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ORIGINAL ARTICLE

Exploring the application of biostimulation strategy for bacteria in the bioremediation of industrial effluent

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Abstract

The purpose of this study was to isolate and characterise toxic element-resistant bacteria from acid mine drainage water and to apply them in the bioremediation of industrial effluent, as well as to identify optimal effluent:nutrient concentration for onsite biostimulation strategy. Wastewater samples were collected from acid mine drainage and industry. Inductively coupled plasma optical emission spectroscopy (ICP-OES) was employed for elemental composition analysis. Isolated bacterial strains were characterised using molecular methods. Bioremediation assays were employed to determine the extent of bacterial tolerance and removal of toxic elements using a biostimulation strategy employing minimal salt medium (MSM) at varied concentrations and positive and negative controls of only MSM and industrial effluent, respectively. Two bacterial strains demonstrated resistance to toxic elements, *Bacillus* sp. MGI101 and *Lysinibacillus* sp. MGI102 both isolated from the AMD sites. However, no observable growth of toxic metal-resistant bacteria was obtained from the industrial effluents. Bacterial strains MGI101 and MGI102 demonstrated high resistance to target toxic elements during the screening and tolerance assays. Remarkably, *Bacillus* sp. MGI101 demonstrated greater ability to remove toxic elements including arsenic, chromium, zinc, copper and aluminium in undiluted solutions of the industrial effluent, with its highest removal capacity observed at > 60% for arsenic and aluminium. Both *Bacillus* sp.MGI101 and *Lysinibacillus* sp. MGI102 demonstrated varied abilities for the removal of toxic elements from dilution concentration of effluent mixed with MSM. However, the optimal dilution ratio observed in this experiment was 5:15 (effluent:MSM). Overall results demonstrated that isolated bacterial strains have the potential to be employed in bioremediation programmes of acid mine drainages and multi-element contaminated water.

Keywords Industrial effluents · Toxic elements · Bacteria · Biostimulation · Bioremediation

Introduction

The process of extracting precious metals such as gold, copper, nickel and platinum has the undesired consequence of producing acid mine drainage (AMD). Acid mine drainage results from the oxidation reactions of pyrite (iron disulphide) in a two-stage process which forms sulphuric acid and ferric oxides (Taylor et al. 2005; Tutu 2012). This is in addition to other toxic compounds such as cyanides and arsenic which are generally associated with metal extraction operations (Chihomvu et al. 2015; Vieira and Volesky 2000). However, the mining industry is by no means the only source of environmental pollution as several other industries including the agriculture sector (agricultural chemicals), energy sector (combustion of coal), metal plating industry and tanneries generate equally large concentrations of these toxic, environmentally persistent elements (Fu and Wang 2011; Karnika

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Alluri et al. 2007; Matlock et al. 2002; Valdman et al. 2001). The most commonly produced potentially toxic elements include chromium (Cr), lead (Pb), mercury (Hg), uranium (U), selenium (Se), arsenic (As), zinc (Zn), silver (Ag), cadmium (Cd), gold (Au) and nickel (Ni) (Wang and Chen 2006). Although some metals such as iron (Fe), copper (Cu), nickel and zinc are needed in small quantities by living organisms for metabolic functioning, other toxic metals including mercury, silver and cadmium have no biological role and are detrimental to organisms even when present in miniscule concentrations (de Limae Silva et al. 2012). An increase in the concentrations of these potentially toxic elements and a decrease in the pH of the water profile will in time lead to the death of microorganisms as evident in the inevitable 'lifelessness' of water bodies that are acid mine drains (Munnik et al. 2010).

The severity of the effects of toxic element contamination in both soil and aquatic environments can be attributed to their non-biodegradability, causing the accumulation of toxic levels throughout the food chain to be inevitable (Malik 2004). This accumulation is evident in the biomagnification of these elements in living organisms within the food chain (David et al. 2012). Some toxic elements such as Cd, As and Ni can bind to the protein molecules of microorganisms and prevent DNA repair pathways and therefore the DNA replication process (Morales et al. 2016). The absence of DNA replication has the implication of preventing further cell division and consequently growth and DNA repair (Yoshida et al. 2005). Similarly, the accumulation of these toxic elements in human bodies has been associated with causing cancer and damage to organs (Barakat 2011). Furthermore, elements such as Cd, As, Cr and Hg have also been implicated in some types of cancer (Tchounwou et al. 2012; Sharma et al. 2015) even if they accumulate in low concentrations. It is undeniable that these potentially toxic elements within the environment can have an effect on the ecosystem, which can be observed in the modification of biomass and in significant changes within microbial communities as well as in the cycling of elements (Roane and Pepper 1999; Sobolev and Begonia 2008). However, some microorganisms, mainly bacteria, have evolved mechanisms to survive the acidic and toxic effects of acid mine drainage in their natural ecosystem (Rosen 1999; Huang et al. 2016) through different mechanisms such as (i) precipitation of metal particles through nucleation reactions (Beveridge and Murray 1980; Beveridge and Fyfe 1985; Mugwar and Harbottle 2016), (ii) active efflux of the metals from cells (Nies and Silver 1995), (iii) ion exchange reactions with peptidoglycan and teichoic acid (Monachese et al. 2012), (iv) exclusion of metal particles by changes in permeable membrane barriers (Bruins et al. 2000), (v) intracellular sequestration of the metal through protein-metal complexation (Ianeva 2009), (vi) extracellular sequestration and enzymatic detoxification of metals and (vii) reduction in metal sensitivity of cellular targets (Bruins et al. 2000; Ianeva 2009). However, it is generally more accepted that microbial resistance to persistent element poisoning is largely dependent on biosorption (Syed and Chinthala 2015; Vieira and Volesky 2000). This technique of biosorption employs the inherent characteristics of living organisms, whereinbiomass is applied in the adsorption of elements (Gavrilescu 2004; Wang and Chen 2009). A possible explanation for this biosorption mechanism is the affinity of hydroxylated and carboxylic functional group molecules found on bacterial cell walls for such elements leading to their adsorption and precipitation. The process is recognised as being passive and does not involve metabolism of these elements (Gadd 2008; Syed and Chinthala 2015). The absence of energy requirements in the adsorption process and the ease of release due to weak chemical bonds could be the reason, and it is the most readily applied mechanism of element removal in most living organisms.

Biostimulation is a common strategy employed in in situ bioremediation of contaminated water bodies and involves the supply of growth-limiting nutrients such as nitrogen and phosphorus to facilitate the interaction of either exogenous or autochthonous microorganisms in the degradation of polluted environments (Dixit et al. 2015; Macaulay and Rees 2014). This strategy has previously been used mainly for treatment of hydrocarbon-polluted sites to stimulate the growth of indigenous microorganisms and to facilitate the rapid breakdown of crude oil (Macaulay and Rees 2014). Most polluted water bodies are characterised by growth rate–limiting nutrients that prevent metabolism of both indigenous and exogenous microorganisms and the consequential increase in biomass which is a necessary pre-requisite for the biosorption and biodegradation of these pollutants.

With the understanding that pollution is an inevitable aspect of global industrialisation, the need to ameliorate its effects is the premise of most bioprospecting studies in extreme environments, particularly those contaminated with acid mine drainage. Several studies including Tchounwou et al. (2012) have reported that bacteria are capable of detecting even low concentration changes in toxic elements within their environment and make swift adjustments to cope with these changes. When microorganisms are repeatedly exposed to toxic elements, they develop a tolerance and resistance to the stress of these variations in their environment (Ahemad 2012; Issazadeh et al. 2013). This microbial adaptation to polluted environments can be employed as a bioindicator and tool to determine the extent of pollution within the particular environments (Selvin et al. 2009; Sumampouw and Risjani 2014).

Industrial processes and temporary storage measures employed by industries do not provide adequate contact time for potential resident microorganisms to evolve strategies for the degradation of toxic elements and xenobiotic compounds. The standard practice in most countries is that wastewater is temporarily stored and diluted at industrial sites until it meets acceptable municipal standards. It is then transported to a

wastewater treatment facility for treatment and disposal into large waterbodies (Carter et al. 1999; USEPA, 2004; Doorn et al. 2006; Dhote et al. 2012). The major limitation of wastewater treatment processes is that they are designed to reduce biochemical oxygen demand (BOD) and therefore focus primarily on degradation of organic matter (Campillo 2016; Momba et al. 2006). As a result, such treatments do not reduce or remove toxic element contamination with the implication that presumably treated water often still contains significantly high quantities of toxic elements that inadvertently enter into the receiving water bodies. One strategy that could possibly ameliorate this problem is to expose industrial effluents in situ to monocultures or consortia of microorganisms that would reduce or remove these toxic elements before conveyance to the sewerage system and ultimately the waterbodies which are the ultimate sinks for such effluents.

In the face of increasing pollution of already limited water resources, there is an increasing need to elucidate the fate of potentially toxic contaminants in the environment and to develop cost-efficient methods of removing them from different environmental media. Current physicochemical methods such as the use of ion-exchange resins, ultrafiltration, nanofiltration, electrodialysis and reverse osmosis (Karnika Alluri et al. 2007;Wang and Chen 2009; Fu and Wang 2011; Kumar et al. 2011) present cost implications that are not sustainable or eco-friendly. It would be useful to find microbial strains that are able to grow and metabolise in the presence of potentially toxic elements. Toxic element-resistant bacteria have the potential to be used as bioremediation tools for contaminated water and can be incorporated into existing waste treatment programmes. Such alternatives will also allow for effective and cost-efficient wastewater treatment and bioremediation systems (Filali et al. 2000; Kumar et al. 2011; Sharma 2012). It is therefore of vital importance to identify microorganisms that can tolerate and possibly reduce the concentrations of these toxic elements from environmental milieu. Thus, the purpose of this study was to isolate and characterise toxic element-resistant bacteria from acid mine drainage water, apply them in the bioremediation of industrial effluent of similar chemical profiles and ascertain the effectiveness of the bioremediation employing the biostimulation strategy.

Material and methods

Sample collection

Acid mine drainage water was collected from two common drainage sites (25° 50′ 22.018″ S 27°7′ 11.103″ E and 26° 7′ 30.151″ S 27°7′ 11.103″ E) of a conglomeration of active gold mines located in Gauteng, South Africa. Sample bottles intended for sample collection for elemental analysis were soaked overnight in 2% nitric acid (Merck, South Africa) and rinsed twice with double-distilled water and dried in a clean, metal-free cabinet. Industrial effluent samples at point source were collected from an anonymously protected industrial site. All samples were stored at 4 °C prior to chemical and microbiological analysis.

Elemental composition analysis

Elemental concentrations such as Al, As, Fe, Cu, Zn, Cr and Ni in the AMD water and industrial effluent samples were analysed using inductively coupled plasma optical emission spectrometry (ICP-OES) (PerkinElmer Optima 5300 DV, Germany). Samples were digested using Aqua regia (Sigma Aldrich, South Africa) following the protocol provided by USEPA (1996). All digested samples were filtered using 0.45 μm filter paper (Whatman, USA), prior to ICP-OES analysis. The target elements were analysed by direct aspiration into the ICP-OES. Data inclusion was based on regression coefficient readings of > 0.999 benchmarked against the standard calibration curves for each of the element standards at the respective absorbance wavelength, taking into consideration their respective method detection limits.

Isolation and screening of bacteria

The isolation of bacteria was achieved by amending 1-lnutrient agar with the following quantities of salts before sterilisation: $Al(NO_3)_3{\cdot}9H_2O$ (34 mg), $CuSO_4{\cdot}5H_2O$ (0.83 mg), $Fe(NO_3)_3{\cdot}7H_2O$ (24 mg), $MgSO_4$ (750 mg), $KMnO_4$ (230 mg), $Zn(NO_3)_2$ (5.9 mg), $Pb(NO_3)_2$ (0.32 mg), $Ni(NO_3)_2{\cdot}6H_2O$ (9.2 mg), K_2CrO_4 (0.75 mg) and $Cd(NO_3)_2{\cdot}4H_2O$ (0.55 mg). The initial concentrations of salts were determined based on initial concentrations of the target elements in the AMD water samples, with the intent of simulating the chemical profile of the water samples. Triplicate media plates containing these salts were inoculated with 0.1 ml of each AMD water sample and incubated at 30 °C for 48 h under aerobic conditions. Colonies were purified by transfer into fresh media plates of the same constitution until axenic cultures were obtained. Nutrient agar plates that did not contain salts of the test elements were also inoculated with 0.1 ml of AMD water samples and incubated under the same conditions to serve as experimental controls. Isolates from these non-amended plates were later transferred to fresh plates containing concentrations of salts of the test elements as indicated previously and incubated under the same conditions.

Molecular characterisation and phylogenetic analysis

The genomic DNA of axenic cultures of bacterial isolates was extracted using the ZR Fungal/Bacterial DNA Kit™ (Zymo Research, Irvine, CA) following the manufacturer's instructions. The obtained DNA was then amplified using the 16S

rRNA universal gene primer set (27F and 1492R) under the following cycling conditions: an initial denaturation step at 98 °C for 3 min followed by 32 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min followed by final extension at 72 °C for 10 min. The final PCR products were then purified and sequenced in the forward and reverse directions on the ABI PRISM™ 3500xl Genetic Analyser. The obtained sequences were subjected to BLAST analysis for the identification of bacterial taxa and submitted to NCBI GenBank for the generation of accession numbers. The accession numbers of the submitted sequences are KX641888 and KX641889. Phylogenetic analysis was performed using the Molecular Evolutionary Genetics Analysis v7 (MEGA7) software (Tamura et al. 2013) using an alignment created with SINA Aligner (Pruesse et al. 2012).

Tolerance tests and minimum inhibitory concentrations

Bacterial isolates that demonstrated an ability to grow on the initial simulated resident concentrations of target elements obtained from the initial ICP-OES readings on amended nutrient agar plates were further exposed to incremental quantities of the initial readings by employing 10% incremental inclusions to the nutrient agar, using the same salts of the target elements employed previously until growth was almost non-existent on plates.

Bioremediation assays

The isolates that demonstrated the greatest minimum inhibitory concentrations (MICs) were introduced to the industrial effluent sample to ascertain whether this observed tolerance implied the removal of only the test element or possibly other toxic elements from the polluted water sample. Minimal salt medium (MSM) was used to provide all other growth-limiting nutrient requirements for the selected isolates. MSM composition in 1000 ml of distilled water used is as follows: $Na_2HPO_4{\cdot}7H_2O$ (12.8 g), KH_2PO_4 (3.0 g), NaCl (0.5 g), NH_4Cl (1.0 g), 1 M $MgSO_4$ (0.4 ml), 1 M $CaCl_2$ (0.02 ml) and glucose (4 g). The triplicates of each test solution were prepared by mixing industrial effluents with liquid MSM at the following ratios: 1:19, 2:18, 3:17, 4:16, 5:15, 6:14, 7:13, 8:12, 9:11, 10:10, 20:0 and 0:20. The 20:0 as well the 0:20 formulations served as the negative and positive controls, respectively. Growth patterns were monitored during the 72-hincubation (at 30 °C) by withdrawing samples at intervals of 12 h and determining the absorbance at 600 nm using a T60 UV-VIS Spectrophotometer. Samples at the end of the 72-hincubation period were analysed using ICP-OES to determine the final elemental composition after microbial treatment.

Statistical analysis

Paired *t*test analysis was used to determine the significant differences between the bacterial isolates and the amount of toxic element reduction in different treatment ratios at a 95% confidence level. These percentages were deemed significant when *p*values were less than 0.05. Graphs of these percentages of toxic element removal were constructed for each dilution ratio of effluent:MSM formulation that was employed to determine the extent of biostimulation required for effective bioremediation.

Results

Elemental composition analysis

A total of eight toxic elements in different concentrations were consistently identified in the three water samples (Table 1). Results of the AMD water samples showed that AMD site 1 had the higher concentration of most of the target elements monitored in this study. In addition, analysis of the industrial effluent demonstrated very high concentrations of Cu and Zn with quantities of 1340 ppm and 1830 ppm, respectively. Furthermore, As was only found in negligible quantities in AMD water samples from both sites. However, in the industrial effluent sample, it was detected in very low quantity (18 ppm).

Screening and isolation of bacteria

A total of six bacterial strains were initially isolated from the AMD water samples based on their ability to grow on all target elements included on nutrient agar plates. No growth was observed on plates inoculated with the industrial effluent. From the non-amended nutrient agar plates, 10 bacterial

Table 1 Initial concentration of toxic elements in the AMD water and industrial effluent samples

Elements	ICP-OES Readings (ppm) AMD site 1	ICP-OES Readings (ppm) AMD site 2	ICP-OES Readings (ppm) Industrial effluent
Al	2.43	<0.20	1.57
As	<0.20	<0.20	18
Fe	3.30	<0.20	0.74
Cu	0.21	<0.20	1340
Zn	2.03	0.75	1830
Cr	<0.20	<0.20	49
Ni	1.86	<0.20	304
Pb	<0.20	<0.20	1.33

<0.20 – below the detection limit

strains were isolated. Those 10 strains showed no positive growth when transferred to plates containing the target elements. Of the six strains that had demonstrated tolerance to the resident toxic elements of the AMDs, only two strains demonstrated high tolerance to incremental quantities of the same target elements. Table 2 summarises the minimum inhibitory concentrations survived by the two bacterial strains that demonstrated the highest tolerance before observable extinction on plates during exposure to the target elements.

Molecular characterisation

Although six different pure bacterial isolates were obtained from the collected acid mine water samples, only the two isolates that demonstrated high potential for toxic element resistance were subjected to molecular identification. Phylogenetic comparison of PCR-amplified 16S rDNA sequence data of each isolate with the database of known species using the NCBI database revealed that one of the isolates was closely related to *Bacillus cereus*and while the other was closely related to *Lysinibacillus fusiformis*.The pairwise nucleotide sequence similarities of both isolates were 99 and 100%, respectively. In order to evaluate their phylogenetic positions, the 16S rRNA gene sequence of each strain was analysed and a phylogenetic tree was constructed using Mega 7 software. The phylogenetic tree showed that isolates belong to their respective closest similarities (Fig. 1).

Tolerance test and minimum inhibitory concentrations

Bacillus sp. MGI101 and *Lysinibacillus* sp. MGI102 demonstrated high tolerance to target element concentrations, with often greater than 100-fold tolerance for the target elements compared with the initial resident concentrations. Isolate *Lysinibacillus* sp. MGI102 demonstrated no growth on the plates with 240 ppm concentration of manganese (Mn) salt. Further increments also showed no visible colony formation. The increments of target element salts in nutrient agar plates above the concentrations shown in Table 2 caused extinction on plates with no observable growth after 72 h.

Bioremediation assays

For bioremediation assays, industrial effluents were mixed with MSM to compensate for any growth rate nutrient limitations. On the basis of direct correlation between biomass and absorbance, the isolate *Lysinibacillus* sp. MGI102 did not demonstrate an appreciable biomass increase when cultured only in industrial effluent (as represented in absorbance values in Fig. 2) in the 72-h period of monitoring growth. However, biomass growth (in terms of absorbance values) for *Bacillus* sp. MGI101 appeared to peak at 60 h. Varying growth patterns were observed in different concentrations of industrial effluent:MSM formulations. It is remarkable that with increasing concentration of industrial effluent, growth of *Bacillus* sp. MGI101 appeared to increase while that of *Lysinibacillus* sp. MGI102 steadily declined.

Percentage removal of each element was determined by computing the difference between the initial and final concentration after 72 h of incubation. The focus in relation to feasible industrial application was placed on ratios with increased concentrations of the toxic elements from the effluent samples. Therefore, the result summary provided in this section excluded any significant findings within the lower concentrations of the effluent:MSM formulations as it was deemed not to be economically viable to introduce such large quantities of MSM or growth-limiting nutrients into the effluent. Nevertheless, while*Bacillus* sp. MGI101 demonstrated an ability to remove most of the target elements from the varied concentrations, it also significantly removed toxic elements from the undiluted effluent samples that it was subjected to (Fig. 3a–h). Results of comparison were evaluated on the basis of the Student *t*test.

Table 2 Minimum inhibitory concentration of different target elements for Bacillus sp. MGI101and Lysinibacillus sp. MGI102

Elements	Initial concentration (ppm)	MIC (ppm)	
		Bacillus sp. MGI101	*Lysinibacillus* sp. MGI102
Al	2.43	4.89	4.89
Fe	3.30	36.5	36.5
Cu	0.21	3.29	3.29
Mg	152	303	303
Mn	80.4	240	216
Zn	2.03	18.3	18.3
Cr	0.20	4.27	4.27
Cd	0.20	3.20	3.20
Ni	1.86	3.53	3.53
Pb	0.20	18.2	18.2

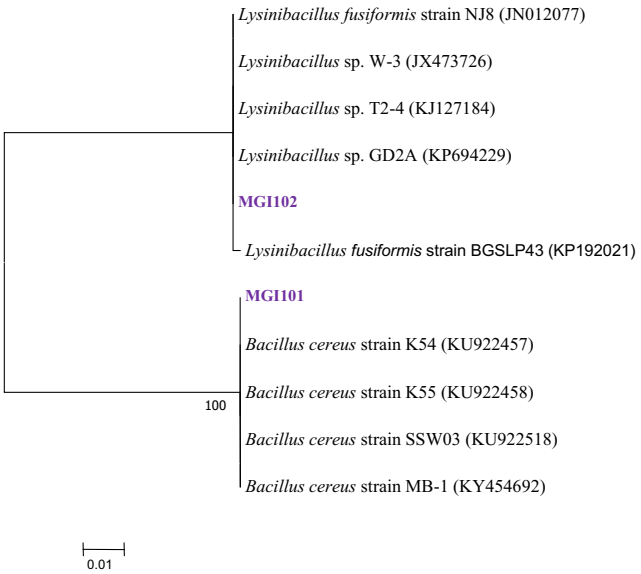

Fig. 1 Phylogenetic tree showing the relationship between *Bacillus* sp. MGI101 and *Lysinibacillus* sp. MGI102 with the most similar sequences based on the 16S rRNA gene

The strain *Bacillus* sp. MGI101 was able to remove As, Cr, Zn, Cu and Al from undiluted effluent solutions that contained no additional growth-limiting nutrient (MSM). The highest removal was observed for As and Al with approximately 63% and 68% removal, respectively. However, the removal capacities of *Lysinibacillus* sp. MGI102 were not evident in undiluted effluent solutions, although it was observed in diluted formulations. Furthermore, MGI102 demonstrated a steady increase in its ability to remove toxic elements with increasing dilution of the effluent using MSM. For example, in diluted sample ratios, *Lysinibacillus* sp. MGI102 demonstrated a steady increase in removal capacities at the higher concentrations of As with the highest removal capacity demonstrated at the ratio of 6:14 (effluent:MSM) where it removed approximately 75% of the As.

Both bacterial strains demonstrated varied but significant removal capacities in diluted effluent:MSM ratios for the different elements including Cr, Zn, Cu and Al. It is also remarkable that the removal capacity at 5:15 (effluent:MSM) was significantly high for Cr, Zn, Cu and Ni with removal percentages of > 60% demonstrated by both bacterial strains.

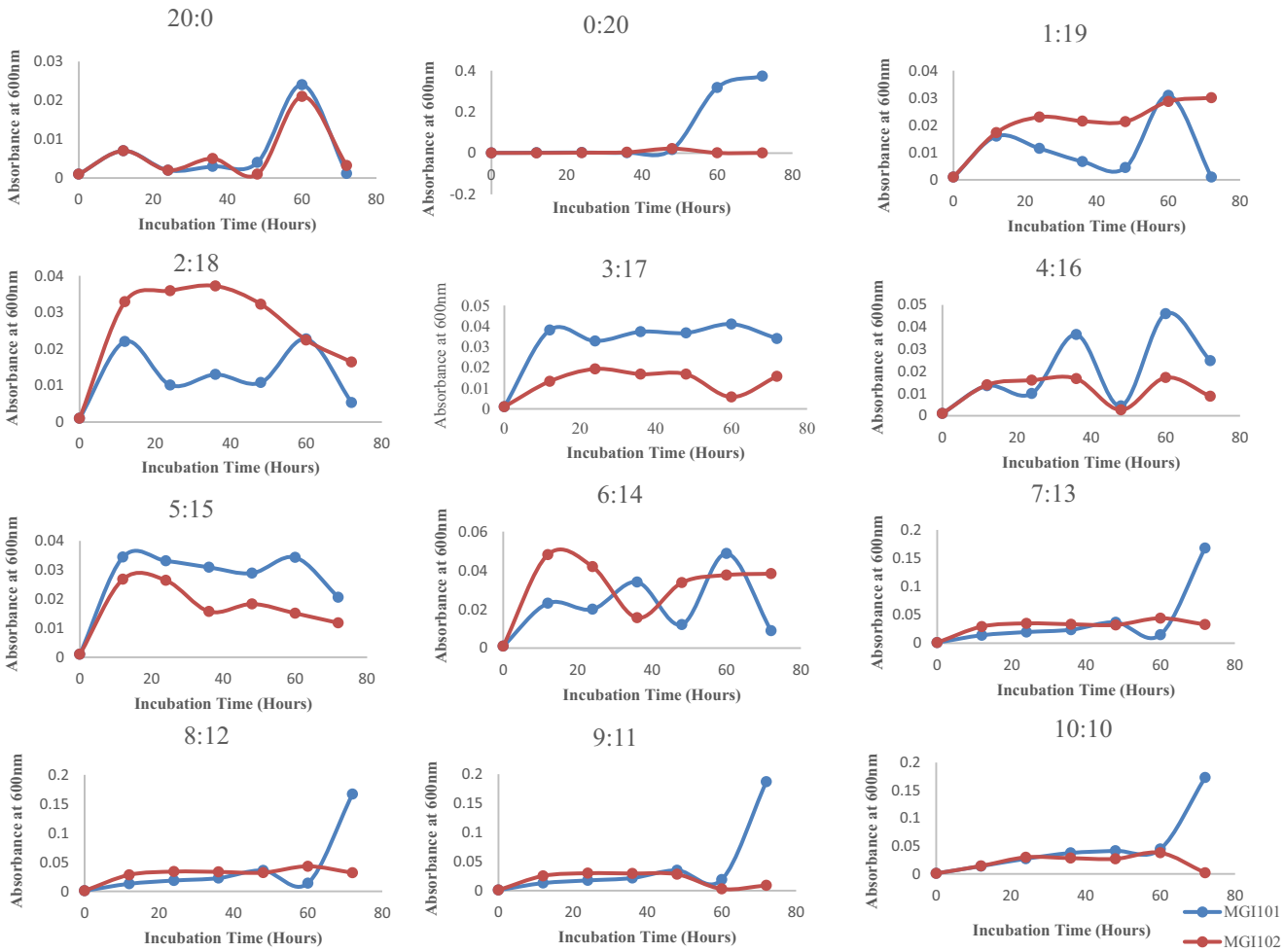

Fig. 2 Growth patterns of *Bacillus* sp. MGI101and *Lysinibacillus* sp. MGI102 using varying concentration ratios of industrial effluent:MSM formulations

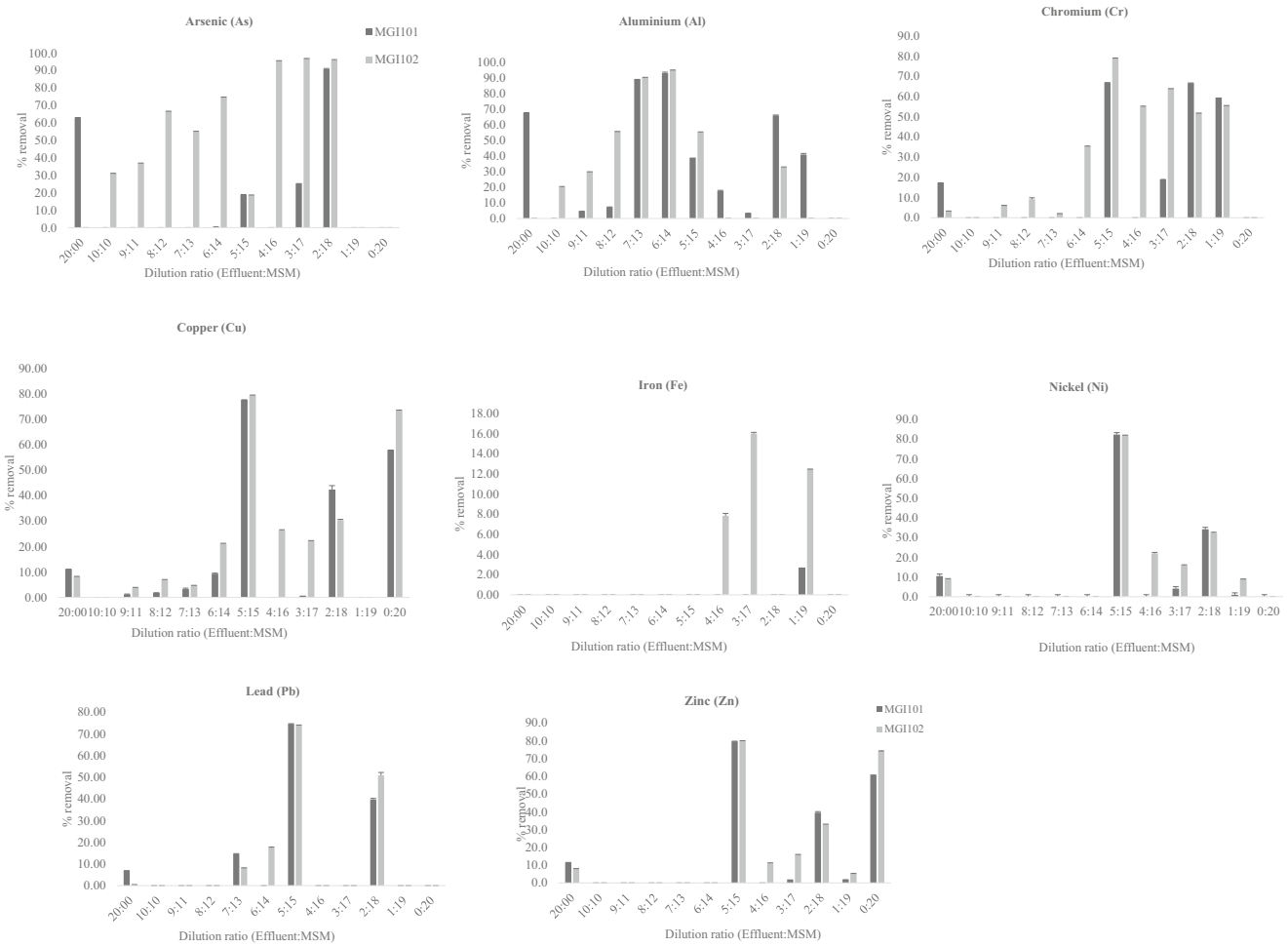

Fig. 3 Percentage removal of potentially toxic elements from industrial effluent samples mixed with MSM (effluent:MSM ratio) by *Bacillus* sp. MGI101 and *Lysinibacillus* sp. MGI102. Values were derived for triplicate samples ($n = 3$) and are reported as mean±SD, with $p$value > 0.05

However, both bacterial strains were unable to demonstrate significant removal of Fe, Ni and Pb above 5:15 (effluent:MSM) ratios.

Discussion

The success of an in situ bioremediation programme is dependent on several factors that include the choice of microorganisms and the provision of growth-limiting nutrient(s) (Sarkar et al. 2016). It is also of importance to consider in bioaugmentation strategies that microorganisms must originate from an environment with a similar chemical profile to that of the polluted water under consideration. This study investigated the effect of concentration of growth-limiting nutrients in the efficiency of two bacterial strains, *Lysinibacillus* sp. MGI102 and *Bacillus* sp. MGI101, for the bioremediation of industrial effluents with a chemical profile similar to acid mine drainage water. The objective was to identify the most suitable concentration that could be suitable for preliminary in situ bioremediation of effluent before municipal collection and further treatment in the local wastewater treatment plant. While information can be found for biostimulation strategies with respect to the bioremediation of hydrocarbon contaminated water (Macaulay and Rees 2014; Sarkar et al. 2016), the same cannot be said for toxic element bioremediation using this strategy before this study.

Both bacterial strains, *Lysinibacillus* sp. MGI102 and *Bacillus* sp. MGI101, were isolated from AMD sites. They were Gram-positive, rod-shaped, endospore-forming strictly aerobic bacteria, profiles that matched those of other bacteria previously isolated from similar niche environments (Gupta et al. 2012; Ka-ot et al. 2017; Liu et al. 2013; Park et al. 2012; Wichlacz et al. 1986). These two isolates showed tremendous resistance and biosorptive abilities toward the target elements employed in this study as indicated by the plate growth and ICP-OES analysis. This is similar to reports from previous findings that employed quantitative analytical methods to determine the abilities of microorganisms to remove toxic elements from contaminated samples. Chang and Chen (1998) designed experiments employing quantitative techniques to

investigate the behaviour of selective adsorption of *Pseudomonas aeroginosa* PU21 (Rip64) with solutions containing Pb, Cu and Cd. In another study by Syed and Chinthala (2015, b), three species of *Bacillus* isolated from solar salterns were screened for their potential to detoxify heavy metals Pb, Cr and Cu by biosorption using Atomic absorption spectroscopy (AAS), ICP-OES and energy dispersive spectroscopy (EDS) to determine the quantities before and after treatment with significant results.

The present study observed that concentrations of the elements and the growth rate–limiting nutrients as provided by MSM in test solutions were pivotal in increasing the removal capacities of the two bacterial strains, particularly for *Lysinibacillus* sp. MGI102. *Bacillus* sp. MGI101 demonstrated great removal capacities at high concentrations of the target elements even without growth rate–limiting nutrients, suggesting that this strain has great potential to remediate polluted water possibly without the need for biostimulation. This is particularly important as there are critics of this strategy that have noted the likely problem of eutrophication associated with biostimulation (Boesch, 2002; Macaulay & Rees, 2014). In addition, the exploitation of microorganisms that can remediate without the need for biostimulation or in environments with low concentrations of nutrients will offer a considerable advantage under such circumstances.

Traditionally, *B. cereus* is usually implicated in food poisoning and food intoxication, making it a human pathogen (DelVecchio et al., 2006; Hoffmaster et al., 2006). However, *B. cereus*SIU1 has previously demonstrated toxic element-resistant ability (Singh et al., 2010), which is likely attributed to its production of thermo-alkaline protease that changes the pH of the environment and substrate. The findings in this study are likely explained by the fact that several strains of *Bacillus* including *B. anthracis* and *B. cereus* have demonstrated the presence of multiple plasmids, particularly the presence of the transposase genes that are located in two large plasmids, as was found in the *B. cereus* ZK strain which is responsible for horizontal gene exchange between plasmids and chromosomes (Rasko et al., 2004). It is very likely that such plasmids are present in the *Bacillus* sp. MGI101 isolated from AMD water site 2 and may account for its increased resistance to toxic elements. It is also likely that their prolonged exposure to the AMD water has allowed them to develop strategies to cope with these routinely present toxic elements (Roane & Pepper, 1999).

The strain *Bacillus* sp. MGI101 demonstrated a high tolerance for As as observed in its removal of between 60 and 90% of these elements from different concentrations of effluent:MSM culture medium formulations. However, it only seemed to be capable of removing low concentrations of Fe (< 1% from the raw industrial effluent sample) (Fig. 3). This is similar to the finding of Kanwal et al. (2004) that reported high removal rate for As compared with Fe. In their study, the isolated strains (*E. coli* and *B. cereus*) were able to survive at 10 ppm concentrations of As, although no growth was observed at the same concentrations of Pb, Cu and Fe.

Information regarding *L. fusiformis* is sparse and, at present,is evolving in its application for bioremediation purposes (Chihomvu et al., 2015; He et al., 2011; Lin et al., 2014).However, the strains *L. fusiformis* ZC1 (He et al., 2011) and *L. fusiformis* ZYM1 (Zhao et al., 2016) have previously been shown to have toxic element resistance to chromium and selenite/tellurite, respectively. This study also confirmed that *Lysinibacillus* sp. MGI102 demonstrated toxic

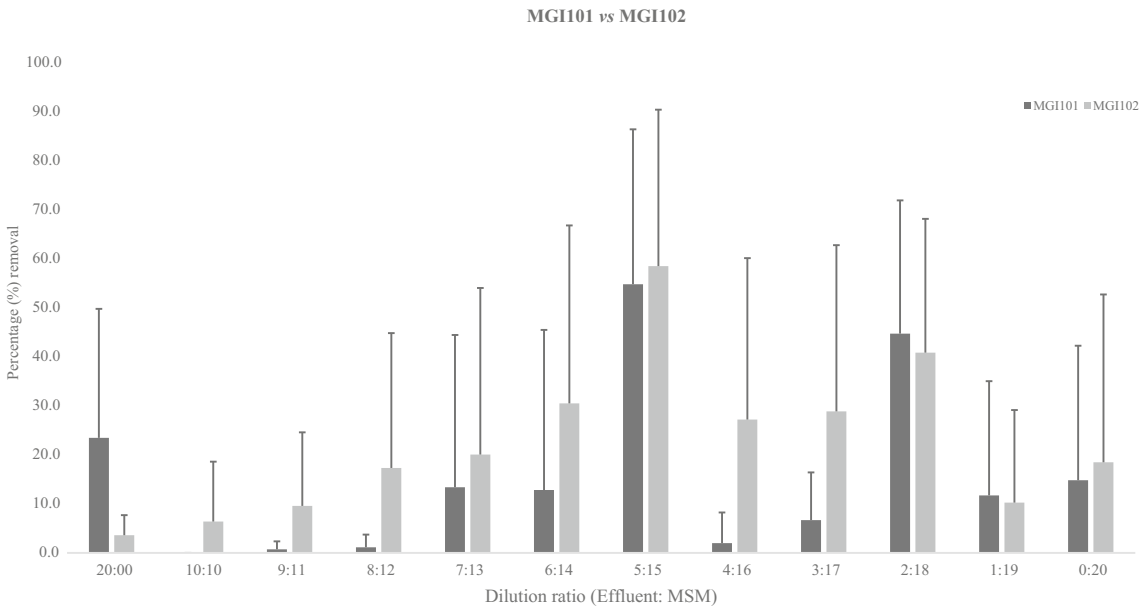

Fig. 4 Average percentage of removal of toxic elements by *Bacillus* sp. MGI101 and *Lysinibacillus* sp. MGI102. Values were derived for triplicate samples ($n = 3$) and are reported as mean±SD, with $p$value > 0.05

elementresistance to varying concentrations of the different toxic elements including As, Cr, Zn, Cu, Ni, Pb and Al. Furthermore, studies done by He et al. (2011) have established the presence of the gene *chrA* in the particular strain of *L. fusiformis* which encodes a Cr(VI) transporter that accounts for the Cr(VI) resistance. This is further supported in the present study by the ability of *Lysinibacillus* MGI102 to remove significantly high quantities of Cr from different concentrations of the industrial effluent. It must, however, be noted that unlike *Bacillus* sp. MGI101, strain *Lysinibacillus* sp. MGI102 required the addition of growth rate–limiting nutrients. Thus, for bioremediation application, it would require strategies involving the biostimulation processes.

On average, it appeared that the most suitable dilution ratio for bioremediation was the 5:15 ratio as seen in Fig. 4. It is pertinent to note that the element removal capacity of the bacterial strains was element-specific and the large standard deviation obtained at the different dilution concentrations is an indication of the varied removal capabilities of the two bacterial strains for the different toxic elements. Moreover, this study observed that the removal of toxic elements is not necessarily in direct proportion with the concentration present within the medium. For instance, both strains*Bacillus* sp. MGI101 and *Lysinibacillus* sp. MGI102 removed 78% of Cu in medium formulation 5:15 while managing to remove 0.6% and 22.3% of Cu, respectively, when the media formulation was 2:18 (effluent:MSM). It will be presumed that a lower concentration will favour a greater efficiency of element removal since there are less elements to remove (Ahirwar et al., 2013; Raghuraman et al., 2013), but this was not the case. This, however, can be explained from the understanding that the higher concentration of Cu implies that there are several free elemental ions present in the solution as compared with lower concentration, where more likely the copper ions present may already be bound in other chemical reactions. According to Nogueira et al. (2015), the amount of free Cu^{2+}ions that result from bacterial activity is a function of the metal speciation which determines the interaction between the microorganism and the metal.

Overall, it is remarkable to note the efficiency of removal of As by the two isolates (Fig. 3) especially since the AMD sites from which the two bacterial strains were isolated did not contain detectable levels of arsenic. This further validates the need for bioprospecting and bioremediation studies as microorganisms are constantly evolving mechanisms to adapt to the changing environment which is a direct consequence of pollution.

Conclusion

In the present study, we evidenced the tolerance of *Bacillus* sp. MGI101 and *Lysinibacillus* sp. MGI102 to various toxic elements. The results showed that the identified bacteria are able to survive in AMD water and have developed mechanisms to tolerate these toxic elements. These toxic elements were effectively removed by the growing cells of both tested isolates, with Fe being the most recalcitrant to biosorption and As, Cr, Pb and Al being the most important toxic elements removed from the effluent due to their health-related consequences. These two isolates demonstrated potential usefulness in bioremediation processes not only for AMD water but also for industrial effluents that have similar chemical profiles. Both strains were effectively applied in the treatment of effluents with the strain *Lysinibacillus* sp. MGI102 demonstrating a higher efficiency in removing toxic elements as compared with*Bacillus* sp. MGI101. However, it must be added that the application of *Lysinibacillus* sp. MGI102 would require the employment of a biostimulation strategy to provide the growth rate–limiting nutrients. A possible bioremediation design may be to introduce *Bacillus* sp. MGI101 to initiate biosorption in the raw industrial effluent, thus reducing the quantities of rate-limiting nutrient requirements that will be later added for *Lysinibacillus* sp. MGI102 to complete the process. Such an adaptation of biostimulation will require further investigation to facilitate efficient bioremediation processes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals N/A

Informed consent N/A

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