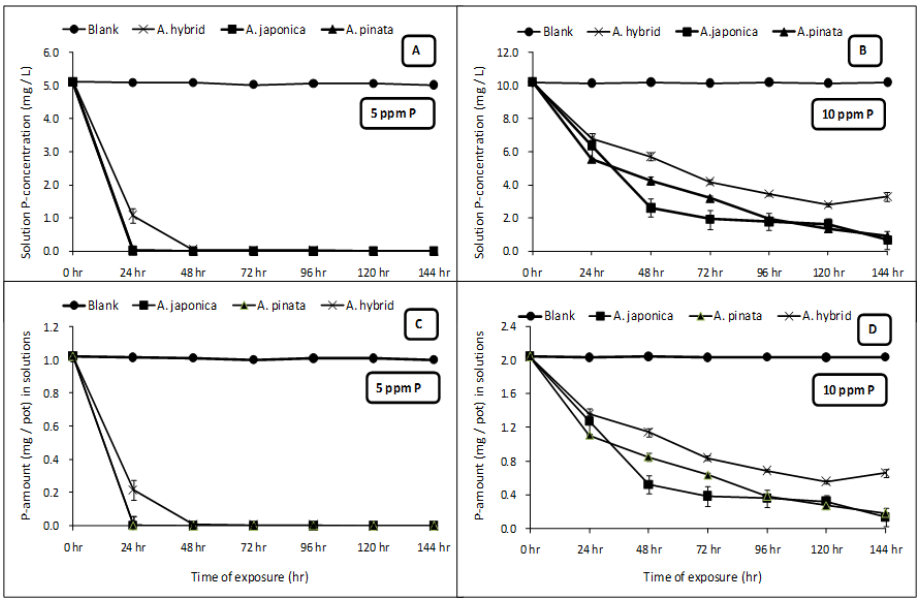

Fig. 3: (A-B) P-concentrations ([P]s), and (C-D) P-amount remained in solutions during 144 h exposure of Azolla in study 1 & 2, respectively along with control; error bars show ± SE (n = 3)

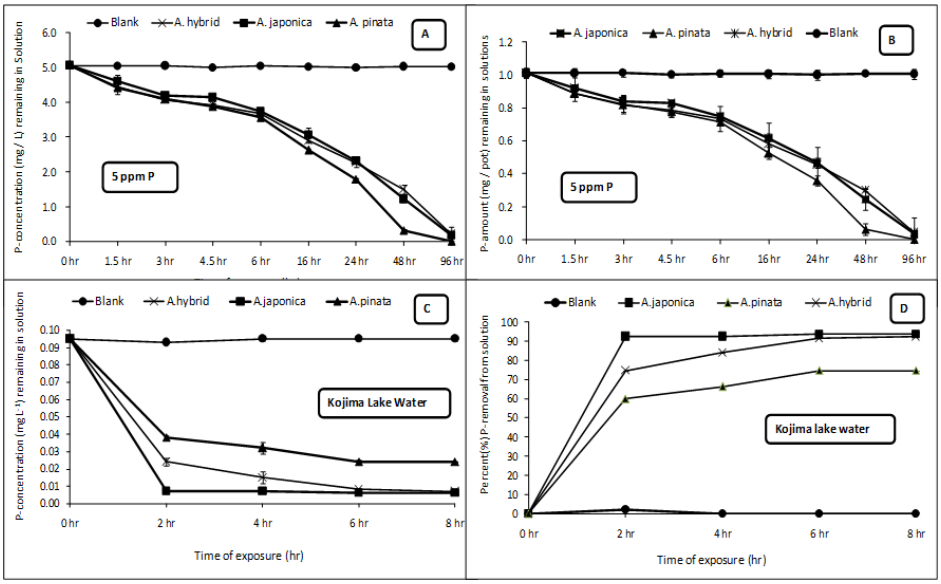

Fig. 4: (A-B) [P]s and P-amounts remained in solutions during 96 h exposure of Azolla without P-hunger to 5 ppm P (study 3), and (C-D) [P]s and P-removal efficiency (%) during 8 h plant`s exposure to water of Kojima Lake Okayama, Japan (study 4); error bars show ± SE (n = 3)

within 2 h exposure to *A. japonica* and within 6 h to *A. hybrid*, whereas, in case of *A. pinnata*, [P] was depleted from 0.095 to 0.03 ppm during 8 h (Fig. 4C). Percent P-removal from water is depicted in Fig. 4D.

P-removed Amounts and Removal Rates

Fig. 5A-B showed removed P-amounts and removal rates in study 1. Higher removed amount of P (120 mg/m^2) was observed in 5 ppm treatment solutions within 24 h after their exposure to *A. Japonica and A. pinnata* that might be due to the higher removal rate (20 mg/m^2/day) during first 24 h compared to the solutions exposed to *A. hybrid* that removed the same P-amount within 48 h due to the lower removal rate (15 mg/m^2/day) during first 24 h of exposure. Solutions with 10 ppm (study 2) exposed to all types of Azolla showed almost similar removed P-amount during first 24 h due to non-significant differences in P-removal rates from these solutions, however, solutions exposed to *A. japonica and A. pinnata* depicted higher removed amount of

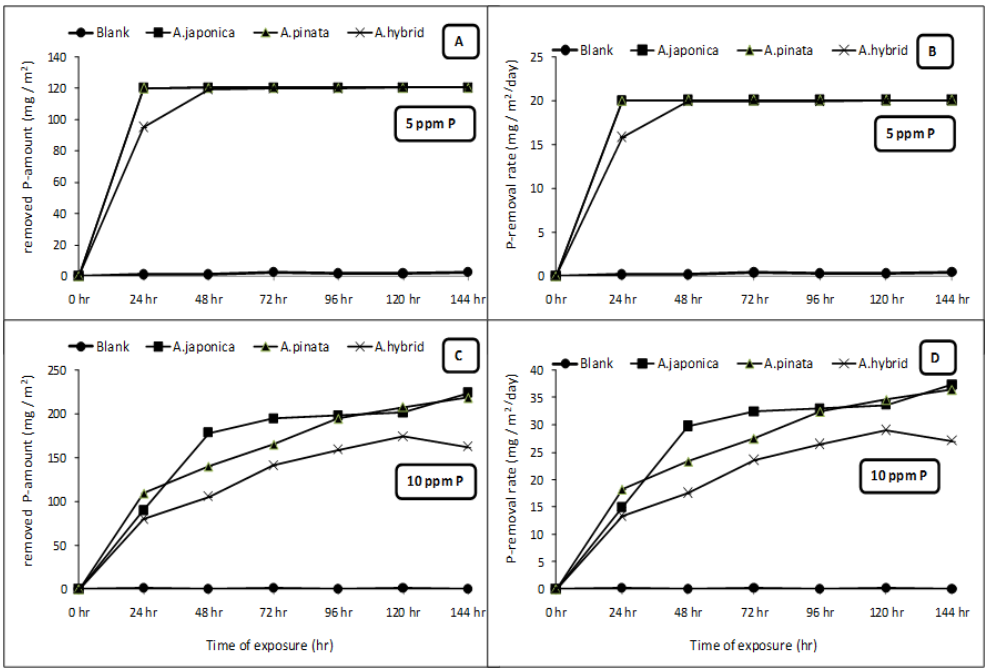

Fig. 5: (A-D) Removed P-amount and P-removal rate during 144 h exposure of Azolla in study 1 and 2, respectively along with control; error bars show ± SE (n =3)

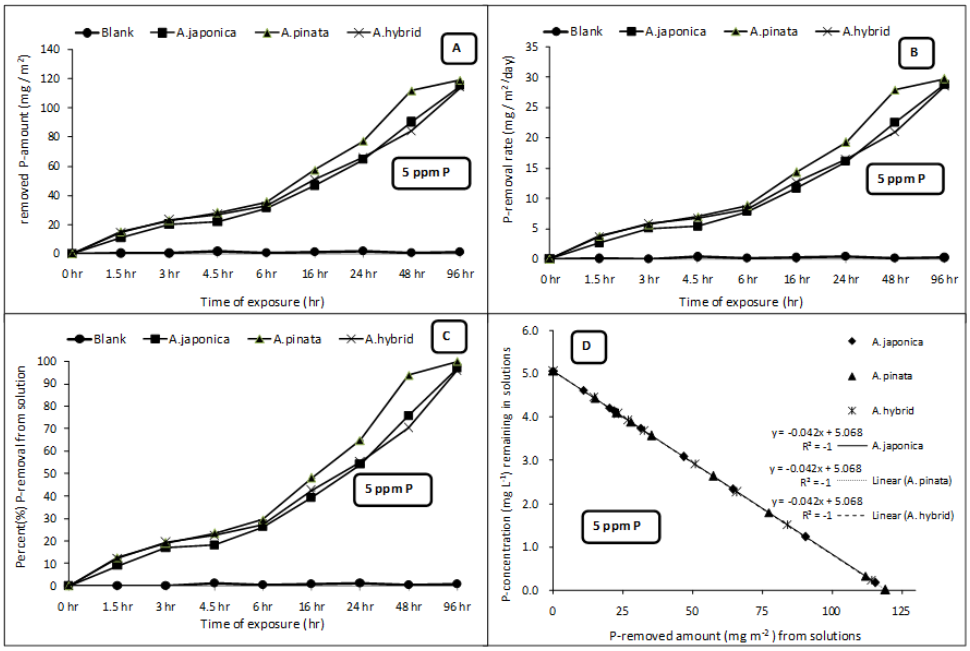

Fig. 6: (A) Removed P-amount, (B) P-removal rate, (C) % P-removal, (D) relationships between P-removed amount and [P]s remained in solutions during 96 h exposure in study 3

P than solution with *A. hybrid* after 24 h due to their higher removal rates during the same incubation periods (Figs. 5C-D). In study 3, solutions with Azolla plants without P-hunger showed almost similar trend in removed P-amounts and P-removal rates except for solution with *A. japonica* that showed slightly higher P-removed amounts and rates after 16 h of exposure (Figs. 6A-B). Percent P-removal from solution is depicted in Fig. 6C. Relationships between P-removed amounts and remained [P]s in solutions during exposure to Azolla plants showed highly significant correlations between these parameters (Fig. 6D).

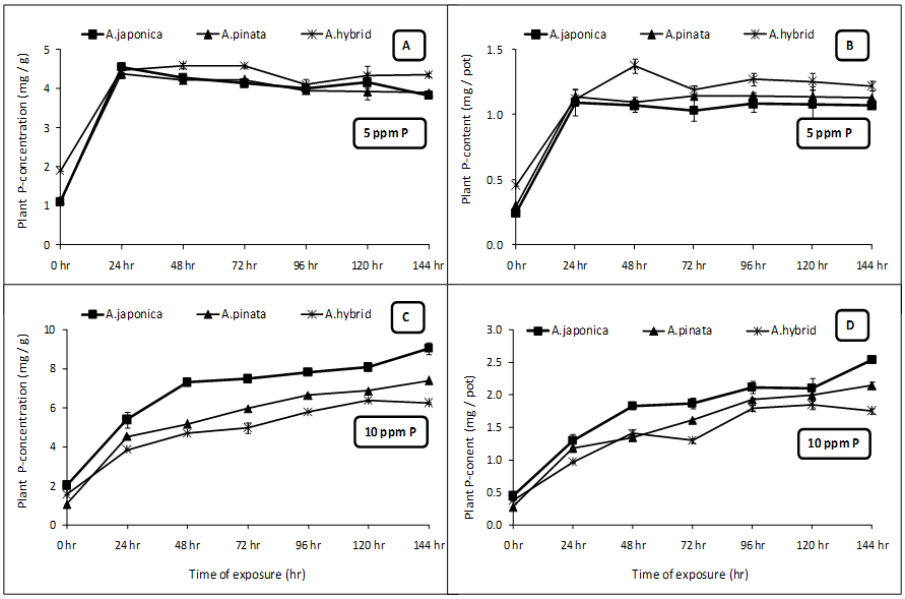

Fig. 7: (A-D) Plant [P]s and P-contents of Azolla during 144 h exposure to 5 ppm (study 1) and 10 ppm P (study 2), respectively along with control at 0 h; error bars show ± SE (n =3)

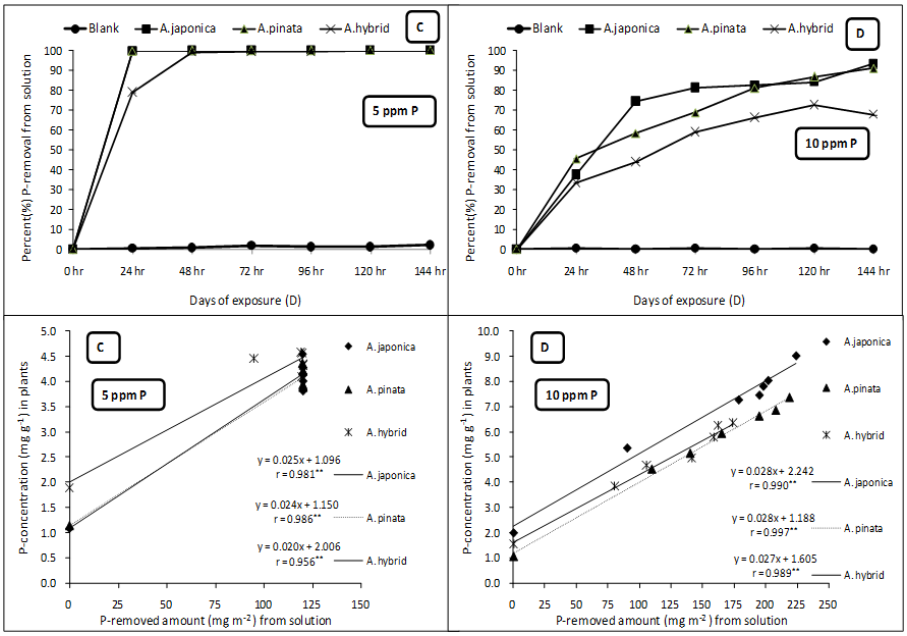

Fig. 8: (A-B) P-removal efficiency (%), and (C-D) relationships between P-removed amount and plant [P]s during 144 h of exposure in study 1and 2, respectively along with control

P-concentrations and Contents in Plants

Plant-[P]s and contents of P-treated plants in study 1 and 2 along with control plants harvested at 0 hr are depicted in Fig. 7. Compared to the control plants, [P]s and P-contents of plants increased more than 3-folds within 24 h of exposure followed by similar trend in the remaining incubation time (Figs. 7A-B). Fig. 7C-D showed a gradual increase in [P]s and P-contents of Azolla plants in study 2.

P-removal Efficiency and Relations between Related Parameters

Percent P-removal from solutions by Azolla along with control treatments in study 1 and 2 are depicted in Fig. 8A-B. Fig. 8A showed that P is almost 100% depleted within 24 h from 5 ppm P solutions exposed to *A. japonica and A. pinnata*, whereas in case of *A. hybrid*, P is approximately 80% depleted within 24 hr followed by complete P-

depletion within 48 h. In study 2, more than 90% P is depleted in solutions exposed to *A. japonica and A. pinnata* during 144 h, whereas about 70% depleted in case of *A. hybrid* within the same incubation period. Nevertheless, when plants were exposed to the natural lake water in study 4, *A. japonica* depleted 94% P from solution within 2 h followed by *A. hybrid* that depleted 93% P within 6 h, whereas *A. pinnata* depleted about 75% P form water within 6 h (Fig. 4B). Relationships between P-removed amounts from solutions and [P]s in biomass of plants in study 1 and 2 are depicted in Fig. 8C-D.

Discussion

Biomass accumulation is an important plant parameter in growth analysis. Phosphorus is an important nutrient for Azolla growth (Sadeghi *et al.,* 2013), and rapid increase in biomass can display high potential for accumulation of contaminants (Zhang *et al.,* 2010). Differential growth response of Azolla plants in study 1 and 2 (Figs. 2A-B) also showed that P is an essential and limiting nutrient for Azolla growth. El Katony *et al.* (1996) also reported that phosphorus is an important nutrient for successful and adequate growth of Azolla species. Decrease in solution pH during plant`s exposure (Figs. 2C-D) might be ascribed to H^+-efflux by ATPase pumps in root plasamalemma during nutrient uptake process, and might be useful, particularly with high pH solutions, to bring pH under favorable range for plant P-uptake, In addition, various origins of pH changes normally due to cation-anion exchange balance, carboxylates extrusion, root exudation and respiration and redox-coupled processes (Hinsinger *et al.,* 2003). Results about decrease in pH were in agreement with Akhtar *et al.* (2009) who also reported a decrease in solution pH when *Bassica* cultivars were grown with sparingly soluble P-sources to estimate P-acquisition under deficiently buffered P-stress environments. Although, pH values of 4.5 to 7.5 is optimum for growth of most Azolla species (Cary and Weerts, 1992), however Azolla can survive even at pH values ranging from 3.5 to 10 (Serag *et al.,* 2000) indicating a wide pH range for Azolla growth. More than 1-fold average decrease in solution pH by tested Azolla species indicating their adaptability for better P-uptake by adjusting pH during incubation.

Although, Azolla plants showed almost similar trend in removing [P]s from solutions in study 1 and 2, however P-depletion time after plant`s exposure was more in study 2 (Fig. 3). Nevertheless, all types of Azolla were effective in removing [P]s; however, *A. japonica and A. pinnata* were more efficient than *A. hybrid* during 144 h of incubation period (Fig. 3A-B). Statistically non-significant changes in [P]s in control treatments throughout incubation period highlighted that [P]s removed from solutions is due to the Azolla plants in the culture medium. These results are in agreement with Song *et al.* (2012) who reported that water fern effectively removed nitrogen, phosphorus and iron when exposed to three sources of polluted water along with standard solutions. Results about P-amount indicated that [P]s in solutions exposed to *A. japonica* and *A. pinnata* were removed faster than with solutions exposed to *A. hybrid* indicating more removal ability of naturally growing *A. japonica* and *A. pinnata* in study 1 and 2. Pandey and Singh (2011) also reported that naturally growing plant species are more suitable phytoremediators in comparison to the introduced plants. The removal of [P]s in solutions exposed to plants without P-hunger was slow compared to the solutions exposed to plants with P-hunger in study 1 (Fig. 3). Therefore, fern plants with P-hunger might be more effective than plants without P-hunger period for practical purposes of environmental cleanup of P-driven eutrophication by phytoremediation. In Study 4, by considering the safe limit of P (0.02 ppm P) (Sharpley *et al.,* 2003) to avoid P-eutrophication, *A. japonica* proved to be the most efficient (> 90%) in P-removal followed by *A. hybrid*. *A. pinnata* exhibited 70% P-removal efficiency during the same exposure time (Fig. 4D). Removal of solution [P]s in different studies highlighted that Azolla plants showed substantial P-removal ability from P-eutrophicated waters. Due to fast growth and high remediation efficiency, Azolla plants are potential candidates for phytoremediation (Sood *et al.,* 2012; Nazim *et al.,* 2014), confirmed the present studies. P-amount (120 mg/m^2) in solutions without P-hunger plants in study 3 (Fig. 6A) was removed in longer incubation time when compared to the same removed P-amount in solutions exposed with P-hunger plants in study 1 (Fig. 5A) indicating that P-hunger plants might remove P more efficiently than plants without hunger period.

Results about plant [P]s and P-contents (Fig. 7) confirmed that depletion of [P]s in 5 ppp P solutions within 24 h is due to fast uptake of P by Azolla plants. Maximum P-content of *A. japonica* and *A. pinnata* at 24 h is due to P-removal from solutions at 24 h, and of *A. hybrid* at 48 h is due to P-removal from solution at 48 h. A gradual increase in plant [P] and P-content in study 2 confirming the reciprocal trend for removal of [P]s from solutions exposed to Azolla. *A. japonica* showed high plant P-content compared to other Azolla types. These results showed that P-removal from solutions is due to plant P-uptake, and removed [P]s are accumulated into plants. Because the total pollutant extraction is the product of biomass and tissue concentration (Comis, 1998; Valderrama *et al.,* 2013), hence the success of phytoremediation mainly depends on the plant growth rate and bioaccumulation ability. As the tested Azolla plants showed both these characteristics, therefore, Azolla macrophytes might be the effective plants for phytoremedation of P-eutrophicated waters.

Results about P-removal efficiency confirmed that *A. japonica and A. pinnata* were more efficient than *A. hybrid* during the same incubation period in study 1 and 2 (Figs. 8A-B). Nevertheless, when plants were exposed to the natural lake water in study 4, *A. japonica and A. hybrid*

were more efficient in P-removal than *A. pinnata*. These results indicated the differential P-removal ability of Azolla exposed to different P-solutions. Statistically significant correlations between P-removed amounts and plant [P]s (Figs. 8A-B) indicated that P-removed from P-eutrophicated solutions is actually accumulated in plant biomass. This type of phytoremediation is termed as phytoextraction or phytoaccumulation (Pilon-Smits, 2005), where contaminants, particularly non-degradable inorganics, are removed by the plant parts. Therefore, on the basis of the results obtained in the present experiments, it is plausible to conclude that Azolla had higher phytoremediation ability to remove P from P-eutrophicated water and can decrease P-concentration considerably in aquatic ecosystems.

Conclusion

Plants of tested Azolla types showed differential growth response and sufficient P-removal efficiency when exposed to the solutions with different P-concentrations and different incubations periods under controlled environmental conditions. *A. japonica* exhibited highest P-removal ability in all experiments. Plants without P-hunger removed P slowly than plants with P-hunger period. About 1-fold decrease in pH of solutions might be ascribed to H^+-efflux by ATPase pumps in plasamalemma of roots during nutrient acquisition and uptake process that might be helpful in adjusting pH of high pH solutions in a favorable range for plant uptake. Phytoaccumulation was the possible mechanism for P-removal from solutions. Results obtained in the present experiments revealed that Azolla plants might be the best phytoremediator plants for P removal form P-eutrophicated water and can be deployed for P-removal efficiently in aquatic systems.

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