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Influence of environmental factors on arsenite transformation and fate in the *Hydrilla verticillata* (L.f.) royle - Medium system

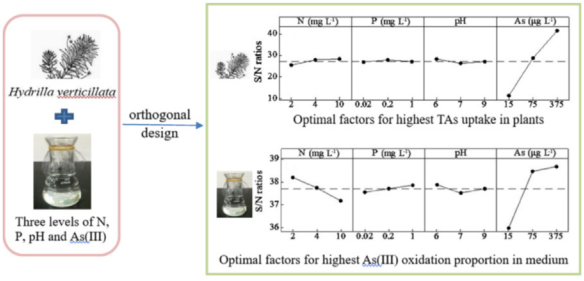
Yuan Zhao [a, b], Changzhou Yan [a, *], Zhuo Zhen [a, b]

[a] Key Laboratory of Urban Environment and Health, Fujian Key Laboratory of Watershed Ecology, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen, 361021, China
[b] University of Chinese Academy of Sciences, Beijing, 100049, China

HIGHLIGHTS

- Orthogonal design was used to determine optimum conditions for As uptake and biotransformation.
- High N and As(III) were advantageous for As uptake in plants.
- High P and As(III) displayed positive effect on As(III) transformation in the med-ium.

GRAPHICAL ABSTRACT



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ABSTRACT

***Hydrilla verticillata* (L.f.) Royle** has a great ability to accumulate large amounts of arsenic (As). We studied the influence of phosphorus (P), nitrogen (N), pH, and arsenite (As(III)) on As transformation and fate in the *H. verticillata* - medium system via orthogonal experimental design. The results showed highest plant growth was under intermediate As(III) in the medium, with Chlorophyll *a* and Chlorophyll *b* contents in plant diminishing after 96 h treatment. Exposure to high N, high As(III), intermediate P, and low pH in the medium, the highest total arsenic uptake by plants were 169.1 ± 5.5 μg g^{-1} dry weight, with As(III) as the predominant speciation (49.1 ± 4.8% to 88.5 ± 0.2%) in plants. Meanwhile, trace As (mainly arsenate (As(V))) was adsorbed on the surface of *H. verticillata*, and the adsorption amounts of As(V) increased with increasing As(III) concentrations in the medium. The dominant As species was As(V) in the medium although plant was supplied with As(III), and highest As(III) oxidation proportion in the medium would occur when low N and pH associated with high P and As(III). Collectively, As(III) uptake and transformation by *H. verticillata* cannot be overlooked in the biogeochemical cycling of As in aquatic environment.

1. Introduction

Arsenic (As) is a toxic, unnecessary element, has been classified as a pollutant (Islam et al., 2004), Arsenic contamination is becoming a serious health concern in the world (Zhao et al., 2009; Arco-Lázaro et al., 2018; Saifullah et al., 2018). Fresh water is an important part of the hydrosphere, maintaining regional ecological system balance and biodiversity. However, in recently years, waste discharge, rock weathering, mining, manufacturing, fertilizer and pesticide using have been causing serious eutrophication and As

* Corresponding author.
E-mail address: czyan@iue.ac.cn (C. Yan).

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pollution (Li et al., 2020; Niu et al., 2020) in fresh water. Arsenate (As(V)) and arsenite (As(III)) are the most common species of As found in freshwater (Srivastava et al., 2007; Hasegawa et al., 2010), in which maintaining a dynamic equilibrium of As(V) and As(III) among uptake, adsorption, release, mobility and transform by aquatic organisms or sediments. As(V) is taken up by phosphate transporters while As(III) uptake shares the highly efficient silicon (Si), water and glycerine channels of entry to cells (Zhao et al., 2009; Zhang et al., 2013), they also can be biotransformed to methyl and organoarsenic species by aquatic organisms (Rahman et al., 2012; Li et al., 2015). These are all the critical process of As biogeochemical cycle (Wang et al., 2014a; Xie et al., 2014) in aquatic environment, influencing As species and concentration presenting in fresh water. So far, although some studies have involved in As transformation and fate in water-plant system (e.g., *Pteris vittata* (L.f.) vittata, *Dunaliella salina*, and *Microcystis aeruginosa*) (Su et al., 2008; Wang et al., 2016a, 2017), most studies involving in As transfer and fate still focused on the soil-plant system (e.g., rice, winter wheat (*Triticum aestivum* (L.) Carolus)) and summer corn (*Zea mays* (L.) Carolus)) (Wang et al., 2016b; Yang et al., 2016; Lin et al., 2018), soil-magnetite and hematite etc (Giménez et al., 2007; Yang et al., 2017), marine waters (Rahman et al., 2012; Foster and Maher, 2016) and sediments (Keon et al., 2001). Only few studies existing have paid attention to As transfer and transformation in fresh water. Additionally, mostly of previous studies still focused on the individual factor effect on As uptake and transformation by plants or microalgae, including P (Baker and Wallschläger, 2016) and nutrient level (oligotrophic or eutrophic) (Krayem et al., 2016), which imposing a restriction on the interpretation of As biogeochemical cycle in fresh water without considering the integrated and systematic effects of As, N, and P concentrations and pH levels.

Some previous studies reported that submerged macrophytes have shown considerable potential tolerance, accompanying with high ability to uptake and transform As, such as *Hydrilla verticillata* (L.f.) Royle (Xue and Yan, 2011), *Vallisneria neotropicalis* (V.) Marie (Lafabrie et al., 2011), *Ceratophyllum demersum* (L.) Coontail (Xue et al., 2012) and *Myriophyllum alterniflorum* (DC.) Haloragaceae (Krayem et al., 2016). However, both of As(V) and As(III) are toxic to plants in freshwater. The toxicity of As(V) depends on the damage of P metabolisms in plants (Miot et al., 2009), while the combination with GSH is responsible for the toxicity of As(III), impacting the normal functions of several enzymes (Wang et al., 2014a). Arsenic would lead to growth inhibition, photosynthesis reduction, and malondialdehyde content increasing in plants (Krayem et al., 2016), which consequently impact As uptake and transformation by plants. In addition, various physicochemical and biological factors influence As uptake and transformation in plant and As species distribution in water, including P (Kao et al., 2011; Srivastava et al., 2018), N (Srivastava et al., 2019), pH (Chen et al., 2014), microorganisms (Wang et al., 2012), plant species (Favas et al., 2012) etc. Yamaoka et al. (1996) showed that the Rhaphydophyceae *Chattonella antiqua* (H.) Ono growth was inhibited when the levels of As higher than 10 mg L^{-1}, as well as As accumulation in *C. antiqua* was limited when the concentration of N was more than 18 mg L^{-1}. Conversely, Srivastava et al. (2019) found that high N stimulated As uptake in rice. As(V) uptake and reduction were improved by higher As(V) concentration in cultures grown at a low concentration of P (Knauer and Hemond, 2000; Wang et al., 2013a). Additionally, increasing P concentration resulted in significant decrease in intracellular As after exposure to both As(III) or As(V), and P-deficiency may lead to methylation in *Chlorella salina* (Karadjova et al., 2008). What cannot be ignored is that, pH also plays the critical roles on As uptake in plant, three-fold As(V) accumulation was found in *Stichococcus bacillaris* at pH 6.8 than that at pH 8.2 (Pawlik-Skowrońska et al., 2004). Some researchers have studied the systematic and integrated effects of P, N, pH and As(V) on P uptake by *P. vittata* (Tu and Ma, 2003) and As uptake by *M. aeruginosa* (Wang et al., 2017). However, until now, little has been studied regarding the influence of environmental factors on As(III) uptake and transformation by submerged plants in fresh water. Therefore, the impact of environmental factors on As(III) transformation and fate in the plant - medium system remains unclearly and warrant further research.

This paper is a development and enlarging of our earlier paper (Zhao et al., 2020), where the same experiment was performed for As(V) under same environmental factors with this study. The destination of this study was to investigate the influence of As(III), N, P and pH on the growth of plants, As uptake and speciation in *H. verticillata* and the changes of As concentration and species in the medium, providing insights into As(III) transformation and fate in the *H. verticillata* - medium system.

2. Materials and methods

2.1. Plant maintenance and incubation

H. verticillata samples were collected from the eastern section of Lake Taihu (120°20′41.6″ E, 30°56′55.1″ N) located in Wuxi City, China in June 2017, then were maintained in a greenhouse more 5 months. We selected the fresh shoot apical meristem (approx. 5–7 cm) of plants in January 2018 and washed gently to remove soil debris or epiphytes, thereafter, the meristem was incubated for 7 d in artificial freshwater solutions with addition of N (KNO_3 as N source) at 4 mg L^{-1} and P ($NaH_2PO_4 \cdot 2H_2O$ as P source) at 0.2 mg L^{-1} according to eutrophic conditions for plants growth well in a controlled environment growth chamber. The composition of the artificial freshwater solutions was as follows (mg L^{-1}): 22.7 $MgSO_4 \cdot 7H_2O$, 30.7 $MgCl_2 \cdot 2H_2O$, 20.4 $CaCl_2 \cdot 2H_2O$, 45.7 NaCl, 26.0 $NaHCO_3$, 3.61 KCl, 1.41 $FeCl_3 \cdot 6H_2O$, 0.97 $Al_2(SO_4)_3 \cdot 18H_2O$, 0.19 $MnCl_2 \cdot 4H_2O$, and (μg/L): 3.86 $ZnSO_4 \cdot 7H_2O$, 2.17 $CuCl_2 \cdot 2H_2O$ (Xue and Yan, 2011). The temperature was kept constant at 27 ± 2 °C under a 14 h photoperiod with a light intensity of 9600 lx.

2.2. Experimental design

To investigate the effects of four major environmental factors (As(III), N, P, pH) on As(III) uptake and speciation in *H. verticillata* and As(III) transformation in the medium, orthogonal experiment was established using Minitab 17 as orthogonal Table 1 including four factors and three levels, accordingly, nine experimental schemes L_9 (3^4)in Table 2. Additionally, three control treatments (without plants) were also incorporated, thereby, 12 treatments and three replications in total in Table 2.

The levels of N and P referred to the maximum values of surface water environmental quality standards in China to represent mesotrophic, eutrophic, and hypereutrophic aquatic systems in surface water (Wang et al., 2017), and the pH level were selected based on the actual pH ranges of freshwater (Yan et al., 2016) and were adjusted with NaOH or HCl according to the design in Table 2 using a pH meter (PH 7110, WTW, Germany). The levels of As(III)

Table 1
The factors (N, P, pH and As(III)) of orthogonal test.

Level	N (KNO_3, mg L^{-1})	P ($NaH_2PO_4 \cdot 2H_2O$, mg L^{-1})	pH	As(III) (μg L^{-1})
1	2	0.02	6	15
2	4	0.2	7	75
3	10	1	9	375

Table 2
The design of orthogonal test.

Experiment design	Treatments	N (KNO_3, mg L^{-1})	P ($NaH_2PO_4 \cdot 2H_2O$, mg L^{-1})	pH	As(III) (μg L^{-1})
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

($NaAsO_2$) used were the same with our previous study based on the As concentration range in fresh water (Caumette et al., 2011, Che et al., 2018), and the tolerance of *H. verticillata* to As without significant restricting plant growth. Then, 0.5 g *H. verticillata* were cultured in 100 mL sterile artificial freshwater solution in triangular flask excluding control treatments. Water samples were obtained at 1, 3, 6, 12, 24, 36, 48, 72 and 96 h exposure using for As analysis and plant samples were harvested at 96 h for plant growth, chlorophyll, and As test.

The significance of environmental factors on As uptake in plants and As species in mediums, and comparison among treatments were determined by multi-way analysis of variance (MANOVA). Signal to noise (S/N) ratios (shown in Eq. (1)) were calculated as optimal environmental factor levels to maximize the As uptake in plants and As species transformation in the medium.

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

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

2.3. Plant relative growth

Fresh weight of plant in each treatment was recorded before and after experiment, the plant relative growth was presented as follows:

$$\text{Plant relative growth}\ (\%) = \left(W_{final} - W_{initial}\right)/W_{initial} \times 100\% \quad (2)$$

where, $W_{initial}$ represents the fresh weight (g) of plant at the beginning of the experiment, W_{final} is the fresh weight (g) of plant after 96 h incubation.

2.4. Chlorophyll test

60 mg fresh weight of leaf material was cut into filaments and added into tubes with plugs, simultaneously, 4 mL acetone 80% (v:v) and 4 mL ethanol were applied to Chlorophyll extraction in the dark for a night until the leaves fade to white. After complete extraction, the mixture was filtered and the volume was adjusted to 10 mL with acetone 80% (v/v) and ethanol. The mixture of acetone 80% (v/v) and ethanol was recorded as controls. The absorbance of the extract was read at 663 and 645 nm using UVPROBE (VU-2450, Daojin, Japan) and pigment concentrations were calculated according to the following formula in Meher et al. (2018):

$$\text{Chlorophyll 'a'}\ (mg/g) = (12.7 \times A663 - 2.69 \times A645)\ V/W \quad (3)$$

$$\text{Chlorophyll 'b'}\ (mg/g) = (22.9 \times A645 - 4.68 \times A663)\ V/W \quad (4)$$

where, 12.7, 2.69, 22.9, and 4.68 are the constants, A means the absorbance at specific wavelengths, V is the final volume of chlorophyll extract in 80% acetone, W represents fresh weight of tissue extracted.

2.5. Analysis of As species in the medium

After 0.5 mL nutrient medium were taken from each vessel, they were immediately diluted with a phosphate buffer solution (PBS) (Xue et al., 2012) of 2 mM NaH_2PO_4 and 0.2 mM Na_2EDTA (pH 6.0), EDTA was used to prevent As species changing in water samples (Bednar et al., 2002; Xu et al., 2007). Thereafter, water samples were filtered through 0.45-μm filters before analysis of As species. We analyze As species content in these medium samples with High-performance liquid chromatography (HPLC, Agilent LC1200 series, Agilent Technologies, USA) associated with inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700a, Agilent Technologies, USA). 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) under the mobile phase comprised by 10 mM $(NH_4)_2HPO_4$ and 10 mM NH_4NO_3 (pH 6.25), the pH at 6.25 was regulated with HNO_3 or $NH_3 \cdot H_2O$ (Guaranteed reagent, Chemical Reagent Purchasing and Supply Station, China), and run isocratically at 1.0 mL min^{-1}. Identification of As morphology and calculation of As concentration in the samples were realized by comparing with the retention times of standard compounds and external calibration curves with peak areas. The planks were applied during analysis.

2.6. Speciation analysis of As adsorption and uptake by plants

Plant samples harvested after 96 h As(III) exposure were rinsed briefly with Milli-Q water and then immersed for 10 min in 50 mL an ice-cold desorption solution consisted of 1 mM K_2HPO_4, 0.5 mM $Ca(NO_3)_2 \cdot 4H_2O$ and 5 mM MES (pH 6.0) to obtain the extracts of As adsorption on the surface of plant. Thereafter plant samples were dry and frozen to constant weight, and then were ground in a mortar and pestle with liquid nitrogen (LN_2). To obtain the extracts of As uptake in plant, about 0.03 g of the ground materials were extracted in 5 mL PBS at 4 °C for 1 h under sonication. Then we filtered extracts of As adsorption and uptake through four layers of muslin cloth, followed by filtration through 0.45-μm filters for As speciation content analysis as that in water samples. Additionally, the initial As(V) and As(III) concentrations determined before

experiment starting in *H. verticillata* were small enough to make no statistical difference.

2.7. Total arsenic analysis in the medium and plant samples

0.02 g plant samples grinded were digested with HNO_3:H_2O_2 (2:1, v:v) using Auto Digestion Unit (X-42 A+, Shengsheng, China) for total arsenic (TAs) analysis in plant samples. We raised the temperature to 55 °C for 15 min, then raised again to 75 °C for 15 min, finally held at 120 °C for 2 h, then cooled these samples to ambient temperature to obtain the supernate of plant samples. Both supernate and water samples filtered through 0.45 μm filters were prepared for TAs analysis. TAs analysis was conducted by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500cx, Agilent Technologies, USA), the planks were also included. To verify the accuracy of the analysis of metals/metalloids in plant, certified reference materials (Bush twigs and leaves, GBW 07602 GSV-1; National Research Center for Standards, China) were included. Recoveries were 82.1–96.1% for TAs.

No difference was noted between TAs content by digesting and the sum of As(V) and As(III) concentrations in water and plant samples. Therefore, we calculated TAs content by the sum of As(V) and As(III) concentrations in this study.

2.8. Data analysis

All values (means ± standard deviations) were presented as of triplicate in Microsoft Excel (2016). OriginPro 8.5.0 SR1 software (Origin Lab Corporation, 1991–2010) was applied to show concentrations of As species. Multi-way analysis of variance (MANOVA) and comparison among treatments was employed using R.3.5.2 to examine significant differences among the treatments, the value of $P < 0.05$ was considered as significant. S/N ratios of optimal environmental factor levels for highest As uptake in plant and As transformation in the medium were processed in Minitab 17.

3. Results and discussions

3.1. Plant relative growth and chlorophyll content

It has been reported that the tolerance of plant to As(III) can be evaluated by plant growth (Wang et al., 2013c; Xu et al., 2016), and it affected uptake and accumulation of As in plants. Moreover, Chlorophyll is directly connected to the photosynthetic capacity of plants, so that Chlorophyll could influence plant growth (Meher et al., 2018) and indirectly related to tolerance of plant to As. In this study, when supplemented with As(III) in the medium, both plant relative growth and Chlorophyll in *H. verticillata* were monitored and then plotted in Fig. 1, which further indicated plant growth was found after 96 h incubation, simultaneously, variation of plant growth was influenced by N, P, pH, and As(III) levels from Table A1. We can also concluded that As(III) concentrations in the medium had the most influential role on plant relative growth (lowest *P* value in Table A1), this conclusion was conversely from the result in Tu and Ma (2003), they confirmed that P ranked first, this maybe resulted from the consequence of higher As tolerance of *P. vittata* than that of *H. verticillata*. Additionally, plant relative growth was higher in intermediate As(III) than that in low and high levels, indicating that intermediate As(III) in the medium could stimulate plant growth compared to the other two levels, which is consistent with the result in Xue et al. (2012), they declared that high As might cause toxicity to plant.

Compared with the initial Chlorophyll concentration in plants, both Chlorophyll *a* in E5, E2, and E8 and Chlorophyll *b* in E5, E2, E8, and E4 decreased significantly (see Fig. 1b and c). Strangely, plant relative growth in E5, E2, and E4 were higher than that in the other two treatments under the same As(III) level. In view of this, maybe the proper N and P was the primary reason leading to faster plant growth in E5 (Liu et al., 2016; Srivastava et al., 2019). Specifically, Fig. 1 b and **1c** provided the result for low Chlorophyll *a* and *b* at high pH in E5, this was opposite with the result in Chen et al. (2014), showing more Chlorophyll loss in *Vallisneria natans* (L.) Hara under low pH, however, it was similar to result in Tu and Ma (2003), in which increased biomass in *P. vittata* was found when pH increasing in the medium. These different conclusions may originated from the plant species or complicated interaction of N, P, pH and As combination in different treatments based on the results from Table A.2 to **A.5**, in which N, P, pH and As(III) played no statistically significant individual effect on Chlorophyll in *H. verticillata* leaves, but different treatment (different factors combination) had a significant influence on Chlorophyll in plant. Additionally, N is the predominant element of Chlorophyll, however, a previous study considered that high N may lead to injures restraining growth in plants (Krayem et al., 2016), so that leading to high Chlorophyll and low growth in E9 in this study. As for E8, high As(III) associated with severer stress was the most reasonable explanation for low plant growth and Chlorophyll according to the study in Srivastava et al. (2013).

3.2. As uptake and speciation in plants

The result of As uptake (Fig. 2) indicated that, after treatment of

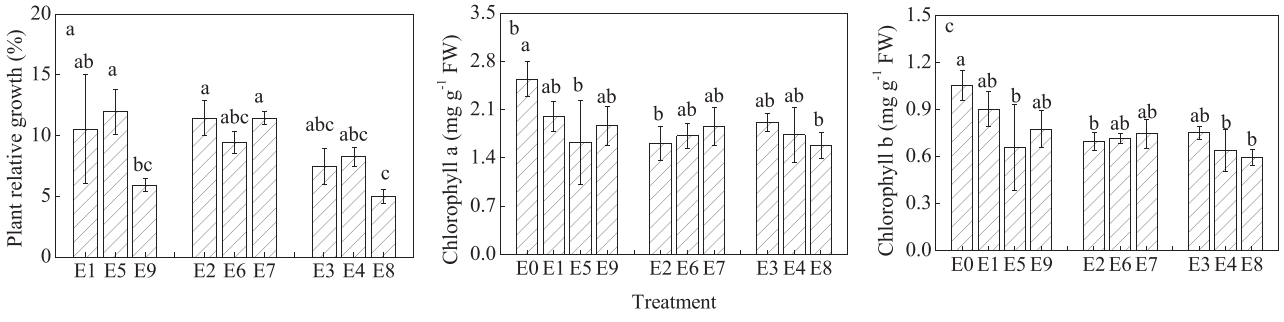

Fig. 1. Plant relative growth (a), Chlorophyll *a* (b) and Chlorophyll *b* (c) in plants. E1: N 2 mg L^{-1}, P 0.02 mg L^{-1}, pH 6, As(III) 15 μg L^{-1}; E5: N 4 mg L^{-1}, P 0.2 mg L^{-1}, pH 9, As(III) 15 μg L^{-1}; E9: N 10 mg L^{-1}, P 1 mg L^{-1}, pH 7, As(III) 15 μg L^{-1}; E2: N 2 mg L^{-1}, P 0.2 mg L^{-1}, pH 7, As(III) 75 μg L^{-1}; E6: N 4 mg L^{-1}, P 1 mg L^{-1}, pH 6, As(III) 75 μg L^{-1}; E7: N 10 mg L^{-1}, P 0.02 mg L^{-1}, pH 9, As(III) 75 μg L^{-1}; E3: N 2 mg L^{-1}, P 1 mg L^{-1}, pH 9, As(III) 375 μg L^{-1}; E4: N 4 mg L^{-1}, P 0.02 mg L^{-1}, pH 7, As(III) 375 μg L^{-1}; E8: N 10 mg L^{-1}, P 0.2 mg L^{-1}, pH 6, As(III) 375 μg L^{-1}. E0 is the initial Chlorophll a and Chlorophll b contents in plant. FW: Fresh weight. $n = 3$, data are mean ± standard deviation. Lowercase letters indicate the significance of plant relative growth, Chlorophll a and Chlorophll b, respectively.

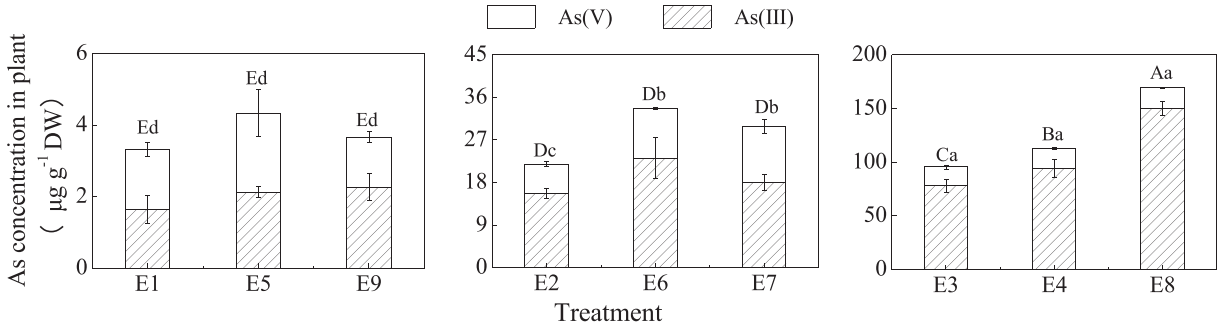

Fig. 2. As speciation and concentration in plants after 96 h. E1: N 2 mg L^{-1}, P 0.02 mg L^{-1}, pH 6, As(III) 15 μg L^{-1}; E5: N 4 mg L^{-1}, P 0.2 mg L^{-1}, pH 9, As(III) 15 μg L^{-1}; E9: N 10 mg L^{-1}, P 1 mg L^{-1}, pH 7, As(III) 15 μg L^{-1}; E2: N 2 mg L^{-1}, P 0.2 mg L^{-1}, pH 7, As(III) 75 μg L^{-1}; E6: N 4 mg L^{-1}, P 1 mg L^{-1}, pH 6, As(III) 75 μg L^{-1}; E7: N 10 mg L^{-1}, P 0.02 mg L^{-1}, pH 9, As(III) 75 μg L^{-1}; E3: N 2 mg L^{-1}, P 1 mg L^{-1}, pH 9, As(III) 375 μg L^{-1}; E4: N 4 mg L^{-1}, P 0.02 mg L^{-1}, pH 7, As(III) 375 μg L^{-1}; E8: N 10 mg L^{-1}, P 0.2 mg L^{-1}, pH 6, As(III) 375 μg L^{-1}. TAs = As(III) + As (V). DW: Dry weight. $n = 3$, data are mean ± standard deviation. Capital and lowercase letters indicate the significance of As(III) and As (V), respectively.

H. verticillata for 96 h with As(III), As(III) was the predominant As speciation in plants, the highest TAs uptake in plants was 169.1 ± 5.5 μg g^{-1} DW, the proportion of As(III) in TAs ranged from 49.1 ± 4.8% to 61.7 ± 0.2% in E1, E5 and E9, from 60.4 ± 1.2% to 72.1 ± 2.2% in E2, E6 and E7, from 81.6 ± 1.7% to 88.5 ± 0.2% in E3, E4 and E8 in *H. verticillata*, respectively. No organic As speciation was detected in plants. The result of ANOVA (Table A.6) showed that As(III) concentration in the medium was the most influential factor for As(III) uptake in plants. Since As(III) can be inactive to form no toxic complexes with low molecular organics, such as glutathione (GSH) or phytochelatins in plant vacuoles (Wang et al., 2013b), more As(III) was taken up into plant with the gradually increasing As(III) concentration in the medium. Additionally, based on the result of comparison among given in Fig. 2, remarkedly difference of As(III) concentration in *H. verticillata* was observed when As(III) was supplemented at level 3, which reflected that the concentration of As(III) in *H. verticillata* was obviously affected by N, P and pH levels at high initial As(III) level in the medium. At the same time, As(V) concentration in plants was also monitored, ranging from 1.4 ± 0.2 to 19.4 ± 0.9 μg g^{-1} DW in *H. verticillata*. Interestingly, a previous study indicated that regardless of the form of As supplied was As(III) or As(V), in leaves of *H. verticillata* the dominant form was As(III) (Xue and Yan, 2011). Therefore, we summarized that As(V) in plants mainly be attributed to the uptake of As(V) into plants in the medium, rather than oxidation of As(III) in plant. Table A.7 provided the ANOVA results, which reflected that the effects of N, P, pH and As(III) levels on the concentration of As(V) in *H. verticillata* were statistically significant ($P < 0.05$). However, the influence of P was remarkedly smaller than that of As(III), N and pH based on the relative high *P* value. We speculated that this may be due to the fact that the effect of P on As(III) oxidation induced by epiphytic bacteria was lower than other environmental factors (As(III), N and pH). Up to now, little information is available in the literature on the effect of environmental factors on As(III) transformation in the medium, so it is worth studying in the near future.

Maximum S/N ratio revealed the optimal factors combination for greatest As uptake in plants. The results in Fig. 3a and c showed highest As(III) and TAs concentration in *H. verticillata* under the combination (exactly E8) of N at level 3 (10 mg L^{-1}), P at level 2 (0.2 mg L^{-1}), pH at level 1 (6), and As(III) at level 3 (375 μg L^{-1}). The highest As(V) concentration in *H. verticillata* occurred during the combination of N at level 2 (4 mg L^{-1}), P at level 1 (0.02 mg L^{-1}), pH at level 3 (9), and As(III) at level 3 (375 μg L^{-1}) in Fig. 3b. It has been well documented that N is a vital element for plant growth and its optimum supply is required for plant healthy growth (Srivastava et al., 2019). Additionally, N is the main component of phytochaletins (Lou and Shen, 2001), and As(III) has a high affinity to glutathione (GSH) and phytochelatins and can be transported to vacuoles for storage in plants (Levy et al., 2005; Nigam et al., 2013; Punshon et al., 2017). Conversely, an excess supply of N has been found to decline the Cd accumulation in soybean plant roots (Konotop et al., 2012). Likewise, Yamaoka et al. (1996) certified that As accumulation was inhibited at N levels more than 18 mg L^{-1}. However, the high N level (10 mg L^{-1}) was lower than that in Yamaoka et al. (1996). Consequently, the highest As(III) and TAs uptake in *H. verticillata* generated during high N level in our study. It was also evidenced that high N was found to enhance biotransformation of As(V) to As(III) in plants (Che et al., 2018). We concluded intermediate N was most advantageous for the highest concentration of As(V) in *H. verticillata* in this study. However, this result differed from our previous study compared to exposing with As(V), showing that highest As(V) was found in plants under low N, since N is related to not only the uptake of As(III) supplemented in the medium, but also preserving in plant cells in this study.

Two explanations for the result that intermediate P exhibited the most positive effect on highest As(III) and TAs in plants illustrated as follows. Phosphate is a major element to plants growth (Huang et al., 2004; Gonzaga et al., 2008); on the contrary, the P-limited cells might synthesize transporters with higher binding affinity to uptake more As(III) (Wang et al., 2014a), accordingly, the phenomenon that high P might restrain the reduction of As(V) in cells was also found by Slaughter et al. (2012). The optimal P was at level 1 for highest As(V) uptake in plants in our study, in agreement with the study of Wang et al. (2013a) and Wang et al. (2017). This was thought to be due to both the less growth under low P level (Chauhan et al., 2018; Karadjova et al., 2008) and high As(V) uptake resulted from low inhibition by low P acting as the chemical analog (Wang et al., 2014b; Baker and Wallschläger, 2016) in the medium.

The result of greatest As(III) and TAs uptake in plant was found during low pH level, it was in disagreement with our previous study (intermediate pH level), while high pH was most beneficial for highest As(V) uptake in *H. verticillata*, this is conformed with our previous study. Easily forming stable As-PC complexes in acidic environments may be one of the predominant reasons reported by Liu et al. (2012) in this study. However, a similar previous study of Pawlik-Skowrońska et al. (2004) reported, stronger toxic effects of As(V) than As(III) were observed on both *S. bacillaris* growth and chlorophyll content at pH 6.8 than 8.2. However, Taboada-de la Calzada et al. (1999) showed high pH was necessary to obtain significant As(III) accumulation in *Chlorella vulgaris*. The explanation of variety results may be attributed to the different plant species or As species availability relating to the pH in the medium, which remains largely unstudied and need more investigation.

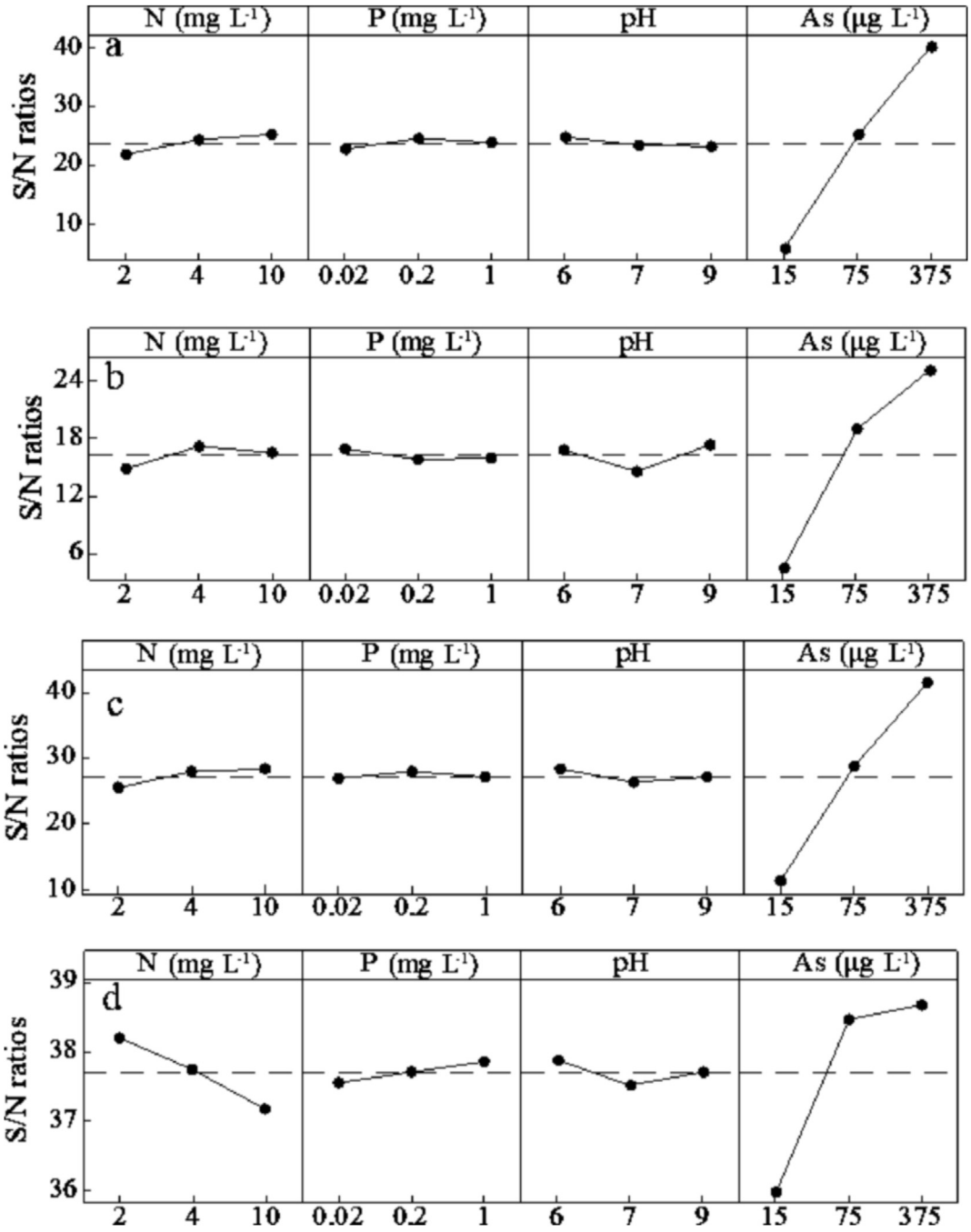

Fig. 3. Mean S/N ratios for (a) As(III), (b) As (V), (c) TAs uptake in plants and (d) highest As(III) oxidation proportion in the medium as affected by As, N, P and pH.

3.3. *As adsorption by plants*

The description of As adsorption by plants in Fig. 4 suggested that more TAs, As(III) and As(V) were adsorbed on the surface of *H. verticillata* with increasing As(III) concentration in the medium, this in accordance with the result in Table A.8, revealing that the leading factor to affect TAs adsorption among four factors was As(III). Specifically, TAs adsorption by plants in E3 was significantly higher than that in E4 and E8 during the same As(III) level (high level), indicating remarkedly effect on TAs adsorption by environmental factors was observed under high As(III) level in the medium. Additionally, although plant was supplied with As(III) in the medium, the predominant As speciation on the surface of *H. verticillata* was As(V). On account of the result in Figs. 2 and 5, chemical oxidation of As(III) in the medium is very little in a shorter time, and *H. verticillata* has no ability to oxidize As(III) to As(V) (Xue and Yan, 2011). Consequently, As(V) presented on the surface of plant must be related to epiphytic bacterial of *H. verticillata*, regardless of whether As(V) adsorption by plants came from oxidation of As(III) either in the medium or on the surface of *H. verticillata*. The result in Table A.9 revealed, every factor had statistically significant effect on As(V) adsorption, however, only As(III) concentration imposed the significant effect on As(III) adsorption (Table A.10). The possible reason for this inconformity is that As(III) adsorption was mainly originated from As(III) supplied in the medium, however, As(V) adsorption might result from firstly As(V) in the medium in which

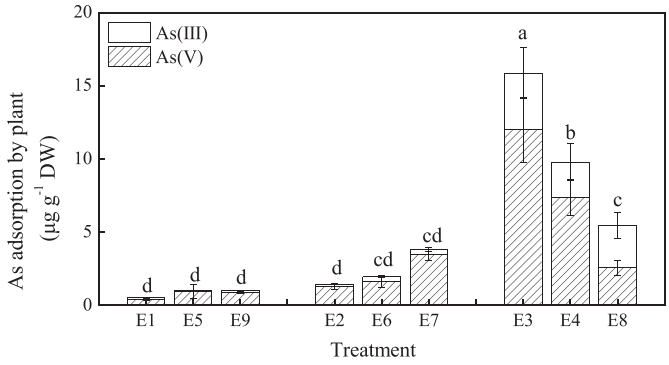

Fig. 4. As adsorption on the surface of plant. E1: N 2 mg L^{-1}, P 0.02 mg L^{-1}, pH 6, As(III) 15 μg L^{-1}; E5: N 4 mg L^{-1}, P 0.2 mg L^{-1}, pH 9, As(III) 15 μg L^{-1}; E9: N 10 mg L^{-1}, P 1 mg L^{-1}, pH 7, As(III) 15 μg L^{-1}; E2: N 2 mg L^{-1}, P 0.2 mg L^{-1}, pH 7, As(III) 75 μg L^{-1}; E6: N 4 mg L^{-1}, P 1 mg L^{-1}, pH 6, As(III) 75 μg L^{-1}; E7: N 10 mg L^{-1}, P 0.02 mg L^{-1}, pH 9, As(III) 75 μg L^{-1}; E3: N 2 mg L^{-1}, P 1 mg L^{-1}, pH 9, As(III) 375 μg L^{-1}; E4: N 4 mg L^{-1}, P 0.02 mg L^{-1}, pH 7, As(III) 375 μg L^{-1}; E8: N 10 mg L^{-1}, P 0.2 mg L^{-1}, pH 6, As(III) 375 μg L^{-1}. TAs = As(III) + As (V). DW: Dry weight. $n = 3$, data are mean ± standard deviation. Lowercase letters indicate the significance of TAs adsorption by plant.

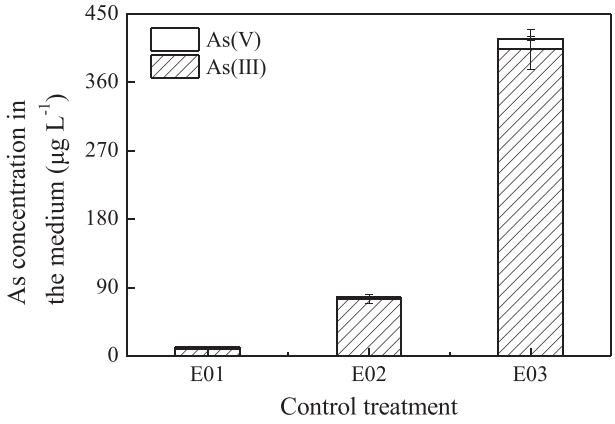

Fig. 5. As species and concentration in the medium of control treatments. E01: N 2 mg L^{-1}, P 0.02 mg L^{-1}, pH 6, As(III) 15 μg L^{-1}; E02: N 4 mg L^{-1}, P 0.2 mg L^{-1}, pH 7, As(III) 75 μg L^{-1}; E03: N 10 mg L^{-1}, P 1 mg L^{-1}, pH 9, As(III) 375 μg L^{-1} $n = 3$, data are mean ± standard deviation.

competing of P and As(V) for adsorption site was presented (Wang et al., 2014b, 2018). Secondly, As(III) adsorbed on the surface of *H. verticillata* was oxidized. Consequently, the difference of TAs adsorption in different treatments mainly be attributed to the effects of N, P, pH on As(V) adsorption by plants under high As(III) level in the medium. The solution pH is an important factor that affects the adsorption efficiency of adsorbent by influencing the amount of charge on the adsorbent surface and As species in the medium (Gong et al., 2019). The optimum pH values of ions, metal species and adsorbents are inconsistence (Altundoğan et al., 2000; Gong et al., 2019). The plentiful presence of H^+ at low pH will inhibit metal adsorption. High pH increased TAs adsorption in E3, E4 and E8 in this study. E3 treatment did not stimulate the combination of As(III) adsorbed with GSH (Bleeker et al., 2006) to be stored in plant cells was due to low N. Meanwhile, high P restrained the As(V) uptake into plant cells due to competition with As(V) (Zhao et al., 2009), therefore both low N and high P may enhance the oxidation of As(III) on the surface of plant by remaining more As adsorbing on the surface of plants. However, this explanation did not fully apply to intermediate and low As(III) level in the medium, much lower As adsorption than that under high As(III) level may responsible for the contradiction in this study. It's worth noting that, in E3, E4 and E8, an opposite trend between TAs uptake and adsorption was observed, this was thought to be due to a balance between As content in plants and plants biomass, based on the result that no significant plant biomass difference was observed in E3, E4 and E8 (Fig. 1a). Additionally, oxidation genes have been extracted from the epiphytes, and our group has identified that epiphytes separated from the surface of *H. verticillata* can oxidize As(III) in the medium (Zhen et al., 2020).

3.4. As species transformation in the medium

As(III) concentrations decreased gradually with the time increasing, contrastingly, As(V) concentrations came to increasing in the medium at the same time, the concentrations of As(III) and As(V) were tested to be equal during 24–36 h in E1, E5 and E9, 12–36 h in E2, E6 and E7, and 36–48 h in E3, E4 and E8, then the concentrations of As(V) reached higher than As(III) in the medium, namely, As(V) served as the dominant As species in the medium at later stage (Fig. 6). The time of As uptake equilibrium point was shorter under intermediate As(III) level than that under low and high As(III) level, which revealed that As(III) was taken up into plants at a faster speed during intermediate As(III) level compared to low and high levels in the medium. This finding was consistent with the study in Xue and Yan (2011), certifying that As accumulation displayed an increasing then decreasing trend with the As concentration accelerating in the medium.

After treatment of *H. verticillata* with As(III) for 96 h, the concentrations of As(III) ranged from 2.4 ± 0.1 to 22.1 ± 0.3 μg L^{-1} in nine treatments in the medium, accounting for 7.4 ± 1.8 to 41.0 ± 1.6% in the medium, meant that 59.0 ± 1.6% to 92.6 ± 1.8% of As(III) was transformed to As(V) in the medium. It was more three times than 2.5 ± 0.2 to 20.1 ± 4.0% in the controls (Fig. 5). Therefore, we concluded the oxidation of As(III) in the medium was mainly due to the present of plants. However, a previous study (Xue and Yan, 2011) and our results (Fig. 2) proved that there was no great oxidation of As(III) in *H. verticillata*. Consequently, the possible explanation for the oxidation of As(III) was affected by microorganisms or secretions of plant. Specifically, the proportion of As(V) in TAs in the medium were from 59.0 ± 1.6 to 67.0 ± 2.6% in E1, E5, and E9, from 77.5 ± 0.6 to 87.3 ± 0.7% in E2, E6, and E7, and from 82.9 ± 1.0% to 92.6 ± 1.8% in E3, E4, and E8, which illustrated that percentage of As(V) in TAs increased with the initial concentrations of As(III) increasing in the medium. However, under high As(III) level in the medium plant growth was lowest. More importantly, Wang et al. (2016a) confirmed that secretion of microalgae had no effect on the transformation of As morphology in the medium. In addition, Su et al. (2017) considered that a majority of proteins involved in metabolism, transport, and oxidative stress were upregulated in fungi *Trichoderma asperellum* SM-12F1 after As exposure. As a result, we suggested that As(V) was dominant As species owe to oxidation role of epiphytic bacterial.

ANOVA result and S/N ratios showed that four environmental factors all displayed significant effect on the oxidation of As(III) in the medium, and the factors combination for highest oxidation of As(III) was when N at level 1 (2 mg L^{-1}), P at level 3 (1 mg L^{-1}), pH at level 1 (6), and As(III) at level 3 (375 μg L^{-1}), given in Table A.11 and Fig. 3d, respectively. Low N and high P inhibited As(V) uptake into *H. verticillata* (Fig. 3b). It was due to plant growth limiting resulted from low N and uptake competition between P and As(V) (Xu et al., 2006; Wang et al., 2018). Low pH and high As(III) enhanced the As(III) assimilation by plants (Fig. 3a). Both low N associated with high P and low pH coexisting with high As(III) indirectly caused more proportion of As(V) remaining in the medium.

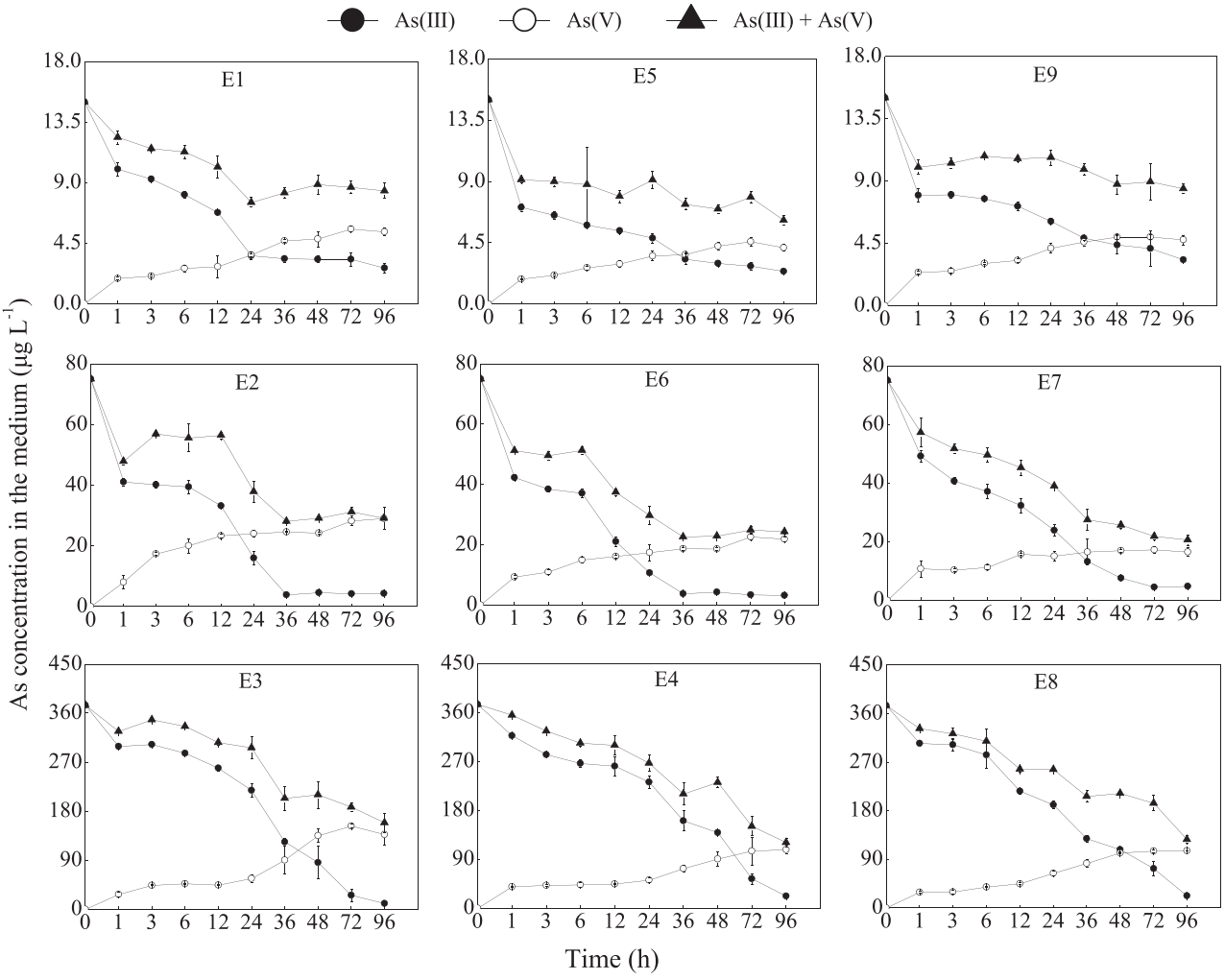

Fig. 6. As(III), As(V) and TAs (As(III) + As(V)) concentrations in the medium with time increasing. E1: N 2 mg L^{-1}, P 0.02 mg L^{-1}, pH 6, As(III) 15 μg L^{-1}; E5: N 4 mg L^{-1}, P 0.2 mg L^{-1}, pH 9, As(III) 15 μg L^{-1}; E9: N 10 mg L^{-1}, P 1 mg L^{-1}, pH 7, As(III) 15 μg L^{-1}; E2: N 2 mg L^{-1}, P 0.2 mg L^{-1}, pH 7, As(III) 75 μg L^{-1}; E6: N 4 mg L^{-1}, P 1 mg L^{-1}, pH 6, As(III) 75 μg L^{-1}; E7: N 10 mg L^{-1}, P 0.02 mg L^{-1}, pH 9, As(III) 75 μg L^{-1}; E3: N 2 mg L^{-1}, P 1 mg L^{-1}, pH 9, As(III) 375 μg L^{-1}; E4: N 4 mg L^{-1}, P 0.02 mg L^{-1}, pH 7, As(III) 375 μg L^{-1}; E8: N 10 mg L^{-1}, P 0.2 mg L^{-1}, pH 6, As(III) 375 μg L^{-1} $n = 3$, data are mean ± standard deviation.

4. Conclusions

Arsenic uptake and transformation of aquatic plants were affected not only by the specific As species but also by ambient environmental factors in freshwater systems. The aims of this study were to investigate As(III) transformation and fate in the *H. verticillata*-medium system under different As(III), N, P and pH levels via orthogonal design. Arsenite revealed to be the most important factor impacting As adsorption and uptake by plants, as well as plant growth. Also, high level of N and As(III) with intermediate P under weak acid pH conditions can improve As uptake in plants. High P and As(III) concentrations associated with low N and pH can accelerate the oxidation of As(III) to As(V). Being the first report on how these ambient environmental factors influence As(III) transformation and fate in the aquatic plant-medium system, this study provides new insight into how ambient environmental factors could be used to regulate As metabolism. Our findings can also benefit our understanding of As biogeochemical cycle in eutrophic environment, as well as practical applications of key environmental factors, particularly those related to aquatic phytoremediation in As polluted water. However, we must also consider in detail the statistical assumptions and limitations involving in no cross term effects while using orthogonal design method (Parks, 2001; Wang et al., 2017).

Author contribution

Zhao Yuan: Literature survey, Experimental design, Experimental material preparation, Experimental implementation, Data Formal analysis, Figure and table making, Writing-Original draft preparation. Yan Changzhou: Proposal of research ideas, Improvement of experimental design, Writing-Reviewing and Editing. Zhen Zhuo: Experimental material preparation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.127442.

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