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Influence of environmental factors on arsenic accumulation and biotransformation using the aquatic plant species *Hydrilla verticillata*

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ABSTRACT

Hydrilla verticillata (waterthyme) has been successfully used for phytoremediation in arsenic (As) contaminated water. To evaluate the effects of environmental factors on phytoremediation, this study conducted a series of orthogonal design experiments to determine optimal conditions, including phosphorus (P), nitrogen (N), and arsenate (As(V)) concentrations and initial pH levels, for As accumulation and biotransformation using this aquatic plant species, while also analyzing As species transformation in culture media after 96-hr exposure. Analysis of variance and the signal-to-noise ratio were used to identify both the effects of these environmental factors and their optimal conditions for this purpose. Results indicated that both N and P significantly impacted accumulation, and N was essential in As species transformation. High N and intermediate P levels were critical to As accumulation and biotransformation by *H. verticillata*, while high N and low P levels were beneficial to As species transformation in culture media. The highest total arsenic accumulation was (197.2 ± 17.4) μg/g dry weight when As(V) was at level 3 (375 μg/L), N at level 2 (4 mg/L), P at level 1 (0.02 mg/L), and pH at level 2 (7). Although *H. verticillata* is highly efficient in removing As(V) from aquatic environments, its use could be potentially harmful to both humans and the natural environment due to its release of highly toxic arsenite. For cost-effective and ecofriendly phytoremediation of As-contaminated water, both N and P are helpful in regulating As accumulation and transformation in plants.

Introduction

Arsenic (As) enters the environment through natural processes and anthropogenic activities, such as volcanic activity, mineral rock erosion, pesticides, fertilizers, preservatives, etc. (Gonzaga et al., 2008), posing potential human health risks through the consumption of crops irrigated with As-rich water as well as drinking water (Arco-Lázaro et al.,

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2018; Saifullah et al., 2018). Inorganic As is ubiquitous in the environment and is considered a carcinogen by the International Agency for Research on Cancer (IARC), causing hyperpigmentation and hyperkeratosis as well as skin and lung cancers. The maximum concentration limit recommended for potable water by the World Health Organization (WHO) is 10 μg/L. However, in 2001, WHO estimated that approximately 130 million people worldwide were exposed to As concentrations above 50 μg/L in groundwater, including countries such as Bangladesh, India, China, and the United States of America (van Halem et al., 2009; Wang et al., 2014a; de Oliveira et al., 2017). Accordingly, the need for remediation of As-contaminated water is both universal and in urgent need of resolution, and water quality improvements require immediate action. Among the available developed techniques, phytoremediation is widely acknowledged to be a simple and viable solution (Alvarado et al., 2008; Favas et al., 2012; Farnese et al., 2013; Rahman et al., 2014). Many studies have been conducted on As accumulation and transformation mechanisms associated with various plant species (e.g., *Acer platanoides*, *Tilia cordata*, and *Pteris vittata*) (Caille et al., 2005; Budzyńska et al., 2018; Budzyńska et al., 2019). However, until now, studies on As accumulation and transformation using submerged aquatic plants have been limited. With respect to aquatic plants, research by Xue et al. (2015) indicated that the ability of submerged plants to take up As-contaminated water is strong and typically higher than that of emerged plants and floating plants. This is because the intake from stems and leaves of submerged plants is as robust as that by their roots. Furthermore, submerged plants significantly differ from terrestrial plants in their rootless physiology and the fact that no translocation barriers exist between roots and shoots, which makes such plants model species of interest in the investigation of As metabolism (Xue et al., 2012). *Hydrilla verticillata*, a fast-growing submerged plant species found throughout the world, has been widely considered an ideal candidate for phytoremediation.

However, As phytoremediation in aquatic environments is affected by environmental factors, such as As content, nitrogen (N) and phosphorus (P) concentrations, and pH levels (Tu and Ma, 2003; Wang et al., 2017). Moreover, N and P play varying roles in the environment, being the main elements that drive eutrophication and plant growth, while also aiding in the accumulation of major elements and the efficiency of transporter activity (Tu and Ma, 2003; Karadjova et al., 2008; Wang et al., 2014b, 2018; Fu et al., 2017; Srivastava et al., 2018). For example, P has long been reported to inhibit arsenate (As(V)) uptake in cells due to its analogous molecular structure with As(V), and P-limited *Chlorella salina* cells were found to be more sensitive to As(V) exposure (Karadjova et al., 2008; Xue et al., 2012). Additionally, N has been shown to affect As accumulation and biotransformation by plants in culture media (Wang et al., 2017). Moreover, pH is one of the most important factors that controls As speciation, and is closely correlated to As bioavailability and accumulation in plants (Karadjova et al., 2008; Favas et al., 2012). Many studies have investigated the individual effects of N, P, and pH on As metabolic processes (Wang et al., 2017); however, the integrated and systematic effects of As, N, and P concentrations and pH levels on As accumulation and transformation by submerged aquatic plant species remain unclear and therefore require further investigation.

The main objective of this study was to investigate the effects of As, N, and P concentrations and initial pH levels on As accumulation and biotransformation by *H. verticillata*. Furthermore, we applied an orthogonal design under the relevant statistical assumptions of these factors to determine optimum environmental conditions for As accumulation and transformation by *H. verticillata*. The method used in this study can provide design parameters for use by other studies related to As-contaminated aquatic phytoremediation treatments.

1. Materials and methods

1.1. Plant growth

We collected *H. verticillata* samples from the eastern section of Lake Taihu (30°56.918′N and 120°20.693′E), Wuxi City, China, in June 2017. These samples were then cultivated in a greenhouse for at least 5 months. The fresh shoot apical meristem (approx. 5–7 cm) of plants was selected in December 2017 and washed gently to remove soil debris or epiphytes, after which these samples were acclimatized in artificial freshwater solutions for 7 days prior to the experiment. The composition of the artificial freshwater solutions was as follows: 22.7 mg/L $MgSO_4 \cdot 7H_2O$, 30.7 mg/L $MgCl_2 \cdot 2H_2O$, 20.4 mg/L $CaCl_2 \cdot 2H_2O$, 45.7 mg/L NaCl, 26.0 mg/L $NaHCO_3$, 3.61 mg/L KCl, 1.41 mg/L $FeCl_3 \cdot 6H_2O$, 0.97 mg/L $Al_2(SO_4)_3 \cdot 18H_2O$, 0.19 mg/L $MnCl_2 \cdot 4H_2O$, 3.86 μg/L $ZnSO_4 \cdot 7H_2O$, and 2.17 μg/L $CuCl_2 \cdot 2H_2O$ (Xue and Yan, 2011). Furthermore, N and P were added to simulate mesotrophic conditions using KNO_3 and $NaH_2PO_4 \cdot 2H_2O$ for N (4 mg/L) and P (0.2 mg/L), respectively, to ensure healthy plant growth. The above chemicals were of analytical reagent grade, and were purchased from Chemical Reagent Purchasing and Supply Station, China. All experiments were conducted inside a controlled environment growth chamber under the following conditions: 14-hr photoperiod with light intensity of 9600 lx where temperature was kept constant (27 ± 2)°C throughout the day and night.

1.2. Experimental design

Experiments were designed according to 4 factors and 3 levels (as shown in Table 1) following orthogonal methods. Table 2 lists 9 experimental schemes (L_9 (3^4)). This study also included 3 additional control treatments (i.e., treatments devoid of plants) at low, intermediate, and high As levels to analyze changes in As species in media without the influence of plants. All treatments were conducted in triplicate.

In this study, As(V) ($Na_3AsO_4 \cdot 12H_2O$, analytical reagent, Chemical Reagent Purchasing and Supply Station, China) concentrations were similar to those measured in natural aquatic systems under low, intermediate, and high contamination levels (Caumette et al., 2011). Additionally, N and P

Table 1 – Factors levels (nitrogen (N), phosphorus (P), pH and arsenate (As(V)) in the orthogonal test.

Level	KNO_3 (counted as N, mg/L)	$NaH_2PO_4 \cdot 2H_2O$ (counted as P, mg/L)	pH	As(V) (μg/L)
1	2	0.02	6	15
2	4	0.2	7	75
3	10	1	9	37

levels were selected according to maximum surface water environmental quality standard values in China to represent mesotrophic, eutrophic, and hypereutrophic conditions in surface water (Wang et al., 2017). pH levels were selected to represent actual pH ranges in freshwater (Yan et al., 2016) and the pH values of the solutions were measured using a pH meter (PH 7110, WTW, Germany). Except for the control treatments, each treatment consisted of 3 plant (*H. verticillata*) replicates (0.5 g) cultured in 100 mL synthetic freshwater media inside triangular flasks. Both water and plant samples were collected from each flask after a 96-hr incubation period, providing a total of 36 water samples and 36 plant samples for As analysis. To investigate the effects of 4 key environmental factors (i.e., As(V), N, P, and pH) on As(V) accumulation and biotransformation by *H. verticillata*, experimental data were as analyzed to determine the optimal environmental factor levels according to the signal-to-noise (S/N) ratios to maximize As accumulation and species transformation in plants and culture media. The significance of the environmental factors was determined by multivariate analysis of variance (MANOVA), which was used to evaluate the influence of each factor on As accumulation and species transformation, while the percentage contribution (PC) was calculated to compare the rank order of each factor.

1.3. Analysis of arsenic species in culture media

During the harvesting phase, 0.5 mL of the nutrient solution was taken from each flask after 96-hr incubation. Following this, nutrient solutions were diluted with a Phosphate-buffered solution (PBS) (Xue et al., 2012) containing 2 mmol/L NaH_2PO_4 and 0.2 mmol/L Na_2EDTA (pH 6), after which the solutions were filtered through 0.45 μm filters and stored on ice before being analyzed within a 24-hr period. High-performance liquid chromatography (HPLC, Agilent LC1200 series, Agilent Technologies, USA) coupled with inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700a, Agilent Technologies, USA) was used to analyze As species in these solutions. We also used ethylenediaminetetraacetic acid (EDTA), which has been shown to preserve As species in water samples (Bednar et al., 2002; Xu et al., 2007). Different As species were separated using a precolumn (11.2 mm, from 12 to 20 mm, Hamilton, CA, USA) coupled with a PRX-100 anion-exchange column (10 mm, 250 × 4.1 mm, Hamilton, CA, USA). As species were separated with a mobile phase of 10 mmol/L $(NH_4)_2HPO_4$ and 10 mmol/L NH_4NO_3; the pH was regulated to 6.25 using HNO_3 or $NH_3 \cdot H_2O$ (Guaranteed reagent, Chemical Reagent Purchasing and Supply Station, China). The mobile phase was pumped through the column at 1 mL/min using isostatic pressing.

1.4. Analysis of arsenic speciation in plants

Plants were harvested after As(V) exposure for 96 hr, rinsed briefly with Milli-Q water and then immersed for 10 min in an ice-cold desorption solution containing 1 mmol/L K_2HPO_4, 0.5 mmol/L $Ca(NO_3)_2 \cdot 4H_2O$, and 5 mmol/L MES (pH 6) to remove apoplastic As. After being dried and frozen to a constant weight, plant samples were ground with liquid nitrogen (LN_2) in a mortar and pestle. Approximately 0.03 g of the ground material was extracted with 5 mL of PBS (2 mmol/L NaH_2PO_4 and 0.2 mmol/L Na_2EDTA at pH 6) for 1 hr at 4°C under sonication. The extracts were filtered through 4 layers of muslin cloth, followed by filtration through 0.45 μm filters prior to As speciation analysis (Xu et al., 2007). The method we used to analyze As speciation was similar to the method we used for species analysis in the culture media. The initial As(V) and arsenite (As(III)) concentrations in *H. verticillata* specimens were (0.1 ± 0.0) μg/g dry weight (dw) and (0.3 ± 0.1) μg/g dw, respectively, which we determined prior to the start of the experiment. Final plant concentrations were ascertained by the detected concentrations minus the initial concentrations.

Table 2 – Design of the orthogonal test.

Experiment design	Treatment	KNO_3 (counted as N, mg/L)	$NaH_2PO_4 \cdot 2H_2O$ (counted as P, mg/L)	pH	As(V) (μg/L)
Experiments	E1	2	0.02	6	15
	E2	2	0.2	7	75
	E3	2	1	9	375
	E4	4	0.02	7	375
	E5	4	0.2	9	15
	E6	4	1	6	75
	E7	10	0.02	9	75
	E8	10	0.2	6	375
	E9	10	1	7	15
Controls	E01	2	0.02	6	15
	E02	4	0.2	7	75
	E03	10	1	9	375

1.5. Analysis of total arsenic in culture media and plant specimens

To determine total arsenic concentrations in plant samples, 0.02 g of the ground plant samples was digested in HNO_3:H_2O_2 (analytical reagent, Chemical Reagent Purchasing and Supply Station, China) (2:1, V/V) using Auto Digestion Units (X-42A+, Shengsheng, China). The temperature was raised to 55°C for 15 min, then raised again to 75°C for 15 min, and finally held at 120°C for 2 hr. Following this, both supernatant and water samples were filtered through 0.45 μm filters before total arsenic (TAs) analysis. Inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500cx, Agilent Technologies, USA) was use to analyze TAs. To verify the accuracy of the analysis of metals/metalloids, we used certified reference material (i.e., bush twigs and leaves, GBW 07602 GSV-1; the National Research Center for Standards, China). Recoveries were 82.1%–96.1% for TAs.

We found no differences in results between TAs analyses determined by digestion and the sum of As(V) and As(III) species. Consequently, TAs concentrations in this study are shown as the sum of As(V) and As(III) in culture media and plants.

1.6. Quality assurance and data analysis

All chemical solutions were prepared using ultrapure water (18 MΩ cm, Milli-Q water). All values are presented as means ± standard deviations in triplicate determined using Microsoft Excel 2016, and blanks were simultaneously applied for correction according to the blanks evaluated using ICP-MS (Agilent 7500cx, Agilent Technologies, USA) and HPLC-ICP-MS (HPLC, Agilent LC1200 series, Agilent Technologies, USA and ICP-MS, Agilent 7700a, Agilent Technologies, USA) in relation to quality assurance samples. OriginPro 8.5.0 SR1 software (Origin Lab Corporation, 1991–2010) was used to reveal the monitored As species concentrations. MANOVA and comparison among treatments in R version 3.4.4 (The R foundation for statistical computing, 2017) were employed, which allowed us to examine significant differences among treatments, wherein $p < 0.05$ was considered significant. Furthermore, PC values were calculated according to Eq. (1) to express the rank orders of N, P, pH, and As. Minitab 17 statistical software was used to measure S/N ratios according to Eq. (2), to determine optimal factor combinations of the highest accumulation and As species transformation by plants.

$$PC = \frac{SS_F - (DOF - V_{ER})}{SS_T} \times 100\% \quad (1)$$

where, SS_T is the total sum of squares; SS_F is the factorial sum of squares; V_{ER} is the variance of error; and DOF is the degree of freedom. Analysis of variance was used to determine DOF, SS_T, SS_F, and V_{ER} values.

$$S/N = -10 \times \log\left[\frac{\sum_{i=1}^{n}\left(\frac{1}{y_i}\right)^2}{n}\right] \quad (2)$$

where, n is the number of repeated measurements under the same experimental conditions, and y_i represents the measured value.

2. Results and discussion

2.1. Arsenic accumulation and biotransformation in plants

In this study, TAs accumulation was (1.5 ± 0.1) - (197.2 ± 17.4) μg/g dw, and As(III) was the dominant As species, accounting for (43.0 ± 4.1)% - (95.3 ± 0.7)% in *H. verticillata* specimens, which demonstrated that the effects N, P, and pH on TAs accumulation and transformation of As(V) were statistically significant (Fig. 1, Appendix A Tables S1 and S2; $p < 0.05$). We detected no organic As species in the plants. This suggested that the ability of *H. verticillata* cells to convert As(V) to As(III) was strong, for which significant amounts of As(III) were then reserved in vacuoles (Xue and Yan, 2011; Xue et al., 2012). The S/N ratios clearly showed that optimal factors for maximal As(III) and TAs accumulation were As(V) at level 3 (375 μg/L), N at level 3 (10 mg/L), P at level 2 (0.2 mg/L), and pH at level 2 (7) as shown in Fig. 2a and c. The PC values of TAs accumulation by *H. verticillata* were: (1) As(V) (95.5%), (2) P (1.6%), (3) pH (1.2%), and (4) N (0.6%), while corresponding values of As(III) accumulation were: (1) As(V) (94.4%), (2) P (2.0%), (3) pH (1.5%), and (4) N (0.9%) as shown in Appendix A Table S3. This indicated that the As(V) concentration effect was the most influential among the 4 factors on TAs and As(III) accumulation by *H. verticillata*. Similar findings showed that cadmium (Cd) and lead (Pb) concentrations in culture media primarily resulted in Cd and Pb accumulation in *Lemna minor* rather than N or P (Yu et al., 2000). Additionally, findings from Wang et al. (2017) demonstrated that the PC values of As(V) concentrations were highest for TAs accumulation in *Microcystis aeruginosa*, accounting for 56.80%. However, their results were lower than that found in our study, whereas their PC values for N, P, and pH were higher than those found in our study, indicating that P, pH, and N levels in culture media should be regulated according to the needs of different plant species during phytoremediation.

Furthermore, TAs concentrations in *H. verticillata* indicated that TAs accumulation increased with increasing As(V) concentration in culture media (Fig. 1). This was supported by a study by Xue and Yan (2011), whereas As accumulation in *H. verticillata* exceeded 400 μg/g dw under the same experimental conditions, which was higher than the highest accumulation (197.2 ± 17.4) μg/g dw detected in our study. A potential explanation for this discrepancy is that the presence of P caused an inhibition effect to occur (Wang and Duan, 2009). Interestingly, our study found that an intermediate P level was optimal for TAs and As(III) accumulation (Fig. 2). On the one hand, it has been confirmed that P is chemically analogous to As(V) (Duman et al., 2010; Mathews et al., 2011; Baker and Wallschläger, 2016; Tang et al., 2018); on the other hand, P is essential for plant growth during phytoremediation (Huang et al., 2004; Alvarado et al., 2008; Gonzaga et al., 2008; Karadjova et al., 2008). However, this has been challenged by

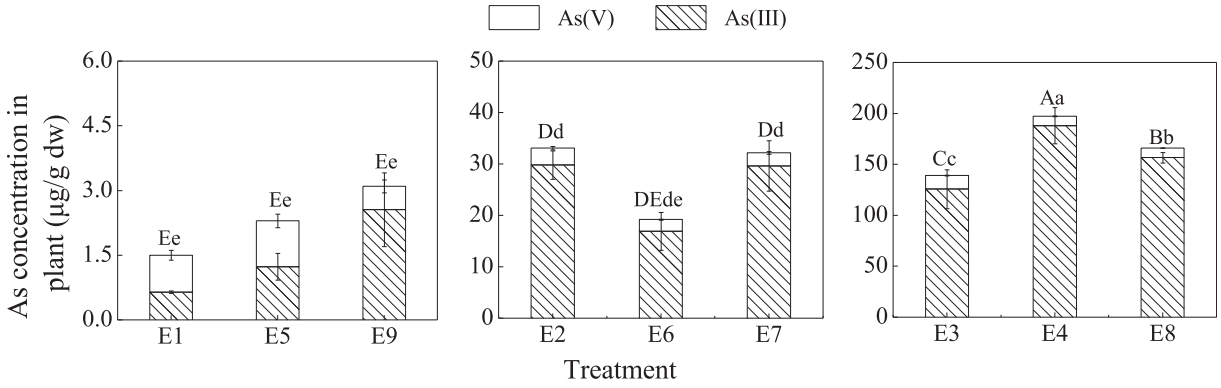

Fig. 1 – Arsenite (As(III)), arsenate (As(V)) and total arsenic (TAs) concentrations in plants at 96 hr. TAs = As(III) + As(V). E1: N 2 mg/L, P 0.02 mg/L, pH 6, As(V) 15 μg/L; E5: N 4 mg/L, P 0.2 mg/L, pH 9, As(V) 15 μg/L; E9: N 10 mg/L, P 1 mg/L, pH 7, As(V) 15 μg/L; E2: N 2 mg/L, P 0.2 mg/L, pH 7, As(V) 75 μg/L; E6: N 4 mg/L, P 1 mg/L, pH 6, As(V) 75 μg/L; E7: N 10 mg/L, P 0.02 mg/L, pH 9, As(V) 75 μg/L; E3: N 2 mg/L, P 1 mg/L, pH 9, As(V) 375 μg/L; E4: N 4 mg/L, P 0.02 mg/L, pH 7, As(V) 375 μg/L; E8: N 10 mg/L, P 0.2 mg/L, pH 6, As(V) 375 μg/L. Capital and lowercase letters indicate the significance of TAs and As(III) in 9 treatments, respectively. $n = 3$, data are means ± standard deviation. dw: dry weight.

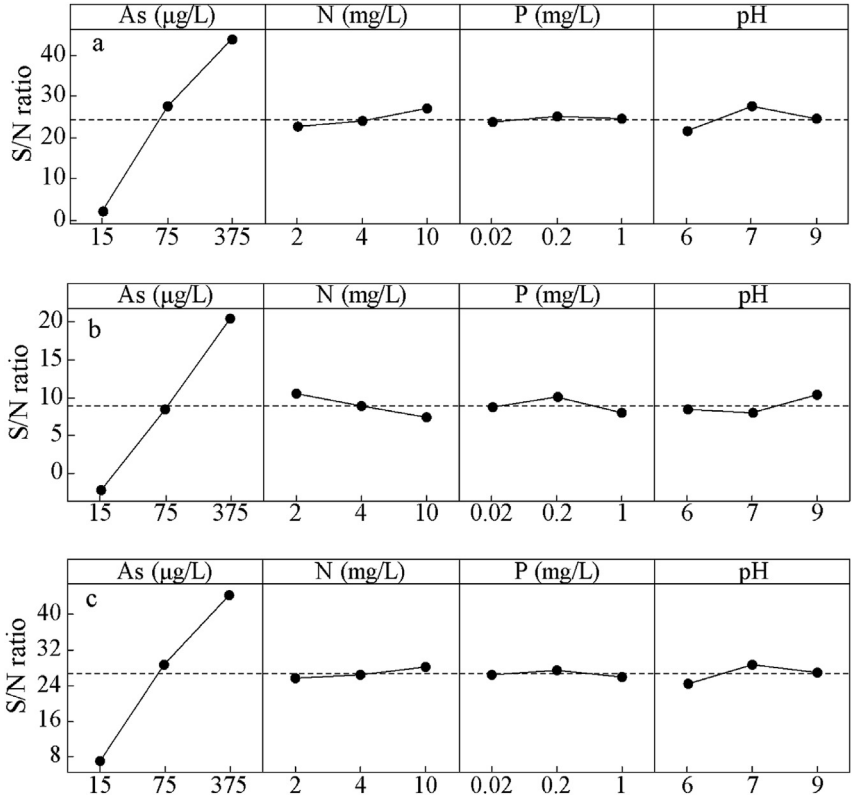

Fig. 2 – Mean signal-to-noise (S/N) ratios for (a) As(III), (b)As(V), (c) TAs in plant as affected by As, N, P and pH.

a study of Wang et al. (2017), who showed that maximum TAs, As(V), and As(III) accumulation in *M. aeruginosa* occurred under a low P level, and this was because the growth of *H. verticillata* is more dependent on P supply compared to *M. aeruginosa*. Namely, the cell density of *M. aeruginosa* increased by a factor of 1.44 under the same P level; nevertheless, *H. verticillata* biomass only increased by approximately 10% (data not shown) in our study. Previous studies revealed that *M. aeruginosa* can store large amounts of P in cells, while lower P availability was adequate in external media. Endogenous P is then mainly used for purposes of growth, whereas exogenous P is used during the initial stage of the culture media (Zheng et al., 2012; Su et al., 2013; Luo et al., 2015), indicating that the P requirement for *M. aeruginosa* growth is less than that of *H. verticillata*. Results from our study can potentially provide a feasible strategy for the remediation of As-contaminated water, while the overall efficiency of phytoremediation in As-contaminated water can be improved by the rational

control of P concentrations (i.e., maintaining an intermediate P level).

The maximal As(V) accumulation (i.e., N at level 1) (Fig. 2b) determined by our study indicated that a low N level is adequate for As(V) accumulation by *H. verticillata*. Krayem et al. (2016) reported a similar finding; namely, they found greater copper (Cu) and As accumulation in Myriophyllum alterniflorum under oligotrophic conditions. However, it is worth noting that the highest TAs accumulation by *H. verticillata* in our study was for N at level 3 (Fig. 2c), and this was because the greatest reduction from As(V) to As(III) and As(III) accumulation by *H. verticillata* was with N at level 3 (Fig. 2a), indicating that high N levels may have an important influence on phytoremediation due to their significant effect in reducing As(V) to As(III) (Che et al., 2018) as well as As(III) accumulation. On the one hand, N is the main component of phytochelatins; on the other hand, the synthesis of phytochelatins is induced in *H. verticillata* in culture media in the presence of As (Lou and Shen, 2001). Additionally, As(III) has a high affinity to phytochelatins and can be transported to vacuoles for storage, whereas As(V) does not bind to phytochelatins (Shukla et al., 2012; Nigam et al., 2013). As a result, high levels of N will enhance the tolerance, accumulation, and transformation of As in plants (Lou and Shen, 2001). Increased N levels in As-contaminated water could potentially be a promising and effective strategy to increase phytoremediation efficiency in As-contaminated water.

The maximal As(III) and TAs accumulation by *H. verticillata* were detected at intermediate pH (7) (Fig. 2a and c), indicating that in our study an intermediate pH level benefited TAs and As(III) accumulation in *H. verticillata*. This is because pH is firstly an important parameter for plant growth through its photosynthetic effect on submerged plants, while low pH levels will result in greater chlorophyll loss compared to high pH levels. Secondly, pH controls As species in culture media, while AsO_4^{3-} is the main species at high pH levels. Furthermore, the affinity of As/P transporters is higher in the presence of highly electronegative AsO_4^{3-} compared to $HAsO_4^{2-}$ and $H_2AsO_4^-$, and high pH could enhance As accumulation in plants. However, high pH levels do not aid in the reduction of As(V), and this is due to plant damage resulting from high TAs accumulation (Chen et al., 2014). Conversely, results from our study disagree with results from Tu and Ma (2003), who reported that As uptake in *P. vittata* increased at pH levels <5.21 in a 0.2 Hoagland solution. Moreover, a previous study reported the successful growth of *M. aeruginosa* at pH 6, and maximum intracellular As accumulation occurred at pH 10 in a BG11 medium (Wang et al., 2017). These results indicated that the effects of pH on As accumulation in plants are complex; moreover, pH is not only correlated to N and P in culture media but is also associated with individual plant species. However, precise mechanisms associated with accumulation and phytoremediation are unclear and require further investigation.

2.2. Arsenic species in culture media

As(V) concentrations significantly decreased by (62.2 ± 1.6)% - (72.0 ± 4.8)% in E1, E5, and E9, by (65.6 ± 0.8)% - (76.1 ± 2.1)% in E2, E6, and E7, and by (59.7 ± 3.2)% - (90.1 ± 0.4)% in E3, E4, and E8 after 96-hr incubation, respectively, when compared to initial culture concentrations; however, As(V) remained the dominant As species with trace amounts of As(III) in culture media (Fig. 3). Analysis of variance results (Appendix A Table S4) indicated that the initial As(V) concentration exhibited the most influential effect. Additionally, the most significant As(V) differences in culture media were observed under the same initial As(V) concentration, indicating that the effects of N, P, and pH on As(V) concentrations in culture media after 96-hr exposure were statistically significant (Fig. 3). The PC values of these factors (Appendix A Table S5) revealed that the rank order for As(V) uptake in culture media was: As(V) (61.0%), P (14.8%), N (11.5%), and pH (11.6%). According to the S/N ratios, the highest As(V) concentration in culture media was As(V) at level 3 (375 μg/L), N at level 1 (2 mg/L), P at level 3 (1 mg/L), and pH at level 3 (9) at the conclusion of the experiment (Fig. 4b).

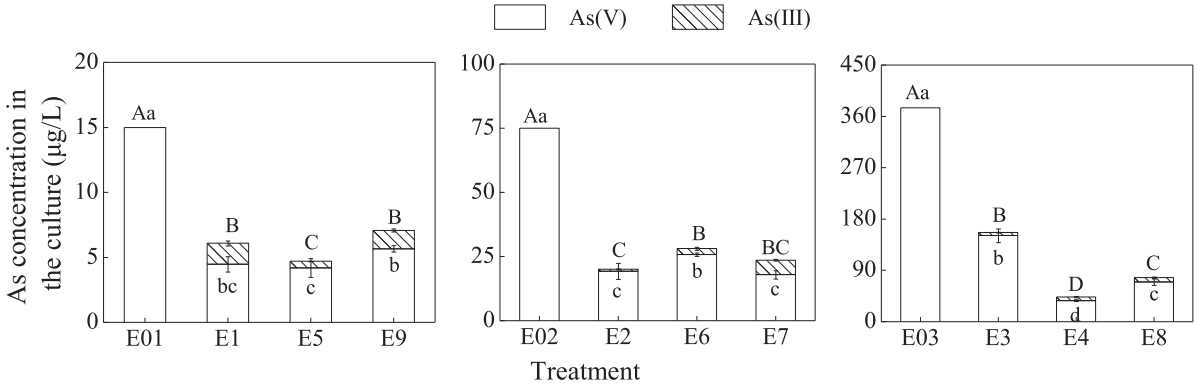

Fig. 3 – As(III), As(V) and TAs concentrations in the culture at 96 hr. TAs = As(III) + As(V). E01: As(V) 15 μg/L; E1: N 2 mg/L, P 0.02 mg/L, pH 6, As(V) 15 μg/L; E5: N 4 mg/L, P 0.2 mg/L, pH 9, As(V) 15 μg/L; E9: N 10 mg/L, P 1 mg/L, pH 7, As(V) 15 μg/L; E02: As(V) 75 μg/L; E2: N 2 mg/L, P 0.2 mg/L, pH 7, As(V) 75 μg/L; E6: N 4 mg/L, P 1 mg/L, pH 6, As(V) 75 μg/L; E7: N 10 mg/L, P 0.02 mg/L, pH 9, As(V) 75 μg/L; E03: As(V) 375 μg/L; E3: N 2 mg/L, P 1 mg/L, pH 9, As(V) 375 μg/L; E4: N 4 mg/L, P 0.02 mg/L, pH 7, As(V) 375 μg/L; E8: N 10 mg/L, P 0.2 mg/L, pH 6, As(V) 375 μg/L. Capital and lowercase letters indicate the significance of TAs and As(V) in 4 treatments, respectively. $n = 3$, data are means ± standard deviation.

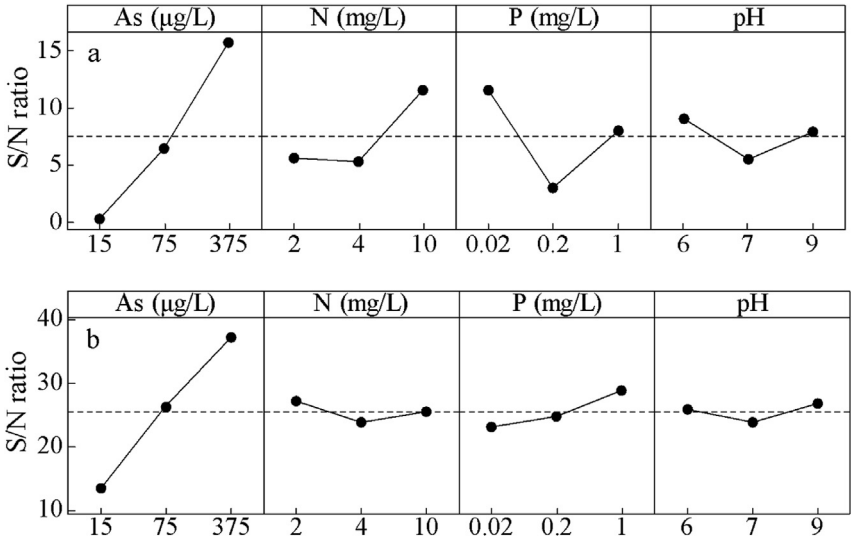

Fig. 4 – Mean S/N ratios for (a) As(III), (b) As(V) in the culture as affected by As, N, P and pH.

High As(V), P, and pH with low N were optimal conditions for attaining the highest As(V) concentration in culture media in this study. On the one hand, P can inhibit As(V) uptake owing to competition between P and As(V) for transporter sites on cell membranes (Panuccio et al., 2012). On the other hand, a portion of As(V) in culture media may derive from plant efflux through passive leakage, as confirmed by a study by Xue et al. (2012) on *Ceratophyllum demersum*. In our study, low N and high pH indirectly accelerated As(V) concentrations in plants by limiting its reduction (Fig. 2b), which likely caused greater As(V) efflux into external solutions.

Besides As(V), only As(III) was detected in culture media at the end of the experiment. It is noteworthy that As species showed no transformation in our plant-free control experiments after 96-hr incubation (Fig. 5). Additionally, previous studies reported that As(V) reduction by microbes and shoot exudates was slight in culture media (Xu et al., 2007; Xue and Yan, 2011). This indicated that As(III) in culture media primarily derives from transformation processes and efflux during phytoremediation by *H. verticillata*. This was also confirmed in *Azolla*, *Wolffia globose*, and *C. demersum*, suggesting that As(V) in plant cells is rapidly reduced to As(III), whereby plants subsequently release As(III) into the external solution (Xue et al., 2012; Zhang et al., 2008, 2009). Additionally, As(III) accounted for (10.8 ± 1.5)% - (26.6 ± 4.5)% in E1, E5, and E9, (4.2 ± 0.2)% - (24.1 ± 1.3)% in E2, E6, and E7, and (3.6 ± 0.4)% - (14.5 ± 2.7)% in E3, E4, and E8. Although As(III) concentrations increased, its overall proportion decreased with an increase in As(V) in culture media at the conclusion of the experiment, and this was due to the high initial As(V) level. Results provided in Fig. 3 and Appendix A Table S6 show the effects of N, P, and pH on As(III) in culture media under different As(V) levels. At the conclusion of the experiment, the proportion of As(III) accounted for (3.6 ± 0.4)% - (26.6 ± 4.5)% of TAs for the different treatments, which was lower than the As(III) efflux (>60%) reported in a study by Xue et al. (2012). The presence of P and N in our study may have been the main reason for the higher As accumulation in *H. verticillata* vacuoles and the lower efflux when compared to studies under low P and N. Additionally, P and N are essential to plant growth, and N is critical for As(V) reduction and As(III) accumulation in plants (Che et al., 2018), indicating that As species transformation is affected not only by As but also by N and P concentrations and pH levels in ambient aquatic environments. Moreover, the PC values of these factors in Appendix A Table S5 show that the rank order for As(III) in culture media was: As(V) (69.6%), N (12.3%), P (7.4%), and pH (2.6%), while growth and transformation affected by N were more influential than P and pH after As(V) levels in culture media in this study. Additionally, according to the S/N ratios (Fig. 4a), the highest As(III) concentration in culture media was for As(V) at level 3 (375 μg/L), N at level 3 (10 mg/L), P at level 1 (0.02 mg/L), and pH at level 1 (6), indicating that high As(V) and N and low P and pH

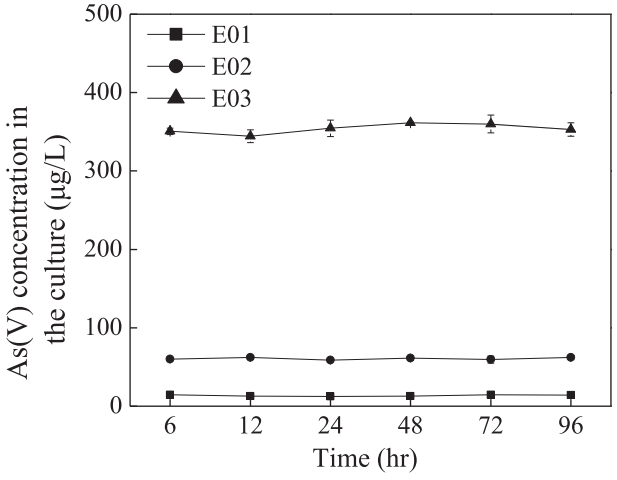

Fig. 5 – As(V) concentrations in the culture during 96-hr incubation. E01: N 2 mg/L, P 0.02 mg/L, pH 6, As(V) 15 μg/L; E02: N 4 mg/L, P 0.2 mg/L, pH 7, As(V) 75 μg/L; E03: N 10 mg/L, P 1 mg/L, pH 9, As(V) 375 μg/L $n = 3$, data are means ± standard deviation.

are in combination the most beneficial to As(III) efflux in external solutions by means of promoting As uptake and transformation by plants. Although high As(III) concentrations were primarily caused by high As(V) concentrations, the optimal environmental factors for As(III) and As(V) in culture media were inconsistent. This was due to the fact that As(III) in culture media resulted from a reduction in As(V) and subsequent As(III) efflux from *H. verticillata* cells; namely, it did not directly derive from the initial As(V) added to the culture media. It is important to note that As(III) toxicity in natural water is considerably more severe than that of As(V); therefore, the presence of As(III) in external solutions may pose a threat to organisms regardless of concentration level. Higher As(III) accumulation and lower efflux in culture media, regulated by N and P, are the conditions most beneficial to highly efficient phytoremediation of aquatic environments.

3. Conclusions

Among the many available methods, bioremediation has proven to be both an efficient and ecofriendly option. However, we found that environmental factors are complex and have significant influence on phytoremediation processes. This study offers insight into As accumulation and transformation in plants and culture media using different N, P, As(V) concentrations and initial pH, employing *H. verticillata* as the phytoremediation species under an orthogonal design. Our experiment resulted in a decrease in As(V) concentrations under various conditions as well as the appearance of As(III) under different N and P concentrations and pH levels. A combination of high N and intermediate P levels was best for greater As(V) accumulation and biotransformation. However, a combined high N and low P level resulted in higher As(III) efflux in culture media during phytoremediation by *H. verticillata*. Therefore, an increase in N under a controlled P concentration is an effective strategy to improve As phytoremediation efficiency in aquatic environments.

Lastly, it is particularly important to understand that even though the regulation of environmental factors (i.e., N, P, pH, etc.) is beneficial to As accumulation in plants, selecting the most effective plant species remains the first priority in phytoremediation.

Conflict of interest

The authors declared that they have no conflicts of interest to this work.

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jes.2019.12.010.

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