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Assessment of Heavy Metal Contamination and Tolerant Bacteria Associated with Halophyte *Arthrocnemum Macrostachyum* in Lake Manzala, Egypt

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Abstract

In Egypt, Lake Manzala is the largest and the most polluted lake due to its use as sink for agricultural drainage, industrial effluents, and sanitary wastes. Pollution indices and associated bacteria of the halophyte *Arthrocnemum macrostachyum* were assessed in up and down streams represent metal-polluted and non-polluted sites, respectively. The concentrations of heavy metal in upstream soil significantly exceeding those in downstream one. The highest concentration in upstream soil was recorded by Fe (> 200000 mgkg^{-1}), followed by Al, Mn and Zn (143733.3, 5032.67 and 1036.43 mgkg^{-1}, respectively). Concerning the plant organs, the levels of all elements in root were higher than those in shoot at both up and down streams. The pollution indices of upstream soil were higher than those of downstream for all metals except for Mo. *A. macrostachyum* has the ability as hyperaccumulator for Al, Fe and V in both up and downstreams and for Cr and Cu in downstream. Plant juice culture media of the halophyte was succeeded to develop translucent and slimy colonies. More than 50% of plant juice culture media diluted with upstream water isolates were tolerant to Al, Cr, Cu and Zn at maximum tolerable concentrations 71.26, 12.5, 0.32 and 0.74 mM, respectively. Nine heavy metal-tolerant bacterial isolates were identified according to cell morphology and API microtube systems. Four isolates were matching *Burkholderia cepacia*, 2 isolates belonged to *Providencia retgeri*, and the rest isolates referring to *Bacillus circulanc*, *Bacillus lentus* and *Raoultella ornithinoltica*. The study suggests that *A. macrostachyum* could be classified as an appropriate candidate for phytoremediation efficiency of extremely heavy metal polluted soils.

Key words *Arthrocnemum macrostachyum,* Heavy metals, Lake Manzala, Plant juice culture media, Rhizobacteria

1. Introduction

Several heavy metal ions and metalloids such as copper, manganese, iron and zinc play significant roles as cofactors for many enzymes and are essential components of living organisms. But their higher concentrations pose critical threats to the environment and public health due to their high toxicity, persistence and accumulation [1]. The rapid urbanization and industrial activities have resulted in the continuous disposal of heavy metals which causing great impact on the structure and function of coastal ecosystems [2–4]. In addition, the anthropogenic activities related to disposal of numerous domestic, industrial and agricultural wastes into water bodies, e.g. rivers, lakes and seas are known to have great impacts towards water quality and aquatic life [1,5]. Unfortunately, heavy metals are present in many environments beyond their permissible limits without degradation and thus accumulate in the environment and food chains for unlimited periods [6,7].

In saline environments, halophytic plant species are adapted to salinity through several morphological, physiological and biochemical strategies that are dependent on species and surrounding environment [8,9]. High concentrations of salts are associated with high concentration of bioavailable heavy metals such as in coastal areas and lakes where urban activities are contributing to the release heavy metals in these saline habitats with their biota [10,11]. Therefore, identification of biota that are adapted to both salinity and heavy metals stress is essential to bioremediation of contaminated habitats especially in arid and semi-arid regions. In this respect, halophytic plant species and their associated bacteria are now receiving increasing attention for bioremediation of the highly contaminated heavy-metal sites [12,13]. In such effective biological approach, halophytic plants play a significant role in removal of heavy metals by both direct accumulation and/or translocation or through enhancing the degradation activity of associated bacteria [12–14]. The role of halophytic plant-associated bacteria in removal of heavy metals is confined to their unique properties and metabolic activities [15,16].

The halophytic perennial shrub *Arthocnemum macrostachyum* (Moric) C. Koch (Chenopodiaceae) is known not only for its ability to grow in a wide range of saline soils, but it is also extremely well adapted to hypersaline conditions [19]. It has the ability to accumulate metals inside its tissues and, therefore, is recommended as a model candidate for metal phytoremediation in highly polluted coastal sediments [17,18]. It has been reported that the internal tissues of *A. macrostachyum* seemed a proper environment for the colonization of moderately halophytic bacterial communities [19]. These associated bacteria might affect positively or negatively the fitness and development of *A. macrostachyum.* These plant-associated bacteria reside on both phyllosphere and rhizosphere compartments which their composition and diversity are influenced by prevailing environmental conditions [23,24] . The former is under fluctuations of temperature, humidity, UV radiation and wind, while the latter is strongly affected by soil water, salinity and pollution [24,25]. Several species of rhizobacteria have evolved a number of mechanisms to tackle heavy metal toxicity and succeed to have normal physiological growth and persistence in highly polluted areas [12,17] . Therefore, analyzing the composition and diversity of root associated bacteria is essential to highlight their role in removal of heavy metals in highly contaminated ecosystem.

Cultivation of the various environmental microbiomes has selective effects, and thus yields results that are greatly not representative for the whole microbial communities [18,19]. Consequently, this is why the last two decades witnessed unlimited efforts towards tailoring culture media satisfying the nutritional requirements for increasing culturability of microbiomes. In this respect, many studies indicated that plant materials such as infusions, extracts, crude slurry homogenates, juices and saps without any supplements successfully supported culturability of plant associated microbiome [20,21]. Using plant materials for culturing rhizobacteria associated to halophytic plants in salt- and heavy metal-stressed environments hardly exists in the literature.

Lake Manzala along the Mediterranean coast of Egypt, is the largest and the most polluted lake in the country due to its use as sink for agricultural drainage, industrial effluents and sanitary wastes [22–27]. Such pollutants and heavy metals are not easily degraded and therefore, accumulate in water, soil, bottom sediments, and biota which vary considerably in their tolerance to different pollutant sources [26]. They can alter the ecosystem process and function, and consequently the growth, development and survival of biota. Therefore, it is important to monitor and assess their levels in the existed biota such as plant species and associated bacteria. *A. macrostachyum* occurs and forms pure stands along the shores of the lake [28]. However, nothing is known about its ability for metal uptake and accumulation and its associated bacteria. We hypothesized that the growth of *A. macrostachyum* in highly contaminated sites of Lake Manzala would enhance its tolerance and ability to uptake and accumulate of heavy metals with interaction of its root associated bacteria. To test such hypothesis, we first assessed the heavy metal concentrations in soil, and plant organs of *A. macrostachyum* in up and down streams, and then compared the differences in bacterial abundance and diversity. Secondly, we isolated and identified the tolerant bacteria towards the existed heavy metals. Thirdly, we assessed the growth of isolated bacteria on culture media of *A. macrostachyum* collected from up and down streams.

2 Materials and Methods

2.1 Study area and sampling

Lake Manzala lies between latitudes 31°07`N and 31°30`N and longitudes 31°48`E and 32°17`E. It is bordered by Suez Canal from east, Nile-Damietta branch from west and Mediterranean Sea from north. It locates on the north-eastern edge of the Nile Delta, *ca*. 170 km from Cairo and *ca*. 15 km west of Port Said. The lake is separated from the Mediterranean Sea by a sandy beach ridge that has three open connections between the lake and the sea. It is the largest in area among the Egyptian lagoons. By 1988, its surface area reduced to 770 km^2 from 1698 km^2, and expected to be less than 500 km^2 in future due to land reclamation projects and urban crawl [29]. The lake is brackish, eutrophic and shallow with depths ranging from 1.2 to 1.5 m. The mean annual temperature ranges from 15.7 ºC (January) to 28.1 ºC (August). The annual relative humidity varies from 72.2 % in August to 57.8 % in December. Evaporation is greater during summer than in winter months, it decreases from 10.4 mm/day in August to 6.1 mm/day in December. The annual rainfall ranges from 38.7 mm to 97.4 mm at Port Said.

We chose two sampling sites representing heavy metal-contaminated area (upstream) that close to the outlet of Bahr El-Baqar drainage (31°17'.095"N 32°13'0.026"E), and downstream area on the shore of Boughaz El-Gamel inlet (31°13'.165"N 32°14'0.195"E) at the Mediterranean Sea. At each site, soil and *A. macrostachyum* samples were collected from five different places. Ten soil and *A. macrostachyum* samples containing roots with adjacent soil (rhizosphere) and shoots were collected using sterilized tools and in a separate plastic bag at both sites. All samples were transported to the laboratory for analysis.

2.2 Soil and plant analyses

Plant shoot and root samples were digested adopting the method of [30]Kruis (2007), after oven drying at 80 ^oC. The procedure recommended by [31]Ehi-Eromosele *et al.* (2012) was used for soil sample digestion after air-drying and sieving. All digested samples were transferred to glass containers and kept at 4 ºC until use. The heavy metals Al, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb, V and Zn of the various digest samples were measured by inductively coupled plasma-atomic emission spectroscopy (ICP – AES) (ARL Fisons 3410, USA) according to [16,32].

2.2.1 Heavy metals indices and uptake

The index of geoaccumulation (Igeo) of soil is computed using [33]Muller, (1960) equation $Igeo = \log_2 (C_n/1.5B_n)$ where; C_n is the concentration of the heavy metal in sample and B_n is the geochemical background values for each metal. The contamination factor [35] was used $CF= C_n/B_n$ where C_n is the mean concentration of the heavy metal in sample and B_n is the geochemical background values for each metal. [36]Bhutiani *et al.* (2017) equation was applied to quantify soil contamination (QoC) as follow; $QoC= (C_n – B_n)/ Cn \times 100$ where Cn is the mean concentration of the metal in the soil under investigation, and Bn is the geochemical background values for each metal. The potential ecological risk of a given contaminant (Er) was calculated by the equation of [35]Hakanson (1980): $Er =Tr \times C_f$ where T_r is the toxic-response factor for a given substance, C_f is the contamination factor. However, his ecological risk index caused by multiple heavy metals (RI) was calculated using the following formula: $RI = \sum^n_i Er$ where n= the number of studied heavy metals and Er—single index of the ecological risk factor. The degree of contamination index for a given site [37] is the sum of all contamination factors, and calculated as follows: $C_{deg}= \sum^n_i C_f$, where; C_f contamination factor and n the number of analyzed heavy metals.

The metal uptake efficiency by *A. macrostachyum* root or shoot was evaluated by bioaccumulation (BF) and translocation (TF) factors. The bioaccumulation factor (BF) is the ratio of metal content in roots to those in corresponding soil used to estimate the ability of plant to accumulate metals from soil [38]. The translocation factor (TF) was obtained by dividing the total element content in shoots by the total element content in roots [39]. In addition, transfer coefficient was calculated according to Madejón *et al.*, (2009)[7]. The metal accumulation efficiency by *A. macrostachyum* was compared with the threshold concentration of the toxic metal ion [40].

2.3 Isolation and quantification of root-associated bacteria

Under aseptic conditions, root associated bacteria were isolated from root samples of both sampled sites. Five grams of *A. mcrostachyum* fresh roots were washed with sterilized tap water, sliced and blended with minimum amount of sterile saline solution then squeezed in a sterilized mortar. The resulting root parts and suspension were transformed to flask and was shaken for 30 min at 110 rpm, this suspension was used for the preparation of serial dilutions. Isolation was on solid media using spread plate technique. Triplicates were conducted, incubation was at 30 ºC for 7–14 days [21]. Colony forming units (CFUs) including micro-colonies, were counted throughout colony counter and total count was presented as Mean log CFU [41].

Four media were used to assess the growth and abundance of bacteria, nutrient agar medium and three *A. mcrostachyum* juice-based culture media. 1/10 strength, Nutrient agar medium (NA), [42] was used as standard culture medium supplemented with 3% NaCl [41]. *A. mcrostachyum* juice- based culture media was prepared by slicing and blending shoots for 5 min in a Waring blender with minimum amounts of distilled water. The resulting crude juices were filtered through gauze and stored in freezer for further use. The *A. mcrostachyum* juice-based culture media were diluted by three diluents to 2% v/v (juice/diluent). The first diluent was distilled water (J), the second was lake water of **D**own **S**tream (JDS) and the third was lake water from **UP S**tream (JUPS). The pH of the three media was adjusted to 7 ± 0.2, then agar was added (1.7%) and autoclaved for 20 min at 121 ºC [20]. Based on morphological characteristics, different colonies were picked and purified and then were preserved as slopes at 4ºC.

2.4 Screening for heavy metal tolerance of selected isolates

The tolerance of the recovered root associated bacteria isolates were tested against Al, Cr, Cu and Zn. These metals were selected based on determination of accumulation and translocation concentrations of heavy metals. The tested metals were used as $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, $K_2Cr_2O_7$ and $AlCl_3$. A stock solution for each metal were prepared and then diluted to the desired concentrations. The tested concentration of each metal was 1x, as x symbolizes metal concentration in plant root which previously measured by ICP. The tested concentration was decreased (0.75x, 0.25x and 0.1x) or raised (10x) according to results, the equivalent mM unit of the tested concentrations will be elucidated in results. Spot inoculation technique on plant based agar plates supplemented with metals were incubated at 28ºC for 10 days [43]. Growth diameters were visually compared by the growth on control (medium lacking metal). The maximum tolerable concentration (MTC) of metal was designated as the highest concentration of metal that allowed growth [44].

2.5 Identification of heavy metal tolerant bacterial isolates

Tolerant isolates were characterized by colony and cell morphology, motility and Gram stain. Biochemical test kits (bioMerieux API) were applied for bacterial identification [45]: API 20E for Enterobacteriaceae, API 20NE for non-Enterobacteriaceae and API 50CHB for bacilli. Test results and developed numerical profiles were entered into the online database [46](*API webTM*) for bacterial identification.

2.6 Statistical analyses

To examine the differences in data sets of heavy metal contaminations in soil and plant organs, t-tests were used of paired up and down streams. One-way ANOVA and Tukey's test in Post hoc were used to evaluate the significant differences in bacterial abundance among the different cultural media. All statistical analyses were performed using SPSS software (SPSS 2006).

3 Results

3.1 Heavy metals in soil and plant organs

Heavy metals concentrations in soil and plant organs at both up and down streams are represented in Table 1. Generally, the levels of heavy elements in soil were higher compared to plant organs The concentrations of heavy metal in upstream soil significantly exceeding those in downstream one. The highest concentration in upstream soil was recorded by Fe (> 200000 mgkg^{-1}), followed by Al, Mn and Zn (143733.3, 5032.67 and 1036.43 mgkg^{-1}, respectively). Concerning the plant organs, the levels of all elements in root were higher than those in shoot at both up and down streams (Table 1). In both organs as in soil, Fe, Al, Mn and Zn were the dominant concentrations. In contrast, the three elements cadmium, cobalt and molybdenum were detected with lowest levels in soil and plant organs (Table 1).

3.1.1 Indices of soil contamination

The evaluated various pollution indices based on soil analyses are presented in Table 2. The indices values of upstream soil were higher than those of downstream for all metals except for Mo. In upstream soil, the lowest and highest values of I_{geo} were less than 1 (Al) and above 4 (Cd) which indicated “unpolluted to moderately polluted” and “heavily to extremely polluted” classes, respectively. The I_{geo} values for Cu, Fe and V classified as “moderately polluted”. The I_{geo} class of Cr, Co, Mn and Ni were “moderately to heavily polluted”; while that of Pb, Mo, Zn were “heavily polluted”. The CF values for Cd, Cr, Co, Pb, Mn, Mo, Ni and Zn in upstream soil showed a “very high contamination”, while the CF values for Al, Cu, Fe, and V indicated a “moderate contamination”. In upstream soil, Cd had the highest CF value of 37.83, followed by Mo and Pb with values of 21.06 and 16.39, respectively. Once more, Cd had the highest Er values 129.13 and 1135 in down and upstream, respectively, while Cu and Zn had the lowest ones in downstream 8.14 and 4.79, respectively. In up and downstream, QoC% showed positive values for all examined metals except for Al which exhibit negative value in downstream. The potential ecological risk (RI) values in both up and down stream soil were 213.29 and 1333.59, respectively. The degree of contamination (C_{deg}) was approximately twice in upstream soil than their counterparts of downstream ones.

3.1.2 Plant heavy metals uptake

Plant bioaccumulation (BF), translocation (TF) factors and transfer coefficient are presented in Table 3. In addition, root content and root plus shoot content of heavy metals were comapred with threshold of hyperaccumulatotr plants (Table 3). BF values were < 1 for all metals in both upstream and downstream which had only BF >1 for Cu. TF values of *A.macrostachyum* recorded values >1 for Al, Cd, Co, Fe, Mn, Pb, V and Zn in downstream, and for Cd and Zn in upstream. Transfer coefficient indicated that *A. macrostachyum* could accumulate Cd, Cu and Zn in downstream and Cu in upstream. By comparing roots and shoots heavy metals contents with the threshold for hyperaccumulator plants, *A. macrostachyum* could be considered as hyperaccumulator for Al, Fe and V in both up and downstreams and for Cr and Cu in downstream.

Table 1 Mean ± standard error (second line) of heavy metal in soil root and shoot at up (UPS) and down (DS) streams where *Arthrocnemum macrostachyum* growing along Lake Manzala

Element (mgkg-1)	Soil		Root		Shoot	
	DS	UPS	DS	UPS	DS	UPS
Al	49450^{a}	143733.3^{b}	1068.91^{a}	1922.67^{b}	791.07	680.27
	±5090.6	±13033.6	±242.2	±148.5	±41.74	±8.41
Cd	1.72	15.13	0.48	0.48	0.59	0.24
	±0.64	±5.66	± 0.0	±0.0	±0.14	±0.0
Cr	347.3^{a}	562.5^{b}	75.39^{a}	63.41^{b}	15.42	16.13
	±29.87	±6.92	±3.28	±1.27	±2.18	± 0.83
Co	63.37	126.63	1.89	2.48	0.71	0.40
	± 9.76	± 19.01	± 0.77	± 0.42	± 0.11	± 0.0
Cu	63.37^{a}	206.73^{b}	70.14	83.17	24.91	36.08
	± 0.89	±1.95	±1.04	± 4.29	±4.09	±5.80
Fe	70516.67^{a}	200400^{b}	2138.67	3797.87	1021.20	787.20
	±9255.71	±15635.32	±560.01	±357.02	±22.40	±44.80
Pb	107.57^{a}	442.4^{b}	10.32	15.41	16.48^{a}	4.33^{b}
	±16.31	±46.14	±0.23	± 2.17	±3.09	±0.11
Mn	2561.67^{a}	5032.67^{b}	102.19^{a}	477.25^{b}	116.40^{a}	206.97^{b}
	±225.77	±220.77	±17.29	±33.42	±8.16	±23.96
Mo	23.3	25.17	6.45	6.88	2.71	3.09
	±0.55	±1.38	±0.58	±0.77	±0.08	±0.29
Ni	229.9	327.03	45.92^{a}	29.04^{b}	9.76^{a}	8.12^{b}
	±18.32	±19.8	±2.26	±0.62	±0.12	±0.29
V	232.83^{a}	650.37^{b}	8.0	12.77	4.00	4.00
	±40.09	±73.09	±0.0	±3.38	±0.0	±0.0
Zn	335.57^{a}	1036.43^{b}	129.2	163.44	107.32	90.21
	±44.51	±128.21	±14.22	±13.56	±15.02	±7.29

Means with the different letters are significantly different between up and down streams for each metal detected in the same sample at $p<0.05$ according to paired t- test.

3.2 Arthrocnemum macrostachyum' root associated bacteria

All tested plant juice culture media supported excellent development of macro- and micro-colonies of root associated bacteria compared to the standard 1/10 nutrient agar. Two media, JDS and JUPS supported growth of diverse morphology; numerous transparent/translucent and slimy micro and macro bacterial colonies; while a greater number of smaller colonies developed on the 1/10 NA medium. Root associated bacterial counts were significantly different among juice- based culture media and 1/10 NA of both up and down streams roots, while, there is no significant difference between JDS and 1/10 NA in case of upstream root (Figure 1).

The root associated bacterial count of downstream root on JUPS (8.41± 0.06 log CFU g^{-1} root) was the highest compared to JDS, J and 1/10 NA. For upstream root the highest count was on JDS (6.99 ± 0.21 log CFU g^{-1} root). The lowest counts of both root samples were on plant juice medium diluted with distilled water (J), (5.11± 0.046 and 4.17± 0.033 log CFU g^{-1} root). A total 77 bacterial isolates were recovered, 22, 9, 30 and 13 on J, JDS, JUPS, and 1/10 NA, respectively (Figure 1).

3.3 Screening of selected bacterial isolates for heavy metal tolerance

The tested concentrations of Al were 0.75x and 1x which equivalent 35.63 and 71.26 mM respectively, for Cr 1x and 10x equivalent 1.2 and 12.5 mM respectively. The rest two metal Cu and Zn were tested at three concentrations 0.1, 0.25, 0.75x equivalent 0.13, 0.32 and 0.9 for Cu and 0.3, 0.74 and 2.23 mM for Zn respectively. Resistance percentages of bacterial isolates for Al, Cr, Cu and Zn for each culture media of plant juice culture media was shown in Figure 2. Bacterial growth decreased on medium containing metals, especially copper, zinc and chromium at concentration 12.5mM. The growth of the tolerant isolates was exceptionally weak and slow; it took 7 to 10days. In the case of aluminum at the concentration of 35.63 mM and chromium at 1.25 mM, growth was quite like control in terms of its speed and size. On higher concentration of Cr (12.5mM), tolerant isolates developed small individual colonies after approximately seven days' incubation (Figure 3).

All isolates recovered from J and 1/10 NA were sensitive to all tested concentrations of metals except 35.63 mM of Al. Most tolerant isolates were recovered from JUPS culture medium while, some were representing JDS isolates. About 27% of JUPS isolates were tolerant to 12.5 mM Cr, 21% to 71.26 mM Al, 6% to 0.32 Cu and 3% to 0.74mM Zn. Nine isolates representing the two sites on JUPS culture medium were considered the most tolerant and were identified. According to tolerance results, MTC for Al, Cr, Cu and Zn were 71.26, 12.5, 0.32 and 0.74 mM, respectively and it was bacterial isolates dependent.

Table 2 Comparisons of pollution indices for soil calculated at up (UPS) and down (DS) streams where *Arthrocnemum macrostachyum* growing in Lake Manzala

Metal	Site	Soil			
		Igeo	CF	Er	QoC(%)
Al	DS	-0.60	0.99	-	-1.11
	UPS	0.94	2.87	-	65.21
Cd	DS	1.52	4.30	129.13	76.77
	UPS	4.66	37.83	1135	97.36
Cr	DS	1.96	5.84	11.67	82.87
	UPS	2.66	9.45	18.91	89.42
Co	DS	1.90	5.61	-	82.17
	UPS	2.90	11.21	-	91.08
Cu	DS	0.12	1.63	8.14	38.61
	UPS	1.82	5.31	26.57	81.19
Fe	DS	0.43	2.01	-	50.37
	UPS	1.93	5.73	-	82.53
Pb	DS	1.41	3.98	19.92	74.89
	UPS	3.45	16.39	81.93	93.89
Mn	DS	1.81	5.25	-	80.95
	UPS	2.78	10.31	-	90.30
Mo	DS	3.94	23	-	95.65
	UPS	3.81	21.06	-	95.25
Ni	DS	2.40	7.93	39.64	87.39
	UPS	2.91	11.28	56.39	91.13
V	DS	0.27	1.80	-	44.59
	UPS	1.75	5.04	-	80.17
Zn	DS	1.67	4.79	4.79	79.14
	UPS	3.30	14.81	14.81	93.25
RI	DS	213.29			
	UPS	1333.59			
C_{deg}	DS	67.14			
	UPS	151.29			

Table 3 Mean concentrations of heavy metals in plant organs (root and root+ shoot) (mg kg-1 d.w.) compared with threshold for hyperaccumulator plants

Metal	**Threshold for Hyperaccumulator plants ($mg\ kg^{-1}$)**	**Root**		**(Root + Shoot)**		**BF**		**TF**		**Transfer coefficient**	
		Down stream	Up stream	Down stream	Up stream	Down stream	Up stream	Down stream	Up stream	Down stream	Up stream
Al	1000 [1]	1068.91	1922.67	1859.97	2602.93	0.02	0.01	0.89	0.35	0.04	0.02
Cd	100 [2]	0.48	0.48	1.07	0.72	0.35	0.05	1.22	0.5	0.86	0.07
Co	300 [3]	1.89	2.48	2.6	2.88	0.03	0.02	0.75	0.18	0.04	0.02
Cr	100 [2]	142.05	63.41	188.04	79.55	0.09	0.11	0.02	0.26	0.27	0.14
Cu	300 [3]	317.52	83.17	389.16	119.25	1.74	0.40	0.25	0.42	2.13	0.58
Fe	1000–3000 [4]	2138.67	3797.87	3725.07	4739.6	0.03	0.02	1.13	0.25	0.05	0.03
Mn	10 000 [2]	102.19	410.59	218.59	617.57	0.04	0.10	1.22	0.43	0.08	0.14
Mo	up to 1000[6]	6.45	6.88	9.16	9.97	0.26	0.31	0.43	0.47	0.36	0.44
Ni	1000 [2]	58.64	29.04	76.51	37.16	0.28	0.09	0.37	0.28	0.36	0.11
Pb	100 [2]	10.32	15.41	26.8	19.75	0.1	0.04	1.62	0.31	0.26	0.05
V	Up to 2 [5]	8.0	12.77	115.32	102.2	0.04	0.02	0.5	0.39	0.06	0.03
Zn	10 000 [2]	129.20	163.44	212.71	253.65	0.42	0.17	0.94	0.56	0.73	0.26

1[47], **2**[48], **3**[49,50], **4**[51], **5**[52], **6**[53]

3.4 Identification of heavy metal-tolerant bacterial isolates

The majority of the selected isolates were Gram negative short rods and non-motile (Figure 4). According to API analysis, four isolates were identified as *Burkholderia cepaci*a, two as *Providencia retgeri* and the rest isolates as *Bacillus circulanc, Bacillus lentus* and *Raoultella ornithinoltica* (Table 4).

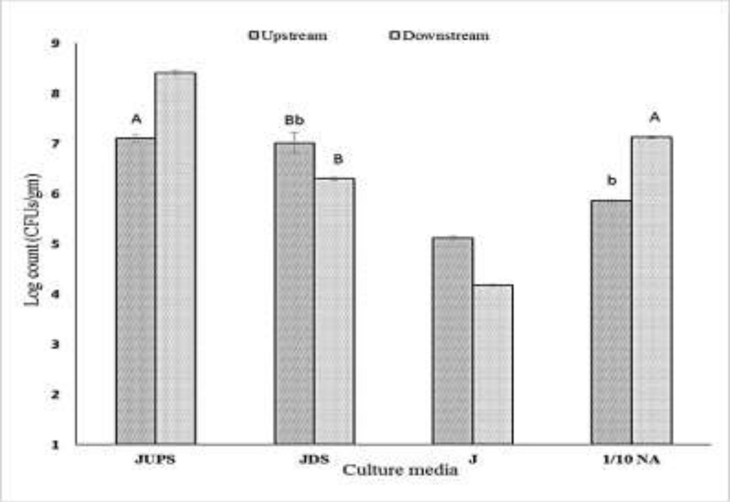

Figure 1 Total cultivable root associated bacterial counts (CFUs) of *Arthrocnemum macrostachyum* from up and down streams developed on the plant juice culture media (J: **J**uice diluted with distilled water; JDS: **J**uice diluted with **D**own**S**tream water; JUPS: **J**uice diluted with **UPS**tream water) compared to **N**utrient **A**gar medium (1/10 NA). Vertical bars represent ± standard errors. The bars with the same small letters were non significantly different within the same site and with capital letters were non significantly different between roots from up and down streams at (P value ≤ 0.05, n= 6).

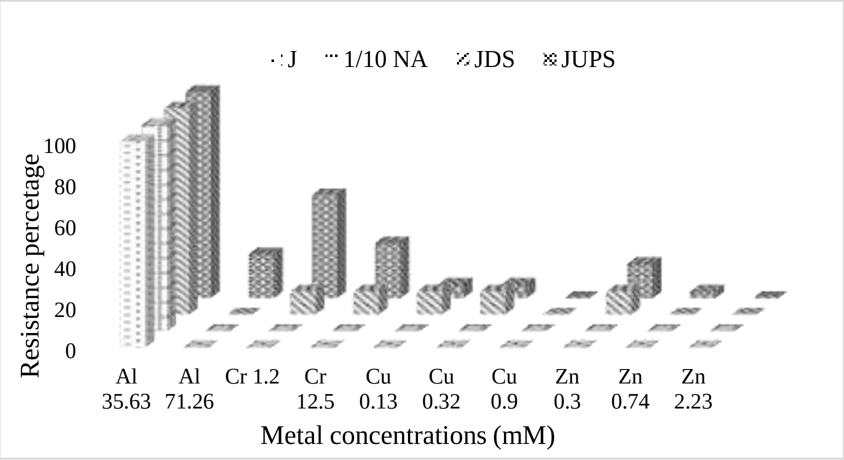

Figure 2 Resistance percentages of bacterial isolates for Al, Cr, Cu and Zn for each culture media of plant juice culture media (J: **J**uice diluted with distilled water; JDS: **J**uice diluted with **D**own**S**tream water; JUPS: **J**uice diluted with **UPS**tream water) and nutrient agar medium (1/10 NA).

4 Discussion

Results indicated that the metal load, in general, was sampling site-dependent where soil taken from upstream area contained extraordinary levels compared to the downstream.

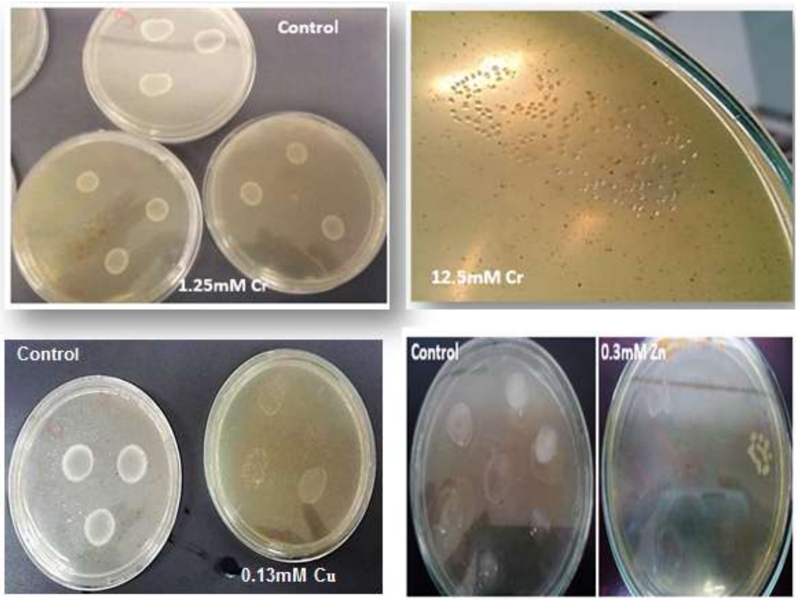

Figure 3 Development of bacterial colonies on plate-based culture media supplemented with various concentrations of heavy metals.

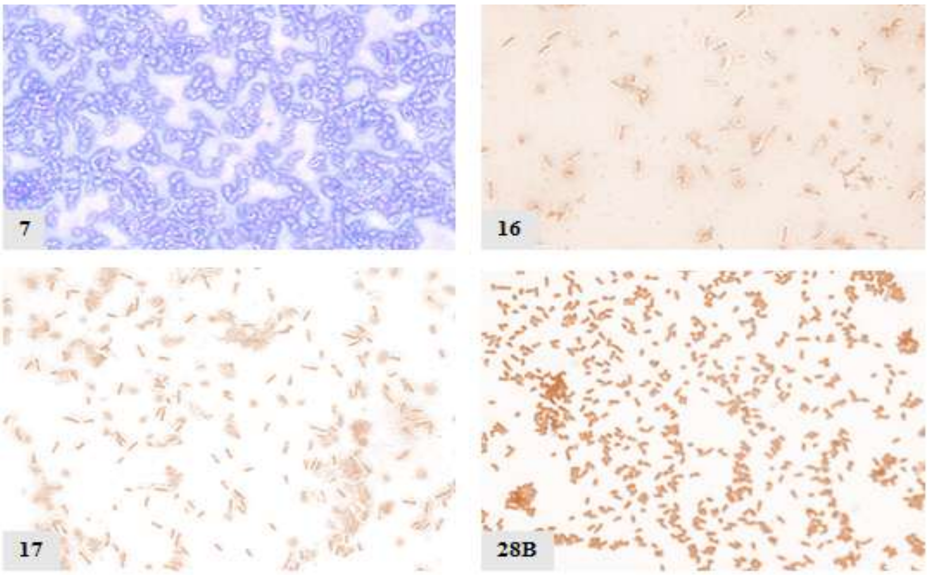

Figure 4 Cell morphology of the selected heavy metal-tolerant root associated bacteria, some cells seemed to be surrounded by polysaccharide layers (7, 16 and 17).

The analysis of soil and plant samples for heavy metal revealed that upstream contained high Fe, Al, Mn and Zn concentrations with descending order of rhizospheric soil, roots and shoots. Such order could be attributed to the fact that soil is considered as the main sink of metals and roots could accumulate more concentrations of heavy metals than shoots due to the thick parenchyma tissue of their cortex that comprise extensive intercellular spaces [54]. The higher concentrations of all measured heavy metals (except Cr and Ni) in *A. macrostachyum* roots of upstream in comparison with their counterparts of downstream could be due to nearby effluents from Bahr El Baqar drainage which is the main source of pollutants and heavy metals in lake Manzala [22,26,55]. El-Amier *et al.* (2020) [56] recorded higher level of Ni in the downstream and that might be attributed to its low mobility [57] and it is association with organic compounds which is higher in upstream due to Bahr El Baqar drainage. Also, among the potential sources of chromium and nickel are the propylene and polypropylene factories close to the downstream, where they are used in plating processes, as well as plastic recycling manufactory. For downstream, Fe, Al and Mn were the most abundant in soil and shoots, but Zn was found in higher concentration than Mn in roots. It was relevance for Fe to be the most dominant element as it is the most important metal and one of the major constituents of the lithosphere as its average content of the Earth's crust is about 5% [4,51] and it is an essential component of clay minerals of lake sediments [58]. It was revealed that Fe and Mn were synchronized which could be related to their chemical behavior in the forms of oxides and hydroxides [59]. The noticed trend of Fe, Mn and Zn concentrations complies with many previous studies on Lake Manzala [3,55]. The high concentrations of Al in soils of up and down streams could be related to siliceous nature of soils where Al represents either its main or secondary components [60]. However, the lowest concentrations of cadmium concentration in soils of both up and down streams could be attributed to its lower content in natural soils (less than 1 mg kg^{-1}) as a result of the weathering of the parent constituents of soils [6].

Table 4 Morphological and taxonomical profiles of the most heavy metal-tolerant bacterial isolates associated with the halophyte *A. macrostachyum*

Isolate Code.	Gram Reaction	Cell-Morphology	Motility	Strains Identification	Identification
7	-ve	Short rods	Motile	*Bukhoolderia cepacian*	Very good
16	-ve	Short rods	Non- motile	*Raoultella ornithinolytica.*	Excellent
17	-ve	Short rods	Motile	*Bukhoolderia cepacian*	Very good
26	-ve	Short rods	Motile	*Bukhoolderia cepacia*	Very good
28A	-ve	Short rods	Non- motile	*Providencia retgeri*	Excellent
28B	+ve	Short rods	Non- motile	*Bacillus circulanc*	Good
30A	-ve	Short rods	Non- motile	*Providencia retgeri*	Excellent
30B	+ve	Long rods	Motile	*Bacillus lentus*	Very good
31	-ve	Short rods	Non- motile	*Bukhoolderia cepacia*	Good

Metal concentrations reported in the present study were compared to the quality references recommended by [51,61,62]Turekian and Wedepohl (1961), Kabata-Pendias (2011) and Fernandes *et al.* (2018) to estimate the prospect adverse biological effects and the soil toxicity of the examined rhizosoils. The metals Cr, Mn, Ni, Pb, V and Zn exceeded the permissible limits, while Cd, Co, Cu and Mo were falling in the recommended levels of quality. Iron concentration in both sampling areas was far from the acceptable limits, but aluminum was in the permissible estimates only in the downstream area (Table 5). The high heavy metal load particularly in the polluted area is most probably attributed to irrigation by Bahr El-Baqar wastewater and over-use of chemical fertilizers as previously reported by Shalaby *et al.* (2017)[27]. ECB (2007) and ATSDR (2008)[64,65] documented that the anthropogenic sources of heavy metals are much more significant than natural emissions and account for their ubiquitous presence in soil. The continental dust flux is among the natural sources of pollution, but much larger amounts of metals are released by human activities. These encompass metal industries, combustion of oil and coal, cement works, waste incineration, vehicular emissions, besides fugitive emissions from road dusts. Indeed, at some industrial and waste disposal locations, several heavy metals are possibly released to the surrounding environments through leakage, and poor storage during manufacturing and/or inappropriate disposal practices [66].

Table ٥ Guidelines for the maximum permissible limit values of heavy metals in soil by three different standards

Quality reference values (background concentrations)	**Al**	**Fe**	**Cd**	**Co**	**Cr**	**Cu**	**Mn**	**Mo**	**Ni**	**Pb**	**Zn**	**V**	**References**
	(gKg^{-1})		(mg Kg^{-1})										
Eastern Amazon, Brazil	5.9	7.1	0.4	-	24.1	9.9	72	0.05	1.4	4.8	7.2	-	[62]
World-soil Average abundance of metals in surface soils	50	35	0.4	11.3	59.5	38.9	488	1.1	29	27	70	129	[51]
Abundances of the metals in the Earth's lithic crust (sedimentary rocks shales)	80	47.2	0.3	19	90	45	850	2.6	68	20	95	130	[61]

In this study, several indices were applied for comparing risks to ecosystem health such as contamination factor (C$_f$), index of geo-accumulation (I$_{geo}$), quantification of anthropogenic concentration of metal (QoC), ecological risk factor (Er), potential ecological risk (RI) and degree of contamination (C$_{deg}$) [67,68]. The obtained values of the contamination factor (CF) indicate that downstream is considered as very low contaminated for Al; while it is very high contaminated for Mo and Ni. Upstream shows very high contamination in case of Cd, Cr, Co, Pb, Mn, Mo, Ni and Zn. Results of the index of geoaccumulation (I_{geo}) of soil show that downstream is unpolluted with Al, Fe, Cu and V and heavy polluted with Mo; while upstream is highly polluted with Pb, Mo and Zn and heavy polluted with Cd. The potential ecological risk (Er) of downstream indicates low potential risk of Cr, Cu, Pb, Ni and Zn to considerable potential risk for Cd; while upstream exhibit low potential risk for Cr, Cu and Zn, moderate potential risk for Ni, considerable potential risk for Pb and very high for Cd. It was noticed that the potential ecological risk increases in upstream than downstream and upstream suffer considerable to very high risk levels for Pb and Cd which all are likely attributed to untreated local drain points associated with agricultural and industrial wastes [24,26]. Phosphatic fertilizers and atmospheric deposition represent the important sources of Cd pollution [64,65]. However, the major sources of Pb in current soil include tannery effluents, motorcars exhaust fumes, paint/pigments industry, leather manufactories and less possible from atmospheric deposition [24,29,66]. Quantification of soil contamination (QoC %) express quantification of the potential source a given contaminant whether anthropogenic or lithogenic origin [36]. The obtained negative value of QoC % for Al in downstream suggests its non-anthropogenic source; while the positive values of other metals in both down and upstreams indicate their anthropogenic sources [36]. Potential ecological risk (RI) is the index applicable for the assessment of the degree of ecological risk caused by multiple heavy metals. RI indicated that downstream showed moderate ecological risk while upstream showed very high ecological risk. The degree of contamination index is the sum of all contamination factors for a given site [35]. Up and down streams were considered as very high degree of contamination sites. Therefore, the remediation procedures and efforts should be pointed towards reducing metal concentrations in the soil, with a view to minimize the health hazards.

A. macrostachyum has exhibited a great efficiency for accumulating heavy metals in its tissues [67]. The results of bioaccumulation factor (BF) and comparing with threshold of hyperaccumulator plant species suggest the classification of *A. macrostachyum* as hyperaccumulator of Al, Fe, Cu, Cr and V as reported by Madejón *et al.* (2009) and Navarro-Torre *et al.* (2017)[7,16]. The lower values of BF for the other metals could be related to high organic matter content in soil which affecting heavy metals uptake as metals complexed or fixed inside organic substances in immobile form [69,70] and become available for uptake after decomposition [51]. The translocation factor (TF) results suggest *A. macrostachyum* have high ability to translocate Al, Cd, Co, Fe, Mn, Pb, V and Zn in its shoot as documented by other studies [7,17,18,71].

Original results and evidence on the sole use of plant-based-sea water culture medium for in vitro cultivation of the plant associated halotolerant microbiome have been provided[41]. In their study, a number of halophytes of the western north coast of Egypt was experimented for the diversity of halotolerant bacteria that the majority of their communities belonged to the genera *Bacillus* spp., *Halomonas* spp. and *Kocuria* spp. As mentioned by Shelake *et al.* (2018)[14], some plant-bacteria interweaves are greatly beneficial under stresses induced by heavy metals, thereby, improving uptake, translocation, distribution and detoxification by either or both the interacting partners. In the present study, juices of the halophyte *A. macrostachyum* did support excellent development of macro- and micro-colonies of its associated microbiome comparing with standard medium. The plant-based culture medium seemed an appropriate substrate for culturing rhizobacteria recovered from both up and down streams areas. Based on cell morphology and API microtube systems, the majority of secured bacterial isolates were identified as *Burkholderia cepacia* (4) isolates, 2 isolates belonged to *Providencia retgeri* and the rest isolates as *Bacillus circulanc, Bacillus lentus* and *Raoultella ornithinltica*. Most of bacteria were gram negative which characterized by cell wall lipopolysaccharide layer that have efficient role in protection against unfavorable conditions particularly heavy metals stress. And some of cultivated bacteria secreted exopolysaccharide layer (EPS) like a slime or capsule surrounded cells to provide additional protection mechanisms. EPS provide various characters including complexing toxic heavy metals, decreasing their mobility in the soils and their availability for plants and so reduce their absorption by plants [72,73]. Such reduction may explain lower accumulation and translocation of metals in *A. macrostachyum* of polluted upstream area, strongly binding potentially toxic metals and entrapping precipitated metal sulfides and oxides, causing the formation of organic metal complexes and subsequently increasing heavy metal resistance [74,75].

Plant root associated bacterial strains help some plants to ignore the toxic effects of heavy metals [13,76] as they accumulate them inside their vacuoles suggests the possibility of immobilization of metals to plants absorption techniques [51]**.** Heavy metals tolerant bacteria were very important in bioremediation and phytoremediation applications [12,16,44], so a great attention should be given to obtaining natural tolerant plant associated bacteria having the ability of tolerate multiple metals and consequently remediate them or improve phytoremediation pathways/ abilities of host or non-host plants. Resistant bacteria on agar media supplemented with heavy metals occasionally showed two important features: the first one is lower size of bacterial inoculated spot than control and the second one is emerging separate colonies of low viable cells could tolerate metals and these cells were the persisted cells which go deeper into dormancy and lose their culturability as becoming viable but not culturing (VBNC) according to the dormancy continuum hypothesis [77]. The obtained heavy metals tolerant bacteria recorded high MTC of examined metals and this well be valuable in future bioremediation applications. It was observed that most heavy metal tolerant bacteria were originally isolated on JUPS culture media from upstream area which is highly polluted with heavy metals. This consistent with the principle of domesticating of heavy metals tolerant bacteria in metals polluted environments provided by many researchers [32,78–80]. This reflect the potential adequacy of JUP selective media which comprise not only plant juice of the host plant that bacteria associated with or affected by its exudates but also it was diluted with heavy metal polluted lake water to mimic the autochthonous environment. The most resistant seven isolates belonged to: *Raoultella ornithinolytica, Providencia rettgeri, Burkholderia cepacia, Bacillus circulans and Bacillus lentus* which previously defined as heavy metals tolerant and perfect candidates as bio-remediators, to restrict the level of heavy metal pollution of the lake as well as similar environments [78,81]. Consequently *A. macrostachyum* has been considered as an appropriate candidate for phytoremediation efficiency of extremely heavy metal polluted soils of various toxic elements.

In this area of study, it should be realized that, the halophytic microbial microbiome associated to the plant certainly benefits the latter not only for heavy metal tolerance, but also for supplementary functions as well. This includes improving the stress-related characteristics and nutrient availability as well as production of several beneficial metabolites, consequently resulting in much better plant development, growth and survival in metal-contaminated habitats. Besides, the isolation from halophytes and application to the non-halophytic plants might have potential benefits since these microbiotas are of multifunctional merits encompassing both plant growth promotion and reduction of toxic metal translocation. Additional advantage is the unique capability of these micro-residents to alleviate heavy metal toxicity of polluted watercourses via bioremediation of such environments.

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