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Arsenic Contamination in Agricultural Soil Reduces Metabolic Activity of Total and Free-Living Nitrogen-Fixing Bacteria as Revealed by Real-Time qPCR

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ABSTRACT

Laboratory microcosms were used to assess the impact of arsenic (As) contamination on agricultural soil bacterial activity focusing free-living nitrogen fixers for three months. Periodically collected microcosm samples were analyzed by RT–qPCR following extraction of total RNA and cDNA preparation for assessing the metabolically active bacterial population. RT–qPCR data showed the gradual increase of 16S rRNA and nifH gene expression, and relative activity of diazotrophs in the enriched soil bacterial consortia under short time As exposure up to 20 ppm and 10 ppm, respectively. A similar trend of these variables was also noticed but up to 1 ppm As when incubating the bulk soil for the same duration. Reduced bacterial activity was noticed at higher concentration of As although in short time exposure. Extending the As exposure time, the bacterial activities in both enriched consortia and bulk soil were decreased. Although, the relative activity of diazotrophs in enriched consortia was increased in presence of 10 ppm As, the same was decreased in bulk soil when exposed to >1 ppm As for long time indicating susceptibility of nitrogen fixer to As contamination in soil. PCA of the data obtained also indicated a negative correlation between As concentrations and diazotrophic activity.

KEYWORDS

Arsenic contamination; agricultural soil; bacterial consortia; 16S rRNA; *nifH* gene expression; real time qPCR

1. Introduction

Arsenic (As) contamination of soil and water poses a severe threat to human health and livestock around the world (Mandal and Suzuki, 2002; Wang and Mulligan, 2006; Rahman and Singh, 2016; Ng *et al.*, 2017). The concentrations of As in uncontaminated soil range from 1 to 40 mg kg^{-1} but due to anthropogenic activities such as mining, the use of As-containing pesticides, herbicides, and irrigation with As-rich groundwater, it may be elevated beyond the threshold level (Smedley and Kinniburgh, 2002; Meharg *et al.*, 2009; Cornejo-Ponce and Acarapi-Cartes 2011; Punshon *et al.*, 2016). Apart from drinking the As contaminated water, translocation of As in soil to plant parts represents one of the major routes for human contact to As (Xia and Liu, 2004). An elevated level of As in soil not only increases the risk of its transfer to plant but also disturbs sustainable agriculture

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by decreasing crop production and soil fertility (Brammer and Ravenscroft, 2009; Srivastava *et al.*, 2013).

The fertility of the soil is largely monitored by the inhabitant microbial community, characterized by species diversity, community structure, and microbial biomass which are influenced by the edaphic factors (Wang *et al.*, 2007; Islam *et al.*, 2011; Reed *et al.*, 2011). The diverse soil bacteria sustain the homeostatic condition of agroecosystems by regulating the recycling of soil nutrients, maintaining soil structure, and promoting the plant growth. It is well-established that microbial community with proper structure, diversity, and function acts as significant factor in maintaining the sustainability and productivity of ecosystems. Considering this fact, microbial community management is now in focus to sustain the soil fertility in present agricultural practices (Das *et al.*, 2016). Therefore, any alteration of soil composition leads to restructuring the inhabitant microbial community and hence changes in soil fertility (Azarbad *et al.* 2016; Silva *et al.*, 2013).

Diazotrophs, the microorganisms capable of assimilating and fixing atmospheric nitrogen (N_2), plays a pivotal role in soil nitrogen input. Changes in any physical or chemical properties of soil lead to diazotrophic response and are thus considered as a biomarker of soil fertility (Hsu and Buckley, 2009; Huhe *et al.*, 2014). It could be assumed that arsenic contamination leads to the destruction of soil microbial community structure and function and might result in a shift in diazotrophic activity. In contrast to this assumption, few previous investigations noticed plant growth stimulation by a low concentration of As (Woolson *et al.*, 1971; Miteva, 2002; Garg and Singla, 2011; Finnegan and Chen, 2012). Singh *et al.* (2014) recently reported the effect of As on symbiotic nitrogen fixers. But the comprehensive studies about the effect of this metalloid on free-living diazotrophs that plays a vital role in bulk soil nitrogen cycling devoid of nonleguminosae plants are still scanty (Mirza *et al.*, 2014, Biswas *et al.*, 2017).

The aim of this study was to find out how the free-living N_2 fixers respond to As contamination in soil. Laboratory microcosms based study was conducted by artificially contaminating an agricultural soil with As and the bacterial abundances and activities were monitored for three months. Emphasis has been given to follow the dynamics of metabolically active diazotrophs during the incubation. As the majority of diazotrophs are recalcitrant to laboratory cultivation and little is known about them, we used culture-independent molecular methods targeting dinitrogenase reductase gene (*nifH*) expression in soil (Gaby and Buckley, 2012; Hsu and Buckley, 2009; Huhe *et al.*, 2014). Analysis of expressed RNA provides the better resolution of metabolically active soil microbial community function and dynamics under perturbed condition (Hugoni *et al.*, 2013; Salter *et al.*, 2015; Li *et al.*, 2016). The activity of the total and nitrogen-fixing bacterial community was estimated by total RNA extraction followed by reverse transcription and quantitative real-time Polymerase Chain Reaction (qPCR). The data generated in this study might be useful to build the cultivation strategy in As contaminated soil and to develop As mitigation option.

2. Materials and methods

2.1. Soil sampling and characterization

The soil sample was collected from the agricultural field of Baruipara village (22°45′33″N 88°14′26″E), Hooghly, West Bengal where rice is rotationally cultivated with other crops. As the groundwater without arsenic contamination history was used to irrigate the selected

land, the collected soil was assumed to be uncontaminated. Soil samples were collected during drying cycle from five randomly selected spots (~7 cm deep), composited, kept on ice and transported into the laboratory. One aliquot of the composite sample was analyzed for soil physicochemical properties and other was used to evaluate the effect of As on culture-independent analysis of microbial community.

An aliquot of soil sample was air dried and ground to obtain powder samples. Part of powdered soil samples was acid digested (nitric, sulphuric, and perchloric acid in a volume ratio of 3:1:1) and arsenic concentration was estimated by Atomic Absorption Spectrophotometer (Thermo Scientific™ iCE™ 3000 Series) attached with vapour generator (VP100). Total organic carbon was estimated using standard Walkley–Black method (Walkley and Black, 1934). Ammonium (NH_4–N) and nitrate (NO_3–N), total phosphorus was measured following standard techniques (Jackson, 1967). Soil pH and electrical conductivity were measured by pH meter and conductivity meter following resuspending the powdered soil in deionized water. The moisture content of the soil was also measured by oven drying the soil for two days. Sand, silt, and clay content in the collected samples were determined by standard procedure (Lu, 1999).

2.2. Microcosm study

Two types of microcosm were set. In type (I) five sets of microcosm each containing 2 g soil in 50 ml medium, incubated for two days followed by exposure to 0.0 ppm (microcosm A1), 1 ppm (microcosm A2), 10 ppm (microcosm A3), 20 ppm (microcosm A4), and 50 ppm (microcosm A5) As. In type (II) five sets of microcosm each containing 50 g soil in 25 ml medium exposed to different concentration of As, 0.0 ppm (microcosm B1), 1 ppm (microcosm B2), 10 ppm (microcosm B3), 20 ppm (microcosm B4), and 50 ppm (microcosm B5) (Figure 1). The used nitrogen-free medium was composed of ((gl^{-1}): K_2HPO_4, 0.8; KH_2PO_4, 0.2; $MgSO_4.7H_2O$, 0.2; $MnSO_4.4H_2O$, 0.002; NaCl, 0.1; $Na_2MoO_4.2H_2O$, 0.002; Na-Vanadate, 0.002; $FeCl_3$, 0.01; Mannitol, 15; $CaCl_2.2H_2O$, 0.02 and Sucrose, 20). Each microcosm was set in 250 ml Erlenmeyer flask plugged with cotton in duplicate and incubated at 30°C without shaking for the variable time period. Destructive sampling was performed for each treatment at the end of one week and three months incubation. Sampling details are presented in Table 1.

2.3. RNA extraction and qPCR

Type (I) microcosm sample (liquid culture) was centrifuged and the pellet was used to recover total RNA using TRIzol™ Reagent (Invitrogen Carlsbad, CA, USA) according to manufacturer instruction. Total RNA from type (II) microcosm sample (2 g soil) was extracted using PowerSoil™ total RNA extraction kit (MoBio, Carlsbad, CA), following manufacturer's instructions. All extractions were done in duplicate and extracted RNA were quantified by Nanodrop-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) divided into aliquots and preserved at −80°C until use.

2.4. Preparation of complementary DNA (cDNA)

Complementary DNA (cDNA) was prepared from aliquots of all RNA extract. Coextracted DNA was eliminated by DNase (Fermentas) treatment according to the manufacturer's protocol, with following modification: the reaction mixture was incubated at 37°C for 45 min

Figure 1. Photograph of a set of microcosms prepared for assessment of the impact of Arsenic on total and diazotrophic bacterial activity. In each microcosm agricultural soil was incubated in presence or absence of variable concentration of arsenic. Please see Table 1 for detail description of the microcosms.

Table 1. Microcosms and sampling details.

Microcosm	Treatment	Samples	Incubation time
A1	2 g Soil + 50 ml medium	S0	One week
		S1	Three months
A2	2 g Soil + 50 ml medium + 1 ppm As	S2	One week
		S3	Three months
A3	2 g Soil + 50 ml medium + 10 ppm As	S4	One week
		S5	Three months
A4	2 g Soil + 50 ml medium + 20 ppm As	S6	One week
		S7	Three months
A5	2 g Soil + 50 ml medium + 50 ppm As	S8	One week
		S9	Three months
B1	50 g Soil + 25 ml medium	S10	One week
		S11	Three months
B2	50 g Soil + 25 ml medium + 1 ppm As	S12	One week
		S13	Three months
B3	50 g Soil + 25 ml medium + 10 ppm As	S14	One week
		S15	Three months
B4	50 g Soil + 25 ml medium + 20 ppm As	S16	One week
		S17	Three months
B5	50 g Soil + 25 ml medium + 50 ppm As	S18	One week
		S19	Three months

and enzyme was inactivated at 65°C for 10 min. RevertAid™ first strand cDNA synthesis kit with Moloney murine leukemia virus reverse transcriptase (M-MuLV RT) and random hexamer primer (Fermentas) was used to prepare cDNA. Negative controls omitting the reverse transcriptase in the cDNA synthesis reactions were prepared and used to verify genomic DNA contamination by normal PCR with 16S rRNA gene primers. All the tests were done in triplicate to get reproducibility.

2.5. Production of standard for qPCR analysis

Extracted genomic DNA from known pure culture was used as a source of 16S rRNA and *nifH* gene. Plasmid standards were generated by ligation of PCR amplified 16S rDNA and *nifH* gene with pGEM-T Easy vector (Promega, Madison, WI, USA) followed by cloning into *E. coli* JM109. PCR amplification was performed using 27F and 1492R (16S rRNA gene) (Islam and Sar, 2011) and polF and polR (*nifH* gene) (Poly *et al.*, 2001) primer set in 50 μl reaction volume with the following component (final concentration): 1 × PCR buffer, 600 μM of each dNTP, 2.5 mM $MgCl_2$, 1 U *Taq* DNA polymerase, 10 pmol of each primer and 20 ng genomic DNA. Plasmid DNA from the positive clones were extracted using Qiaprep® Spin Miniprep kit (Qiagen, GmbH), analyzed for proper insert by PCR using vector-specific primer T7 and Sp6 and quantified by NanoDrop® ND-1000 spectrophotometer. Plasmid DNA concentrations ranging from 1.0–0.0001ng/μl were used to generate the qPCR standard curves.

2.6. Real-time PCR based quantification of 16S rRNA and nifH gene copies

qPCR was performed targeting the 16S rRNA gene and the *nifH* gene. Universal primer set 341F and 518R (Muyzer *et al.*, 1993) were used for quantification of 16S rRNA gene and primer set polF and polR (Poly *et al.*, 2001) for the *nifH* gene. Both the genes were quantified using absolute quantification for cDNA against linearized plasmid standards. All qPCR assays were run in a 96 well plate on iQ5 Real-Time PCR System (BioRad). Both the standard plasmid and cDNA were serially diluted and all dilutions were used as template in the PCR reaction. Each 20 μl reaction mixture was prepared using the Quantifast SYBR Green master mix (Qiagen, India) in duplicate with the following components: 10.0 μl Master mix (2 ×), 1.25 μl of each primer (5 pmol), 4.0 μl template DNA, and 3.5 μl PCR-grade water. The thermal cycling protocol was as follows: initial denaturation for 3 min at 95°C followed by 40 cycles of 10 s at 95°C and 30 s at 60°C (55°C for *nifH*). The fluorescence signal was measured at the end of each annealing step. Standard curves and linear regression data for each assay were calculated, as well as the standard deviation for each range of cross threshold (Ct) values produced. Gene copy number per nanogram DNA was determined based on the size of the linearized plasmid used as the qPCR standard (Lee *et al.* 2008).

2.7. Statistical analysis

Principal component analysis (PCA) using XLSTAT 2014 was performed to build the relationship between the treatment regimes and the real-time PCR data. We used one way ANOVA to analyze the effect of As on *nifH* and 16S rRNA gene expression. Unless otherwise mentioned, we used triplicate data to estimate statistical significance. All other statistical calculation was performed with OriginPro 8.

3. Results and discussion

Agricultural activities are being practiced in As-contaminated agricultural land knowing the fact that high level of As in soil increases the risk of food chain contamination and soil infertility (Wang *et al.*, 2011). The reduction of soil fertility might be due to the shift in structure and activity of soil microbial community caused by a change in soil chemistry as a result of As contamination. To understand the effect of As contamination on soil microbial community with As supplement we characterized the selected soil in terms of total As content and physicochemical properties (Table 2). The silty clay loam soil with moderate medium subangular blocky structure was found to contain 4.5 mg As/kg soil. The slightly acidic soil (pH 5.1) was found to contain a higher level of NO_3-N than NH_4-N. The arsenic content is corroborated well by the background concentration found naturally in soil, therefore the selected soil was considered as uncontaminated (Das *et al.*, 2014).

Autochthonous microbial community especially the free-living N_2 fixers are the main players in regulating the soil fertility where leguminous plants were not propagated (Hsu and Buckley, 2009; Huhe *et al.*, 2014). Therefore, this study evaluated the effect of As contamination on the microbial community especially on free-living N_2 fixers as our experimental system was devoid of plants. It is well reported that change of physical, chemical, and biological factors results in microbial community shift (Chunleuchanon *et al.*, 2003; Orr *et al.*, 2011; Silva *et al.*, 2013; Wang *et al.*, 2016). Thus to understand the effect of As on diazotrophs, it is important to reduce so many soil variables from experimental set up. There are arsenic labile diazotrophs in soil that also need to be encouraged. Keeping these facts in mind we have incubated the soil in type (I) microcosms in larger volume of medium without amending the As. Generally, as soil system harbor both As oxidizer and As reducer, equal proportion of As (III) and As (V) was supplied in microcosms and the overall As concentration was considered by summing of these two species. We monitored the dynamics of metabolically active total and free-living N_2 fixing bacteria in the microcosms through the analysis of expressed RNA followed by cDNA preparation and qPCR. The standard curve obtained for qPCR analysis is presented in Figure 2.

3.1. Response of enriched total bacterial population under graded arsenic concentration

Real-time qPCR data (Figure 3) showed, compared to control, expressed 16S rRNA copy increased with the increasing concentration of As exposure up to 20 ppm in type (I) microcosms following one-week incubation. But in type (II) microcosms, increase in total bacterial

Table 2. Physical and chemical characteristics of soil sample analyzed in this study.

Parameter	Value
Sampling date	20.11.2015
Soil texture	Silty clay loam, moderate medium sub-angular blocky structure
Moisture content (%)	26.32
Total As (mg/kg)	4.5
NH_4-N (ppm)	110.4
NO_3-N (ppm)	235.4
SO_4 (ppm)	84
PO_4 (ppm)	1.4
TOC (%)	0.88
pH	5.1
Electrical conductivity (μs/cm)	117.7

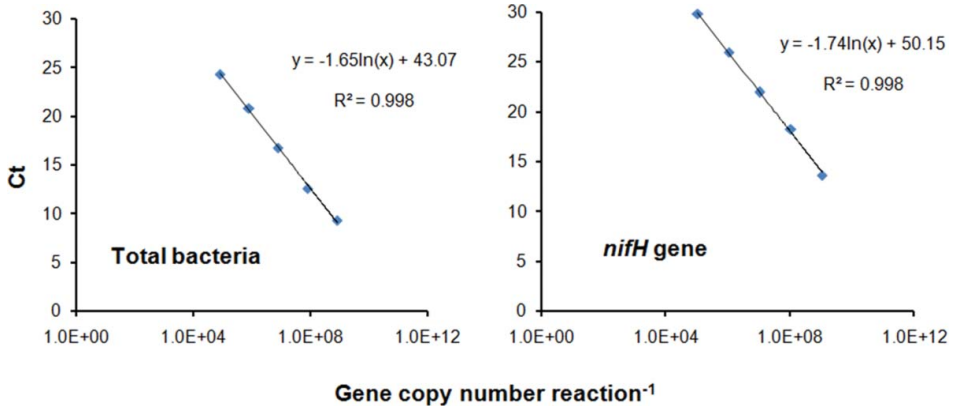

Figure 2. Real-time PCR standard curves for 16S rRNA gene of total bacteria, *nifH* gene of free-living diazotrophs. Copy number of linearized plasmid of cloned *nifH* and 16S rRNA genes in known graded concentration of DNA solution was estimated. The cycle threshold (Ct) of each qPCR reaction was estimated as a function of DNA copy number.

activity was only noticed in presence of 1.0 ppm As compared to control following a same period of incubation. This increased activity of bacterial population in presence of lower concentration of As corroborated well with the findings of Greaves, 1917. He reported increased bacterial activity in soil with a small quantity of As than in soil without As treatment.

Increasing the incubation time up to three months, in both type (I) and type (II) microcosm, expressed 16S rRNA copy decreased in control (microcosm A1 and B1) indicating loss of active enriched bacterial population without any As stress. This might be due to the depletion of nutrient from the batch system used. Similar but drastic fall of bacterial activity was also observed in all As exposed type (I) microcosms (microcosm A3–A5) except in presence of 1 ppm (microcosm A2) As indicating long-term incubation with a higher concentration of As severely affects the total bacterial population when proliferating outside the soil. Total bacterial activity decreased by 100 orders of magnitude in all type (II) microcosms irrespective of As-exposed and unexposed compared to the first week but the magnitude of the decrease is lesser than the type (I) microcosms. In type (II) microcosm (except Microcosm B1) the bacteria were enriched under the selective pressure of As, therefore, only As tolerant bacteria get flourished and hence exhibit higher total bacterial activity throughout the graded concentrations of As compared to type (I) microcosms.

3.2. Response of free-living N_2-fixing bacterial population under arsenic stress

Real-time PCR data based on *nifH* expression indicated, insignificant change in activity of nitrogen fixer in presence of 1 ppm As (microcosm A2) compared to control in type (I) microcosm after one week incubation, however, more than two order of increase in magnitude for expressed *nifH* was noticed in presence of 10 ppm As (microcosm A3) along the same period of incubation (Figure 4). A drastic fall in the expression of that gene was observed under elevated As stress (microcosm A5). In type (II) microcosm, a similar pattern of *nifH* gene expression was observed, however, the declining of the expression started from more than 1 ppm of As stress (microcosm B2). Extending the incubation period up to

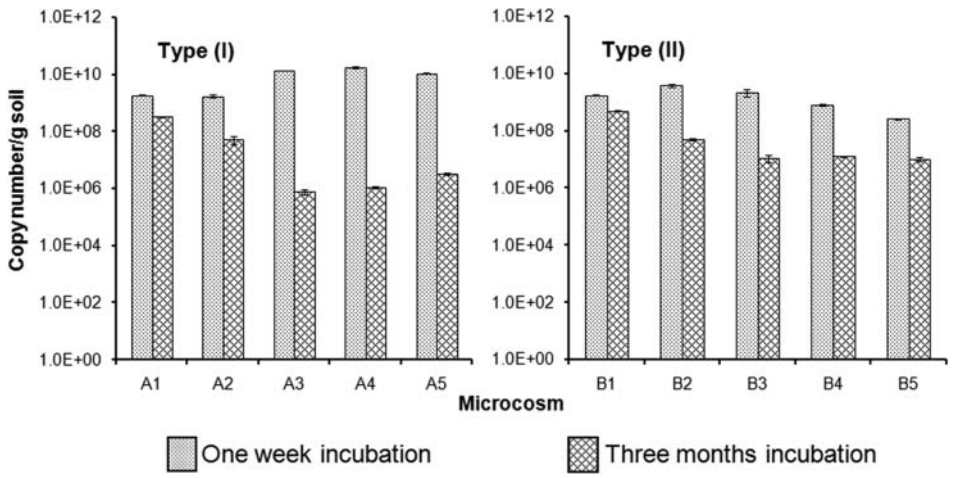

Figure 3. Changes of absolute activity of total bacteria in terms of level of expressed 16S rRNA in different microcosm samples. The level of expressed genes was determined by the estimation of copy number in DNA solution by qPCR. Primer set 341F and 518R were used to amplify the DNA. Datasets and results of analysis of variance (ANOVA) in the microcosms soils are presented ($n = 3$; error bars represent standard deviations).

3 months, a gradual decrease of *nifH* copy number with increasing concentration of As was observed in all As treated microcosms.

3.3. Relative activity of free-living diazotrophs and PCA

Each type of microcosm contained different levels of As (from control to 50 ppm). There was a difference between total bacterial activities in every treatment. In this circumstance, the study of the absolute activity of nitrogen-fixing bacterial population is unpredictable. Therefore, the effort was also made to compare the fluctuation of diazotrophic bacterial activity with respect to the fluctuation of total bacterial activity in each treatment. In this aspect abundance of the expressed *nifH* copy was expressed as per copy of 16S rRNA.

In type (1) microcosms, there was an increased relative activity of nitrogen fixer in presence of 1 ppm (microcosm A2) and 10 ppm As (microcosm A3) compared to control following one-week incubation (Figure 5). But this activity was rapidly decreased in higher concentrations of As (microcosm A5). Higher relative expression of the same gene in presence of 1 ppm As (microcosm B2) was observed compared to control (microcosm B1) in type (II) microcosm. Higher than 1 ppm As concentration showed very low relative expression of *nifH* (microcosm B3-B5).

It could be assumed that with the fall of the total active bacterial population, the abundance of active nitrogen fixer will go down proportionately during longer incubation in presence or absence of As, however, it is not obvious that the rate of expression of the *nifH* gene will also be altered. But our data of relative expression indicated fall of *nifH* gene expression under elevated As stress in shorter exposure time. In longer arsenic exposure although we could observe the lower level of 16S rRNA gene expression, declined level of *nifH* gene expression suggested the effect of both nutrient limitation and As toxicity on diazotrophs. Previous investigations showed higher concentrations of As (>10 mg/kg) lower the soil

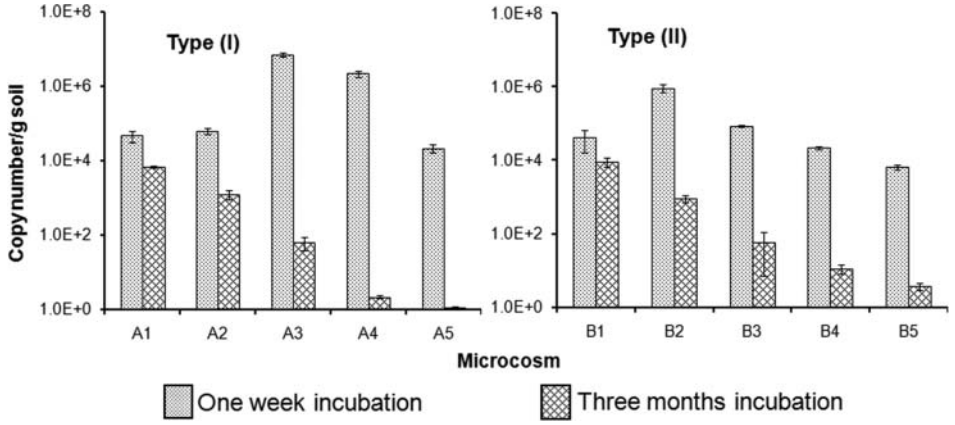

Figure 4. Changes of activity of nitrogen fixer in terms of expressed *nifH* in different microcosms. The level of expressed genes was determined by the estimation of copy number in DNA solution by qPCR. Primer set polF and polR were used to amplify the DNA. Datasets and results of analysis of variance (ANOVA) in the microcosms soils are presented ($n = 3$; error bars represent standard deviations).

fertility and hence significantly limit the growth of plants (Yan-Chu, 1994; Smith *et al.*, 1998; Abedin *et al.*, 2002; Khan *et al.*, 2010; Das *et al.*, 2013a; Das *et al.*, 2013b;). In this regard, our study demonstrated that the significant exposure of As in soil declines the expression of *nifH* gene which might result in decrease of soil fertility.

PCA based on treatment regime and the molecular data obtained from real-time PCR analysis was performed to assess variability between the arsenic concentration in the microcosms and the level of expression of the selected genes. The first two principal components (PC1 and PC2) in Figure 6 represented 77.69% of the variation of gene expression between treatment regimes. PCA revealed the negative correlation between arsenic concentration and the relative expression of *nifH*. This indicates that the activity of diazotrophs is adversely affected by As contamination.

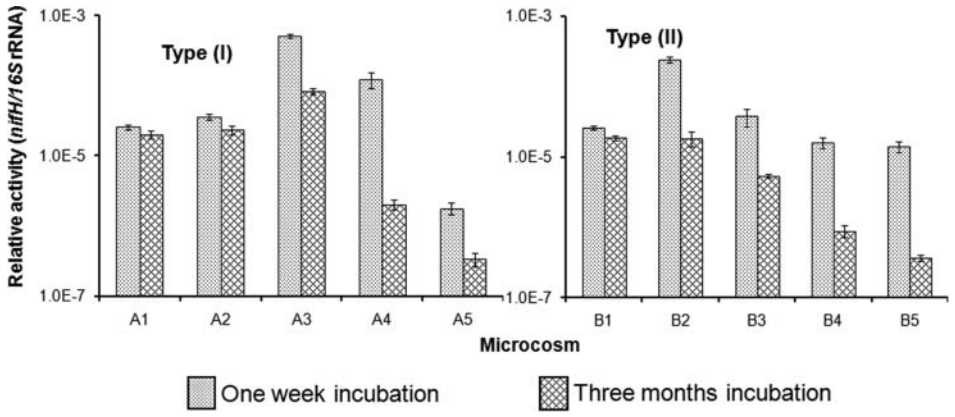

Figure 5. Changes of relative activity of nitrogen fixer in different microcosms. The level of expressed *nifH* gene is presented per unit copy of expressed 16S rRNA gene. Please see Section 2.2 microcosm study for details of Type (I) and Type (II) microcosms.

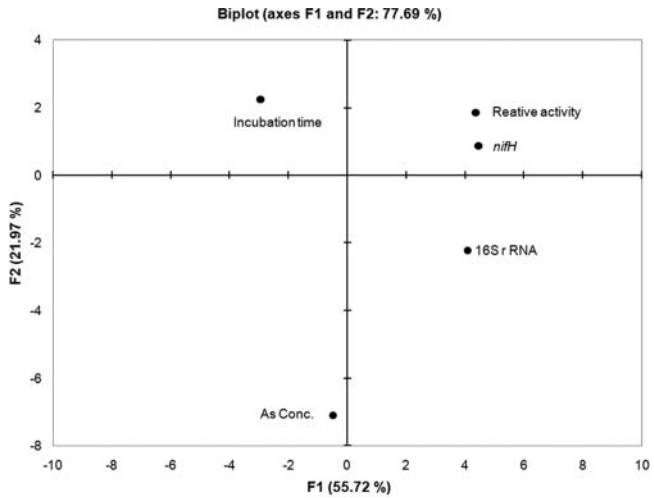

Figure 6. Principle component analysis (PCA). The relationship of 16S rRNA and *nifH* genes data to different treatment regimes in microcosms is used to find out the positive or negative correlation between the variables.

4. Conclusions

In summary, the present study revealed that the low concentration of As did not inhibit expression of *nifH* and 16S rRNA gene rather it stimulated at a certain level. But under high As stress, decreased expression of these genes was observed. Reduced 16S rRNA gene copy and absence of *nifH* gene expression due to long-term exposure of As was also noticed. Although the increase of relative expression of *nifH* to 16S rRNA genes at the lower level of As for short duration suggested the stimulating effect of As, the deleterious effect of high concentration of As on the level of *nifH* expression indicated impaired nitrogen fixation process by free-living diazotrophic soil bacterial population. Although the present study was performed in laboratory condition under graded As stress, this study enlightens on the survival of diazotrophic microbial populations in soils with different level of As contamination. The studies based on enrichment system might encourage some particular groups of bacteria. It might not represent the total bacterial diversity as a whole. Generally, in laboratory condition, experiments are conducted by amending high level of As, which is far from a real-world As exposure in the soil. However, more research with different types of diazotrophic bacterial isolates in the laboratory as well as field condition might confirm our observation. So, the findings of this study could be exploited in the agricultural practice in As-contaminated area considering the plant growth, As translocation in the plant as well as As mitigation.

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References

Abedin, M. J., Cresser, M. S., Meharg, A. A., Feldmann, J., and Cotter-Howells, J. 2002. Arsenic accumulation and metabolism in rice (*Oryza sativa* L,). *Environ. Sci. Technol.* **36**, 962–968. doi:10.1021/es0101678.

Azarbad, H., van Straalen, N. M., Ryszard, L., Karolina, N., Röling, W.F.M., and Niklinska, M. 2016. Susceptibility to additional stressors in metal-tolerant soil microbial communities from two pollution gradients. *Appl. Soil Ecol.* **98**, 233–242. doi:10.1016/j.apsoil.2015.10.020.

Biswas, J. K., Mondal, M., Rinklebe, J., Sarkar, S. K., Chaudhuri, P., Rai, M., Shaheen, S.M., Song, H., and Rizwan, M. 2017. Multi-metal resistance and plant growth promotion potential of a wastewater bacterium *Pseudomonas aeruginosa* and its synergistic benefits. *Environ. Geochem. Health*. doi:10.1007/s10653-017-9950-5.

Brammer, H. and Ravenscroft, P. 2009. Arsenic in groundwater: a threat to sustainable agriculture in South and South-east Asia. *Environ. Int.* **35**, 647–654. doi:10.1016/j.envint.2008.10.004.

Chunleuchanon, S., Sooksawang, A., Teaumroong, N., and Boonkerd, N. 2003. Diversity of nitrogen-fixing cyanobacteria under various ecosystems of Thailand: population dynamics as affected by environmental factors. *World J. Microbiol. Biotechnol.* **19**, 167–173. doi:10.1023/A:1023286823958.

Cornejo-Ponce, L. and Acarapi-Cartes, J. 2011. Fractionation and bioavailability of arsenic in agricultural soils: Solvent extraction tests and their relevance in risk assessment. *J. Environ. Sci. Health, Part A:Toxic/Hazard. Subst. Environ. Eng.* **46**, 1247–1258. doi:10.1080/10934529.2011.598807.

Das, I., Ghosh, K., Das, D. K., and Sanyal, S. K. 2013a. Assessment of arsenic toxicity in rice plants in areas of West Bengal. *Chem. Spec. Bioavailab.* **25**, 201–208. doi:10.3184/095422913X13785717162124.

Das, S., Chou, M. L., Jean, J. S., Liu, C. C., and Yang, H. J. 2016. Water management impacts on arsenic behavior and rhizosphere bacterial communities and activities in a rice agro-ecosystem. *Sci. Total Environ.* **542**, 642–652. doi:10.1016/j.scitotenv.2015.10.122.

Das, S., Jean, J. S., Kar, S., and Liu, C. C. 2013b. Changes in bacterial community structure and abundance in agricultural soils under varying levels of arsenic contamination. *Geomicrobiol. J.* **30**, 635–644. doi:10.1080/01490451.2012.746407.

Das, S., Jean, J. S., Kar, S., Chou, M. L., and Chen, C. Y. 2014. Screening of plant growth-promoting traits in arsenic-resistant bacteria isolated from agricultural soil and their potential implication for arsenic bioremediation. *J. Hazard. Mater.* **272**, 112–120. doi:10.1016/j.jhazmat.2014.03.012.

Finnegan, P. M. and Chen, W. 2012. Arsenic toxicity: The effects on plant metabolism. *Front. Physiol.* **3**, 182. doi:10.3389/fphys.2012.00182.

Gaby, J. C. and Buckley, D. H. 2012. A comprehensive evaluation of PCR primers to amplify the *nifH* gene of nitrogenase. *PLoS ONE* **7**, e42149. doi:10.1371/journal.pone.0042149.

Garg, N. and Singla, P. 2011. Arsenic toxicity in crop plants: physiological effects and tolerance mechanisms. *Environ. Chem. Lett.* **9**, 303–321. doi:10.1007/s10311-011-0313-7.

Greaves, J. 1917. The influence of arsenic on the bacterial activities of a soil. *Sci. Mon.* **5**, 204–209.

Hsu, S. F. and Buckley, D. H. 2009. Evidence for the functional significance of diazotroph community structure in soil. *ISME J.* **3**, 124–136. doi:10.1038/ismej.2008.82.

Hugoni, M., Taib, N., Debroas, D., Domaizon, I., Dufournel, I. J., Bronner, G. et al. 2013. Structure of the rare archaeal biosphere and seasonal dynamics of active ecotypes in surface coastal waters. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 6004–6009. doi:10.1073/pnas.1216863110.

Huhe Borjigin, S., Cheng, Y., Nomura, N., Nakajima, T., Nakamura, T., and Uchiyama, H. 2014. Effect of abandonment on diversity and abundance of free-living nitrogen-fixing bacteria and total bacteria in the cropland Soils of Hulun Buir Inner Mongolia. *PLoS ONE* **9**, e106714. doi:10.1371/journal.pone.0106714.

Islam, E. and Sar, P. 2011. Molecular assessment on impact of uranium ore contamination in soil bacterial diversity. *Int. Biodeterior. Biodegrad.* **65**, 1043–1051. doi:10.1016/j.ibiod.2011.08.005.

Islam, E., Dhal, P., Kazy, S. K., and Sar, P. 2011. Molecular analysis of bacterial communities in uranium ores and surrounding soils from Banduhurang open cast uranium mine India: a comparative study. *J. Environ. Sci. Health A Tox. Hazard Subst. Environ. Eng.* **46**, 271–280. doi:10.1080/10934529.2011.535433.

Jackson, M. L. 1967. *Soil Chemical Analysis*. New Delhi, Prentice Hall of India Pvt. Ltd., pp. 498.
Khan, M. A., Stroud, J. L., Zhu, Y. G., McGrath, S. P., and Zhao, F. J. 2010. Arsenic bioavailability to rice is elevated in Bangladeshi paddy soils. *Environ. Sci. Technol.* **44**, 8515–8521. doi:10.1021/es101952f.
Lee, C., Lee, S., Shin, S., and Hwang, S. 2008. Real-time PCR determination of rRNA gene copy number: Absolute and relative quantification assays with *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **78**, 371–376. doi:10.1007/s00253-007-1300-6.
Li, F., Henderson, G., Sun, X., Cox, F., Janssen, P. H., and Guan, L. L. 2016. Taxonomic assessment of rumen microbiota using total RNA and targeted amplicon sequencing approaches. *Front. Microbiol.* **7**, 987.
Lu, R. 1999. *Analytical Methods of Soil Agrochemistry*. Beijing, Science and Technology Press.
Mandal, B. K. and Suzuki, K. T. 2002. Arsenic round the world: A review. *Talanta* **58**, 201–235. doi:10.1016/S0039-9140(02)00268-0.
Meharg, A. A., Williams, P. N., Adomako, A., Lawgali, Y. Y., Deacon, C., Villada, A., Cambell, R. C. J., Sun, G., Zhu, Y. G., Feldmann, J., Rabb, A., Zhao, F. J., Islam, R., Hossain, S., and Yanai, J. 2009. Geographical variation in total and inorganic arsenic content of polished (white) rice. *Environ. Sci. Technol.* **43**, 1612–1617. doi:10.1021/es802612a.
Mirza, B. S., Potisap, C., Nüsslein, K., Bohannan, B. J. M., and Rodrigues, J. L. M. 2014. Response of free-living nitrogen-fixing microorganisms to land use change in the Amazon rainforest. *Appl. Environ. Microbiol.* **80**, 281–288. doi:10.1128/AEM.02362-13.
Miteva, E. 2002. Accumulation and effect of arsenic on tomatoes. *Commun. Soil Sci. Plant Anal.* **33**, 1917–1926. doi:10.1081/CSS-120004832.
Muyzer, G., de Waal, E. C., and Uitterlinden, A. G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59**, 695–700.
Ng, C. A., Wong, L. Y., Lo, P. K., Bashir, M. J., Chin, S. J., Tan, S. P., Chong, C. Y., and Yong, L. K. 2017. Performance of duckweed and effective microbes in reducing arsenic in paddy and paddy soil. In *AIP Conf. Proc.* **1828**, 020031.
Orr, C. H., James, A., Leifert, C., Cooper, J. M., and Cummings, S. P. 2011. Diversity and activity of free-living nitrogen-fixing bacteria and total bacteria in organic and conventionally managed soils. *Appl. Environ. Microbiol.* **77**, 911–919. doi:10.1128/AEM.01250-10.
Poly, F., Ranjard, L., Nazaret, S., Gourbière, F., and Monrozier, L. J. 2001. Comparison of *nifH* gene pools between soils and between soil microenvironments of contrasting properties. *Appl. Environ. Microbiol.* **67**, 2255–2267. doi:10.1128/AEM.67.5.2255-2262.2001.
Punshon, T., Jackson, B. P., Meharg, A. A., Warczack, T., Scheckel, K., and Guerinot, M. L. 2016. Understanding arsenic dynamics in agronomic systems to predict and prevent uptake by crop plants. *Sci. Total Environ.* **581-582** (2017), 209–220.
Rahman, Z. and Singh, V. P. 2016. Assessment of heavy metal contamination and Hg-resistant bacteria in surface water from different regions of Delhi, India. *Saudi J. Biol. Sci.* doi:10.1016/j.sjbs.2016.09.018.
Reed, S. C., Cleveland, C. C., and Townsend, A. R. 2011. Functional ecology of free-living nitrogen fixation: a contemporary perspective. *Annu. Rev. Ecol. Evol. Syst.* **42**, 489–512. doi:10.1146/annurev-ecolsys-102710-145034.
Salter, I., Galand, P. E., Fagervold, S. K., Lebaron, P., Obernosterer, I., Oliver, M. J., Suzuki, M. T., and Tricoire, C. 2015. Seasonal dynamics of active SAR11 ecotypes in the oligotrophic Northwest Mediterranean Sea. *ISME J.* **9**, 347–360. doi:10.1038/ismej.2014.129.
Silva, M. C. P., Hai, B. S., Schloter, M., Elsas, J. D. V., and Salles, J. F. 2013. Temporal dynamics of abundance and composition of nitrogen-fixing communities across agricultural soils. *PLoS ONE* **8**, e74500. doi:10.1371/journal.pone.0074500.
Singh, S., Shrivastava, A. K., and Singh, V. K. 2014. Arsenic and cadmium are inhibitors of cyanobacterial dinitrogenase reductase (*nifH*1) gene. *Funct. Integr. Genomics* **14**, 571–580. doi:10.1007/s10142-014-0375-2.
Smedley, P. L. and Kinniburgh, D. G. 2002. A review of the source, behaviour and distribution of arsenic in natural waters. *Appl. Geochem.* **17**, 517–568. doi:10.1016/S0883-2927(02)00018-5.

Smith, E., Naidu, R., and Alston, A. M. 1998. Arsenic in the soil environment: a review. *Adv. Agron.* **64**, 149–195. doi:10.1016/S0065-2113(08)60504-0.

Srivastava, P. K., Singh, M., Singh, N., and Tripathi, R. D. 2013. Soil arsenic pollution: a threat to crops. *J. Biorem. Biodegrad.* **4**, 7.

Walkley, A. and Black, I. A. 1934. An examination of degtjareff method for determining soil organic matter and a proposed modification of the chromic acid titration method. *Soil Sci.* **37**, 29–37. doi:10.1097/00010694-193401000-00003.

Wang, J., Zhang, D., Zhang, L., Li, J., Raza, W., Huang, Q and Shen, Q. 2016. Temporal variation of diazotrophic community abundance and structure in surface and subsoil under four fertilization regimes during a wheat growing season. *Agric., Ecosyst. Environ.* **216**, 116–124. doi:10.1016/j.agee.2015.09.039.

Wang, Q., He, M. and Wang, Y. 2011. Influence of combined pollution of antimony and arsenic on culturable soil microbial populations and enzyme activities. *Ecotoxicology* **20**, 9–19. doi:10.1007/s10646-010-0551-7.

Wang, S. and Mulligan, C. N. 2006. Occurrence of arsenic contamination in Canada: Sources, behavior and distribution. *Sci. Total Environ.* **366**, 701–721. doi:10.1016/j.scitotenv.2005.09.005.

Wang, Y., Shi, J., Wang, H., Lin, Q., Chen, X., and Chen, Y. 2007. The influence of soil heavy metals pollution on soil microbial biomass enzyme activity and community composition near a copper smelter. *Ecotoxicol. Environ. Saf.* **67**, 75–81. doi:10.1016/j.ecoenv.2006.03.007.

Woolson, E. A., Axley, J. H., and Kearney, P. C. 1971. The chemistry and phytotoxicity of arsenic in soils: I. Contaminated field soils. *Soil Sci. Soc. Am. Proc.* **35**, 938–943. doi:10.2136/sssaj1971.03615995003500060027x.

Xia, Y. J. and Liu, J. 2004. An overview on chronic arsenism via drinking water in PR China. *Toxicology* **198**, 25–29. doi:10.1016/j.tox.2004.01.016.

Yan-Chu, H. 1994. Arsenic distribution in soils. In: Nriagu, J. O. (ed) *Arsenic in the Environment, Part I: Cycling and Characterization*. New York, John Wiley & Sons Inc, pp. 17–51.