BIOSORPTION OF Cd^{2+} BY WILDTYPE AND CADMIUM RESISTANT, CdR-99 CELL LINE OF *CHLORELLA VULGARIS*

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ABSTRACT: Removal of heavy metals is very important in wastewater treatment process, due to their toxic effects on the environment. Biological treatment has attracted researchers for years since it has many advantages over physical and chemical methods for removing heavy metals from wastewater. The purpose of this research was to assess the biosorption of Cd^{2+} by wildtype (WT) and CdR-99 resistant line of *Chlorella vulgaris* confirming mechanisms of resistance to Cd^{2+} toxicity and the effect of the variable concentrations of Cd^{2+} on their growth. Exposure of both algal cell lines to increasing Cd^{2+} concentrations resulted in progressive inhibition of growth as revealed by growth experiments. The higher ID_{50} value (38 μM Cd^{2+}) of CdR-99 resistant line exhibited some degree of resistance to Cd^{2+} toxicity. Metal content was determined by flame atomic absorption spectrometry (FAAS). When exposed to the growth medium containing 50 μM Cd^{2+}, CdR-99 resistant isolate proved to be efficient cell line compared to the WT, in terms of adsorption and removal of Cd^{2+} at 15 min and 48 hr interval of time respectively. Extracellular Cd^{2+} adsorption was found significantly higher than intracellular uptake in both the tested cell lines. Total Cd^{2+} accumulation and distribution between the external and internal cell fractions of the CdR-99 were significantly higher to the WT. Thus, the CdR-99 cell line appeared more resistant to Cd^{2+} toxicity and hence may be used for wastewater treatment and remediation of metal contaminated sites.

Keywords: Biosorption; Heavy metals; *Chlorella vulgaris*; Pollution.

INTRODUCTION

Heavy metals such as Cu, Cd, Pb, Zn, As, Ni and Cr infiltrate to the environment from a variety of anthropogenic sources that involve the metallurgical, energy, mining and transport industries[1]. They directly affect proper development and stability of individual ecosystems since they are accumulated in plants and animals. Environmental pollution by heavy metals has, therefore become a serious threat to the living organisms in an ecosystem[2,3] and the metal toxicity is of great environmental concern because of their persistent and non-biodegradability in nature[4,5]. The toxic effects posed by the metals may include breaking fatal enzymatic functions, destructing ion regulation, and directly affecting the formation of DNA as well as protein[6,7]. The physiological and biochemical properties of microorganisms can be altered by the presence of heavy metals. This harmfulness is generated by the displacement of metals from their

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Received: 3 Nov 2020; First Review: 7 Nov 2020; Second Review: 15 Nov 2020; Accepted: 16 Dec 2020.
Doi: https://doi.org/10.3126/sw.v14i14.35022

native binding sites or ligand interactions[8].

Removal of heavy metals from wastewater is very important due to their hazardous effects. There are various chemical and physical methods including ion exchange, precipitation, reverse osmosis, electrodialysis and ultra-filtration for cleaning heavy metals from wastewater but they are considered to be quite expensive, less eco-friendly and inefficient. Therefore, biological treatment has gained much attention for years because of its eco-friendly nature as well as economic and treatment operation efficiencies[9,10]. The treatment involves the process of using specific microorganisms to convert harmful pollutants present in water to harmless products[11]. Microalgae seem to be promising due to their favorable capabilities, including the substantial capability of absorbing heavy metals and eliminating them from wastewater[12]. In addition, they exhibit a variety of mechanisms of metal tolerance which include sorption of metal ions to cell wall components[13], excretion of organic compounds that form extracellular complexes with metal ions[14], and production of intracellular metal-binding proteins which may function in detoxification of these metals[15]. Besides, cheap cultivation, effective biological treatment and production of valuable biomass of the microalgae made it appropriate method with many advantages. One of the most ideal microalgae species for this purpose is *Chlorella vulgaris*[16, 17].

Chlorella vulgaris is a unicellular green microalga, having spherical cells with a diameter of 2 to 10 µm. The algal species belongs to C*hlorellaceae* family and this photosynthetic microorganism was first discovered by Willen Beijrenick, a Dutch researcher in 1980. The easy and flexibility of culture conditions and resistance to unfavorable factors makes its growth possible in different wastewater environments and that is the reason for vast investigations on this field aiming to use *Chlorella vulgaris* in wastewater treatment[18]. The ability of this microalga to thrive in environments that are polluted by heavy metals and the mechanisms that enable them to grow under these conditions are of interest. Isolation or selection of algal strain that are tolerant to the toxic effects of heavy metals, and analysis of the mechanisms that contribute to metal tolerance, may help to improve the efficiency of waste water processing as well as give insight into the processes involved in adaptation to this form of environmental stress. In the research presented here, we describe a strain of *Chlorella vulgaris* that displays high resistance to Cd^{2+}. The growth characteristics of the wild type culture and the selected CdR-99 resistant line under different Cd^{2+} concentrations are described. Besides, biosorption of Cd^{2+} by the algal cell lines including extracellular metal binding and intracellular uptake is also discussed.

MATERIALS AND METHODS

Growth conditions

The wild type (WT) and a cadmium resistant (CdR-99) isolate of *Chlorella vulgaris* were obtained as gift from Israel. Actually, the CdR-99 as the Cd^{2+} resistant line was selected by increasing the Cd^{2+} concentration in the growth medium following EMS (Ethyl methane Sulphonate) mutagenesis in the WT *Chlorella vulgaris*[19]. In our laboratory, both the gifted algal cultures were grown on BG-11 mineral medium. While the CdR-99 resistant line was grown on the medium supplemented with 25 µM Cd^{2+}, the WT culture was grown on the same medium without Cd^{2+}. All experiments were performed in 250-ml Erlenmeyer flasks containing 100 mL of medium, placed on a gyratory shaker (180 rev./min) at 27ºC. The surface of the flasks was exposed to a continuous illumination of 150 $mmol/m^2/s^{-1}$ provided by cool white fluorescent lamps. The growth of the algal cultures was monitored time to time. When the cultures reached the stationary phase of growth, they were further inoculated into fresh growth medium of same volume as described to keep them growing continuously.

Preparation of (0.1 M) standard solution of Cd^{2+}

Standard solution of (0.1M) $Cd(NO_3)_{2..}4H_2O$ of analytical grade (Sigma-Aldrich) was prepared by dissolving a calculated amount of the tetrahydrate salt in double distilled water and then filter sterilized. This stock solution was preserved in a refrigerator at 4 ºC until further use.

Growth rate experiment

The experiment involving effect of Cd^{2+} on growth rate of both the algal cultures was monitored at the exponential phase. In the growth experiment, two separate series of sterilized Erlenmeyers each containing 100 mL of BG-11 medium were prepared for the algal cultures and each series supplemented with 0, 1, 10, 50 and 100 µM Cd^{2+} respectively to maintain similar experimental condition. Then, the WT and CdR-99 resistant line were inoculated in each of the respective series in the range of 5.0 - 5.5 x 10^5 cells/mL of the mineral medium. The growth was monitored by change in cell density measured in spectrophotometer at maximum absorbance of 540 nm. Cell density was determined at the time of inoculation and each day thereafter for 7 days by measuring turbidity. Growth rate (increase in absorbance at 540 nm/day) of both the algal cultures was calculated from the change in cell density between the 2nd and 6th days of exponential phase of growth[20].

Determination of extracellular binding of Cd^{2+} ions (Adsorption kinetics)

The CdR-99 resistant line initially grown in BG-11 mineral medium containing 25 µM Cd^{2+} was inoculated into fresh mineral medium without Cd^{2+} and allowing them to grow for 5 days. The process was repeated three times to ensure that the metal ions were completely desorbed from the cells. Then, the CdR-99 cells at the stationary phase of growth were collected by centrifugation. The pelleted cells were re-suspended in 10 mL of fresh mineral medium and counted in Hemacytometer to note the actual cell numbers. Three sterilized Erlenmeyers containing fresh mineral medium were inoculated with the dense cell suspension in such a way that each flask counted 10^9 cells per 100 mL of the mineral medium. A parallel set of three Erlenmeyers were also prepared for similar experiment with the WT algal culture as control.

To study adsorption kinetics at different time intervals, the stock solution of Cd^{2+} was added to each set of Erlenmeyers maintaining a final concentration of 50 µM. From each of the metal added Erlenmeyers, a 10 mL sample was pipetted immediately representing a zero hour sample; however it took 15 min to proceed through a complete treatment. Hence, 15 minute was considered as zero hour sampling in each case. The samples were drawn in a similar way at an interval of 0.5, 1.0, 2.0, 4.0, 8.0, 12.0, 24.0 and 48.0 hr respectively. The Erlenmeyers were placed back to the shaker after each sample drawn. The samples drawn at each of these time intervals were spun down in a bench centrifuge (3500 rpm, 10 min) and the supernatants collected separately for metal analysis in order to determine the residual metal left over the medium. The cell pellets collected after centrifugation was then washed with 5 mL of EDTA (10 g/L) three times[21]. Each time, the cells were spun down (3500 rpm, 10 min) and the supernatants collected for metal analysis in order to determine the amount of metal adsorbed to the cell walls at different time interval.

Determination of intracellular Cd^{2+} ions uptake (Absorption kinetics)

Each aliquot of cell pellets left over after EDTA treatment was re-suspended in 10 mL of double distilled water by gentle vortex. The cells were spun down and re-suspended in double distilled water repeatedly for three times following the process of washing. Each of the washed cells was then re-suspended in 1 mL of the water and transferred to digestion tubes. The cell suspensions in each digestion tube were treated with 2 mL of conc. nitric acid. The mixture was placed in a chemical hood overnight so as to ensure a complete dissolution and to prevent foaming during subsequent digestion process. Each sample was digested at 100 ºC for 1 hr followed by gradually increasing the temperature up to 230 ºC. The digestion was continued for approximately 3 hr until the solution became completely clear and transparent. After digestion, the digestion tubes were cooled and the content transferred into volumetric flask of 10 mL capacity. Each tube was rinsed with double distilled water, transferred into the respective flask and adjusted to a final volume of 10 mL with the water. Then, flame atomic absorption spectrophotometer (Perkin Elmer 1100 B) was used to determine Cd^{2+} concentrations in the samples[22].

Statistical analysis

All statistical analyses and data processing in this study were performed on an IBM-PC computer. Descriptive statistics (mean, percentage and standard deviation) were performed and student's t-tests were used to determine significant differences between means where significant main effects were found. For the statistical test, significance was defined at P = 0.05.

RESULTS AND DISCUSSION

Effect of Cd^{2+} on growth of the algal cell lines

Figure 1 shows the effect of increasing Cd^{2+} concentrations on growth of the two algal cell lines. The growth rate at any given Cd^{2+} concentration was determined from the increase in cell density between day 2 and day 6 since this was the period of exponential growth. Exposure of *Chlorella* sp. to increasing Cd^{2+} concentrations resulted in progressive inhibition of growth (Fig. 1).

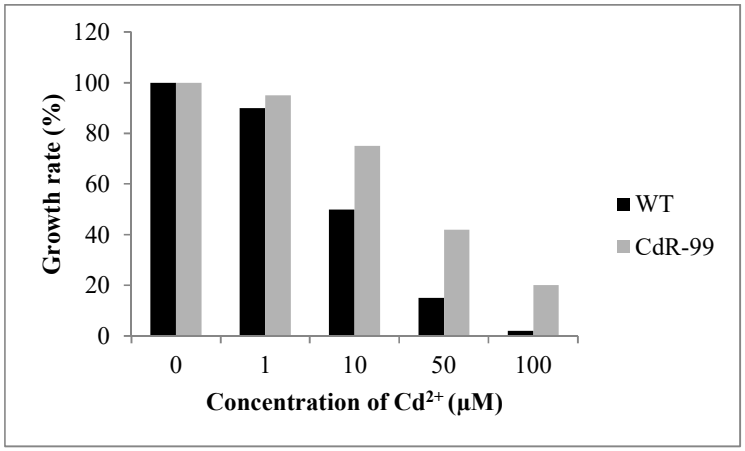

Figure 1: Percentage growth rate of WT and CdR-99 resistant line at different Cd^{2+} concentration.

The concentration level of 1 µM Cd^{2+} showed least inhibitory effect on both the algal lines grown under similar experimental condition while the presence of 10µM inhibited the growth by 50 and 25% in the WT and CdR-99 lines respectively. Similarly, the CdR-99 resistant line sustained less inhibitory effect (58%) compared to that of the WT (85%) in the presence of 50 µM. However, the growth of both the algal cultures was significantly arrested by the presence of 100 µM Cd^{2+} in the liquid growth medium. Accordingly, the CdR-99 resistant line sustained inhibitory effect by 80% while the WT was by 98%. The inhibition of 50% in growth rate (ID_{50}) calculated on the basis of the growth rate was observed at concentrations of 12 and 38 µM Cd^{2+} in the WT and CdR-99 resistant line, respectively. These ID_{50} values demonstrated three fold higher degree of resistance in the CdR-99 resistant line to Cd^{2+} toxicity compared to the WT. The higher resistance capacity of the CdR-99 resistant line to the effect of metal ions on its growth may be attributed to EMS mutagenesis[19]. The findings are in agreement with several studies which also showed resistant to growth rate by metal ion effect due to mutagenic strains[20,23].

Adsorption and removal of Cd^{2+} from growth medium by the algal cell lines

Before performing experiment on adsorption kinetics of Cd^{2+} on the algal cell walls *i.e.*, extracellular binding of the metal ions, it was necessary to find cell numbers before and after exposure of the algal cell lines to the liquid growth medium containing 50 µM Cd^{2+} up to 48 hr treatment. Table 1 shows the initial and final cell numbers of the WT and CdR-99 resistant line in the liquid growth medium. Results revealed that the difference of initial and

final (48 hr of treatment with Cd^{2+}) cell numbers between the tested algal cell lines was not found significant (t-test, $p>0.05$). Therefore, the cell numbers as shown in Table 1 were assumed for all time intervals throughout the kinetic experiment.

Table 1: Cell numbers per 100 mL of liquid growth medium

Cell lines	*Initial	**Final
WT	5.4×10^9	5.0×10^9
CdR-99	6.5×10^9	6.2×10^9

*Initial: Before exposure to 50 µM Cd^{2+} for 48 hr;

** Final: After exposure to 50 µM Cd^{2+} for 48 hr

Figure 2 shows the adsorption kinetics and percentage removal of Cd^{2+} from the liquid growth medium by the WT culture. Upon exposure to the growth medium containing Cd^{2+}, it was found that the metal binding to cell wall of the WT was rapid during the first few hours, increasing gradually up to 12 hr and then reached steady state thereafter (Fig. 2A). On the contrary, the residual concentration of Cd^{2+} in the medium was found to be decreased with the progress of treatment hours. Results revealed that Cd^{2+} association to the WT cell walls during the first hour was found to be 37%. Similarly, the rate of the metal ion removal from the medium was rapid during the first few hours, increasing gradually up to 12 hr and then reached to stationary state thereafter (Fig. 2B). Initially, Cd^{2+} removal from the medium reached to27% at 15 min and then increased to 45% till the first hour of treatment (Fig. 2B). Finally, percentage removal of Cd^{2+} from the medium reached to 72% at 48 hr of treatment time of which 56% was externally bound to cell walls of the WT algal culture.

Figure 3 shows the adsorption kinetics and percentage removal of Cd^{2+} from the liquid growth medium by the CdR-99 resistant line. In contrast to the WT algal cells, CdR-99 resistant line demonstrated a distinct pattern in terms of its' adsorption and removal kinetics of Cd^{2+} when exposed to the liquid growth medium under similar experimental condition (Fig. 3). It was found that the resistant line demonstrated a very rapid increase in metal adsorption compared to the WT type. The adsorption of Cd^{2+} ions to the cell walls was very rapid during the first few minutes, increasing gradually up to 8 hr and then reached to a saturation point of adsorption (Fig. 3A). Conversely, the residual concentration of Cd^{2+} in the medium also decreased simultaneously with the progress of treatment hours.

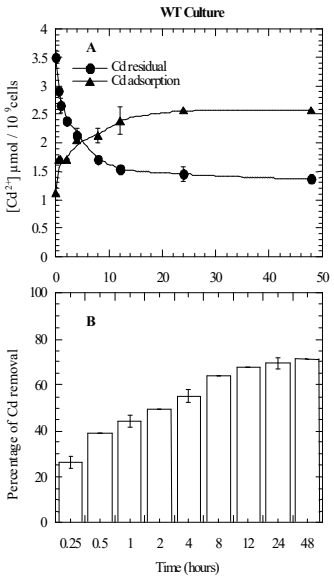

Figure 2: Extracellular adsorption and residual concentration of Cd^{2+} at different time intervals (A) and percentage removal of Cd^{2+} from the growth medium (B) in WT culture (Mean±SD; n=3)

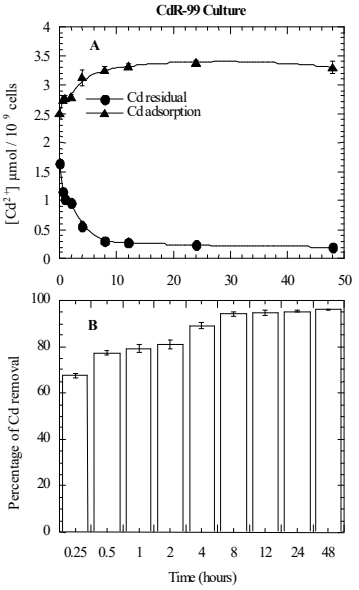

Figure 3: Extracellular adsorption and residual concentration of Cd2+ at different time intervals (A) and percentage removal of Cd2+ from the growth medium (B) in CdR-99 resistant line (Mean±SD; n=3).

Results also revealed very rapid Cd^{2+} removal from the medium attaining 80% within the first few hours of exposure. At 15 min, Cd^{2+} removal from the growth medium was found to be 67% of which 50% was contributed by the cell wall binding alone. This figure is markedly different to that of the WT in terms of Cd^{2+} removal and adsorption at 15 min. Till 48 hr of treatment, the CdR-99 resistant line showed 96% Cd^{2+} removal from the medium (Fig. 3B) of which 66% was found externally bound to the algal cell walls which is appreciably higher compared to the WT culture.

Apparently, both the algal cell lines upon exposure to the metal ions showed rapid adsorption rate within the first few minutes and then reached a point of saturation with the progress of treatment hour in consistent with previous reports[24,25]. Microorganisms adopt different mechanisms to interact and survive in the presence of inorganic metals. Several microorganisms like algae, bacteria and fungi have carboxyl, hydroxyl, sulfydryl, sulfonate, alcohol, phosphoryl, amine, ester, thioether, and thiol groups as the negatively charged sites on cell surface that play role in adsorption of metal[26]. As far as metal binding mechanism is concerned, ionic charge and covalent bonding are hypothesized. Although various types of tolerance mechanisms have been reported in these microorganisms for heavy metal stress, Cd detoxification has only been restricted to efflux pumps in bacteria. The most important aspect of Cd ions is that they covalently bind to sulfhydryl groups[27]. A similar mechanism of tolerance may be expected in *Chlorella vulgaris* as well despite limited evidences. However, a plausible explanation for rapid adsorption in the present study may be due to availability of negatively charged binding sites on algal cell wall that empowers them to bind to positively charged metal ions[26]. The adsorption process takes place more rapidly and also simultaneously with a slow intracellular metal uptake. Microbial cells exhibit biosorption mechanisms for the uptake of heavy metals; the process can be classified into metabolism-independent biosorption, which mostly occurs on the cells exterior and metabolism-dependent bioaccumulation, which comprises sequestration, redox reaction, and species-transformation methods[28]. Biosorption can be carried out by living cells or dead biomass as passive uptake through surface complexation onto the cell wall and surface layers, however a variety of chemical, physical, and biological factors may influence the bioaccumulation process[29]. Besides, microorganisms can decontaminate metals by valence conversion, volatilization, or extracellular chemical precipitation[30].

Total Cd^{2+} bioaccumulation and distribution in external and internal cell fractions of the algal cell lines

(Tables 2 & 3) show total Cd^{2+} bioaccumulation and distribution between external and internal cell fractions of the WT and CdR-99 resistant line *via* adsorption and absorption mechanisms at different time intervals. It was found that the WT showed a gradual accumulation of Cd^{2+} with increasing time of exposure to the medium containing 50 μM Cd^{2+} (Table 2). Total Cd^{2+} accumulation reached a steady state after about 12 hr while the accumulation was rapid during the first few hours. The distribution of Cd^{2+} between the external and internal cell fractions also increased with duration of exposure indicating that the process of adsorption and absorption occurred simultaneously. The amount of Cd^{2+} associated with the external cell fractions was higher than the internal at each time interval. While the extracellular adsorption was found gradually increased, intracellular uptake appeared fluctuating at different time intervals although the tendency of Cd^{2+} association was of increasing order. Of the total Cd^{2+} accumulated (3.46 μmol per 10^9 cells) at 48 hr, 75% was externally bound to the cell surface whereas nearly 25% was found inside the cells.

Table 2: Total Cd^{2+} bioaccumulation and distribution between external and internal cell fractions of the WT at different time intervals (Mean±SD; n=3).

Cell line	Time Interval (hr.)	Cd^{2+} association in cells (μmol / 10^9 cells)		
		External (Adsorption)	Internal (Absorption)	Total bioaccumulation
WT	0.25	1.15 (0.15)	0.18 (0.04)	1.33
	0.5	1.72 (0.00)	0.20 (0.06)	1.92
	1.0	1.72 (0.00)	0.45 (0.12)	2.17
	2.0	1.72 (0.00)	0.70 (0.00)	2.42
	4.0	2.10 (0.00)	0.78 (0.15)	2.88
	8.0	2.15 (0.15)	0.82 (0.14)	2.97
	12.0	2.40 (0.30)	0.88 (0.06)	3.28
	24.0	2.60 (0.00)	0.86 (0.12)	3.46
	48.0	2.60 (0.00)	0.86 (0.03)	3.46

Table 3: Total Cd^{2+} bioaccumulation and distribution between external and internal cell fractions of the CdR-99 resistant line at different time intervals (Mean ±SD; n = 3).

Cell line	Time Interval (hr.)	Cd^{2+} association in cells (μmol / 10^9 cells)		
		External (Adsorption)	Internal (Absorption)	Total bioaccumulation
CdR-99	0.25	2.52 (0.02)	0.90 (0.02)	3.42
	0.5	2.78 (0.02)	1.20 (0.06)	3.98
	1.0	2.84 (0.02)	1.38 (0.02)	4.22
	2.0	2.84 (0.09)	1.38 (0.02)	4.22
	4.0	3.15 (0.00)	1.50 (0.07)	4.65
	8.0	3.36 (0.12)	1.52 (0.00)	4.88
	12.0	3.38 (0.30)	1.52 (0.14)	4.90
	24.0	3.38 (0.15)	1.54 (0.00)	4.92
	48.0	3.35 (0.09)	1.60 (0.12)	4.95

The CdR-99 resistant line demonstrated a higher accumulation of Cd^{2+} per 10^9 cells compared to that of the WT (Table 3). Besides, the distribution of Cd^{2+} between the external and internal cell fractions of the resistant line was also higher to the WT. Evidently; the resistant line accumulated almost all the metal ions supplemented within the first few hours showing a saturation point at early treatment hours. The Cd^{2+} association in external and internal cell fractions increased with treatment hours in the growth medium. However, the algal culture was found to be saturated with the metal ions during the first few hours showing that the resistant line promptly responded to the Cd^{2+} toxicity. This further indicates that the CdR-99 line demonstrated more resistance to Cd^{2+} toxicity. Of the total

metal accumulated (4.95 μmol /10^9 cells) by the resistant line at 48 hr, nearly 68 and 32% of Cd^{2+} were externally bound and inside the cells respectively.

The results of the present study are in agreement with several studies in which microbes demonstrate various types of defense mechanisms in response to metal toxicity[31,32,33]. These may include biotransformation, extrusion, use of enzymes, production of exopolysaccharide (EPS) and synthesis of metallothioneins etc. Intracellular sequestration is the complexation of metal ions by various compounds in the cell cytoplasm. The concentration of metals within microalgal cells can result from interaction with surface ligands followed by slow transport into the cell. The ability of cells to accumulate metals intracellular has been exploited in practices, predominantly in the treatment of effluent treatment. However, there are several factors that influence biological heavy metals removal including temperature, biomass concentration, environment pH, ionic strength and the initial concentration of the metal ions. Hence, further investigation is required in this field. In addition, genetic engineering and chemical modification could alter the components of cells surface and can efficiently improve the adsorption capacity and selectivity to target-metal species. These would facilitate the development of improved techniques for the bioremediation of heavy metals in the ecosystem.

AKNOWLEDGEMENTS

We are very much grateful to the Department of Environmental Hydrology and Microbiology (EHM), The Jacob Blaustein Institutes for Desert Research, Ben-Gurion University, The State of Israel for gifting us a cadmium resistant strain of *Chlorella vulgaris* for our research purpose.

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