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Remedial Approaches against Arsenic Pollution

Gia Khatisashvili, Tamar Varazi, Maritsa Kurashvili, Marina Pruidze, Evgeni Bunin, Kakha Didebulidze, Tinatin Butkhuzi, Elina Bakradze, Nino Asatiani, Tamar Kartvelishvili and Nelly Sapojnikova

Abstract

The study is devoted to a very urgent and acute problem for Georgia – remediation/restoration of the arsenic (As) mining and storage sites. The approach of a given work is based on using capabilities of nature itself, which has a great adaptive potential to chemical environmental pollution. The aim of the study is to identify the bacterial strains from the endemic soil microbiota, characteristic to a specific localization of arsenic contaminated sites and able to resist to the toxicant. To determine the level of arsenic contamination, soil samples have been analyzed using Inductively Coupled Plasma - Optical Emission Spectrometry method. The distribution of arsenic in soil samples splits them into categories according to the degree of contamination, ranging from 50 ppm to 13000 ppm. The local bacteria community has been studied using conventional cultivation method along with modern method of bioindication – a biochip. The low density biochip contains the relevant probes for the identification of the bacterial consortium in soil microbiota. Chemical and microbiological analysis was based on the standards and methodologies developed by International Standards Organizations – ISO and Environmental Protection Agency – EPA. It is prospected that bioremediation can become essential part of remediation against arsenic pollution in the context of circular economy.

Keywords: arsenic pollution, inductively coupled plasma - optical emission spectrometry method, biochip, soil microbiota, bioremediation, PCR amplification

1. Introduction

The study deals with chemical pollution of the environment, in particular arsenic contamination of soil, which is a highly pressing problem for both Georgia and the world. Arsenic is one of the toxic metalloids that exist in more than 200 mineral forms, where 60% of them are normally arsenates, 20% are sulfosalts and sulfides and 20% are arsenite, oxides, arsenide, silicates and elemental arsenic. Arsenic mainly exists in the environment as arsine (As^{-3}), elemental arsenic (As^0), arsenite (As^{+3}) and arsenate (As^{+5}). Among all these forms only arsenite and arsenate are more abundant in natural environment than the other two [1]. Arsenic is naturally present in the lithosphere (earth crusts, soil, rock and sediment), hydrosphere (surface water, aquifers, deep well and oceans) and biosphere (food chain and

ecosystems) [2, 3]. Arsenic is one of the top five toxic chemicals that were listed in the US Comprehensive Environmental Response, Compensation, and Liability (CERCLA) act of hazardous substances [4]. The toxicity of arsenic to living organisms is due to its functional affinity for phosphorus and its ability to form covalent bonds with sulfur. Arsenate replaces phosphate in phosphorylation reactions, and arsenite interacts with thiol groups of proteins, causing serious disturbances in cell metabolism [5]. Arsenic species are deposited in the skin, lungs, kidney, liver, etc. and cause several severe diseases by oxidative stress, altered DNA methylation, altered DNA repair, mitochondrial damage, cell proliferation, tumor promotion and co-carcinogenesis [6]. Arsenic exists both in toxic inorganic (associated with iron, cobalt, nickel coupled with sulfate minerals) and comprehensively less toxic organic (associated with carbon and hydrogen) forms. It is released into the environment by natural phenomenon (geogenic) or by anthropogenic activities. Soil texture is an important characteristic that affects arsenic chemistry. Arsenic levels in soil depend on climate, pH and redox potential. Arsenic can be chemically transformed in soils through several mechanisms. These include oxidation, reduction, adsorption, dissolution, precipitation, and volatilization. The trivalent form (As^{+3}) is easily absorbed to ferric and aluminum oxides in the soil environment and oxidized to the pentavalent form in aerobic condition and reduced back to the trivalent form by reduction [7]. Under aerobic environment, the inorganic form of arsenic can easily bind to inorganic and organic materials in soil such as clay, iron and manganese dioxide, and exists in the pentavalent state (arsenate AsO_4^{3-}). Under anaerobic environment, anaerobic bacteria transform it into less toxic volatile forms such as dimethyl arsenic acid and monomethyl arsenic acid [8]. Oxidation and reduction of arsenic species are carried out both chemically and biologically in soil and water. The acid/base chemistry plays a major role in the types of arsenical compounds present in the soils. Naturally, arsenic can be released into the soil environment by weathering and erosion (hydrolysis and oxidation process) of primary sulfide mineral (arsenopyrite). Organic content, iron and aluminum oxides, hydroxides, and phosphate ion play an important role in the speciation as well as mobility and sorption of arsenic in sediments [9]. Arsenic retention in soil media is mainly dictated by adsorption and desorption reactions, presence of ligands and soil redox conditions [10]. Bioavailability plays an important role in uptake of arsenic from soil to plants. The widespread contamination of the inorganic form of arsenic and its level of toxicity is becoming a global problem as the metalloid does not degrade, and cannot be destroyed. It is reported that conventional methods such as oxidation or reduction, chemical precipitation, filtration, ion exchange, reverse osmosis and evaporation recovery of cleaning contaminated area are too expensive and laborious. There is need to develop eco-friendly and low cost technique to mitigate the arsenic contamination. It is well documented that bioremediation is a cost-effective and a comparatively innocuous alternative to physical methods for heavy-metal remediation [11]. Wide varieties of microorganisms are capable of growth in presence of heavy metal ions and can tolerate high concentrations [12]. Although arsenic is generally toxic for life, microorganisms can use arsenic compounds as electron donors, electron acceptors or show arsenic detoxification mechanisms [13]. Furthermore, suggestions have been made about the existence of microorganisms in which arsenic can play the role of phosphorus. These microorganisms are able to live and reproduce in conditions of phosphorus deficiency, replacing phosphorus in DNA with arsenic toxic to other life forms [14]. Various bacteria such as *Acidithiobacillus*, *Bacillus*, *Deinococcus*, *Desulfitobacterium* and *Pseudomonas* have been reported to be resistant to arsenic [15–17]. It has been reported that the strains of *Aeromonas*, *Exiguobacterium*, *Acinetobacter*, *Bacillus* and *Pseudomonas* can tolerate high concentrations of arsenic up to 100 mM arsenate or up to 20 mM arsenite [18]. Molecular-biological studies have shown that they possess arsenic resistance

genes. *ArsA* and *arsB* genes encode transmembrane pumps that remove As^{+3} from the cytoplasm, reducing the concentration of arsenic in the cell. The *arsC* gene encodes an enzyme capable of transforming As^{+5} into As^{+3} . The *arr* gene encodes a periplasmic As^{+5} reductase, which uses As^{+5} as an electron acceptor during anaerobic respiration. The *aox* gene encodes a periplasmic As^{+3} oxidase that oxidizes As^{+3} to As^{+5} . The genes *arsR* и *arsD* are regulatory genes [19, 20]. The ecosystems near arsenic mining industrial areas are characterized with an elevated level of pollutants in Caucasus region; such hot spots are presented in Western Georgia (Uravi, Tsana) abandoned arsenic production facilities and nearby mining tailings are stored in deteriorating conditions that pose a threat to the population. In this study, we evaluated the diverse populations and functioning microbial communities in the soil for natural and external remediation in environmental cleanup; the level of As contamination in the wide area of Western Georgia hot spots; the characteristics of microorganisms cultivated from the highly contaminated regions.

2. Biochip applications

The establishment of the structure of the collective genomes (metagenomes) of environmental microbial communities (microbiomes) has a decisive influence on the development of earth sciences of the 21st century. However, it soon has become apparent that gene sequence data alone is of relatively little use unless it was directly linked to agriculture, alternative energy production, industrial processes, and environmental cleanup relevance. Monitoring of bacterial consortium in contaminated regions will enable to assess in advance the extent of native bioremediation and accordingly suggest a strategy of external bioremediation (detoxification). Diagnostic biochip is able to enumerate bacteria, involved in bioremediation. A biochip is a platform for screening a single sample for multiple purposes. The constructed and applied in the presented study low density DNA-biochip is a small solid matrix with multiple dot markers (probes). Each marker contains genetic DNA-sensors (pico-amounts of oligonucleotides), which is designed according to the genetic map of the studied objects.

2.1 Biochip design: DNA probes and biochip matrix

Effect of toxic metals in natural conditions is rarely revealed by the action of one of them, mainly it is a complex action of several metals (sometimes even non-toxic), and the same concerns *arsenic* contaminated area. The groups of bacteria that can be involved in the arsenic and heavy metals transformation were used as targets for a low-density biochip. The constructed low-density biochip contains 16 probes in duplicate (**Table 1**). Probes 1–3 are derived from 16S rRNA genes. All other probes (4–16) are derived from functional genes. Highly specific probes covering the taxa of interest were selected for subsequent testing from the publications pointed in **Table 1**. The BLAST NCBI database (<http://www.ncbi.nlm.nih.gov>) confirms bacterial specificity for a probe sequence. A crucial step in biochips design is the evaluation of probes hybridization capacity. Even though specific probes for target microorganisms are generated following well-established requirements, this would require an experimental confirmation of their equal hybridization capacity. The experimental cassette approach proves the selected probes equal hybridization capacity [22, 26]. Therefore, the variations in the fluorescent intensities on a biochip, coming from probe's size and nucleotide sequence differences are excluded. The equalized biochip is the basis for the estimation of the microbial proportion in the consortium, assuming that bacterial functional genes are presented in one

N	Probe name	Target species	Gene name	Sequence 5' → 3'	Ref.
1	Archaea1	<i>Archaea</i>	16S rRNA	GTG CTC CCC CGC CAA TTC AT	[21]
2	GeoS	<i>G. sulfurreducens</i>	16S rRNA	TTC GGG CCT CCT GTC TTT C	[22]
3	GeoM	<i>G. metallireducens</i>	16S rRNA	TTC GGG CCT TTT GTC ACC	[22]
4	BacilPhosL	<i>Bacillus</i> spp.	Phospholipase PL	CTA CTG CCG CTC CAT GAA TCC	[23]
5	Pseudomonas	<i>Pseudomonas</i> spp.	Polyketide synthase phlD	GAG GAC GTC GAA GAC CAC CA	[24]
6	napA	<i>Nitrate Reducing Bacteria</i>	nitrate reductase (napA)	CCG CGG CTA TGT GGG TCG AAA AAG	[22]
7	nirS	<i>Nitrate Reducing Bacteria</i>	nitrite reductase (nirS)	CGC TGT TCG TCA AGA CCC ATC CG	[22]
8	Clostridia	<i>Clostridia</i>	Fe-hydrogenase hydA	CGG CGA GCA TGA TCC AGC AAT	[22]
9	Shew	<i>Shewanella</i>	Ni,Fe-hydrogenase hydA	ACA ACT GCC CAA CCG AGC G	[22]
10	FTHFS	<i>Acetogenic bacteria</i>	formyltetrahydrofolate synthetase (fthfs)	TGC ATG GCC AAG ACC CAA TAC AGC	[22]
11	a/bssA	<i>Hydrocarbon-degrading bacteria</i>	alkylsuccinate synthase and benzylsuccinate synthase alpha subunits (assA/bssA)	TCG TCA TTG CCC CAT TTG GGG GC	[22]
12	SRB	<i>Sulfate-reducing bacteria</i>	adenosine 5'-phosphosulfate-reductase APR	CCA GGG CCT GTC CGC CAT CAA TAC	[25]
13	Dsmicrobium1	<i>Desulfomicrobium</i>	hydA (Ni,Fe-hydrogenase)	CCA CAA CCT GGC CAT CCC GGA AAT	[22]
14	Dtomaculum2	<i>Desulfotomaculum</i>	hydA (Fe-hydrogenase)	CAC GCA TCG GGG AGA GGG TGG	[22]
15	Dbulbus	<i>Desulfobulbus</i>	hydA (Ni,Fe-hydrogenase)	GCG CCA CCC TGC CGT TCA AC	[22]
16	Dbacterium1	<i>Desulfobacterium</i>	hynB (periplasmic Ni,Fe hydrogenase)	CAC TGG AAC AGG CGA TCA AG	[22]

Table 1.
Characteristics of probes used in this study.

copy. As the 16S rRNA genes are multiplied in the bacterial genome, the probes 1–3 are not included in the consideration of the bacterial proportion in the samples. The signals from the probes 1–3 can only confirm or disprove the presence of the target species in the soil samples and can show the comparative relation between the studied samples. Biochip dendrimeric matrix technology and conditions of hybridization on a biochip were the same as described previously [26].

2.2 Analysis of bacterial composition using a biochip

The intensive study of heavy metal contaminated areas has revealed special property of bacteria living in these areas. It is their selective metallophilic property. That is largely due to the activity of these organisms to change the oxidation state of soluble heavy metal oxyanions by transformation them to an insoluble form. Metal-reducing bacteria may receive metal ions as soluble oxyanions via the anionic transport system. Some metal-reducing bacteria can play a key role in the mobilization of arsenic in sediments collected from a contaminated aquifer [27]. *Shewanella*, *Clostridia*, *Pseudomonas* spp., *Bacillus* spp., *Geobacter* spp., *Archaea* exhibit metal-resistant, metal-reducing properties. Sulfate reducing bacteria (SRB) are metal-tolerant. They show the potential to remove As and other metals from As mining area [28]. Active nitrate-reducing population in soil may oppose to SRB activity to produce hydrogen sulfide, the key metal precipitating substrate. Activity of acetogenic bacterial population is a source of energy and carbon.

To evaluate and compare bacterial composition of arsenic contaminated area soil profiles were sampled at different points. Three sampling points were selected for a biochip analysis. Samples from the arsenic ore collector area were selecting based on the location: soil from arsenic processing plant (**P**) sample (GPS 42.66760°N, 43.30048°E) and refers to artificial primitive soil; soil from arsenic ore (**O**) sample (GPS 42.66768°N, 43.30021°E) and refers to artificial primitive soil. The sample (GPS 42.62343°N, 43.34136°E) is considered as control sample (**C**), located 6 km far from the ore collector area and refers to brown forest soils. All these soil samples (20–25 cm depth) were collected using sterile materials in hermetic plastic 50 ml flasks, transported to the laboratory at 4°C, and stored at - 20°C. Conditions of soil DNA preparation were the same as described previously. The steps of DNA fragmentation and its fluorescent labelling are very important in the process of biochip visualization and precede the hybridization of DNA fragments with the immobilized probes onto the matrix for the signal registration [22]. The intensity of the fluorescent signal on a biochip is proportional to the level of the bacteria in the assemblage.

2.2.1 Comparison of bacterial functional assemblages

The **Figure 1A** represents the hybridization signal intensities estimated as signal to noise (S/N) ratio for the probes targeting key genes encoding enzymes involved in metabolic processes. Functional gene array is the approach to assess diversity and functional activities of microbial communities in natural environments. The **Figure 1A** expresses the proportion of the issued bacterial species in the samples **C**, **P** and **O** functional assemblage. The DNA of the control (**C**) sample showed the highest level of the fluorescent signals with the functional gene probe targeting *Bacillus* spp. The pointed bacterial species dominate in the studied bacterial consortium. The DNA of the samples from arsenic processing plant (**P**) and from arsenic ore (**O**) revealed the significant increase of the fluorescent signals with the probes targeting metal-tolerant species *Bacillus*, *Shewanella*, *Clostridia*. *Bacillus* spp. are using As^{+5} as an electron acceptor and as a result arsenate is reduced to arsenite. The populations with nitrate/nitrite reduction and acetogenic activities increased dramatically as well.

Figure 1. Comparative analysis of the bacterial profiles of non-contaminated (Control) and arsenic contaminated (Plant) and (Ore) soils, performed by DNA hybridization on biochip. The results of the hybridization are presented as a signal to noise (S/N) ratio. The data presented are mean values $\pm$ SD from three separate sets of experiments

And only the *Pseudomonas* species remain at background levels at all studied areas. If *Pseudomonas* are present in small amounts in soil, it can be said that the content As^{+3} is low in this soil because these bacteria receive metabolic energy by its oxidation.

The hybridization signals from the probes targeting 16S rRNA genes are presented in **Figure 1B**. The presence *Archaea* and *Geobacter* spp. in all samples is confirmed by the fluorescent signals from the subsequent probes (Archaea1, GeoM, and GeoS). However, their content does not differ between the control and arsenic processing areas.

2.2.2 Comparison of sulfate reducing bacteria functional assemblages

Sulfate reducing bacteria take the special attention, as SRB along with methanogens are involved in arsenic methylation and demethylation in soils [29], and microbial arsenic methylation and demethylation are important components of the As biogeochemical cycle. Only a limited number of organisms are well known for their narrowly defined metabolic capabilities, and SRB is such group. The detection of SRB in the bacterial consortium could be carried through functional

genes as *dsr* and *apr* (genes directly associated with the reduction of inorganic sulfate). We use the probe (SRB) based on the highly homologous sequence of *apr* gene as the universal probe for the detection of the wide spectra of SRB [25]. In addition, we expanded the detection of different SRB species using the probes based on the hydrogenase genes for the specific recognition of *Desulfomicrobium*, *Desulfobacterim*, *Desulfotomaculum* and *Desulfobulbus* species in the SRB assemblage. The results of SRB detection in the selected samples (C, P, and O) are presented in **Figure 2**. **Figure 2A** presents the biochip results. **Figure 2B–E** present the result of PCR amplification of the soil DNA with the primers specific for *Desulfomicrobium*, *Desulfobacterim*, *Desulfotomaculum* and *Desulfobulbus* species on the 1.5% agarose gel (1xTAE running buffer, ethidium bromide staining). M - Gene Ruler 100 bp Plus DNA Ladder (Thermo Scientific, USA), C+ – positive control.

PCR primers are listed in **Table 2**. Individual gene-specific PCR primers were designed using Primer3Plus software. The PCR mixture (20 µl) contained 1xiProof High-Fidelity Master Mix (Bio-Rad Laboratories, USA) with 1.5 mM MgCl₂, 200 µM (each) deoxynucleoside triphosphate, 500 nM each primer, 20 ng of DNA template. PCR amplification was conducted using the following conditions identical for each primer pair: an initial denaturation step (30 s, 98°C) was followed by 30 cycles of denaturation (10 s, 98°C), annealing (20 s, 60°C), and extension (15 s, 72°C) and one terminal extension step (10 min, 72°C).

As it follows from the analysis of the SRB assemblages, the use of nothing but the wide spectra probe (SRB) on a biochip does not differentiate the content of SRB in the studied samples. The expansion of the SRB allows distinguishing SRB composition between the samples. Specifically, *Desulfotomaculum* and *Desulfobulbus* spp. are partly inactivated in arsenic processing areas (P and O) in comparison with the control

Figure 2. Comparative analysis of sulfate reducing bacteria profiles, performed by DNA hybridization on biochip (Panel A) and by PCR amplification (Panels B, C, D, E).

Positive control DNA	Target gene GenBank accession no.	PCR Primers name	Sequence 5' → 3'	PCR fragment size (bp)
<i>Desulfobacterium autotrophicum</i> DSM 3382	<i>hynB</i> (periplasmic Ni,Fe hydrogenase) CP001087	For2	CTT GAG ACC ATT TCG GTT GA	150
		Rev2	GGC GCC GTT ATA GGC TGT AG	
<i>Desulfobulbus propionicus</i> DSM2032	<i>hydA</i> (Ni,Fe-hydrogenase); CP002364	For1	CTC TAC AAA CTG GGG TGC AAG	165
		Rev1	GAC CTT TGC CTT GGA AAA CA	
<i>Desulfomicrobium baculatum</i> DSM 4028	<i>hydA</i> (Ni,Fe-hydrogenase); CP001629	For1	GTT TCG CCG AAG AAC ATG A	109
		Rev1	CGC CCA ATC CCT ACA ACC T	
<i>Desulfotomaculum ruminis</i> DSM 2154	<i>hydA</i> (Ni,Fe-hydrogenase); CP002780	For2	GGA AAT TCC GGA CTG GTA CA	170
		Rev2	GAG GGA GTC TTC TCC AAG CA	

Table 2.
DNA and PCR primers.

sample (C). Inhibition of some SRB species correlates with the dramatically increased nitrate-reducing activity in the corresponding samples, as nitrite (product of nitrate reductase) inhibits the enzyme dissimilatory sulfite reductase [30]. *Desulfobacterium* spp. are at the same level in all three samples. As it was mentioned above, *Desulfobacterium* spp. are among the species, which resist As. Only *Desulfomicrobium* spp. are significantly increased in the soil from arsenic processing plant (P). The biochip data coincides with the PCR data. The following elemental analysis, determining the total As content in the selected samples: C – 140 mg/kg soil; P– 6300 mg/kg soil; O – 110 mg/kg soil, points to the highest concentration of the As in the arsenic processing plant soil (P). The increased *Desulfomicrobium* spp. content can be considered as an indicator of highly polluted place even before the elemental analysis.

Taking into consideration biochip data about bacterial composition assessment, it can be said that a biochip is not only diagnostic, but also a predictive instrument.

The detailed analysis of total As content in the contaminated area is presented in the next Section.

3. Total As content analysis

Arsenic contamination in Georgia has many natural and anthropogenic sources. Arsenic ore extraction, processing and production of arsenic-containing

preparations have been carried out on the territory of Georgia for decades. The processing of the ore, which is located in Ambrolauri region, started in 1937. Main products of processing were metallic arsenic of high purity, As_2O_5 , As_2S_5 and tin arsenate. Technological cycle in factory was very simple and included thermal treatment of ore. Amount of waste is about 60 tons, which contains up to 1% As_2O_5 . Waste was not used and it was stored at Kajiani territory in special hydrotechnical building, called tailings. The mining of ore was carried out at Lukhuni ore, processing was performed in the factory nearby village Uravi. Chemical mining factory in village Tsana started working is 1938. The main products were metallic arsenic and refined “white arsenic” (As_2O_3) (I grade – 99.9%, II grade – 99.5%). This substance is poorly soluble in water and permanently can have great negative influence on environment. The factory in Tsana, administrative buildings and warehouse farming are fully destroyed and collapsed. There is no fence around the territory. Arsenic kilns and containers are taken from the ground. Thus the problem is arsenic containing waste and soils. Until today the great amount of toxic waste of arsenic production is stored in villages Uravi and Tsana, near the territory of the factories (more than 120 000 tons waste, containing 4–9% of white arsenic), which is not located safely and there is a high risk of ecological disaster in rivers and soils, especially risks of natural disasters (floods, rockslide, erosion and etc.). It should be mentioned that LEPL National Environmental Agency is conducting permanent monitoring in order to determine arsenic content. The data are not encouraging [31]. Based on this it is necessary to determine arsenic content in ecosystems (soil, water) of this region. The fifty four soil samples encompassing two hectares have been analyzed using Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES) method. Preparation (drying, loosening, sieving, extraction, etc.) of soil samples for pre-treatment and chemical analysis was carried out - ISO-11464; EPA 3050; According to EPA-TCLP-1111 ST methodologies. The results are presented in **Figure 3**. According to **Figure 3**, As content ranges from 47 mg/kg soil to 13000 mg/kg soil.

Figure 3.
Total arsenic content in 54 soil samples.

4. Characteristics of bacterial isolates from As highly contaminated area

4.1 Conditions of soil samples treatment for bacterial isolates recovery

Soil samples for bacterial isolates recovery were taken from the arsenic contaminated area and were selecting based on the location: soil sample from arsenic storage area (SA) (GPS 42.81425°N, 43.11586°E) and refers to brown forest black soil; soil sample from the place nearby the storage containers (SC) (GPS 42.81403°N, 43.11567°E) and refers to brown forest black. The sample (GPS 42.62343°N, 43.34136°E) is considered as control sample (C), located 6 km far from the storage area and refers to brown forest soils. The ICP-OES method determined the total As content in the selected samples: C – 140 mg/kg soil; SA – 7400 mg/kg soil; SC – 10400 mg/kg soil. Therefore, the bacterial isolates recovered from SA and SC samples are from highly contaminated area. Soil samples in the sterile tubes were first thermally treated at 80°C for 10 minutes. Then the samples were processed according to the appropriate methodology, which involves pre-incubation the soil bacteria, both gram-positive and gram-negative, in universal and selective growth medium. In particular, to stimulate the growth of gram-negative microorganisms, MacConcey Broth was used, in which 1 g of soil samples were incubated at different conditions, namely at 4°C, 20–25°C (room temperature), and 42°C for the particular stimulation of growth and further isolation of genus *Shewanella* microorganisms [32]. Three different types of growth medium were prepared for the isolation of bacterial strains. The composition of the first liquid medium was as follows (g/L): $\text{Na}_2\text{HPO}_4 \times 7\text{H}_2\text{O}$ - 12.80; $\text{KH}_2\text{PO}_4 \times 7\text{H}_2\text{O}$ - 3.00; NH_4Cl - 1.00, yeast extract - 2.00; CH_3COONa - 8.20; the second liquid medium was (g/L) LB broth - 10, yeast extract - 5; NaCl - 10; solid nutrient agar was used as the third growth medium. To determine arsenic respiration of the isolates, As^{+3} or As^{+5} salts were added to the growth medium [33]. Specific growth media M2 and M6 were prepared for this purpose. The composition of M2 medium was as follows (g/L): lactate – 0.45; NaCl – 1.17; KCl – 0.30; NH_4Cl – 0.15; $\text{MgCl}_2 \times 6\text{H}_2\text{O}$ – 0.41, CaCl_2 – 0.11, KH_2PO_4 – 0.20; Na_2SO_4 – 0.07, NaHCO_3 – 2.00, NaAsO_2 or Na_2HAsO_4 – 0.007. The composition of M6 medium was as follows (g/L): MgSO_4 – 1.00; NH_4Cl – 1.00; Na_2SO_4 – 1.00; K_2HPO_4 – 0.10; CaCl_2 – 0.05; lactate – 4.00; FeSO_4 – 0.002; NaHCO_3 – 8.00, arsenate or arsenite – 0.00. Incubation of 39 primary unidentified strains under anaerobic conditions at 31°C for 72 hours was performed in M2 and M6 growth media [34]. Microbial masses grown on solid and liquid medium were tested for arsenic oxidation–reduction ability by potassium permanganate method [35]. Nissui Compact Dry diagnostic-selective media were also used for primary screening of microorganisms.

4.2 Microorganisms properties

Bacterial isolates that were motile, had oxidative metabolisms, were oxidase and catalase positive, ornithine decarboxylase positive, and DNase positive, and produced H_2S on triple sugar iron slants within 72 h of incubation were identified as belonging to the phenospecies, respectively based on acid production from sucrose and maltose, growth on SS agar, and growth in the presence of 6.5% NaCl . All tests were performed according to the manufacturer's instructions. Routine biochemical test results were read daily for 72 h; oxidation of various carbohydrates was assessed by Liofilchem® Microbial Identification system after 7 days of incubation. Enzymatic plate assay results were read daily for 7 days; appropriate positive and negative control strains were included for each assay. Some additional enzymatic activities (2 h) and

Soil sample	Determined microorganism	Strain	Catalase test	Oxidase test	Mannitol fermentation	Starch hydrolysis	Nitrate reduction test	Urease test	Indole test	Citrate utilization test	TSI agar test	Gram staining
C	<i>Bacillus spp.</i>	#01	p	p	p	p	n	n	n	p	alk/ acid/ alk	p
		#02	p	p	p	p	p	n	n	p	alk/ acid	p
		#03	p	p	p	p	n	p	n	p	alk/ alk/ H ₂ S	p
		#04	p	p	p	n/d	n	p	n	n/d	alk/ alk/ H ₂ S	p
SA	<i>Bacillus spp.</i>	#05	p	p	p	p	n	n	n	n/d	alk/ alk/ H ₂ S	p
		#06	p	p	p	p	p	n	n	n	alk/ acid	p
SC	<i>Bacillus spp.</i>	#07	p	p	p	n/d	n	n	n	n	alk/ acid	p
		#08	p	p	n/d	p	p	p	n	p	alk/ acid	p
		#09	p	p	p	p	p	n	n	p	alk/ acid	p
		#10	p	p	p	p	p	n	n	n	alk/ acid	p
		#11	p	p	p	p	n	n	n	n	alk/ alk/ H ₂ S	p
<i>p</i> - positive, <i>n</i> - negative. <i>n/d</i> - not determined, <i>alk</i> – alkaline.												

Table 3.
Biochemical activity of Bacillus isolates.

Soil sample	Determined microorganism	Strain	Catalase test	Oxidase test	Mannitol fermentation	Starch hydrolysis	Nitrate reduction test	Urease test	Indole test	Citrate utilization test	TSI agar test	Gram staining
SA	Shewanella spp.	SH01	p	p	p	n/d	p	n	p	p	alk/ alk/ H ₂ S	n
		SH04	p	p	n/d	n/d	n	n	n	n	alk/ acid/	n
SC	Non-specified strains	X01	n	p	n/d	n/d	n	n	n	p	alk/ alk	n
		X02	n	p	n/d	n/d	n	n	n	p	akl/ alk	n
		X06	p	p	n/d	n/d	p	n	n	n	alk/ alk/ H ₂ S	p
		X07	p	p	n/d	n/d	p	n	n	n	alk/ alk/ H ₂ S	n
p - positive, n - negative. n/d - not determined, alk – alkaline.												

Table 4.
Biochemical activity of Shewanella and non-specified isolates.

gelatinase activity (3 days) were estimated using Wee-Tab tablets or gelatin strips (Key Scientific Products, Roundrock, Tex.). Microorganisms have been studied for intracellular and extracellular enzymatic activities such as Oxidase test, Catalase test, Carbohydrate utilization test, Urease test, Indole test, Esculine test, Nitrate reduction test, Citrate utilization test, Gelatin hydrolysis test and Motility test.

4.3 Bacterial isolates identification and characterization

The screening of the bacterial composition using a biochip (Section 2) showed that sulfate-reducing bacteria, along with the representatives of *Bacillus*, *Clostridium* and *Shewanella* spp. predominate and are characterized by high content in the biomass of microorganisms from the As contaminated area. Laboratory cultivation in the above-mentioned media shows those gram-positive and gram-negative microorganisms, revealing sulfate-reducing activity, as *Bacillus cereus*, *Bacillus subtilis*, *Clostridium* spp., *Enterobacter* spp., and *Shewanella* spp., which are prevailing in the studied biomass.

The biochemical characteristics of selected *Bacillus* isolates are presented in **Table 3**. As follows from **Table 3**, the vast majority of *Bacillus* strains show catalase activity, all strains show pronounced oxidase activity. Tests of carbohydrates fermentation show that part of the *Bacillus* spp. strains can completely utilize carbohydrates. Inexplicable data were obtained when analyzing the results of citrate utilization and urease conversion tests. Genus *Bacillus* is known as citrate consumers and has no urease activity, but repeated analysis of the tests reveals that *Bacillus* isolates # 03, # 04 and # 8 are characterized by urease activity, which in our opinion is an exception. A similar trend was observed for the citrate utilization test data, namely, *Bacillus* isolates # 06, # 07, # 10 and # 11 did not utilize citrate and the diagnostic area did not change color. It should be mentioned that the full results of the tests for *Bacillus* isolates #04, #05 could not be evaluated; what may have been caused by the polymorphism of the microorganisms and the altered biochemical properties. The results of gelatin hydrolysis tests of microorganisms of the genus *Bacillus* showed that the vast majority of the selected microorganisms are characterized by the ability to hydrolyze gelatin.

The biochemical characteristics of *Shewanella* and non-specified isolates are presented in **Table 4**. *Shewanella* isolates # SH01, # SH04 and also isolates #X06, #X07 reveal positive both oxidase and catalase activities. In the next phase of the study, the selected microbial isolates were tested for arsenic oxidation–reduction ability. The results of arsenic respiration are presented in **Table 5**.

As follows from **Table 5**, the *Shewanella* isolates #SH01 and #SH04, as well as non-specified isolates #X01, #X02, #X06 and #X07, can oxidize As^{3+} to As^{5+} in the

Soil sample	Determined microorganism	Strain	Arsenic reduction		Arsenic oxidation	
			M2	M6	M2	M6
SA	<i>Shewanella</i> spp.	SH01	n	n	p	n
		SH04	n	n	p	n
SC	Non-specified strains	X01	n	n	p	n
		X02	n	n	p	p
		X06	n	n	p	n
		X07	n	n	p	n

Table 5.
Arsenic respiration by isolated strains.

studied growth conditions. Namely, the isolates exhibit this property in M2 growth medium. At this time the toxic form of arsenic transforms into a less toxic one. This result is very important for the planning and implementation of phytoremediation of arsenic-contaminated soil, because the presence of this microbiota in the soil will facilitate the growth of plants and their absorption of arsenic.

5. Conclusions

Evaluation of arsenic pollution and health risks, prevention of natural soils and waters from pollution in order to provide ecosystem enhancement and population safety is extremely important for the reality of Georgia. Arsenic as the most heavy metals are scattered and unevenly located in the earth's crust. Under natural conditions, in sufficiently high concentrations, it is found in areas of ore deposits. Arsenic toxicity is governed by its high affinity to sulfur and phosphorus, what is extremely dangerous for living organisms. In modern ecological biotechnologies aimed at cleaning up a chemically polluted environment, both from an economic and an ecological point of view, phytoremediation is considered the most effective, which consists in the purposeful planting of specially selected plants in contaminated areas. To use phytoremediation, it is necessary to study not only the level of pollution, but also the physicochemical properties of the soil and, mostly important, the soil microbiota. This is due to that a certain consortium of microorganisms has already formed in the contaminated soil, which, on the one hand, is accustomed to being present in a polluted environment, on the other hand, determines the conditions for plant growth on this soil and, in some cases, leads to the transformation of pollutants. Therefore, the screening of the indigenous bacteria was the first key step on the way of the bioremediation of arsenic contaminated area. Diagnostic biochip is used for the monitoring of the indigenous bacteria. The bacterial composition revealed the prevalence of metal-tolerant species as *Bacillus*, *Shewanella* and *Clostridia* in the arsenic contaminated area. The bacterial interrelation, specifically the significant increase of *Bacillus* spp., *Desulfomicrobium* spp. and a very low level of *Pseudomonas* spp. could indicate that As^{+5} is the predominant arsenic form in the studied sites, as *Bacillus* spp. use As^{+5} as an electron acceptor in bacterial respiration, and *Pseudomonas* spp. may receive of metabolic energy from As^{+3} oxidation. Under aerobic conditions, most of the arsenic in soils is in the As^{+5} form. Microbiological transformation of As^{+5} into As^{+3} is slow, and only up to 0.5% of arsenates are converted into arsenites. However, as a result of transformation, As^{+5} adsorbed on soil particles is released into the soil solution in the form of As^{+3} , which is much more mobile and toxic than As^{+5} . Therefore, microorganisms can increase the mobility of arsenic in the soil, thereby contributing to phytoremediation. Some *Bacillus* and *Shewanella*, strains were isolated from the As-contaminated sites, cultivated and characterized. These strains are the basis for the further phytoremediation, especially *Bacillus* spp., as they as rhizosphere microorganisms may bioabsorb arsenic from contaminated soils and by that to promote and facilitate soil phytoremediation.

Acknowledgements

This work was supported by grant # CARYS-19-179 from Shota Rustaveli National Science Foundation (SRNSF).

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Author details

Gia Khatisashvili¹, Tamar Varazi^{1*}, Maritsa Kurashvili¹, Marina Pruidze¹, Evgeni Bunin¹, Kakha Didebulidze¹, Tinatin Butkhuzi¹, Elina Bakradze², Nino Asatiani³, Tamar Kartvelishvili³ and Nelly Sapojnikova³

¹ Durmishidze Institute of Biochemistry and Biotechnology, Agricultural University of Georgia, Kakha Bendukidze University Campus, Tbilisi, Georgia

² Ministry of Environment Protection and Agriculture, National Environmental Agency, Tbilisi, Georgia

³ Andronikashvili Institute of Physics, I. Javakhishvili Tbilisi State University, Tbilisi, Georgia

*Address all correspondence to: t.varazi@agruni.edu.ge

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