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ORIGINAL ARTICLE

Impact of heavy metals on water quality and indigenous *Bacillus* spp. prevalent in rat-hole coal mines

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Abstract

The present study reports pollution evaluation indices employed to assess the intensity of metal pollution in water systems affected by acid mine drainage from rat-hole coal mines prevalent in North-east India. The concentration of seven eco-toxic metals was evaluated from coal mine waters which showed concentration order of Iron (Fe) > Manganese (Mn) > Zinc (Zn) > Chromium (Cr) > Lead (Pb) > Copper (Cu) > Cadmium (Cd). The water samples were acidic with mean pH 2.67 and burdened with dissolved solids (924.8 mg/L). The heavy metal pollution index (HPI) and heavy metal evaluation index (HEI) displayed high and medium range of pollution level in majority of the water samples. Statistical correlation suggested strong positive correlation between metals such as Cr with Mn ($r = 0.780$), Mn with Fe (r = 0.576), Cr with Fe ($r = 0.680$), Pb with Mn ($r = 0.579$) and Cr with Pb ($r = 0.606$), indicating Mn, Pb, Fe and Cr to be major metal contaminants; an unequivocal affirmation of degradation in water quality. The sampled waters had lower heavy metal concentration during monsoon and post-monsoon seasons. The commonly occurring bacterial species *Bacillus pseudomycoides* and *Bacillus siamensis* were chosen to understand their behavioral responses toward metal contamination. Findings demonstrated that *Bacillus* spp. from control environment had low tolerance to metals stress as evident from their MTC, MIC and growth curve studies. The survival of the native isolates across varying pH, salinity and temperature in the coal mine areas suggest these isolates as promising candidates for reclamation of rat-hole coal mining sites.

Keywords Acid mine drainage · *Bacillus* spp. · Metal pollution · Rat-hole coal mines · Heavy metal pollution index (HPI) · Heavy metal evaluation index (HEI)

Introduction

Fallout from coal excavation sites flag multiple serious environmental concerns, exemplified by disruption of habitat, biodiversity, landscape and land use patterns. Disposal of mine tailings, acid mine drainage (AMD) and effluent discharges, escalate groundwater contamination and exacerbate settlement issues around mines. In addition, loss of site stabilization, climate change, workplace health and safety, changes in river regimes, have been attributed to coal mining activities (Rani et al. 2018). Across the globe, these specific concerns are cardinal to unbridled coal mining to satiate surging energy demands. North-east India has abundant deposits of sub-bituminous tertiary coal, with appreciable sulphur, volatile and vitrinite content, in addition to low ash content. While these features render the coal valuable for industry, the presence of Fe, Cu, Cd, Ni, Pb and Mn in bound

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mineral form are detrimental to the environment (Chabukdhara and Singh 2016).

In north-eastern India, Meghalaya has abundant coal bearing seams in Bapung, Ioksi, Rymbai, Khliehriat and Sutnga, that form the core of coal mining operations within the state (Das and Ramanujam 2011). Even in the third decade of the twenty-first century, the mining operations are conducted in the atavistic 'rat-hole' mining mode, besides being small scale, manual and under private ownership. Rat-hole mining briefly necessitates removal of surface vegetation, and excavation of vertical pits varying in depth from 5 to 100 m^2. Once the coal seams are detected deep within these shafts, horizontal tunnels are hollowed out till large coal seam are reached and quarrying can be initiated. The entire process is manual, with only the aid of baskets or wheel barrows to transport the debris and coal to the surface. The cost–benefit analysis of rat-hole mining though heavily weighed against the environmental fallout, remains undeniably a major source of livelihood and economic prosperity for Meghalaya. Environmental costs of rat-hole mining are linked with the potential deterioration of natural assets that precipitate forest cover reduction, agricultural productivity loss and water scarcity (Swer and Singh 2005).

Deep rat-hole mining of coal drastically affects the water level, quantity and overall quality of the underground water table. Mine tailings in combination with leachates, and other wastes are discharged into the neighborhood and adjacent water bodies; seepage from these sources renders the ground water highly unsafe for domestic use (Khan et al. 2005). The accumulation of heavy metals such as Iron (Fe), Manganese (Mn), Zinc (Zn), Chromium (Cr), Lead (Pb), Copper (Cu) and Cadmium (Cd) in the environment over time has only enhanced and the augmented solubility of such metals has become a grave concern. Levels of metals that constitute micronutrients are augmented beyond permissible limits in potable water making it detrimental for human consumption (Prasanna et al. 2011). Consequently, water quality assessment and impact of contamination caused by heavy metals in coal mine drainage water, acquires near emergency status. The heavy metal pollution index (HPI), heavy metal evaluation index (HEI), are useful indices; they utilize overall water quality with respect weighted arithmetic quality mean and metal concentration in comparison to its acceptable standard (Edet and Offiong 2002). Metal pollution indices, HPI has been classified into three main classes, namely low (<300), medium (300–600) and high level (>600) and HEI as low (<150), medium (150–300) and high (>300), respectively (Bhuiyan et al. 2010; Mahato et al. 2017).

The leaching of acid mine water or run-offs, known as AMD, constitutes a critical threat by increasing accumulation of inorganic matter and soil acidity, while lowering organic content. AMD engendered by sulphide mineral oxidation (the most common being pyrite FeS_2), participates in a series of oxidation reactions producing sulphates and hydroxides, while generating large amounts of acid (Fig. 1) (Plumlee et al. 1999). The inherent metal aggregating capacity of AMD, while encumbered with other mine wastes and residual materials causes breakdown of soil structure, water availability and its biological dynamics (Zhou et al. 2007). The complex process of coal extraction also generates harmful by-products such as mineral dust and toxic heavy metals, with increasing solubility that saturate extended areas adjacent to the mines with toxic metals (Chandra and Jain 2013). The heavy metals from mine wastes get mobilized in the environment and contaminate the soil and river sediments further affecting the natural biota. This build up is harmful to all life forms; especially to the soil microbiota, flora, fauna and the food chain (Yao et al. 2012).

The harsh coal mine environment is the natural habitation of a limited community of highly stress tolerant and adapted microorganisms. The acidic condition and heavy metal contamination curtail the natural microbial profusion of soil microbes (Gogoi et al. 2007). Bacteria isolated from coal mines are related to limited phylogenetic groups having few or no known homologues, as most are yet to be investigated in order to be able to obtain viable cultures (Joseph et al. 2003). Certain native microorganisms in the domains of archaea and bacteria, and some algae and fungi are able to adapt and thrive even in extreme environments like the AMD sites (Méndez-García et al. 2015). *Bacillus* spp. are among the few culturable genera from coal mines sites that reportedly possess metal tolerance capacity (Majumder and Palit 2016; Ka-ot et al. 2017). Such niches are reservoirs for novel metal tolerant *Bacillus* spp. with manifest heavy metal bioremediating ability via extracellular entrapment, membrane transport, biosorption on cell wall and the biogeochemical cycling of metal ions (Luo et al. 2012; Roohi et al. 2014; Kalita and Joshi 2017). In the present study, pollution indices for heavy metals in water samples and the stress tolerance of native *Bacillus* spp., from the rat-hole coal mines has been undertaken. Subsequently, utility of native microbiota in heavy metal bioremediation and eco restoration, with specific reference to the water bodies in the vicinity of rat-hole coal mine sites of Meghalaya, will be critically analyzed.

Materials and methods

Study site and sampling

Khliehriat in East Jaintia hills district of Meghalaya, India, is an important coal deposit of the region with rat-hole coal mining rampantly practiced by the mine owners. Water samples from five distinct niches were collected from the mine sites which comprised of, Side cutting mine

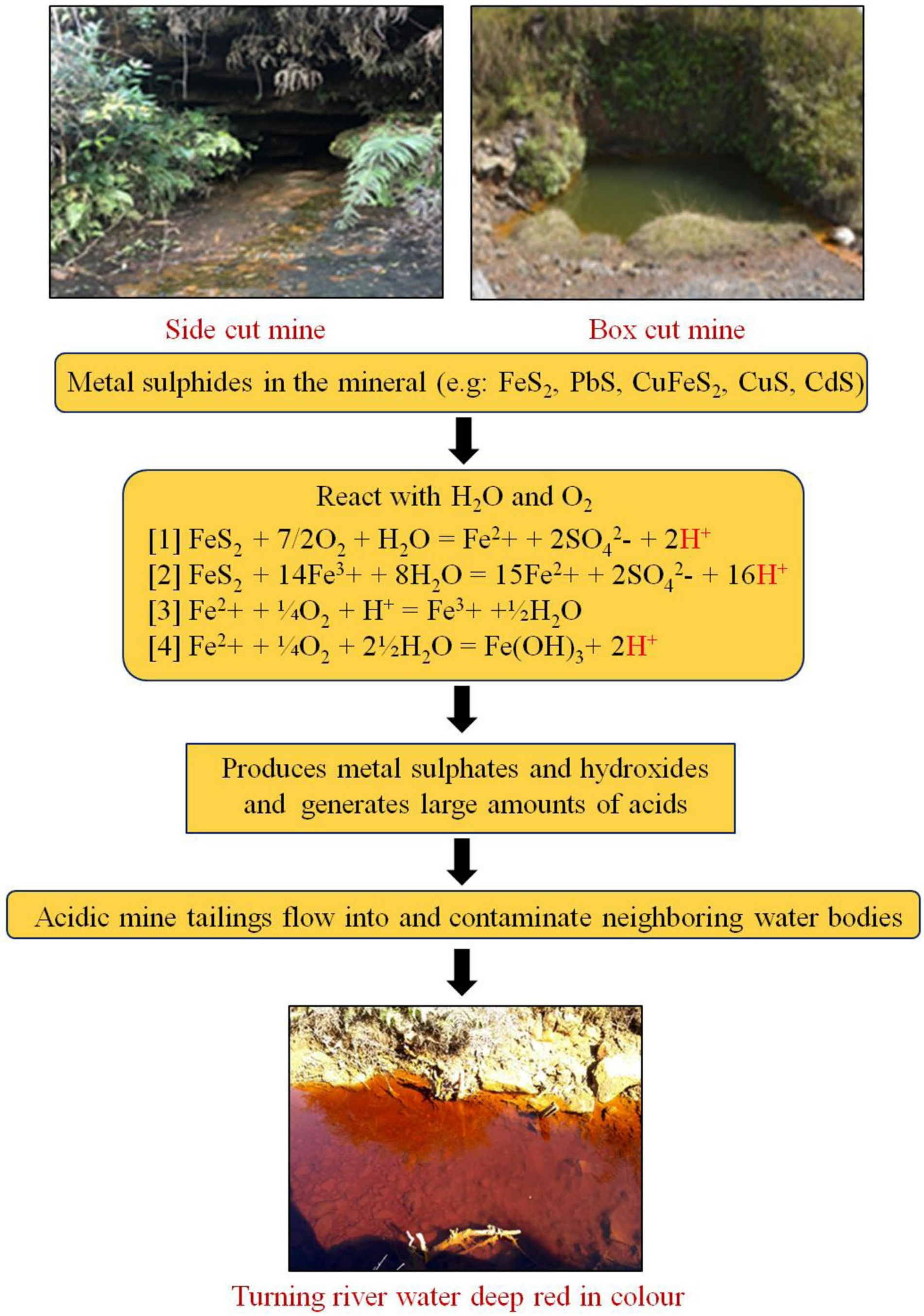

Fig. 1 Hazardous impact of metal sulphides pyrite ores contaminating water bodies adjacent to coal mines

(WS1), Box cutting mine (WS2), Nearby stream (WS3), Acid mine drainage (WS4) and an undisturbed pond taken as control (WC). Water samples were collected in three different seasons; Pre-monsoon, Monsoon and Post-monsoon during the year 2018 and 2019. All samples were aseptically collected in sterile bottles, pre-conditioned with 5% HNO_3 followed by rinsing with double distilled water, by immersing the bottles approximately 10 cm below the water surface and taken to the laboratory for analysis within 24 h.

Determination of pH, EC and TDS

pH of the water samples were recorded by immersion of the pH probe until a stable equilibrium was achieved (Rayment and Higginson 1992). Prior to recording the data, the pH instrument was carefully calibrated using standard buffers and between each measurement the probe was thoroughly washed with sterile deionized water. pH was measured using DIC µ pH meter (GOLD 533, Digital Instrumental Corp). Electrical conductivity (EC) of the samples was determined using DiST® 4 EC Tester HANNA Instruments, wherein the probe was standardized using 0.01 M KCl solution and conductance of the samples measured. Total dissolved solids (TDS) of the water samples were measured with the help of pre-calibrated EcoTestr TDS (Eutech instruments).

Estimation of heavy metals

Eastimation of lead (Pb), manganese (Mn), iron (Fe), chromium (Cr), cobalt (Co), nickel (Ni), copper (Cu), Zinc (Zn), cadmium (Cd) and aluminium (Al) was done by digesting the water samples with aqua regia consisting of 67% HNO_3 and 37% HCl in the ratio 3:1 and adjusted to pH < 2 using HNO_3. The sample solutions were concentrated by evaporating to one-tenth of its original volume and filtering through Whatman filter paper No.42 for analysis. The concentration of trace metals was determined using ICP-OES (Thermo Scientific™ iCAP™ 7600) and expressed as parts per million (Radulescu et al. 2014).

Water pollution indices

The heavy metal pollution index (HPI) is a measure of total water quality with respect to heavy metals. A total of seven metals namely, copper, zinc, lead, Iron, cadmium, chromium and manganese were considered for the HPI measurement. This can be determined by two given equations for HPI and Qi.

$$\mathrm{HPI} = \frac{\sum_{i=1}^{n} W_{\mathrm{i}} Q_{\mathrm{i}}}{\sum_{i=1}^{n} W_{\mathrm{i}}} \tag{1}$$

Where, W_{i} is the unit weightage of i^{th} parameter, a value inversely proportional to the recommended standard Si of the particular metal; Q_{i} is the sub index of the i^{th} parameter; n is number of parameters involved. For determination of sub index Q_{i},

$$Q_{\mathrm{i}} = \sum_{(i=1)}^{n} \frac{\{M_{\mathrm{i}}(-)I_{\mathrm{i}}\}}{(S_{\mathrm{i}} - I_{\mathrm{i}})} 100 \tag{2}$$

Where, M_{i} is the monitored value for the heavy metal, I_{i} the ideal desirable value and S_{i} the standard value of the i^{th} parameter. (−) indicates the numerical difference of the two values, negating the algebraic sign (Prasad et al. 2014).

Heavy metal evaluation index (HEI) is determined by the equation:

$$\mathrm{HEI} = \sum_{i=1}^{n} \frac{H_{\mathrm{c}}}{H_{\mathrm{mac}}}$$

Where, H_{c} is the monitored value and H_{mac} is the maximum admissible concentration (MAC) of the i^{th} parameter.

Isolation and screening of heavy metal tolerant native bacteria

The isolation of bacterial isolates was performed by serial dilution method wherein tenfolds dilutions of sample solutions were prepared and aseptically plated onto sterilized Nutrient Agar medium (HiMedia, India) containing g/L H_2O: beef extract (10 g), peptic digest of animal tissue (10 g), NaCl (5 g), agar (15 g) and pH 7.2 ± 0.3. The plates were incubated at 37 °C for 72 h and based on variation in color and colony morphology, the isolates were streaked on NA plates and stored in glycerol stocks for long-term preservation (Kumar et al. 2011). For heavy metal tolerance, metals stock solutions of Pb, Cr, Mn and Fe were prepared using analytical grade reagents Lead(II) nitrate, Potassium dichromate, Manganese(II) sulphate monohydrate and Iron(II) sulfate heptahydrate (Sigma-Aldrich). Respective metal salts were dissolved in known quantity in sterilized deionized water and filtered to attain 2000 ppm stock solutions for each. These were stored in dark reagent bottles kept at 4 °C and used within a month. Nutrient agar plates supplemented with the test metals, with a working concentration of 100 ppm, were prepared and bacterial isolates were spotted onto the plates and incubated at 37 °C for 24–48 h. The isolates that grew in the presence of these metals were considered and further characterized (Devika et al. 2013).

Screening of bacterial isolates for biochemical and physiological traits

The bacterial isolates were screened for their physiological ability to survive across pH gradients of 4–8, temperature range of 24–45 °C and salt (NaCl) concentration of 1–7%. Pure culture isolates were inoculated onto nutrient broth tubes adjusted to different pH and temperature and salt concentrations. Nutrient broth of pH 7.2 ± 0.3 was used for the growth of the isolates treated with these parameters. Optical density at 600 nm wavelength (Cecil Aquarius: CE 7200, Double Beam Spectrophotometer) was measured after 24 h as a measure of growth. Temperature and pH showing optimum growth of the isolates were considered for further study on the bacteria. The colony morphology was visualized by Gram's staining assay. Biochemical traits such as IMViC test, enzyme (catalase, oxidase, indole) production and sugar utilization abilities were tested for each of the isolates. The tests were carried out in triplicates based on Bergey's Manual of Systematic Bacteriology (Buchanan and Gibbons 1974).

Molecular and phylogenetic analysis of the bacterial isolates

Identification of metal tolerant bacterial isolates was carried out by genomic DNA extraction method (HiPurA™

Bacterial Genomic DNA Purification Kit-MB505). The PCR amplification of 16S rRNA gene form the extracted genomic DNA was carried out using forward primers 27F 5' AGA GTTTGATCMTGGCTCAG 3' and reverse primer 1492R 5' ACGGYTACCTTGTTA CGACTT 3' in a reaction mixture of genomic DNA (30 ng), dNTPs (250 µM each), primers (5 µM each), 1× Taq Buffer, Taq Polymerase (0.5unit), $MgCl_2$ (1.75 mM) using a thermal cycler (Gene Amp 9700: Applied Biosystems, USA) (Weisburg et al. 1991). PCR amplicons were then purified using QIA Quick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced at Macrogen Inc, Seoul, Korea. Nucleotide BLAST similarity search was performed to retrieve related sequences of bacterial lineage from the ezTAXON database of validly published prokaryotic strains (http://www.eztaxon.org/) (Chun et al. 2007). The homologous sequences having close relatedness to the query were selected and aligned using ClustalW of MEGA (version 7.0) software. The phylogenetic relationship of the bacterial isolates was illustrated by generating an unrooted phylogenetic tree based on neighbor-joining algorithm with 1000 bootstrap replications (Tamura et al. 2011).

Determination of MTC and MIC

The bacterial isolates were tested against Mn, Fe, Pb and Cr by spot plate method for determination of their respective maximum tolerable concentration (MTC) and minimum inhibitory concentration (MIC). From stock solution of 10,000 ppm, the metals were supplemented in nutrient agar plates ranging from 100 to 2000 ppm. An inoculum of 10µL freshly revived bacterial culture (OD 0.5 at 600 nm) was spotted onto the plates and incubated at 37 °C for 72 h. The plates were observed for growth every 24 h and results noted. The highest metal concentration at which the isolate could still grow was considered as the MTC; while the lowest concentration that inhibited any visible growth of bacteria within 24 h to 48 h was noted as the MIC (Aleem et al. 2003).

Effect of metals on bacterial growth

The effect of Mn, Fe, Pb and Cr on the growth pattern of the bacterial isolates was assessed by growth curve studies. 100 ml nutrient broth supplemented with 100 ppm each of Mn, Fe, Pb and Cr was inoculated with 1 ml of mid-log phase culture of the bacterial isolate. As a control, nutrient media inoculated with bacteria was prepared in absence of metal in the media. Once inoculated, the flasks were placed in a rotary shaker set at 100 rpm and 37 °C. The cell turbidity as a measure of growth was recorded as the optical density at 600 nm wavelength using UV–Vis spectrophotometer (Cecil Aquarius: CE 7200, Double Beam Spectrophotometer

Table 1 Physicochemical parameters of the water samples

Samples	WS1			WS2			WS3			WS4			WC		
Parameters	PRE	MON	POS	PRE	MON	POS	PRE	MON	POS	PRE	MON	POS	PRE	MON	POS
pH	2.68	3.14	3.01	2.77	3.23	2.89	2.26	2.98	2.07	1.87	2.77	2.48	5.16	5.35	5.23
TDS (mg/L)	519	360	492	401	301	809	1540	990	1012	2421	602	1654	40	10	12
EC (µS/cm)	807	727	665	1597	986	1471	2460	2180	2324	2420	1046	1873	27	26	24
Sample source	Side-cut mine			Box-cut mine			Stream water			AMD site			Non-mine water		
Location															
Latitude	25.3406667			25.3424667			25.342219			25.341567			25.3399523		
Longitude	92.358200			92.3588333			92.3608508			92.3585168			92.3617818		
Elevation (msl)	1139			1138			1141			1135			1139		

TDS total dissolved solids, *EC* electrical conductivity, *PRE* pre-monsoon, *MON* monsoon, *POS* post-monsoon, *Location* GPS decimal readings, *Elevation* msl (metres above sea level) at regular intervals from 0 to 27 h, thereafter at 48 h, 72 h and 96 h. All tests were performed in triplicates (Shakoori et al. 2010).

Statistical analysis

The data obtained for heavy metals in water samples were subjected to statistical interpretation. Graphpad PRISM software (version 8.0) was assessed for any correlation by performing Pearson correlation analysis. The significance of correlation was reported at 5% and 1% level on the basis of the probability value. Standard deviation (± SD), Standard error of mean (± SEM) was carried out for various results that are presented.

Results and discussion

Physicochemical and pollution profile of water

Measured against a mean pH of 5.24 ± 0.09 from the control site, the pH profiles varied with location and season. Thus, a mean pH of 2.67 ± 0.42 was derived from 2.96 recorded for box-cut mine WS2 sample and 2.37 from the AMD of WS4 water sample. Seasonally, the highest pH was observed in monsoon with a mean of 3.49 ± 1.05, followed by post-monsoon with 3.16 ± 1.22 while the lowest was in pre-monsoon season with 2.94 ± 1.28 (Table 1). In a similar finding, AMD tailings contaminated with heavy metals was seen to be highly acidic with mean pH ranging between 2.6 and 3.2 (Hatar et al. 2013), also corroborating with the report of Sahoo et al. (2012). An evident outcome of leaching of the acidic tailings and AMD is the acidification of the water in the vicinity of the mines.

For total dissolved solids (TDS), the mean for the samples was 924.83 ± 647.7 mg/L with the highest in AMD WS4 (1558.33 mg/L) and lowest in water from side cutting mine WS1 (457 mg/L), while in the control it was 20.77 mg/L. Seasonal variations in TDS were recorded, with the highest in pre-monsoon followed by post-monsoon and least in the monsoon season, with mean TDS of 984 ± 977, 795.8 ± 610 and 452.2 ± 366 mg/L, respectively (Table 1). The TDS content in water during post-monsoon season is the least with the mean TDS falling within the acceptable range, while in pre-monsoon and monsoon seasons, it is above the acceptable limit, making it unfit for consumption or domestic use. TDS was found to be within the acceptable limit in all seasons in the control WC. A study on geochemical characteristics of the AMD discharged from mines was found to be similarly on the higher side in the range of 2700 mg/L (Chon and Hwang 2000).

The concentration of individual metals in the samples was in the order Fe > Mn > Zn > Cr > Pb > Cu > Cd. Concentration of Fe exceeded other metals by more than 80% in all the tested samples (Table 2). A similar profile on mine water quality has been reported from coal mining area of Damodar River Basin India (Mahato et al. 2017). The mean HPI and HEI of the mine affected samples were seen to

Table 2 Heavy metal concentration (mg/L) of the water samples in three different seasons

Heavy metals	WS1				WS2				WS3			
	PRE	MON	POS	MCM	PRE	MON	POS	MCM	PRE	MON	POS	MCM
Fe	207.9	54.092	136.8	132.93	178.1	42.036	78.9	99.678	143.6	22.8	40.5	68.967
Mn	3.19	0.279	1.65	1.706	7.16	0.2	4.32	3.893	5.538	0.144	2.34	2.674
Cu	0.004	0.005	0.002	0.004	0.002	0.003	0.004	0.002	0.004	0.002	0.003	0.003
Pb	0.031	0.006	0.026	0.021	0.044	0.008	0.04	0.031	0.098	0.009	0.0119	0.04
Zn	6.75	0.489	3.95	3.73	4.69	0.42	2.77	2.627	4.03	0.055	2.34	2.142
Cd	0.002	0.001	0.001	0.001	0.001	BDL	BDL	0.001	BDL	BDL	0.001	0.001
Cr	2.8	0.216	0.089	1.035	3.4	0.124	0.713	1.412	1.146	0.082	0.146	0.458

Heavy metals	WS4				WC			
	PRE	MON	POS	MCM	PRE	MON	POS	MCM
Fe	259.4	96.7	195.3	183.8	0.765	0.529	0.46	0.585
Mn	5.34	0.201	2.741	2.761	0.055	0.007	0.019	0.027
Cu	0.007	0.005	0.003	0.005	0.001	BDL	0.002	0.001
Pb	0.027	0.006	0.016	0.016	0.002	0.001	0.002	0.001
Zn	5.34	0.922	0.575	2.279	0.26	0.025	0.012	0.099
Cd	0.001	0.001	0.007	0.003	BDL	BDL	BDL	0.0
Cr	3.7	0.194	0.98	1.625	0.022	0.002	0.001	0.009

PRE pre-monsoon, *MON* monsoon, *POS* post-monsoon, *MCM* mean concentration of metal, *BDL* below detection level

Table 3 Mean heavy metal pollution index (HPI) and heavy metal evaluation index (HEI) of the coal mine samples for the three seasons and classification of samples based on the level of pollution

Heavy metals	M_i Mean value (μg/L)	S_i Standard permissible value (μg/L)	I_i Ideal desirable value (μg/L)	W_i Unit weightage	Q_i Sub index	$W_i \times Q_i$	H_c/H_{mac}
Fe	121.344	1000	300	0.001	17,292.0	17.292	121.344
Mn	2758.54	300	100	0.0033	1329.27	4.3865	9.19513
Cu	3.40000	1500	50	0.0006	3.21379	0.0019	0.00226
Pb	26.8916	10	0	0.1	268.916	26.8916	2.68916
Zn	2694.25	15,000	5000	0.00006	23.0575	0.00138	0.17961
Cd	1.20833	10	0	0.1	12.0833	1.20833	0.12083
Cr	1132.50	50	0	0.02	2265.00	45.300	22.6500

$\Sigma\ Wi = 0.22496$; $\Sigma\ WiQi = 95.081$. HPI = 422.661. HEI = 156.181. Standards BIS IS10500: 2012, WHO (2011)

	Category	Pollution level	Samples		
HPI			PRE	MON	POS
	< 300	Low	WC	WS1 WS2 WS3 WS4 WC	WS1 WS3 WC
	300–600	Medium	–	–	WS2 WS4
	> 600	High	WS1 WS2WS3 WS4	–	–
HEI	Category	Pollution level	Samples		
			PRE	MON	POS
	< 150	Low	WC	WS1 WS2 WS3 WS4 WC	WS1 WS2 WS3 WC
	150–300	Medium	WS1 WS2 WS3	–	WS4
	> 300	High	WS4	–	–

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HEI heavy metal evaluation index (Bhuiyan et al. 2010), *PRE* pre-monsoon, *MON* monsoon, *POS* post-monsoon

Table 4 Water pollution indices metal pollution index (HPI) and heavy metal evaluation

	Pre-Monsoon			Monsoon			Post-Monsoon		
Samples	HPI	Mean deviation	% Deviation	HPI	Mean deviation	% Deviation	HPI	Mean deviation	% Deviation
WS1	799.06	62.40	8.47	102.34	22.32	27.90	234.79	−9.85	−4.03
WS2	967.95	231.29	31.40	86.64	6.62	8.28	388.135	143.49	58.66
WS3	772.52	35.86	4.87	67.88	−12.14	−15.17	124.38	−120.26	−49.16
WS4	985.34	248.68	33.76	124.47	44.45	55.56	418.38	173.74	71.02
ACS	881.22	144.56	19.62	95.33	15.31	19.14	291.42	46.78	19.12
WC	13.89	−722.77	−98.11	3.44	−76.58	−95.70	10.74	−233.90	−95.61
	Mean: 736.66			Mean: 80.01			Mean: 244.64		
	Pre-Monsoon			Monsoon			Post-Monsoon		
Samples	HEI	Mean deviation	% Deviation	HEI	Mean deviation	% Deviation	HEI	Mean deviation	% Deviation
WS1	278.28	48.33	21.02	60.01	11.20	22.94	147.06	34.88	31.09
WS2	274.75	44.80	19.48	46.05	−2.76	−5.66	111.8	−0.38	−0.34
WS3	195.1	−34.85	−15.16	25.79	−23.02	−47.17	52.64	−59.54	−53.07
WS4	354.36	124.41	54.10	101.94	53.13	108.83	226.34	114.16	101.77
ACS	275.62	45.67	19.86	58.45	9.64	19.74	134.46	22.28	19.86
WC	1.616	−228.34	−99.30	0.646	−48.17	−98.68	0.772	−111.41	−99.31
	Mean: 229.95			Mean:48.81			Mean: 112.17		

HPI heavy metal pollution index, *HEI* heavy metal evaluation index, *ACS* mean of all coal mine sites affected samples other than control for the particular season

be 422.661 and 156.181, respectively (Table 3). The HPI and HEI indices have been calculated for all the test samples, including control for all the three seasons (Table 4). The mean HPI of the samples was noted to be 736.66 for pre-monsoon, 80.01 for monsoon and 244.64 for the post-monsoon season. HPI recorded for the different samples was in the order of WS4 > WS2 > WS1 > WS3 > WC. In case of HEI, the pre-monsoon, monsoon and post-monsoon season recorded mean of 229.9, 48.8 and 112.1 respectively. In terms of sample, HEI was observed in the order of as WS4 > WS1 > WS2 > WS3 > WC. The pollution indices indicated higher concentration of metals during the pre-monsoon, followed by post-monsoon season and the least in the monsoon season. An earlier study on mining water also suggested a similar lowering of metal concentration in the monsoon and post-monsoon seasons (Singh and Kamal 2017). Based on HPI index, all the samples except control were seen to fall in the high pollution category. HEI index indicated WS4 in the high pollution category only during the pre-monsoon season (Fig. 2). Bhuiyan et al (2010) reported water samples from coal mines to have HPI index in the range of 166.92–827.39 and HEI range of 85.89–367.28, while the present study indicate HPI of 95.33–881.22 and HEI values as 58.45–275.62, for all the three seasons (Table 4). The AMD sample WS4 showed maximum metal pollution while the nearby stream WS3 was found to be least polluted. This could be due to the fact that the WS4 sample was collected from a site where tailings from many different mines merge. Pollution indices such as HPI and HEI have been successfully used in the comparative evaluation studies across developing countries like Africa and Asia (Edet and Offiong 2002; Prasad and Mondal 2008). Pearson correlation matrix suggested strong positive correlation between Zn with Mn ($r = 0.923$) and Cr with Mn ($r = 0.780$) at $p < 0.01$. Positive correlation was noted between Mn with Fe ($r = 0.576$), Cr with Fe ($r = 0.680$), Pb with Mn ($r = 0.579$), Cr with Pb ($r = 0.606$) and Cr with Zn (0.665) at $p < 0.05$ (Table 5). These results indicate that Mn, Pb, Fe and Cr contribute to the majority of the metal contamination in these sites and possibly have a common source of emergence.

Metals tolerance response of *Bacillus* spp.

Culturable bacteria were enumerated by spread plate serial dilution method where the average population of bacteria from the coal mine samples was found to be 3.7, 1.2 and 1.9×10^3 cfu/ml and that of the control sample to be 4.8, 2.1 and 3.3×10^3 cfu/ml during the pre-monsoon, monsoon and post-monsoon seasons, respectively. Bacterial population was found to be highest in the pre-monsoon season compared to the post-monsoon and monsoon season; corroborating the findings of Edwards et al (1999). A total of 32

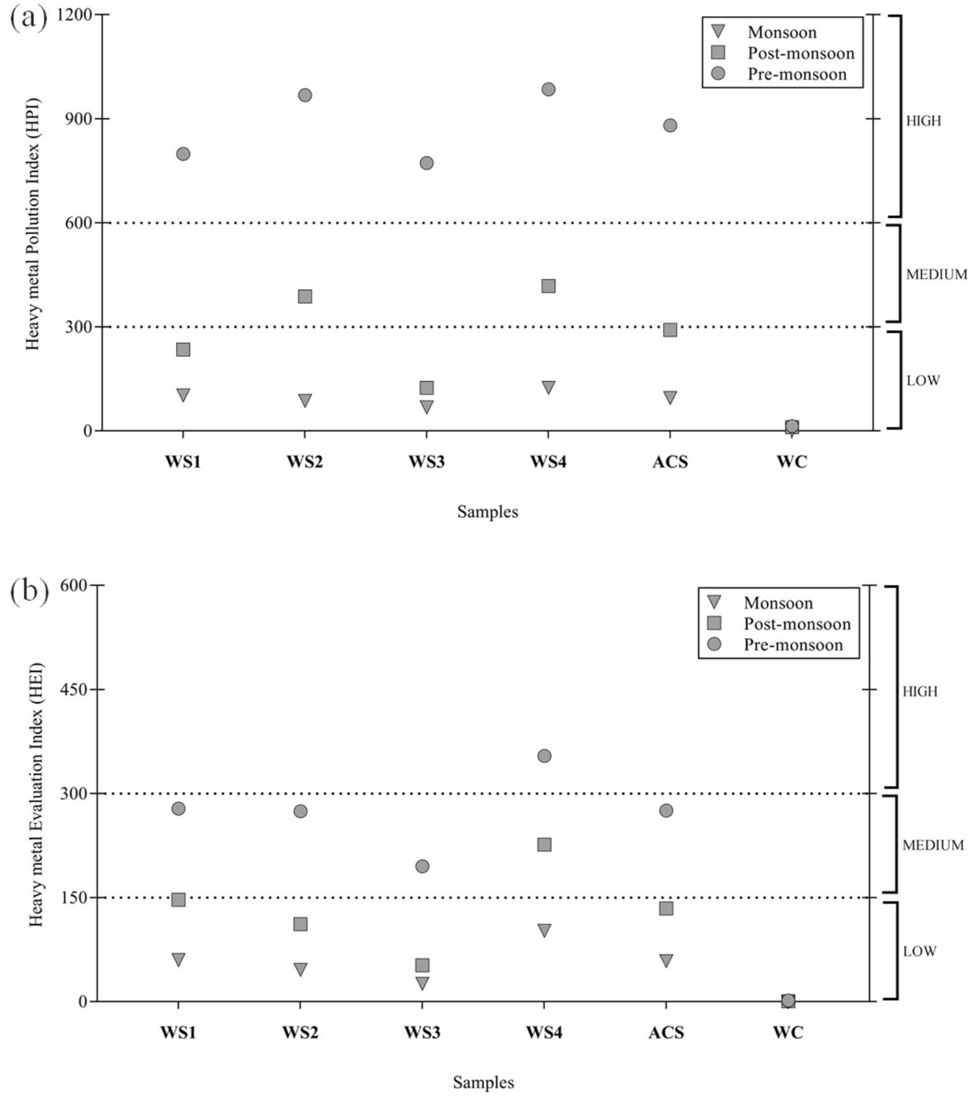

Fig. 2 Classification of water quality across pre-monsoon, monsoon and post-monsoon seasons based on **a** heavy metal pollution index and **b** heavy metal evaluation index for samples WS1, WS2, WS3, WS4, WC and ACS (ACS: mean of all coal mine samples—mean HPI and HEI for all coal mine affected samples excluding control)

Table 5 Pearson's correlation matrix among metals in the studied water samples

	Fe	Mn	Cu	Pb	Zn	Cd	Cr
Fe	1						
Mn	0.576	1					
	0.049*						
Cu	−0.045	0.437	1				
	0.890	0.156					
Pb	0.499	0.579	0.171	1			
	0.099	0.048*	0.595				
Zn	0.569	0.923	0.445	0.542	1		
	0.053	0.0002**	0.148	0.069			
Cd	0.483	0.030	−0.287	−0.039	−0.139	1	
	0.112	0.926	0.367	0.903	0.666		
Cr	0.680	0.780	0.265	0.606	0.665	−0.002	1
	0.015*	0.003**	0.405	0.037*	0.018*	0.995	

*correlation significant at 0.05

**correlation significant at 0.01 level

Fig. 3 Phylogenetic relationship based on 16S rRNA gene sequences of the bacterial isolates SS1-18, C-15, TW2-22 and WC-3 with their closest homologues using neighbor-joining algorithm using MEGA 7 software with *Myxococcus fulvus* (NR_043946.1) as an outgroup

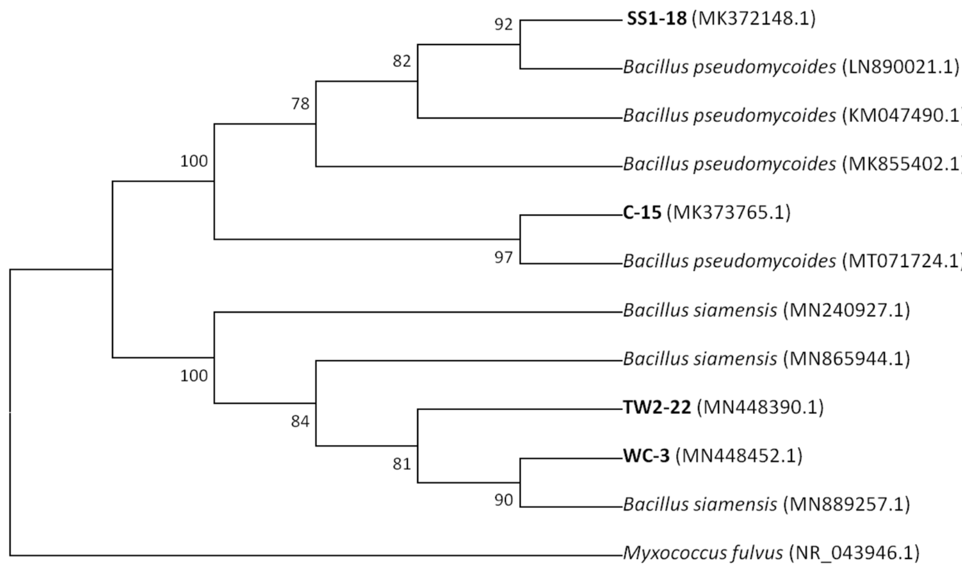

Table 6 Morphological and biochemical traits of the bacterial isolates

Bacterial isolates	SS1-18 *Bacillus Pseudomycoides*	C-15 *Bacillus pseudomycoides*	TW2-22 *Bacillus siamensis*	WC-3 *Bacillus siamensis*
Morphological analysis				
Shape:	Filamentous	Filamentous	Irregular	Irregular
Color:	Creamy	Off-white	Brownish	Whitish
Margin:	Filiform	Filiform	Lobate	Lobate
Elevation:	Flat	Flat	Umbonate	Umbonate
Texture:	Rugose	Dry	Mucoid	Mucoid
Opacity:	Opaque	Opaque	Translucent	Translucent
Gram's staining:	Positive	Positive	Positive	Positive
Cell type:	Rod	Rod	Rod	Rod
Enzyme production assay				
Catalase:	+	+	+	+
Oxidase:	−	−	+	+
Indole:	−	−	+	+
Methyl red:	−	−	−	−
Voges-Proskauer:	+	+	+	+
Citrate:	−	−	−	−

(+) indicates a positive test; (−) indicates a negative test

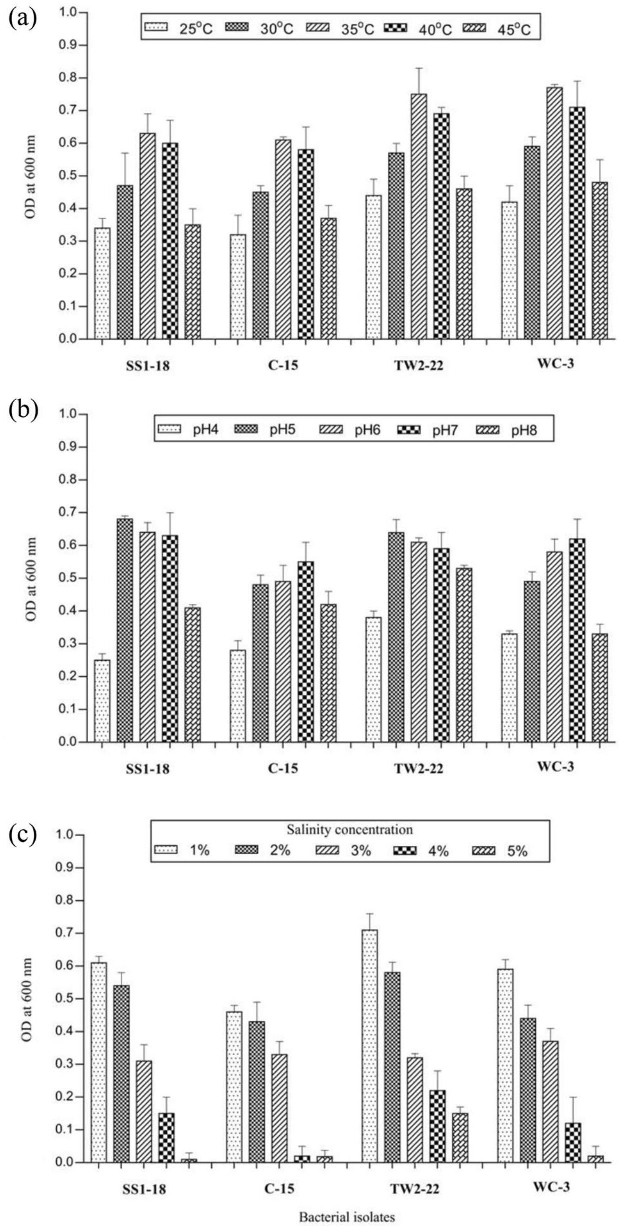

Fig. 4 Effect of varying **a** temperature, **b** pH and **c** salinity conditions on the growth and physiology of the bacterial isolates SS1-18 & C-15 (*Bacillus pseudomycoides*) and TW2-22 & WC-3 (*Bacillus siamensis*)

isolates obtained from the water samples were screened for their ability to grow in the presence of iron, manganese and lead, each at a concentration of 100 ppm. For further studies, 4 isolates belonging to *Bacillus* sp., characterized using biochemical parameters, were found to occur in both mined and control samples with potential tolerance were selected. Molecular characterization of the selected isolates was carried out by amplification and sequencing of 16S rRNA gene, followed by similarity search of related sequences using BLAST tool against ezTAXON database (http://www.eztaxon.org/). The selected homologous sequences were subjected to multiple alignment using ClustalW tool and the phylogenetic tree was constructed based on neighbor-joining algorithm in MEGA software version 7.0 (Fig. 3). Phylogentic analysis showed more than 90% similarity between two mine water isolates (SS1-18 & C-15) belonging to *Bacillus pseudomycoides* and the two control site isolates (TW2-22 & WC-3) from *Bacillus siamensis*. GenBank accession numbers for the submitted sequences of the isolates are MK372148 (SS1-18), MK373765 (C-15), MN448390 (TW2-22) and MN448452 (WC-3). The findings confirm previous reports on *Bacillus* sp. from coal mine samples and their metal tolerance (Jamal et al. 2016; Upadhyay et al. 2017). The isolates exhibited similar features and traits, differing slightly only in their color and texture. All the four isolates were Gram positive with rod-shaped cell type (Table 6). The isolates optimally grew at 35–40 °C, pH of 5–7, and salt concentration of 1–2%. Isolates SS18 and TW2-22 grew better growth at pH 5, whereas the isolates from control site (C-15 & WC-3) grew well at pH7. The growth of bacteria was very low at 4% NaCl, indicating the maximum tolerable limit (Fig. 4). Physiological factors such as pH, temperature, salinity play critical role in enzymatic functions and metabolic efficiency of bacteria, hence determining their ability to thrive in adverse conditions (Samanta et al. 2012).

The selected isolates were analyzed for metal tolerance capacity based on MTC and MIC studies (Fig. 5). The isolate TW2-22 showed highest MTC against all test metals; with Mn at 1600 ppm, Pb at 1400 ppm, Fe at 1000 ppm and Cr at 400 ppm; SS1-18 exhibited the next highest MTC, with Mn at 1400 ppm, Pb at 1200 ppm, Fe at 400 ppm and Cr at 200 ppm (Fig. 6a). Both the isolates from control site exhibited lower values of MTC toward the metals. A similar

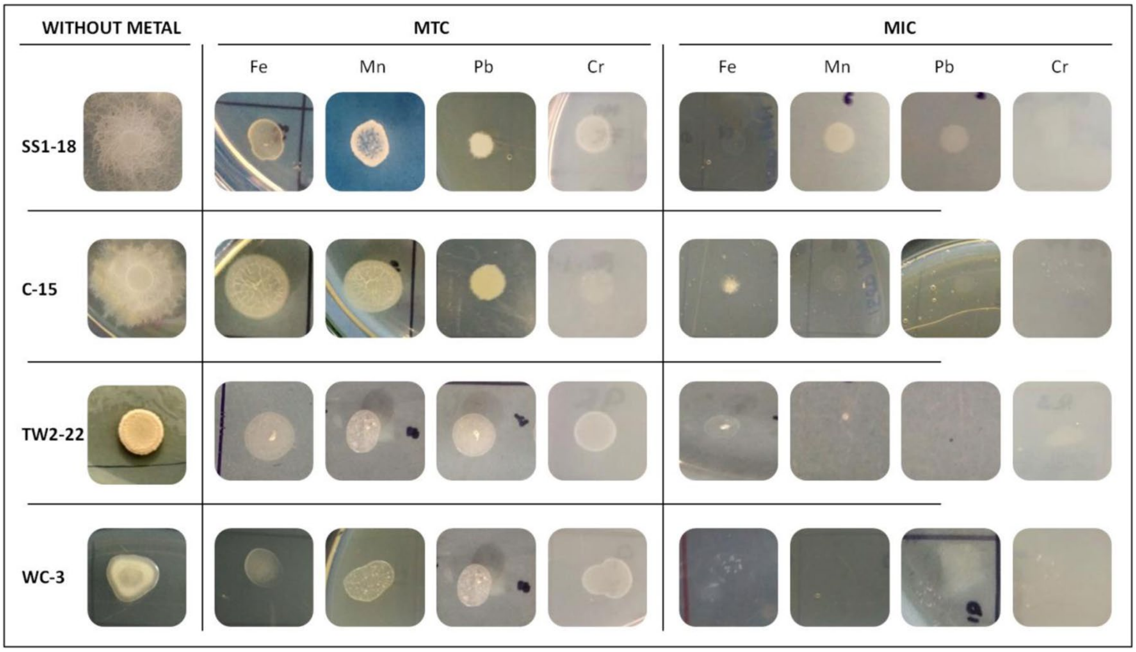

Fig. 5 Spot plate method depicting growth of bacterial isolates SS1-18 & C-15 (*Bacillus pseudomycoides*) and TW2-22 & WC-3 (*Bacillus siamensis*) at Maximum Tolerable Concentration (MTC) and Minimum Inhibitory Concentration (MIC) of Iron (Fe), Manganese (Mn), Lead (Pb) and Chromium (Cr)

pattern was observed in the MIC study, where highest MIC values were shown by TW2-22, followed by SS1-18, as compared to the isolates from control sample. The highest MIC against Mn, Pb, Fe and Cr was 1800, 1600, 1200 and 600 ppm, respectively. The isolates displayed maximum tolerance toward Mn, followed by Pb and Fe, while the least tolerance was toward Cr (Fig. 6b). These results concur with Nongkhlaw et al (2012); they reported *Bacillus* spp. from metal contaminated sites exhibited upto 50% higher MIC for Pb, Cd and Cu as compared to type strains from *Bacillus* spp. (MTCC429 and MTCC430). Strains of *Bacillus* from metal polluted sites are reported to exhibit tolerance toward different heavy metals reflected in their MTC and MIC values (Mohapatra et al. 2019). Another report indicated *Bacillus* from coal mines displayed higher MIC toward chromium compared to *Bacillus cereus* MTCC430 type strain (Ka-ot et al. 2017). Bacteria have the ability to resist metals through resistance mechanisms like segregation through metal complexes and sequestration; or detoxification by expulsion through efflux pumps (Jenkins and Stekel 2010). Bacteria are also able to tolerate heavy metals by immobilization of metals on cell surfaces or converting them to less toxic forms by means of acidification, precipitation or oxidation–reduction mechanisms (Ma et al. 2011).

The isolates exhibited varying growth patterns in presence of metals, over an experimental time period of 3–96 h

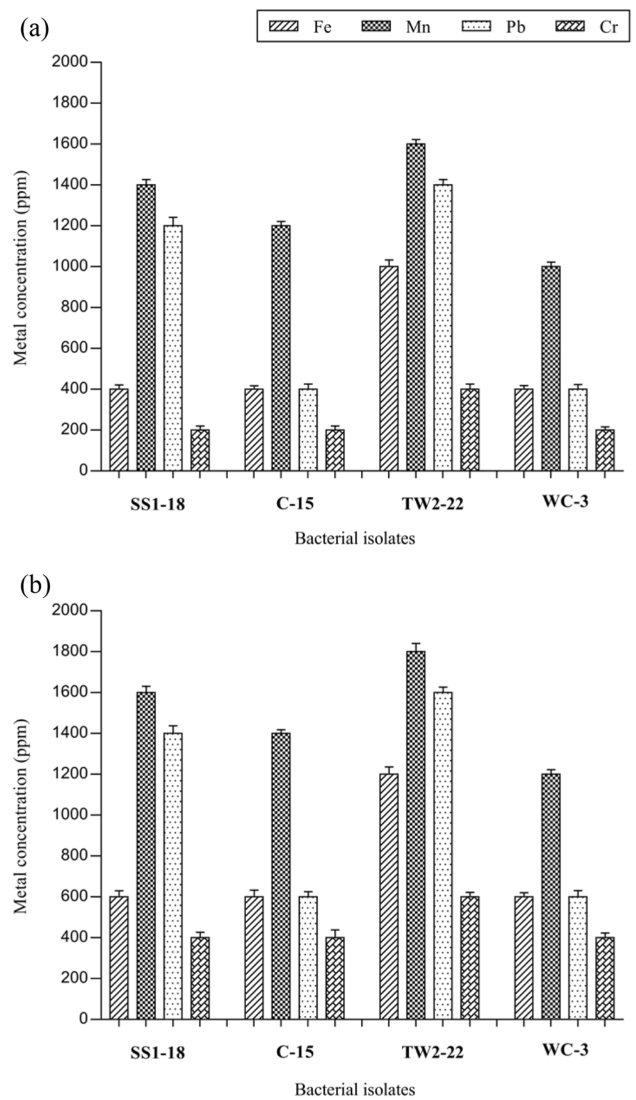

Fig. 6 **a** Maximum Tolerable Concentration (MTC) and **b** Minimum Inhibitory Concentration (MIC) of the bacterial isolates SS1-18 & C-15 (*Bacillus pseudomycoides*) and TW2-22 & WC-3 (*Bacillus siamensis*) in Iron (Fe), Manganese (Mn), Lead (Pb) and Chromium (Cr)

or until the stationary phase was attained. The microbial growth in the absence of metals was also recorded to enable a comparative analysis of the isolates. The lower OD values for biomass growth in presence of metals, underscored a reduction in the biomass growth of the bacterial isolates, in comparison to biomass growth in media that contained no metals. The presence of Fe and Cr was seen to affect the growth pattern more severely than Pb, while Mn was the least toxic to growth. The early transition from the logarithmic to the stationary phase could be due to the inability of bacteria to absorb nutrients in the presence of metals hindering their growth (Monballiu et al. 2015). The growth comparison between SS1-18 and C-15, indicated SS1-18 exhibited better growth in the presence Mn and Pb; however, in the presence of both Fe and Cr, SSI-18 seemed to have lower growth rate, indicating that the isolate has less tolerance to Fe and Cr (Fig. 7). The presence of metals such as iron and chromium are reported to be toxic to *Bacillus* exhibiting inhibitory effect on their growth and proliferation (Kalantari 2008). In the case of the isolates TW2-22 and WC-3, TW2-22 showed better growth in presence of all test metals, and exhibited higher MTC and MIC, compared to other remaining isolates (Fig. 8). In both cases the isolates from mining water samples exhibited better growth in presence of metals, as compared to isolates from control samples. Studies have suggested occurrence of chemical-physical interaction between bacteria and metals, altering their growth behavior and tolerance response by the synthesis of inducible proteins (Cristani et al. 2012; Vashishth and Khanna 2015).

Conclusion

The primary objective of this particular study was to analyze the overall impact of heavy metals on the quality of water, from and around the rat-hole coal mining sites, and its effect on the physiology and response of native *Bacillus* spp. Evaluation of water pollution indices, HPI and HEI placed majority of mine water samples in the high and medium range of pollution level, whereas the control sample was within the safe permissible limit. A strong correlation was noted among Mn, Pb, Fe and Cr; thus designating them as the major pollutants among heavy metals. Water samples were found less contaminated with heavy metals during the monsoon and post-monsoon seasons. The widespread occurrence of *Bacillus* spp. in majority of the coal mine sites, could result in their designation as the indicator organisms of such niches. Findings indicate that *Bacillus* from control environment had lower tolerance

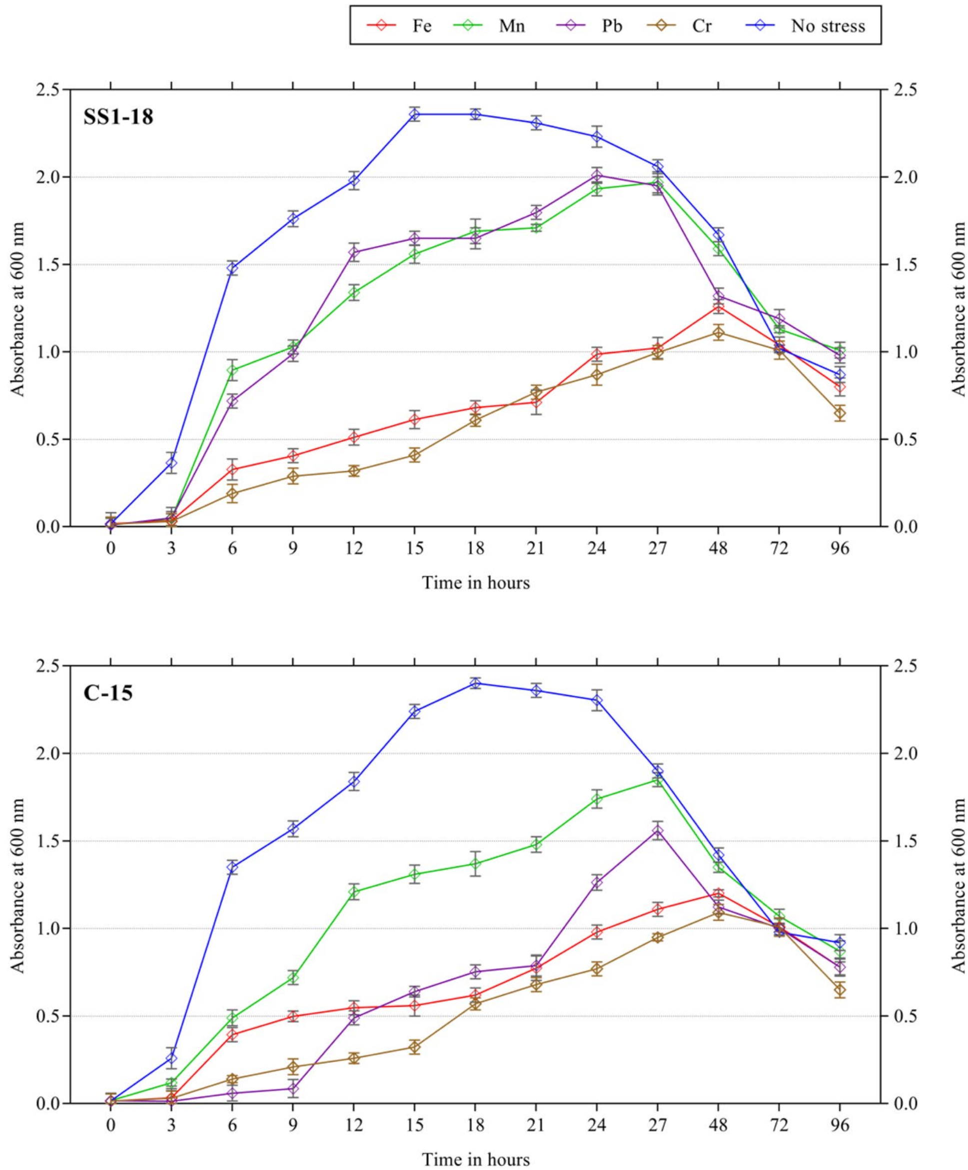

Fig. 7 Bacterial growth curve of *Bacillus pseudomycoides* strains SS1-18 and C-15 in the presence of Iron (Fe), Manganese (Mn), Lead (Pb) and Chromium (Cr)

to metal stress and showed reduced bacterial growth in presence of metals. The physiological endurance of the isolates across varying pH, salinity and temperature gradients and their tolerance capacity toward heavy metals have provided significant insights into their survival strategies in adverse environmental conditions. This study provides baseline information on the water quality and extent of heavy metal contamination in rat-hole coal mine water systems. The native *Bacillus* offers categorical scope for its application in reclamation and remediation strategies for rat-hole coal mining sites.

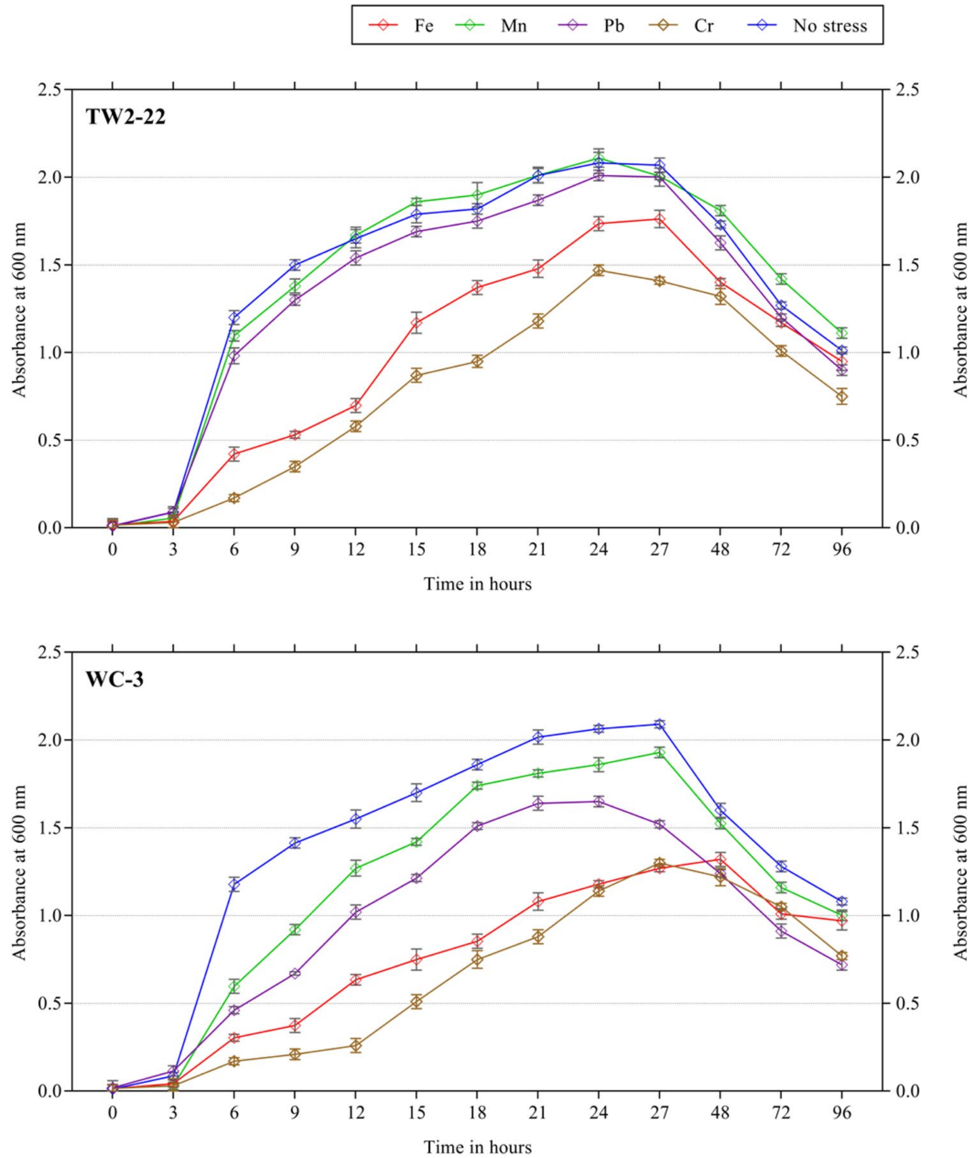

Fig. 8 Bacterial growth curve of *Bacillus siamensis* strains TW2-22 and WC-3 in the presence of Iron (Fe), Manganese (Mn), Lead (Pb) and Chromium (Cr)

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Author contributions LS, DA, AU, HS and NT conducted the study. SRJ, SKB, MDB and KS designed the study. LS and SRJ wrote and analyzed the data.

Declarations

Conflict of interest The authors have no conflict of interest to declare that are relevant to this article.

Ethical approval The sequences of the indigenous Bacillus spp. (SS1-18, C-15, TW2-22, WC-3) analyzed in this study, have been deposited in the Genbank database bearing Accession Numbers—MK372148.1, MK373765.1, MN448390.1 and MN448452.1.

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