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Изолација и карактеризација Cr(VI) толерантних земљишних бактерија

(Isolation and characterization of Cr(VI) tolerant soil bacteria)

-PhD thesis-

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Content

Acknowledgements---V
List of figures ---VI
List of tables---XI
List of abbreviations ---XIII
1. INTRODUCTION---1
2. LITERATURE REVIEW---4
2.1. General characteristics of chromium---5
Natural sources of chromium ---7
Industrial uses of chromium ---8
Chemistry and geochemistry of chromium --- 10
Chromium toxicity--- 12
2.2. Mechanisms of chromium resistance in microorganisms --- 13
Decreased chromate uptake--- 13
Reduction of Cr(VI) to Cr(III) --- 14
Chromate efflux --- 16
DNA repair --- 17
Protection against oxidative stress --- 17
2.3. Bioremediation of chromium polluted environments--- 18
2.4. Influence of chromium on bacterial soil community--- 20
2.5. Chromate tolerant bacteria--- 21
3. AIMS --- 24
4. MATERIALS AND METHODS --- 26
4.1. Sampling design --- 27
Sampling design for cultivable soil bacterial community analyses --- 27
Sampling design for chromate tolerant strains analyses--- 29
Sampling methodology --- 29
Sampling locations --- 30
4.2. Chemical analyses of samples--- 34
4.3. Cultivable soil bacteria community analyses--- 35
Cultivable soil bacteria and chromate tolerant cultivable soil bacteria count 35
Selective enrichment of cultivable bacteria in medium with Cr(VI) --- 35

Proportion of Gram-positive and Gram-negative bacteria ---------------------- 36
4.4. Chromate tolerant bacterial strains analyses -- 36
Media composition and incubation conditions ------------------------------------- 36
Isolation of chromate tolerant strains -- 37
MALDI-TOF analysis -- 38
Chromate tolerance and reduction testing -- 39
DPC test for Cr(VI) concentration determination --------------------------------- 41
Biochemical and morphological characterization -------------------------------- 42
16S rRNA and *pycA* gene sequence analysis and detection of *B. anthracis* virulence genes -- 43
Biofilm formation assay --- 43
Heavy metal tolerance determination --- 44
Detection of chromate transporter gene -- 45
STEM EDS analysis of cells and endospores exposed to Cr ---------------------- 46
4.5. Statistical analysis -- 48
5. RESULTS --- 49
5.1. Influence of chromium on the cultivable soil bacterial community ------------- 52
Chemical characterization of the soil samples ------------------------------------- 52
Cultivable community tolerance to chromate determined by a selective enrichment method --- 55
Enumeration of cultivable bacteria and chromate tolerant cultivable bacteria -- 56
Proportion of Gram-positive and Gram-negative bacteria within the cultivable bacteria soil population -- 62
5.2. Isolation of chromate tolerant bacterial strains ------------------------------------ 64
MALDI TOF identification -- 65
Chromate tolerance and reduction --- 67
5.3. Characterization of selected chromate tolerant *Bacillus cereus* group strains - 72
MALDI TOF identification -- 73
Morphological and biochemical characteristics ----------------------------------- 75
16S rRNA and pycA gene sequence analysis --------------------------------------- 76
Biofilm formation -- 80
Heavy metal tolerance --- 81
MIC (minimal inhibitory concentration) of Cr(VI) of selected *Bacillus cereus* group strains --- 82

Chromate tolerance and chromate reduction testing of selected *Bacillus cereus* group stains ---------- 83
Comparison of chromate tolerance of environmental and reference *Bacillus* genus strains ---------- 89
NCr1a strain characterization ---------- 90
5.4. STEM EDS analysis of chromium content and localization in cells and endospores of strains NCr1a and *B. subtilis* PY79 ---------- 98
Chromium content in cells ---------- 99
Chromium content in endospores ---------- 104
6. DISCUSSION ---------- 108
6.1. Chromium influence on cultivable bacterial soil community ---------- 109
6.2. Chromate tolerance of environmental bacteria ---------- 112
6.3. Chromate tolerance of *Bacillus cereus* group ---------- 117
6.4. Differential response of environmental chromate tolerant *B. cereus* group strain NCr1a and reference strain *B. subtilis* PY79 ---------- 125
7. CONCLUSIONS ---------- 129
8. REFERENCES ---------- 132
9. APPENDIX ---------- 144
9.1. Environmental samples description ---------- 145
9.2. 16S rRNA gene sequences of chromate tolerant *Bacillus* isolates ---------- 147
9.3. *pycA* gene sequences of chromate tolerant *Bacillus* sp. isolates ---------- 150
9.4. Sequence of chromate transporter gene of NCr1a strain ---------- 152
10. EXTENDED ABSTRACT IN SERBIAN LANGUAGE (PROŠIRENI IZVOD NA SRPSKOM JEZIKU) ---------- 153

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LIST OF FIGURES

Figure 1 Physical appearance of different chromium forms: a - chromite mineral ($FeCr_2O_4$), b – crocoite mineral ($PbCrO_4$), c - metal powder of elemental chromium; d - high purity chromium crystals ; e - chromium oxide (Cr_2O_3), f – potassium chromate (K_2CrO_4) (sources: a-e Gray 2018, f - Anger et al. 2000) ... 6
Figure 2 Overview of chromium flow in industrial applications: green – trivalent chromium forms, red – hexavalent chromium forms, blue – zero valence chromium forms (illustration based on data in Stanin 2005) ... 9
Figure 3 Chromium electrochemical behavior at different pH and redox potential values - Eh-pH diagram for the system Cr-O_2-H_2O, for the concentration of Cr = 10^{-6} mol/kg at solid/liquid boundaries (from Dhal et al. 2013) ... 11
Figure 4 Mechanisms of chromate resistance in microorganisms: A – decreased chromate uptake due to the decreased transport through shared sulfate uptake transporters (caused e.g. by mutation in sulfate uptake channel genes), B – extracellular chromate reduction, C – intracellular chromate reduction, D – protection against oxidative stress, E – increased chromate efflux, F – DNA damage repair (source: Ramírez-Díaz et al., 2008) ... 14
Figure 5 Phylogenetic distribution of chromate transporter proteins (family PF02417) (source: EMBL-EBI 2018) ... 18
Figure 6 Locations of soil samples collected for the analyses of Cr influence on the cultivable soil bacterial community: ♦ (blue diamond) background Cr level (group LCr) - samples 1-4; ■ (red square) elevated Cr level of anthropogenic origin (group PCr) – samples 5-8; ●(green circles) elevated Cr level of natural origin (group NCr) – samples 9-17 (interactive KML map available on link: https://drive.google.com/open?id=12Lwi0S5yUbpeylGSSNZcWWsP6qQ&usp=sharing) ... 28
Figure 7 Soil sampling (preparation for soil collection - removal of surface vegetation, stones and fauna on sampling site 12) ... 29
Figure 8 A and B - sites with naturally high level of chromium - serpentine soils, Mt. Zlatibor and Mt. Maljen; B – appearance of serpentine soil samples ... 31
Figure 9 Leather tannery „Panonija“ Subotica – site of the former wastewater lagoon (photo: D. Radnović) ... 32
Figure 10 Chemical factory “Zorka” Subotica (photo: D. Radnović) ... 32
Figure 11 Salt marsh Slano Kopovo (photo: D. Radnović) ... 33
Figure 12 Appearance of the acetate minimal medium with: A - 0, 0.5, 1, 2 mM Cr(VI) before incubation; B - 0, 4, 6 and 8 mM Cr(VI) after incubation. Visible growth (cloudy, turbid appearance) is evident in the 0, 2 and 4 mM tubes, while no growth formed in 8 mM medium ... 37

Figure 13 Determination of Cr(VI) concentration by DPC test – microplate ready for absorbance measurement: 1-4 – four samples with different Cr(VI) concentration as visible from variable intensity of pink color (three replicates for each sample), 5 - abiotic control, ST – standard solutions 42
Figure 14 Biofilm formation assay experimental setup 44
Figure 15 Heavy metal tolerance testing by agar spot inoculation....45
Figure 16 Outline of the research plan: four main research sections and analyses conducted in each section....51
Figure 17 Selective enrichment of soil microbial community on media with increasing Cr(VI) concentrations – number of samples in each sample group showing growth on medium with different Cr(VI) level: NCr – samples with natural geogenic Cr; PCr – samples polluted with Cr; LCr – samples with low background Cr 56
Figure 18 Count of cultivable bacteria in different soil groups: LCr - background Cr level, PCr -elevated Cr level of anthropogenic origin, NCr - elevated Cr level of natural origin 57
Figure 19 Linear correlation of cultivable bacteria counts and Cr content in soil samples with linear trendline and coefficient of determination R^2 58
Figure 20 Count of cultivable bacteria obtained by spread plate method on R2A agar with increasing Cr(VI) concentration: A - background Cr level, B - elevated Cr level of anthropogenic origin, C and D - elevated Cr level of natural origin 59
Figure 21 Percentage of chromate tolerant cultivable bacteria (percentage of cell count on medium with Cr(VI) relative to cell count on medium without Cr(VI) which represents 100%): A - average for each sample group: LCr - background Cr level, PCr - elevated Cr level of anthropogenic origin, NCr - elevated Cr level of natural origin; B – average for all samples 61
Figure 22 Percentage of Gram-positive and Gram-negative bacteria in different soil groups: LCr - background Cr level, PCr - elevated Cr level of anthropogenic origin, NCr - elevated Cr level of natural origin. 62
Figure 23 Linear correlation of Gram-positive bacteria percentage and Cr content in soil samples with linear trendline and coefficient of determination R^2 62
Figure 24 Chromate reduction of 33 environmental isolates - percentage of Cr(VI) reduced in M9 medium with a starting concentration of 0.5mM Cr(VI) after 24 hours of incubation 68
Figure 25 Chromate tolerance of 33 environmental isolates – percentage of growth on M9 medium with: A – 0.5 mM, B – 2 mM and C - 4 mM Cr(VI) compared to no Cr(VI) control....69
Figure 26 Linear correlation between percentage of Cr(VI) reduction and percentage of growth on Cr(VI) contacting media for environmental chromate tolerant isolates ...71
Figure 27 Colony morphology of chromate tolerant environmental *Bacillus cereus* group isolates: A – strain NCr1a, B -representative image of the colony morphology shared by the rest of the isolates. The scale bar represents 5 mm. 75

Figure 28 Phylogenetic tree based on 16S rRNA partial gene sequence, 588 positions showing the relationship of 13 chromate tolerant isolates to closely related *Bacillus cereus* group strains and other *Bacillus* reference strains. *Clostridium acetobutylicum* was used as an out-group. Constructed using Neighbor-Joining method, evolutionary distances were computed using the p-distance method, the bootstrap probabilities calculated from 1000 replications with values greater than 50% indicated at the nodes, the bar represents distance value calculated in MEGA7, GenBank accession no. indicated in brackets 77
Figure 29 Phylogenetic tree based on *pycA* gene partial sequence, 240 positions showing the relationship of 14 chromate tolerant isolates to closely related *Bacillus cereus* group strains and other *Bacillus* reference strains. *Clostridium acetobutylicum* was used as an out-group. Constructed using Neighbor-Joining method, the bootstrap probabilities calculated from 1000 replications with values greater than 50% indicated at the nodes, the bar represents distance value calculated in MEGA7, GenBank accession no. indicated in brackets........ 78
Figure 30 Illustration of biofilm formation potential testing on LB and M9 medium with different concentrations of Cr(VI) after 1 day (X marks faulty well)........ 80
Figure 31 Hexavalent chromium reduction of environmental chromate tolerant *Bacillus cereus* group strains in M9 medium with a starting concentration of 0.2 mM Cr(VI): A – reduction curve for strains with a lower reduction activity (<50 % reduced after 72h), B – reduction curve for strains with a higher reduction activity (>50% reduced after 72h), C – maximum relative growth - percentage of maximum OD600 in medium with Cr(VI) compared to no Cr(VI) medium........ 84
Figure 32 Hexavalent chromium reduction and growth curves with and without Cr(VI) during 72 h of incubation in M9 medium with starting concentration of 0.2 mM Cr(VI); left y axis – Cr(VI) concentration during the experiment expressed as the percentage of the starting concentration (red squares); right y axis – growth curve in medium with Cr(VI) (black triangles) and without Cr(VI) (green circles) measured as OD600 86
Figure 33 Linear correlation of Cr(VI) reduction and growth on Cr(VI) containing media for the *B. cereus* group isolates 87
Figure 34 Hexavalent chromium reduction ability and growth (relative to no Cr(VI) cultures) after 72 h of incubation in M9 medium with different starting Cr(VI) concentrations: A - 0.2 mM; B - 1 mM 88
Figure 35 Growth of chromate tolerant isolates NCr1a and PCr12 and reference *Bacillus* sp. strains measured as OD600 after 24h of incubation in M9 minimal medium with different concentrations of Cr(VI) (maximum relative growth - percentage of maximum OD600 in medium with Cr(VI) compared to no Cr(VI) medium) 89
Figure 36 Colony morphology of strain NCr1a on LB agar plates after different periods of incubation at 28°C (photographs on left are captured from above, photographs on right from below the plate, plate diameter is 90mm) 91

Figure 37 Colony morphology of strain NCr1a on LB agar plates after 2 (left) and 20 days of growth (right) on 28°C, streak inoculation (plate diameter is 90mm) 92
Figure 38 Colony morphology of strain NCr1a on oligotrophic medium R2A – formation of finer, thinner, more widely spreading filaments compared to those formed on nutrient rich LB agar..92
Figure 39 Growth of strain NCr1a in liquid culture - flakey growth with sedimentation indicating lack of motility: A – nutrient rich LB broth, B – minimal M9 medium without Cr(VI), C – minimal M9 medium with 0.2 mM Cr(VI)..93
Figure 40 Scanning electron micrographies of strain NCr1a (Photo: M. Bokorov) 94
Figure 41 Cell and endospore morphology of isolate NCr1a and reference strain *B. pseudomycoides* DSM 12442 observed by brightfield microscopy: A and B - Gram stained; C and D - endospore stained slides (Schaeffer-Fulton staining) (24 h culture on A and C, and 6 day culture on B and D); native slides of culture grown in sporulation medium DSM for 24h: E and F - *B. pseudomycoides* DSM 12442; G and H - isolate NCr1a (Photo E-H: I. Barák)..95
Figure 42 Morphological types of NCr1a cells: A - graphical illustration, B and C - brightfield microscopy micrographs (Photos and graphics: I. Barák and Z. Chromiková)..96
Figure 43 Distance tree of BLASTn search results for chromate transporter *chrA* gene sequence of strain NCr1a (constructed using Fast minimum evolution method after BLAST pairwise alignment) 97
Figure 44 Comparison of growth of strains in minimal M9 medium with different concentrations of Cr(VI): A – chromate resistant environmental isolate NCr1a; B – chromate sensitive laboratory strain *B. subtilis* PY79 99
Figure 45 Cellular chromium content of chromate resistant isolate NCr1a and control laboratory strain *B. subtilis* PY79 (BS) after 4h and 18h of growth in M9 minimal medium with 1 mM Cr(VI) as measured by scanning transmission electron microscopy coupled with energy dispersive spectroscopy (STEM-EDS): A – mean values with standard deviation, B – variability plot with raw data...............................100
Figure 46 STEM EDS images of cells grown for 4h in M9 medium with 1 mM Cr(VI): A – NCr1a, B – *B. subtilis* PY79, C and D –damaged/lysed cells of *B. subtilis* PY79 (HAADF images on the left, elemental maps on the right, Cr colored in purple)........................101
Figure 47 Influence of induction by subihibitory Cr(VI) concentration on Cr cell content of chromate resistant isolate NCr1a and laboratory strain *B. subtilis* PY79. Induced and uninduced cells were collected in exponential phase and exposed to 1 mM Cr(VI) for 1h in PBS...103
Figure 48 Growth of environmental chromate resistant isolate NCr1a and chromate sensitive laboratory strain *B. subtilis* PY79 (BS) in sporulation medium (DSM) with different concentrations of Cr(VI) 104
Figure 49 Chromium content in endospores after different treatments: A - NCr1a, exposure from start of growth, 6 days; B - NCr1a, exposure of mature spores, 1h; C –

B. subtilis PY79, exposure of mature spores, 1h; D - NCr1a, exposure of mature spores, 6 days; E – *B. subtilis* PY79, exposure of mature spores, 6 days 105

Figure 50 Localization of Ca and Cr in NCr1a endospore: A - STEM-EDS image showing Cr falsely colored in green, Ca in red, outer layers marked by a white arrow and core marked by a black arrow; B –Cr content measured in outer layer and core 106

Figure 51 Scanning transmission electron microscopy coupled to energy dispersive spectroscopy (STEM-EDS) images of endospores: A – NCr1a endospores formed in DSM with 1 mM Cr(VI), 6 days of incubation; B – NCr1a endospores formed without Cr(VI) and exposed to 1 mM Cr(VI) for 6 days; C – *B. subtilis* PY79 (BS) endospores formed without Cr(VI) and exposed to 1 mM Cr(VI) for 6 days. Elements false colored in EDS image: Ca green, Cr blue, Mn red 106

LIST OF TABLES

Table 1 General characteristics of chromium (Gray, 2018; Winter, 2018) 7
Table 2 Comparison of Cr(III) and Cr(VI) characteristics (created based on data in Independent Environmental Technical Evaluation Group 2004) 7
Table 3 Overview of some of the chromate tolerant bacterial isolates described in published studies 22
Table 4 Soil sample groups for cultivable soil bacterial community analyses 27
Table 5 Overview of methods used for physical and chemical characterization of samples 34
Table 6 Preparation of standard Cr(VI) solutions used for DPC test 41
Table 7 Testing of heavy metal tolerance of chromate resistant strains: overview of metal forms and concentrations tested 45
Table 8 Chemical characteristics of soil samples used in the cultivable soil bacterial community analyses 53
Table 9 Description of sampling locations: LCr – low background Cr level, PCr - elevated Cr level of anthropogenic origin, NCr - elevated Cr level of natural origin 54
Table 10 Results of the additional chemical analyses of soil samples with elevated Cr level of natural origin (mg/kg) 55
Table 11 Chromate tolerance of cultivable soil microbial communities determined as the ability to grow upon successive transfers in medium containing increasing Cr(VI) concentrations 55
Table 12 Cultivable bacteria counts in different soil groups: LCr - background Cr level, PCr -elevated Cr level of anthropogenic origin, NCr - elevated Cr level of natural origin 57
Table 13 Linear correlation of cultivable bacteria counts (CFU/g), percentage of chromate tolerant cultivable bacteria and Gram-positive bacteria and soil chemical and physical properties determined by Pearson correlation coefficients (r) 58
Table 14 Percentage of cultivable soil bacteria tolerant to various Cr(VI) medium concentrations in soil samples of different groups 60
Table 15 Overview of environmental sample groups collected for the purpose of the isolation of chromate tolerant bacterial strains 64
Table 16 Chromate tolerant bacterial strains isolated from environmental samples with high and low Cr level: overview of isolated strains with the general characteristics of the source of isolation 65
Table 17 Summary of chromate tolerant strains isolated from the environmental samples with different concentration of Cr 66
Table 18 Chromate tolerance and reduction of the 33 environmental bacterial isolates 70

Table 19 List of chromate tolerant *Bacillus cereus* group strains selected for detailed analysis, with the characteristics of the source of isolation 72
Table 20 Interpretation of reliability of MALDI TOF analysis results (Bruker Daltonics Inc., 2018) 74
Table 21 Results of MALDI TOF analysis of chromate tolerant *Bacillus cereus* group isolates and legend for the interpretation of the results according to the manufacturer instructions 74
Table 22 Biochemical and cultural characteristics of chromate tolerant *Bacillus cereus* group isolates 76
Table 23 Selected highest hits recorded on BLASTn search of *pycA* gene sequence of environmental chromate tolerant *Bacillus cereus* group strains (hits with the highest score value shown) (database nucleotide collection nr/nt) 79
Table 24 Biofilm formation potential of chromate tolerant *Bacillus cereus* group strains in various concentrations of Cr(VI) (mM) in LB and M9 medium 81
Table 25 Heavy metal resistance of chromate tolerant *Bacillus cereus* group isolates ... 82
Table 26 Minimal inhibitory concentration of Cr(VI) (mM) for selected chromate resistant *Bacillus cereus* group strains determined on complex nutrient-rich (LB broth) and defined minimal (M9 and acetate minimal medium (AMM)) media 83
Table 27 Comparison of chromate reduction in different assays with starting Cr(VI) concentrations of 0.2 and 0.5 mM 87
Table 28 Chromium cell content in environmental chromate resistant isolate NCr1a and control laboratory strain *B. subtilis* PY79 (BS): A - cells grown in M9 medium with 1 mM Cr(VI) for 4 or 18h (until exponential or stationary phase); B - uninduced or induced cells exposed to 1 mM Cr(VI) for 1h 103
Table 29 Chromium content (at %) in endospores of NCr1a and *B. subtilis* PY79 105

LIST OF ABBREVIATIONS

AMM	Acetate minimal medium
at%	Atomic percent
BC	*Bacillus cereus* ATCC 14579
BOD	biological oxygen demand
BP	*Bacillus pseudomycoides* DSM 12442
BS	*Bacillus subtilis* PY79
BSI	British Standards Institution
CB	cultivable bacteria
CIME	Interdisciplinary centre for electron microscopy (CIME) at the Ecole polytechnique federale de Laussane, Lausanne, Switzerland
COD	chemical oxygen demand
CrCB	chromate tolerant cultivable bacteria
DNA	deoxyribonucleic acid
DPC	diphenylecarbazide
DSM	difco sporulation medium
EC	*Escherichia coli* ATCC 25922
EDS	Energy-dispersive X-ray spectroscopy
EPA	United States Environmental Protection Agency
EPFL	Ecole polytechnique federale de Laussane, Lausanne, Switzerland
EPS	extracellular polymeric substances
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
HAADF	high-angle annular dark-field
HPC	heterotrophic plate count
IARC	International Agency for Research on Cancer
IBA	Important Bird and Biodiversity Area
IMB	Institute of Molecular biology, Slovak Academy of Science's, Bratislava, Slovakia
ISO	International organization for standardization
IUCN	International Union for Conservation of Nature
LB	lysogeny broth
LBA	lysogeny broth agar
LCHR	long chain chromate transporters
LCr	samples with low (background) Cr concentration
MALDI TOF	matrix-assisted laser desorption/ionization time-of-flight
MIC	minimal inhibitory concentration
MS	mass spectrometry
NCr	samples with high Cr concentration of natural origin
OD600	optical density measured at a wavelength of 600 nm
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCr	samples with high Cr concentration of anthropogenic origin
PVA	polyvinyl alcohol
ROS	reactive oxygen species

SCHR	short chain chromate transporters
SEM	scanning electron microscopy
STEM EDS	scanning transmission electron microscopy coupled to energy dispersive X-ray spectroscopy
TEM	transmission electron microscopy
USA	United States of America
USEPA	United States Environmental Protection Agency

1. INTRODUCTION

Chromium (Cr) is a heavy metal used in an array of industrial applications and, as a result, is a common pollutant of soil, sediment, surface and ground water. In the environment, Cr is mostly present in the two stable oxidation states: hexavalent [Cr(VI)] and trivalent [Cr(III)]. Naturally occurring Cr (e.g., serpentine soils derived from ultramafic rocks) is almost exclusively found in trivalent form, while anthropogenic pollution releases hexavalent Cr compounds. Trivalent Cr has very low bioavailability in the environment and shows no or little effect on living organisms, as it forms insoluble hydroxides ($Cr(OH)_3$) and complexes with organic ligands at environmental pH values (Bencheikh-Latmani et al., 2007a). In contrast, hexavalent Cr has notable toxic, mutagenic and teratogenic effects and is classified as a group 1 human carcinogen (International Agency for Research on Cancer, 2012). Hexavalent chromium is highly water soluble, readily bioavailable and easily transported across the cell membrane into the cytoplasm of the cell, which results in a strong toxic effect, mainly through oxidative stress (Ramírez-Díaz et al., 2008).

Certain microorganisms have the ability to not only to grow in the presence of high chromium concentrations, but also to reduce highly toxic hexavalent to the more innocuous trivalent form. Such strains can be employed in bioremediation of contaminated environments in an environmentally friendly and cost-effective way. Lower cost of bioremediation compared to other remediation methods is especially important for less developed countries, in which Cr pollution is a major problem due to the use of obsolete or inadequate methods of industrial production and poor waste management practice. For the successful use of microorganisms in bioremediation, a detailed knowledge of their biology, physiology and metal tolerance mechanisms is of paramount importance.

Effect of chromium on the microbial community of soil and aquatic habitats is less researched compared to the chromium effect on pure cultures of microorganisms (e.g. Cheung and Gu, 2007; Ramírez-Díaz *et al.*, 2008; Dhal *et al.*, 2013; Ahemad, 2014). Current knowledge on the microbial community response to the presence of Cr is mainly based on more or less short term experiments in microcosm conditions. Although results obtained in microcosm experiments give controllable and replicable results, they cannot completely replicate long term chromium exposure which is the norm in an actual field conditions. Therefore, there is an evident need for further studies of microbial community response to Cr in the environmental field conditions.

Long term chromate exposure presumably selects and enriches members of the microbial community that possess high chromate tolerance. Therefore, polluted sites are considered as promising source of bacterial strains which harbour high chromate tolerance. In fact, a majority of studies have been focused on contaminated sites for the isolation of chromate resistant bacteria (e.g. Alam et al., 2011; Camargo et al., 2005; Patra et al., 2010). However, isolation of chromate tolerant strains from unpolluted and pristine environments is also possible (Wani et al., 2007), but scarcely conducted. Therefore, it is possible that chromate tolerance occurs amongst bacteria without

selective pressure for Cr resistance, but also that elevated Cr levels lead to a significant enrichment in chromate tolerant strain proportion.

Investigation of chromate resistance mechanisms and their genetic regulation has been primarily conducted in Gram-negative bacteria such as *Pseudomonas* spp. (Ackerley et al., 2004b; Alvarez et al., 1999), *Shewanella oneidensis* (Middleton et al., 2003), *Cupriavidus metalidurans* (Nies and Silver 1989) and *Ochrobactrum tritici* (Branco et al., 2008), but not in Gram-positive bacteria. This is despite the fact that some studies have suggested that chromate tolerant strains isolated from chromium polluted sites were predominantly Gram-positive bacteria (Branco et al., 2005; Caliz et al., 2012; Viti and Giovannetti, 2001). Furthermore, Gram-positive bacteria are an abundant and important group within the soil microbial community and the ability of some to form endospores enables them survival even in the harshest environmental conditions. Thus, studies of chromate resistance mechanisms in Gram-positive bacteria, including endospore-forming species could provide new valuable insights into bacterial chromium homeostasis.

All things considered, it currently remains to be elucidated how wide spread and which chromate resistance mechanisms are present in the environmental bacteria. Also, long term response of microbial communities to chromium is scarcely studied, while pure culture response is conducted in detail mainly in Gram negative bacteria and bacteria isolated from polluted sites. Clarification of chromate response of microbial community and environmental isolates is of great scientific, as well as of practical bioremediation importance.

2. LITERATURE REVIEW

2.1. GENERAL CHARACTERISTICS OF CHROMIUM

Chromium (Cr) is a metallic pollutant severely hazardous to the environmental and human health, especially in its hexavalent form. Due to its widespread use in diverse industries it is one of the most voluminous pollutants of the environment. Due to its severe negative effects hexavalent chromium is on the US EPA (United States Environmental Protection Agency) list of priority pollutants (United States Environmental Protection Agency, 2014).
Chromium is a transition metal of the group 6 of periodic system. It is shiny, gray, hard and brittle. It was first isolated in 1798 by the French chemist Nicholas-Louis Vauquelin, by heating chromium oxide (Cr_2O_3) with charcoal, whereby reducing chromium to its elemental state. Because of intensive and striking colors of its compounds, Vanquelin named it chromium from Greek word χρωμα (chrôma) meaning color (Motzer, 2005) (Figure 1).
It is found in a wide range of oxidative states from -2 to +6 (Table 1). Oxidative states -2, -1, 0, i +1 are primarily found in synthesized compounds. In environmental conditions it is found only in three states: 0, +3, +6, with two former being the most common. Trivalent and hexavalent forms are characterized by significant differences in charge, physico-chemical properties, solubility, mobility, chemical and biochemical behavior, bioavailability and toxicity (Stanin, 2005) (Table 2).
Trivalent chromium [Cr(III)], the most prevalent form in the natural environment and minerals, is less toxic and hydrosoluble compared to hexavalent form (Motzer, 2005). It easily forms complexes with organic compounds and precipitates in the form of hydroxides, non-dissolvable oxides and sulfates. Contrary, **hexavalent chromium [Cr(VI)]**, is derived from anthropogenic sources and is much more hydrosoluble, bioavailable, mobile, and has strong acute and chronic negative effects on health including carcinogenic, teratogenic, mutagenic, and diverse acute effects (Guertin, 2005; Nickens et al., 2010).

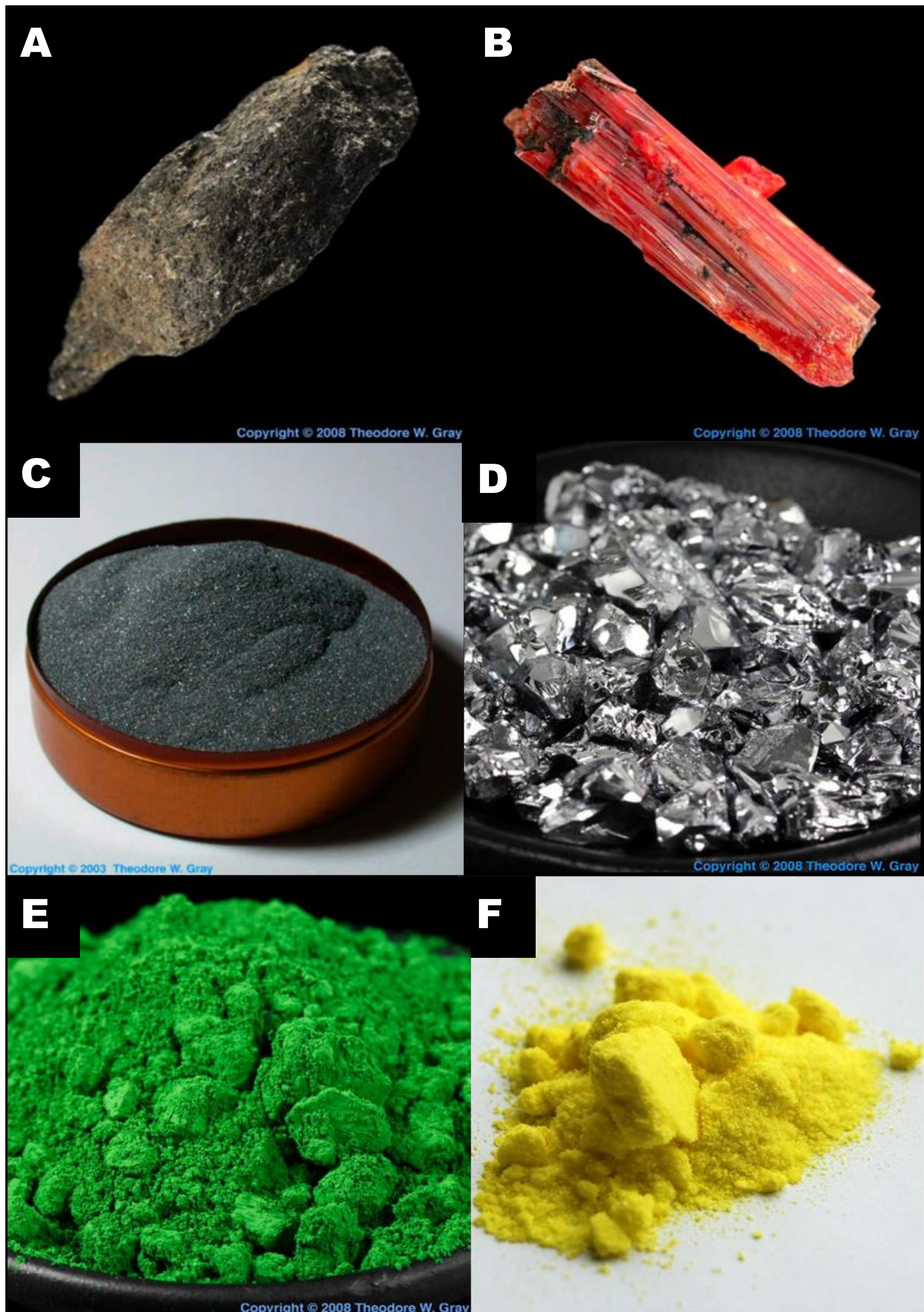

Figure 1 Physical appearance of different chromium forms: a - chromite mineral ($FeCr_2O_4$), b – crocoite mineral ($PbCrO_4$), c - metal powder of elemental chromium; d - high purity chromium crystals ; e - chromium oxide (Cr_2O_3), f – potassium chromate (K_2CrO_4) (sources: a-e Gray 2018, f - Anger et al. 2000)

Table 1 General characteristics of chromium (Gray, 2018; Winter, 2018)

Atomic number	24
Atomic weight	51.9961
Density	7.14 g/cm3
Melting point	1907 °C
Boiling point	2671 °C
Group	6
Period	4
Color	Silver
Electronic configuration	$[Ar]3d^5 4s^1$
Abundance in Earth's Crust (%)	0,014%
Solubility	Oxidation state 0 – nonsoluble in water, soluble in diluted HCl and H_2SO_4, nonsoluble in HNO_3 and aqua regia
Cas. No	7440-47-3
Oxidation state	-2 to +6

Table 2 Comparison of Cr(III) and Cr(VI) characteristics (created based on data in Independent Environmental Technical Evaluation Group 2004)

Characteristic	**Cr(III)**	**Cr(VI)**
Toxicity	Non toxic or low toxicity	Highly toxic
Solubility	Forms compounds with low solubility	Forms easily soluble compounds
Mobility	Immobile in environment	Mobile in environment
Transport into cell	Does not cross cell membrane easily	Easily crosses cell membrane
Bioavailability	Low bioavailability	High bioavailability
Industrial sources	Textile industry (textile printing and coloring) and leather tanning	Metal galvanization (plating) (chromic acid H_2CrO_4), dyes and pigments, wood preservation
Natural sources	Plentiful, chromite mineral	Rare, crocoite mineral

NATURAL SOURCES OF CHROMIUM

World average Cr soil concentration is 200 mg/kg, according to data given in Motzer 2005. However, concentration can vary widely at individual sites. For instance, USA (United States of America) average is 14-70 mg/kg, while individual sites can hold from 1 to 1000 mg/kg (EPA, 1984). Canada’s average is 43 mg/kg (EPA, 1984), while European top soil background average concentration is 21.72 mg/kg (Tóth et al., 2016).

Content in groundwater is dependent on surrounding soil content and in average is less than 1 μg/l (Motzer, 2005). Granites, carbonates and sandy sediment contain the lowest concentrations of chromium. Chromium is naturally found in certain types of parent rocks, such as ultramafic rocks. Ultramafic rocks contain low levels of Si (<45%), Mg, K, and high level of FeO and MgO (>18%). ***Serpentine soils*** are formed on this type of parent rock and are characterized by a high level of Cr and Ni, which can be so elevated in certain locations to cause toxicity to plants and formation of specific vegetation types (e.g. Appalachian Mountains, ultramafic forests of Mount Kinabalu in Malasya, tropical forests of New Caledonia). The most important trivalent Cr ore is ***chromite*** - magnesium-iron-chromium–aluminum oxide $[(Mg,Fe^{2+})(Al,Cr,Fe^{3+})_2O_4]$ – or shortly $FeCr_2O_4$ in which Cr content varies from 15-65% (Motzer, 2005). Main ore deposits are South African Republic, Kazakhstan, India and Turkey, with great majority (up to 95 %) being mined in South African Republic and Kazakhstan. In 2016, leader in production was South African Republic with around 14,000,000 metric tons of chromite ore, which was 48 % of the total world production (U.S. Department of the Interior and U.S. Geological Survey, 2017). The most important hexavalent Cr mineral source is ***crocoite*** - $PbCrO_4$, whose deposits are found in Russia, Brazil, USA and Tasmania (Winter, 2017).

INDUSTRIAL USES OF CHROMIUM

Chromium is one of the most important metals for industrial applications, and its wide use made it the most voluminous metallic pollutant on the Earth with approximately 4,500 kg/day discharged into the environment (Merian, 1984). Leather tanning industry in India alone releases 17,250 to 26,910 tons of Cr into the waste waters, with 45 % of it being in highly toxic hexavalent form (Iyer and Mastorakis, 2006). Total air emission in China in period 1990-2009 was 1.92×10^5 tons, mainly from coal and oil combustion, while total emission to water was 1.34×10^4 tons (Cheng et al., 2014).

The main uses for Cr are in metallurgical (67%), refractory (18%) and chemical industry (15%) (Testa, 2005).

In **metallurgical industry** chromium is an important component of stainless steels and various metal alloys. In fact, majority of Cr industrial application (around ¾) is for alloy manufacture. Chromium in Fe alloys is insoluble with a zero oxidation state and therefore has no environmental significance. However, it can be oxidized and leached from stainless steel into a water-soluble form which can affect the environment and the living organisms (Kimbrough et al., 1999).

Refractory uses of chromium include magnesite-chrome firebrick for metallurgical furnace linings and granular chromite for various other heat-resistant applications.

In the **chemical industry**, chromium is used primarily in chrome plating, leather tanning, paint pigments (chromium compounds can be red, yellow, orange, and green), and wood treatment, while smaller amounts are used for production of catalysts, copy

machine toners, corrosion inhibitors, drilling muds, magnetic tapes, photographic chemicals, safety matches, and in water treatment (Agency for Toxic Substances and Disease Registry, 2008).

Overview of the industrial flow of chromium compounds is given in Figure 2. The industrial use of chromium generally begins with the mining of naturally-occurring chromite ore, usually found in the form of iron(II)chromite ($FeO•Cr_2O_3$ or $FeCr_2O_4$). The ore is either oxidized or reduced during industrial processing, depending on the desired product. The most prevalent products of chromite oxidation are sodium chromate (Na_2CrO_4) and sodium dichromate ($Na_2Cr_2O_7$). Sodium carbonate (Na_2CO_3), calcium oxide (CaO), and calcium chromate ($CaCrO_4$) are obtained as byproducts. Than, Na_2CrO_4 serves as a starting material for many derivatives, such as dichromates ($Na_2Cr_2O_7$ or $K_2Cr_2O_7$), chromium (VI) oxide (CrO_3), chromic acid (H_2CrO_4) and various chromates (potassium, barium, calcium, lead, strontium, and zinc chromate). Reductive processing of chromite ore is conducted by a variety of methods using aluminum, silicon, or carbon as reducing agents. Materials obtained by reduction are used for production of Cr alloys and chrome alum (Stanin, 2005).

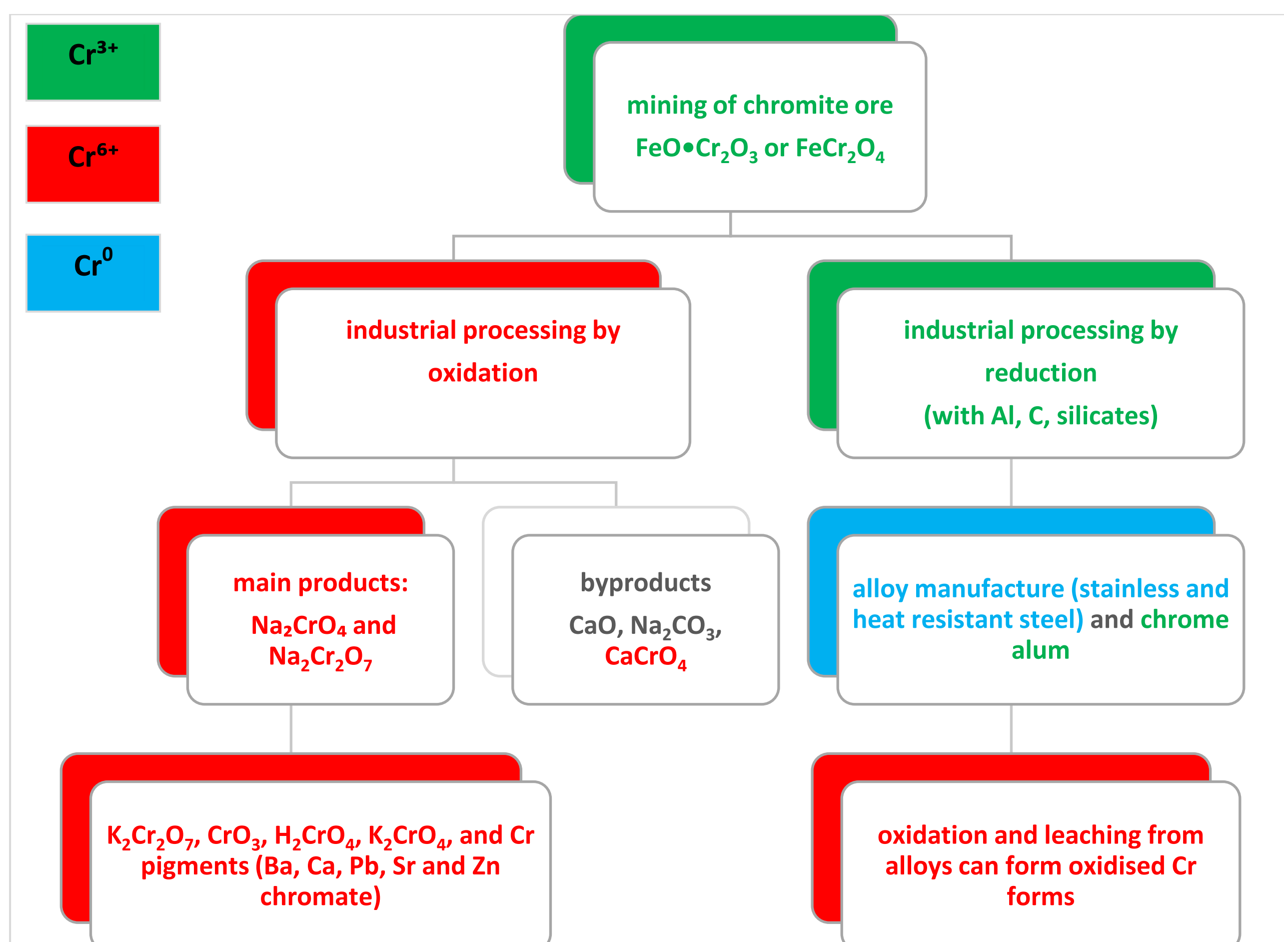

Figure 2 Overview of chromium flow in industrial applications: green – trivalent chromium forms, red – hexavalent chromium forms, blue – zero valence chromium forms (illustration based on data in Stanin 2005)

Chromium pollution can be atmospheric (emissions in air, emissions from storage areas and waste piles), but mostly it is a consequence of solid waste (solid wastes from production, sludge, tailings, slag, plating wastes) or wastewater emissions (Stanin, 2005; Testa, 2005).

Hexavalent Cr compounds are primarily used in metal galvanization (plating) (chromic acid H_2CrO_4), in dyes and pigments and wood preservation. Trivalent Cr is mostly used in textile industry (textile printing and coloring) and leather tanning. Consequently, hexavalent chromium will be released by metallurgical, plating and refractory industries, and pigment production and application (chromate color pigments and corrosion inhibition pigments). Trivalent chromium will be found in wastewaters of leather tanneries, textile printing and dying and decorative plating industries. It should be noted, however, many factors can influence ratio and amount in which Cr(VI) and Cr(III) will be found in wastes (e.g. chromium compound used, pH, Eh, presence of other organic and inorganic compounds). For instance, different chemicals and processes in leather tanning such as sun drying and boiling can lead to oxidation of Cr(III) to Cr(VI) and result in higher amount of Cr(VI) in waste water and other production waste materials. Similarly, Cr(VI) can be reduced to Cr(III) in certain conditions. Other processes involved in distribution of Cr(III) and Cr(VI) include sorption, precipitation and dissolution (Stanin, 2005).

CHEMISTRY AND GEOCHEMISTRY OF CHROMIUM

Chromium can react with non-metals (oxygen, fluorine, and chlorine) or anions such as nitrate, sulfate, and form compounds more or less dissolvable in water. More common are Cr(III) compounds such as tribromide (non-soluble), chromium nitrate (soluble), Cr hydroxides (non-soluble), chromium (III) oxide (non-soluble). In chemical production the most common starting compound is sodium chromate or dichromate, from which further compounds are synthesized (chromic acid, chromium oxides, potassium dichromate) (U.S. Department of the Interior and U.S. Geological Survey, 2017).

Cr(III) has a similar ionic radius (0.064nm) as Fe^{3+} (0,060nm) and Al^{3+} (0,050nm), and often substitutes them in crystal structures of soil. Similarly, chromate CrO_4^{2-} and dichromate $Cr_2O_7^{2-}$ anions can be substituted for sulfate anion SO_4^{2-} which has similar radius (Robertson, 1975).

In general terms, high pH and Eh values favor the presence of hexavalent chromium forms, while low pH and Eh values favor trivalent chromium forms, as can be seen from a Eh-pH diagram (Figure 3).

Trivalent chromium is highly stable in the usual environmental conditions, encompassing wide range of Eh and pH values under normal atmospheric parameters (25°C, 0,987atm) in system Cr-O-H. Hexavalent chromium has much narrower zone of stability both in Cr-O-H and Cr-H_2O-O systems. It forms primarily under oxidizing

(Eh>0) and alkaline (pH>6) conditions. In natural environment, Cr(VI) is never present as a free cation Cr^{6+}, but as chromate and dichromate anions.
Cr(VI) is easily desorbed from contaminated soil, while Cr(III) is mostly in forms not easily desorbed (Davis and Olsen, 1995). Specifically, Cr(III) is quickly, strongly and specifically sorbed to Fe and Mn oxides in soil, as well as to clay minerals and sand. Sorption of Cr(III) is fast (90% is sorbed after 24h on minerals of clay and Fe oxides), increases with pH (Griffin et al., 2008; Rai et al., 1984) and organic matter content (Paya Perez et al., 1988). Organic matter also quickly adsorbs and reduces Cr(VI) to Cr(III). Hexavalent chromium will stay mobile only if its concentration surpasses adsorbing and reducing soil capacity (Bartlett and James, 1979). Kinetic rates are much higher for reduction to trivalent state even in anaerobic conditions, while oxidation requires much more time as determined by kinetic experiments in water, sediment and soil (Saleh et al., 1989). Agents which can lead to oxidation of Cr(III) to Cr(VI) in natural environment are dissolved O_2 and MnO_2, whereby Mn oxides have more pronounced role, while effect of dissolved oxygen is negligible (Eary and Rai, 1987; Johnson and Xyla, 1991; Schroeder and Lee, 1975).

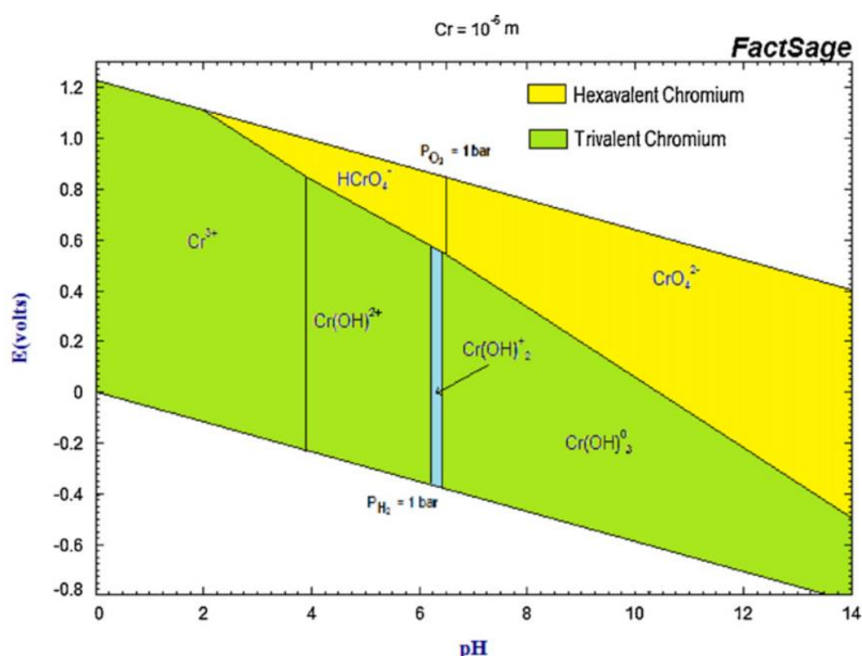

Figure 3 Chromium electrochemical behavior at different pH and redox potential values - Eh-pH diagram for the system $Cr-O_2-H_2O$, for the concentration of Cr = 10^{-6} mol/kg at solid/liquid boundaries (from Dhal et al. 2013)

In aquatic environment, Cr(III) easily forms complexes with numerous inorganic ligands, while Cr(VI) is found in monomeric forms H_2CrO_4, $HCrO_4^-$, CrO_4^{2-}, CrO_3, $Cr_2O_7^{2-}$. Cr(III) ligands include hydroxide (OH^-), sulfate (SO_4^{2-}), ammonium (NH^{4+}), cyanide (CN^-) and sulphocyanide (SCN^-), fluoride (F^-) and chloride (Cl^-) (to a lesser extent), and natural and synthetic ligands (Richard and Bourg, 1991). At low Eh, Cr(III) is dominantly found as Cr^{3+} and $Cr(OH)_2^+$ (Richard and Bourg, 1991). All forms of Cr(VI)

are soluble in water at all pH values. Anions can form salts with certain cations (Ba^{2+}, Sr^{2+}, Pb^{2+} Zn^{2+}, Cu^{2+}), with varying degree of solubility depending on pH (Rai et al., 1987).

CHROMIUM TOXICITY

Toxicity to microorganisms is mostly realized as a consequence of intracellular reduction of Cr(VI) to Cr(III). Hexavalent chromium easily crosses cell membrane and enters cells. Inside of the cell it is specifically or non-specifically reduced to Cr(III) by enzymes or reducing biomolecules. Reduction occurs through a number of steps, generating significant amount of unstable intermediary products and ROSs. Formed Cr(III) can bind to functional groups of biomolecules such as sulfhydryl groups of proteins and phosphate groups of nucleic acids, altering their structure, activity and function. Toxicity of Cr(III), as it cannot be easily transported into the cell, is mostly linked to its precipitation on the surface of the cell, leading to the loss of function and permeability of the cell membrane.

After entering cytoplasm, Cr(VI) can be reduced by a multitude of reducing compounds: NADH, NADPH, $FADH_2$, pentose, cysteine, antioxidants such as ascorbate or glutathione, glutathione reductase etc. (Ahemad, 2014). Reduction produces unstable intermediates Cr^{5+} and Cr^{4+} and ROSs. The resultant intermediates cause oxidative damage to proteins and DNA by forming a range of DNA lesions, together with Cr-DNA adducts, DNA-DNA cross-links, DNA-protein cross-links (Cheung and Gu 2007; Nickens et al. 2010). Cr^{5+} and Cr^{4+} have been shown to play a part in carcinogenicity of Cr(VI) (Salnikow and Zhitkovich, 2008) and apoptosis (Ye et al., 1999). Positively charged Cr^{3+} and Cr^{4+} form electrostatic interactions with phosphate groups of DNA molecules, inhibiting DNA replication and leading to more errors during transcription (Bencheikh-Latmani et al. 2007a; Cervantes and Campos-Garcia 2007; Plaper et al. 2002; Ramírez-Díaz et al. 2008). Cr^{3+} reacts with carboxyl and thiol groups of enzymes influencing their structure and activity (Cervantes et al. 2001).

Health effects on humans. Toxicity of Cr(VI) to humans is mainly linked to occupational exposure. Main routes of exposure are inhalation, ingestion and dermal contact. Acute toxic effects have been registered among workers of industries which use Cr compounds: skin irritation, ulcers, irritation of respiratory system, ulceration and perforation of nasal septum. Hexavalent chromium does not pose risk if ingested in small amounts as it quickly gets reduced to Cr(III) by intestinal juices, which is than excreted by feces. However, ingestion of >1-5 g inflicts serious gastrointestinal disorders, cramping, convulsions, hemorrhage, and even death due to the cardiovascular shock (Motzer, 2005). Hexavalent chromium is a Group 1 human carcinogen according to IARC (International Agency for Research on Cancer). Contrary to Cr(VI), Cr(III) is not considered to be notably toxic to humans and is not considered

carcinogenic (International Agency for Research on Cancer, 2012). Essentiality of Cr as a nutrient for humans is not under scientific consensus. Chromium was proposed as an essential element in 1959 (Schwarz and Mertz, 1959). However, failure to identify the any biomolecules that contain chromium and their mode of action resulted in questioning of its status in recent years (Di Bona et al., 2011; Jeejeebhoy, 2009; Vincent, 2013). The European Food Safety Authority does not recognize Cr as an essential nutrient (EFSA, 2017), while the authorities of the United States of America do (Institute of Medicine, 2001).

2.2. MECHANISMS OF CHROMIUM RESISTANCE IN MICROORGANISMS

Terms resistance and tolerance to metals are mostly viewed as synonyms. However, Gadd (1992) makes distinction among these terms. According to this author, ***heavy metal resistance*** is an ability of microorganism to survive toxic effects of heavy metal with detoxifying mechanisms, which are expressed as a response to metal exposure. Contrary, ***heavy metal tolerance*** is an ability of microorganism to survive heavy metal toxicity with the use of constitutive characteristics, which are expressed regardless of metal exposure (e.g. cell wall impermeability, extracellular polymeric substances, metabolite excretion). Thus, to differentiate between these terms it is necessary to know the exact mechanisms involved in metal homeostasis of a particular organisms. As this is rarely the case, it is difficult to rightfully follow these definitions. Consequently, we also will not make a distinction between the two terms in this thesis.
Several classifications of mechanisms of chromate resistance/tolerance in microorganisms have been proposed. Here we will list mechanisms of chromate transport, toxicity and resistance in bacterial cells proposed by Ramírez-Díaz *et al.* (2008) (Figure 4):

1. Decreased chromate uptake
2. Reduction of Cr(VI) to Cr(III) (extracellular and intracellular)
3. Chromate efflux
4. DNA repair
5. Protection against oxidative stress

DECREASED CHROMATE UPTAKE

Chromate anion, due to its structural similarity to sulfate anion, can use sulfate transport systems to enter the cell (Cervantes & Campos-García, 2007). If mutation in sulfate transport genes occurs, it will lead also to decreased uptake of chromate (Brown et al., 2006; Hu et al., 2005; Ramírez-Díaz et al., 2008).

Reduction of Cr(VI) to Cr(III)

Among all the mechanisms, reductive immobilization has the greatest significance in a practical sense, as it can be employed for bioremediation through reduction of highly toxic Cr(VI) to more innocuous Cr(III).

Hexavalent chromium has a high tendency to be reduced in the common environmental conditions. Thus, in the case of sporadic and low level pollution with Cr(VI), soil, sediment or water receiving pollution will have sufficient reductive capacity to facilitate instant reduction to Cr(III). However, in cases of prolonged pollution with hexavalent chromium compounds, reducing capacity of the environment will be surpassed and increasing amounts of hexavalent chromium will accumulate. Consequently, Cr(VI) will express its toxic effects on the biota. Tolerant members of the community will survive, while sensitive ones will decrease in abundance or take refuge in the microenvironments with locally low Cr(VI) level.

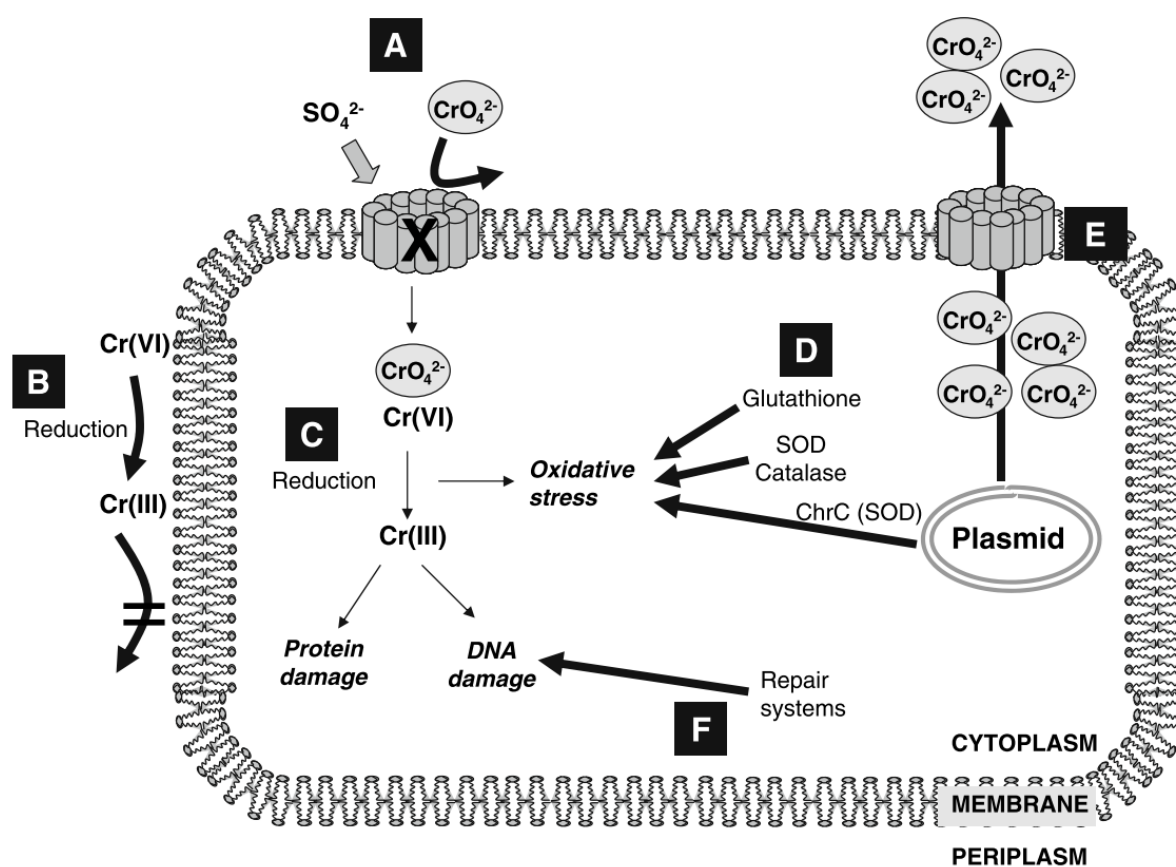

Figure 4 Mechanisms of chromate resistance in microorganisms: A – decreased chromate uptake due to the decreased transport through shared sulfate uptake transporters (caused e.g. by mutation in sulfate uptake channel genes), B – extracellular chromate reduction, C – intracellular chromate reduction, D – protection against oxidative stress, E – increased chromate efflux, F – DNA damage repair (source: Ramírez-Díaz et al., 2008)

Reductive immobilization of Cr(VI) to Cr(III) by microorganisms is realized through one or the combination of the following mechanisms (Cervantes and Campos-Garcia 2007):

a. in aerobic conditions by soluble chromate reductases using NADH or NADPH as cofactors;
b. in anaerobic conditions where Cr(VI) serves as an electron acceptor in electron transport chain (e.g. *Desulfotomaculum reducens* or *Shevanella reducens*); and
c. unspecific reduction by reducing compounds in intracellular or extracellular environment (e.g. amino acids, nucleotides, sugars, organic acids, vitamins, glutathione, etc.).

Mechanism of aerobic Cr(VI) reduction in the presence of oxygen usually encompasses two or three steps with initial reduction of Cr(VI) to unstable intermediates Cr^{5+} and/or Cr^{4+}, before further reduction to stable end product Cr(III) (Cheung and Gu 2007). Different reduction enzymes generate varying amounts of ROSs depending on the reduction pathway. For example, chromate reductase ChrR reduces Cr(VI) to Cr^{5+} by one electron transfer followed by two electron transfer from Cr^{5+} to form Cr(III). Cr^{5+} is highly reactive and oxidizes back to Cr(VI) with the generation of significant amount of ROSs. Some flavoproteins with one-electron transfer reduction are glutathione reductase, lipoyl dehydrogenase and ferredoxin-NADP oxidoreductase. Bacteria containing such enzymes are not appropriate for bioremediation as such reduction pathway produces too much oxidative damage to the cell and Cr(III) is not its end product (Ackerley et al., 2004b). Contrary, soluble flavoprotein YieF from *E. coli* reduces Cr(VI) to Cr(III) directly without redox cycling by presumed four-electron transfer, with notably lower level of generated oxidative stress, thus causing less chromate toxicity. Three electrons are consumed in Cr(VI) reduction, while one is bound to O_2 to produce H_2O_2 (Ackerley et al., 2004a).

Chromate reduction can be discussed based on the localization (intra/extracellular) or based on the mode of reduction (aerobic/anaerobic/indirect).

Intracellular chromate reduction can be realized by intracellular compounds with reductive power such as NADH/NADPH, flavoproteins, glutathione, ascorbic acid, etc. It is damaging to the cell as ROSs are formed in the process of reduction. Therefore, additional mechanisms of DNA repair and protection from ROSs need to be employed to counteract the negative consequences of the intracellular reduction.

Extracellular chromate reduction occurs outside of the cell by enzymes and compounds released from the cell or on the surface of the cell. Excretion of enzymes outside of cell is an energy consuming process. Thus, chromate reducing extracellular enzymes are excreted only if Cr(VI) is present in the environment. For the cell, extracellular reduction is far less damaging than intracellular. Cr(III) formed outside of cell can be precipitated on the surface of the cell, on peptidoglycane components of the cell wall (Thatoi et al., 2014).

Aerobic reduction is cometabolic process meaning microorganism does not gain energy from pollutant degradation, but is degraded as a side reaction, while enzymes or compounds involved, have another primary function (Thatoi et al., 2014). In such process bacteria use a carbon substrate as an electron donor and oxygen as an electron acceptor. Aerobic reduction is mostly linked to soluble proteins, which use NADH or NADPH as electron donors.

Anaerobic reduction presumes use of Cr(VI) as an electron acceptor in the electron transport chain in the absence of oxygen. It can be realized by soluble and membrane-bound enzymes (e.g. flavin reductases, cytochromes and hydrogenases, that can be part of electron transport system) (Thatoi et al., 2014).

Some bacteria can employ ***both aerobic and anaerobic process***. For instance, *P. fluorescens* in anaerobic conditions can use acetate as an electron donor for Cr(VI) reduction, while in aerobic conditions it can use a number of other donors (Bopp and Ehrlich, 1988).

Indirect Cr(VI) reduction **by iron reducing bacteria (IRB) and sulfate reducing bacteria (SRB).** IRB and SRB produce Fe(II) and sulfides, which reduce Cr(VI) even up to 100 faster compared to chromate reducing bacteria (Joutey et al., 2015). SRB produce H_2S which reduces Cr(VI) in three phases: 1) sulfate reduction to sulfides, 2) reduction of Cr(VI) to Cr(III) by sulfides and 3) Cr(III) precipitation by sulfides (Fude et al., 1994; Humphries et al., 2006; Qian et al., 2016). IRB reduce Fe(III) to Fe(II), which then reduces Cr(VI) to Cr(III) (Peng et al., 2015; Wielinga et al., 2001).

Chromate efflux

As mentioned before, chromate anion is structurally and chemically similar to sulfate anion, thus chromate and sulfate share same uptake pathways (Ramírez-Díaz et al., 2008). Chromate efflux is a widespread and efficient resistance mechanism, which prevents accumulation of chromate inside the cell. It has been extensively characterized in *Pseudomonas aeruginosa* (Cervantes & Campos-García, 2007). Plasmid pUM505 of this strain contains genes that code ChrA protein responsible for chromate resistance. ChrA functions as a chemiosmotic pump extruding chromate anion outside of the cytoplasm (Alvarez et al., 1999).

Transposone Tn*OtChr of* chromate resistant strain *Ochrobactrum tritici* 5bvl1 contains genes *chrA*, *chrB*, *chrC* and *chrF.* For establishing chromate resistance *chrA* and *chrB* genes are essential. Promotor of this transposone is upregulated by Cr(VI), while it is inhibited by Cr(III), oxidative agents, sulfate and some other anions. Induction of *chr* operone leads to reduced accumulation of chromium inside the cell, due to the efflux activity of transporter gene *chrA* (Branco et al., 2008).

The chromate ion transporter protein superfamily (CHR), which includes putative ChrA orthologs, contains sequences from all the three domains of life and is divided in two

families: LCHR (long chain chromate transporters) and SCHR (short chain chromate transporters). More than 4000 species of Bacteria, Archaea and eukaryotes (fungi, green plants, algae, protozoa and many animal groups including mammals) contain CHR sequences, as can be viewed by search in EMBL-EBI and NCBI databases (Figure 5) (EMBL-EBI, 2018). SCHR family contains proteins of about 200 amino acids, while LCHR proteins are about 400 amino acids long. It is presumed LCHR were formed by ancient fusion of two SCHR protein-encoding genes (Díaz-Pérez et al., 2007). These two families have separate distribution and different genomic context. CHR proteins probably carry out different functional roles in addition to chromate efflux, which is presumably variable among separate CHR subfamilies.

DNA REPAIR

Chromium can cause oxidative and non-oxidative damage to the DNA (Zhitkovich 2011). Non-oxidative damage is realized through binding of Cr to DNA, mostly in the form of Cr(III) (Zhitkovich et al. 2001). Oxidative damage is realized through action of reactive oxygen species which indirectly lead to mutation and damages to DNA.
Proteomic and transcriptomic studies indicate that a large number of proteins involved in DNA recombination, replication and repair are up-regulated in *S. oneidensis* and *Arthrobacter* sp. FB24 cultures grown in the presence of chromate (Chourey et al., 2006; Kristene L. Henne et al., 2009). This suggests that bacteria subjected to chromate stress have to make additional effort to protect their DNA (Viti et al. 2014). To mention a few: Cr(VI) was proven to upregulate SOS repair system in *E. coli* (Llagostera et al., 1986), DNA helicases (RecG and RuvB) in *P. aeruginosa* (Miranda et al., 2005), endonucleases and RecA protein in *Caulobacter crescentus* (Hu et al., 2005), site-specific recombinase (SO3013), DNA topoisomerase III (TopB, SO3061), type I restriction-modification system subunit (HsdM-2, SO4265), type II restriction endonuclease (SOA0003), and type II DNA modification methyltransferase (SOA0004), helicase genes, DNA repair protein RecO in *Shewanella oneidensis* (Chourey et al., 2006). A detailed account on the subject of DNA metabolism in chromium stress conditions can be found in reviews by Ramírez-Díaz et al. (2008) and Viti et al. (2014).

PROTECTION AGAINST OXIDATIVE STRESS

Upon entering the cytoplasm, chromate toxicity is linked to its intracellular reduction, which produces large amount of ROSs. Namely, reduction occurs via formation of highly unstable Cr(V) (Ackerley et al., 2004). Cr(V) is quickly re-oxidized back to Cr(VI), while transferring one electron to O_2, thus generating reactive oxygen species (ROS). This redox cycling leads to high amount of formed ROSs and oxidative stress. Microorganisms can battle oxidative stress by enzymes such as SOD and catalase. For

example, exposure to Cr causes upregulation of these enzymes in *Escherichia coli* (Ackerley et al., 2004). Also, compounds such as glutathione, thiols and others, can prevent oxidative damage.

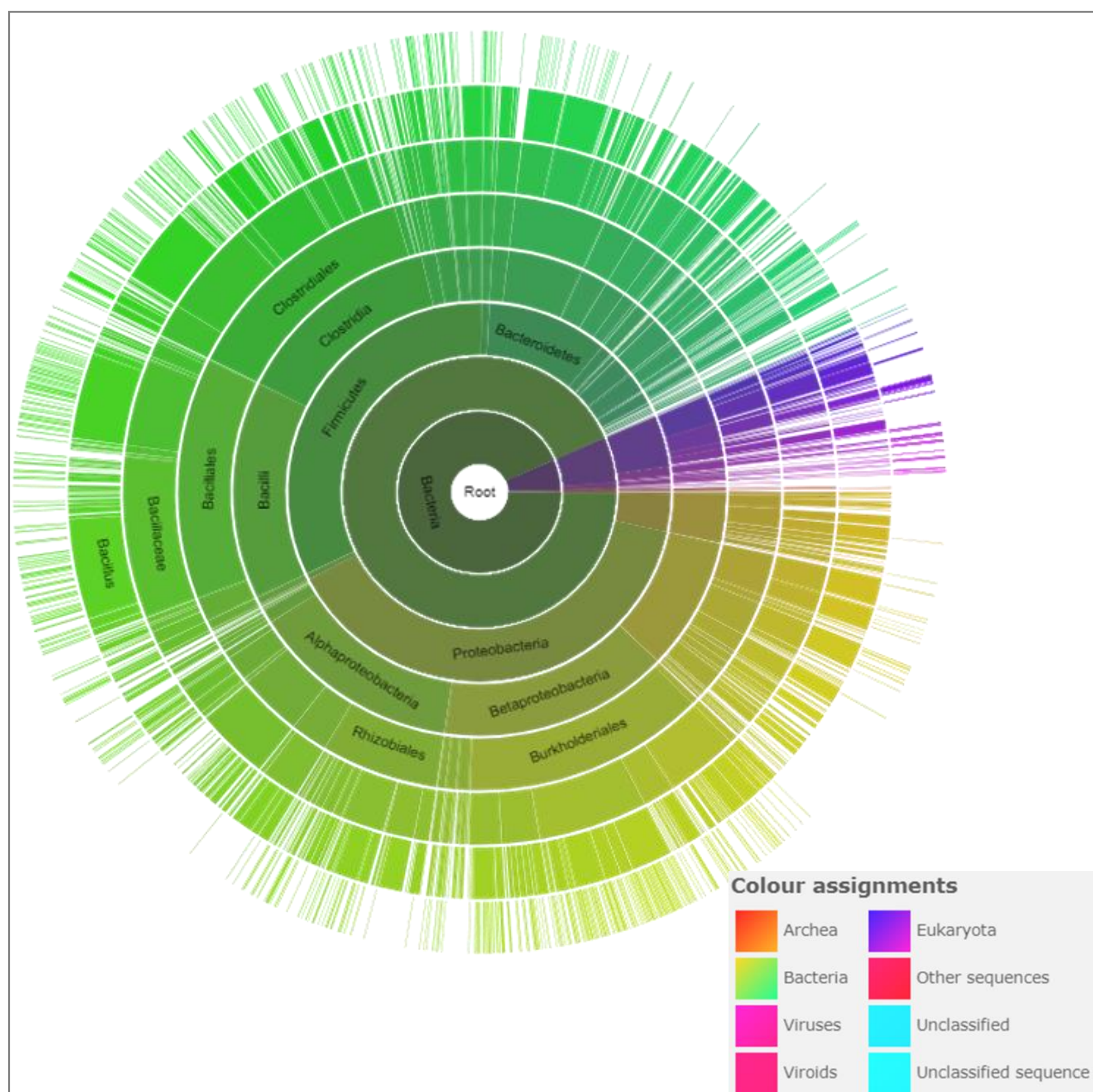

Figure 5 Phylogenetic distribution of chromate transporter proteins (family PF02417) (source: EMBL-EBI 2018)

2.3. BIOREMEDIATION OF CHROMIUM POLLUTED ENVIRONMENTS

Conventional remediation approach for soil and water contaminated with Cr include physico-chemical methods such as precipitation, adsorption, ion exchange, chemical reduction etc. (Dhal et al., 2013). Disadvantages of these methods are high price, high energy and chemicals consumption (Krishna and Philip, 2005). Additionally, safe disposal of generated toxic sludge, incomplete Cr(VI) reduction and formation of other

toxic compounds can also pose a problem in these methods (Dhal et al., 2013; Focardi, et al., 2013).

Innovative technologies are based on bioremediation – the use of microorganism's metabolic potential for removal of toxic metals from the environment. Microorganisms are being employed in bioremediation of Cr(VI) pollution because many of them can reduce Cr(VI) to far less toxic Cr(III).

Some of the advantages of bioremediation are (Dhal et al., 2013):

- *in situ* approach without the need for soil excavation and transport;
- application at sites with high groundwater level;
- continual process of Cr(VI) reduction;
- no adverse effects on bioremediation site.

Bioremediation can be conducted *in situ* or *ex situ* (EPA, 2006). *In situ* bioremediation is treatment of soil or water without removal and transport to another site. Disadvantage of *in situ* approach is limited control over physical and chemical characteristics of the environment. *Ex situ* bioremediation presumes collection and removal of polluted soil/water and their transport to a site on which further treatment or storage will take place.

For bioremediation process indigenous microorganisms already present at the site can be used, or microorganisms can be artificially added. The later approach - *bioaugmentation* - is employed if native microorganisms lack the capacity to biodegrade pollutants or their activity is insufficient.

Biostimulation is optimization of environmental conditions in contaminated soil/water through e.g. aeration, pH correction, nutrient addition, in order to improve microbial activity and bioremediation (Frolkis et al., 1975; Kamaludeen et al., 2003a).

A vast number of studies on Cr(VI) bioremediation with bacteria has been published. These studies indicate that many species have Cr(VI) reductive potential, at least in the laboratory scale experiments. Some examples are *Escherichia coli* (Shen and Wang, 1993), *Pseudomonas putida* (Ishibashi et al., 1990), *Streptomyces* sp. (Polti et al., 2010), *Shewanella* sp. (Brown et al., 2006), *Enterobacter cloacae* (Wang et al. 1990), *Cellulosimicrobium* sp. (Bharagava and Mishra, 2018), *Arthrobacter* sp. (Kristene L Henne et al., 2009) and *Ochrobactrum tritici* (Branco et al., 2004). Pilot and full scale application studies are less numerous. For instance, ChromeBac™ system (Ahmad et al., 2010) combines bioreduction of Cr(VI) by bacteria followed by chemical precipitation. System uses cheap waste materials (liquid pineapple waste) for bacteria cultivation, which increases its cost-effectiveness. Bacterial bioreduction is carried out by A*cinetobacter haemolyticus* cells immobilized onto carrier material inside a 0.2 m^3 bioreactor. Reactor was fed with neutralized electroplating wastewater with 17–81 mg/l Cr(VI) at a rate of 0.11 to 0.33 m^3/h. Complete Cr(VI) reduction to Cr(III) was obtained immediately after the start of bioreactor operation. If flocculation, coagulation and filtration were combined in treatment, outflow concentration of less than 0.02 mg/l

Cr(VI) and 1 mg/l total Cr were obtained. ChromeBac™ had stable performance in variable conditions of pH, Cr(VI) inflow, temperature and nutrient ratio (Ahmad et al., 2010). Similar combination of chemical–biological processes was used for treatment of tannery wastewater for Cr remediation and recycling. Chemical precipitation of Cr(III) using lime and cement dust was combined with biological removal of Cr(VI) by actinomycete strain *Kitasatosporia* sp. The precipitated Cr(III) was recycled for repeated use in tanning industry (Ahmed et al., 2016).

Bacteria can be used not only for removal but also for detection of pollution. Genetic engineering has enabled scientist to create organisms which give easily measurable response to presence of a contaminant. Such microbial reporters or bioreporters have an advantage of responding only to bioavailable portion of a pollutant, while chemical measurements usually measure total concentration which may not be of significance. Branco, Cristóvão and Morais (2013) designed two microbial bioreporters for detecting bioavailable Cr(VI) in wastewater: pCHRGFP1 *Escherichia coli* reporter and pCHRGFP2 *O. tritici* reporter. Designs are based on the expression of *gfp* under the control of the *chr* promoter and the *chrB* regulator gene of Tn*OtChr* determinant from *Ochrobactrum tritici* 5bvl1. Measurement is simply conducted by mixing sample to be tested and bacterial suspension, followed by measurement of fluorescence emitted by the bioreporter. These bioreporters possess high specificity to chromate and high sensitivity (minimal detectable concentration of 100 nM) (Branco et al., 2013).

2.4. INFLUENCE OF CHROMIUM ON BACTERIAL SOIL COMMUNITY

Although the effect of chromium on reference strains and environmental isolates has been studied extensively (e.g. Nies 2003; Bencheikh-Latmani et al. 2007; Cheung & Gu 2007; Ramírez-Díaz et al. 2008; Dhal et al. 2013; Thatoi et al. 2014), its effect on microbial communities is not well documented.

Current knowledge of the interaction of chromium compounds and microorganisms is mostly limited to the response of pure cultures or microcosms to relatively short-term chromium exposure. While laboratory microcosm experiments provide controlled and reproducible conditions, they lack the ability to assess the long-term influence of a contaminant and cannot entirely replicate natural environments. For instance, detailed studies of the effect of chromium on the cultivable microbial community in soil microcosms were performed (Viti et al., 2006; Caliz et al., 2012). Study provided a large amount of data regarding structural and metabolic changes in the community. However, the acute exposure to chromate and the short-term monitoring do not compare well to the field situations of chromium pollution, where decades of continual gradual input of chromium are the norm.

Previous studies of soil and sediment cultivable communities in heavy metal contaminated sites have reported conflicting results regarding the influence of

chromium concentration on bacterial abundance and the dominant bacterial groups that tolerate Cr. On the one hand, heavy metal pollution is well known to impair metabolic activity in soil (Chew et al., 2001; Roane and Kellogg, 1996) and hexavalent chromium was found to inhibit soil biological activity, such as denitrification, sulfatase and phosphatase activity and biomass carbon accumulation (Speir et al., 1995). In contrast, there are reports that high Cr levels do not impact biomass abundance. For instance, high Cr, Cu and As levels in the soil of a wood impregnating plant did not significantly affect heterotrophic plate counts (HPC) compared to unpolluted soil controls (Turpeinen et al., 2004). In addition, there was no significant correlation between Cr(VI) concentrations in polluted river sediments and cultivable counts, diversity at the genus level, or the ability of the microbial community to withstand or to reduce high Cr(VI) concentrations (Branco et al., 2008).

There is a similar ambiguity regarding the relative proportion of Gram-positive and Gram-negative bacteria in microbial communities affected by heavy metal pollution. Some studies show that Gram-negative bacteria exhibited greater metal tolerance (Duxbury and Bicknell, 1983; Kamaludeen et al., 2003; Turpeinen et al., 2004; Sheik et al., 2012), and that Gram-positive bacteria are less abundant in polluted soils (Kelly et al., 2003), while others report that isolated Cr(VI) tolerant strains are predominantly Gram-positive bacteria (Branco et al., 2005; Caliz et al., 2012).

2.5. CHROMATE TOLERANT BACTERIA

As mentioned before, many studies of pure culture's response to chromium have been conducted, including both reference laboratory strains and environmental isolates. Isolates predominantly originated from chromium polluted sites and thus have been previously exposed to chromium related stress. Usual sources for their isolation include tannery wastewaters, soils and sediments affected by tannery wastes, chromite mining affected environments, electroplating industry affected environments, etc. Very few accounts on chromate resistance in strains from non-polluted low chromium environments exist to this date (Wani et al., 2007). Overview of some of the strains characterized in respect of their chromate resistance, together with their origin and main findings is given in the Table 3.

Table 3 Overview of some of the chromate tolerant bacterial isolates described in published studies

Strain	Origin	Cr(VI) tolerance	Experimental conditions and main findings	References
Arthrobacter sp. SUK 1205	Chromite mine tailings	≥*2 mM in minimal Vogel Bonner medium	Cr(VI) reduction increased with cell density and decreased with starting Cr(VI) concentration, maximal at pH 7 and 30°C	Dey and Paul, 2015
Bacillus cereus	Soil contaminated with tannery wastewater	≥1.35 mM in undefined **medium	Almost 100% Cr(VI) reduced at 10-50 mg/l Cr(VI), 96.7% at 60 mg/l Cr(VI), 72.1% at 70 mg/l in undefined medium	Murugavelh and Mohanty, 2013
Bacillus cereus	Chromate slag heap	≥ 2 mM in LB	2 mM Cr(VI) reduced by LB (lysogeny broth) culture supernatant, intact cells show lesser activity	Chen *et al.*, 2012
Bacillus cereus SJ1	Wastewater of a metal electroplating factory	20mM for uninduced, 30 mM for induced cells in LB	Complete bacterial reduction of 1 mM Cr(VI) was achieved within 57 h in LB medium, same activity of induced and uninduced cells; azoreductase gene *azoR* and four nitroreductase genes *nitR* possibly involved in chromate reduction; putative chromate transport operon *chrIA1* identified in genome, expression of adjacent genes *chrA1* and *chrI* was induced in response to Cr(VI), while expression of the other two chromate transporter genes *chrA2* and *chrA3* was constitutive	He *et al.*, 2010
Bacillus megaterium TKW3	Cr contaminated marine sediment	/	Cr(VI) reduction was constitutive, Cr was precipitated on inner side of cell membrane	Cheung and Gu, 2005; Cheung, Lai and Gu, 2006
Bacillus methylotrophicus	Tannery sludge	≥0.5 mM in defined ***medium	Culture supernatant (extracellular medium) has the highest reduction activity compared to negligible reduction of whole cells, homogenized cells and cell wall fraction; 10% (v/v) crude extracellular medium in tannery effluent reduced 91% Cr(VI) after 48 h	Sandana Mala, Sujatha and Rose, 2015
Bacillus mycoides 200AsB1	Arsenate spiked soil	2.4 mM in LB	Biosorption and bioaccumulation remove Cr(VI) from solution, reduction did not occur, N–H and O–H groups from N-methyl-glucamine were involved in Cr(VI) adsorption	Wang *et al.*, 2016
Bacillus sp. strain KSUCr9a	Soda lakes; soil and water samples	75 mM in alkaline undefined agar medium	Cr(VI) reduction optimal at pH 9, 35°C, 0.8 % glucose and without mixing	Ibrahim *et al.*, 2012
Bacillus subtilis 168	Reference strain	≥0.3 mM in M9 minimal medium	Induction had no influence on resistance, indicating it is constitutive; short chain chromate transporter genes *ywrB* and *ywrA* confer chromate resistance and diminish Cr uptake in *Escherichia coli* only when both proteins are expressed, *chrS* gene product acts as negative regulator	Díaz-Magaña *et al.*, 2009; Aguilar-Barajas *et al.*, 2013

Strain	Origin	Cr(VI) tolerance	Experimental conditions and main findings	References
Bacillus subtilis alkaliphilic	Tannery effluent contaminated soil	15.4 mM in CA-M9 Minimal	Reduction by constitutive membrane bound enzymes, optimal at alkaline condition (pH 9)	Mary Mangaiyarkarasi *et al.*, 2011
Bacillus subtilis BYCr-1	Rare-earth mine	MIC 10 mM in M9 medium	*nfrA* gene upregulated and linked to reduction activity, confers reduction activity in *E. coli*; intracellular and membrane Cr(III) precipitation occurs	Zheng *et al.*, 2015
Cellulosimicrobium cellulans KUCr3	Sludge waste canal	MIC 400 mM in minimal medium	Exhibited chromate reduction activity and growth promoting properties to plants grown in chromium-contaminated soil	Chatterjee, Sau and Mukherjee, 2009
Cellulosimicrobium sp. KX710177	Tannery wastewater	MIC 9.61 mM in nutrient agar	Multi-drug and multi-metal resistant strain; >96 % reduction at 50 and 100 mg/L Cr(VI), reduction decreased on higher concentrations of 200 and 300 mg/l (84 and 62 %, respectively); increased cell size in Cr(VI) exposed cells, Cr intracellular content 0.19 wt%	Bharagava and Mishra, 2018
Lysinibacillus fusiformis ZC1	Cr contaminated wastewater of electroplating factory	MIC 60 mM in R2A medium	Almost completely reduced 1 mM K_2CrO_4 in 12 h; chromate resistance was constitutive in both phenotypic and gene expression analyses; contains large number of metal(loid) resistance genes: *chrA* chromate transporter; *yieF* and several reductase genes; constitutive expression of adjacent putative chromate reduction related genes *nitR* and *yieF;* a large number of NADH-dependent chromate reductase genes may be responsible for the rapid chromate reduction	He *et al.*, 2011
Microbacterium liquefaciens MP30	Tannery waste	15 mM in LB	Reduced Cr(VI) only under anaerobic conditions at the expense of acetate as the electron donor; PVA (polyvinyl alcohol)-borate and PVA-alginate cell beads showed a higher rate and extent of chromate reduction than PVA-nitrate cell beads in batch experiments; reduction also tested in continuous-flow system	Pattanapipitpais al, Brown and Macaskie, 2001
Microbacterium sp. GM-1	Activated sludge	2.3 mM in nutrient broth	Employed in biocementation of chromium slag based on microbially induced calcium carbonate precipitation	Lun et al. 2016
Ochrobactrum tritici 5bvl1	treatment plant of Cr contaminated wastewater	10 mM in buffered mineral medium	Tn*OtChr* operon mainly responsible for resistance - 7,189-bp-long; contains *chrB*, *chrA*, *chrC*, and *chrF* genes situated between divergently transcribed resolvase and transposase genes; *chrB* and *chrA* genes, but not *chrF* or *chrC*, were essential for establishment of high resistance	Branco, Alpoim and Morais, 2004; Branco *et al.*, 2008
Rhodococcus erythropolis	Coal mine waste water	1.92 mM	Tolerant to Fe, As, Cu, Ag, Zn, Mn, Mg and Pb; accumulation of Cr(VI) as Cr(III) both within and outside the cell	Banerjee *et al.*, 2017
Rhodococcus erythropolis MTCC 7905	Metal contaminated soil	5.8 mM	Exhibited plant growth promotion and Cr(VI) reduction at psychrophilic conditions	Trivedi, Pandey and Sa, 2007

* ≥ - maximal tested Cr(VI) concentration on which growth occurred; ** medium with high nutrient content and undefined ingredients : ***chemically defined minimal medium

3. AIMS

Chromium is a serious environmental contaminant widely used in a number of industrial activities. Certain microorganisms have the ability to reduce highly toxic hexavalent to less toxic trivalent chromium. Consequently, they can be employed in environmentally viable and cost effective bioremediation of chromium contaminated soil, sediment and wastewater. Hence, isolation and characterization of microorganisms with high chromate tolerance and robust chromate reduction activity is of great environmental and scientific importance.

Having this in mind, **general goal** of this thesis was to investigate the effect chromium on soil bacteria.

Specific goals, through which general goal will be realized, were to:

- determine influence of high chromium concentrations on the cultivable soil bacteria counts, proportion of chromate tolerant cultivable soil bacteria and proportion of Gram-positive and Gram-negative bacteria;
- isolate and characterize chromate tolerant strains from both high Cr and low Cr environments;
- determine chromate tolerance of isolates;
- determine chromate reduction of isolates;
- determine differences in response to Cr(VI) between reference and environmental *Bacillus* sp. strains.

Through realization of these goals, valuable new insights into chromate tolerance and reduction of bacteria will be gained, which will be of importance not only from a fundamental scientific point of view, but also from a practical bioremediation aspect.

4. MATERIALS AND METHODS

4.1. SAMPLING DESIGN

This thesis encompasses two-tier approach to determination of chromium influence on bacteria:

a) Investigation of cultivable soil bacterial community characteristics in regard to Cr;
b) Investigation of chromate tolerant bacterial strains.

Therefore, sampling design was adjusted for the two specific sections. For the first research section, soil samples of the three groups according to their different Cr concentration and origin were collected. For the second research section, in addition to soil samples, diverse types of environmental samples were collected, in order to isolate chromate tolerant strains from various environments.

SAMPLING DESIGN FOR CULTIVABLE SOIL BACTERIAL COMMUNITY ANALYSES

To determine the effect of chromium on cultivable soil bacterial community, samples of the three different groups according to their Cr concentration and Cr origin were collected (Table 4). Two groups encompassed samples with high Cr level of either natural geogenic or anthropogenic origin. Third group encompassed soils with low background level of Cr. In total, 17 samples marked 1 to 17 were collected and tested by chemical and microbial analyses.

Table 4 Soil sample groups for cultivable soil bacterial community analyses

Sample group mark	Cr level (qualitative)	Description of sample group	Sample numbers	No of samples	Range of Cr concentration (mg/kg)
LCr	Low background	Background Cr concentration	1-4	4	25-48
PCr	High	Elevated Cr concentration caused by pollution	5-8	4	112 -818
NCr	High	Elevated Cr concentration of natural origin	9-17	9	342-1287

Samples were collected at various sites in the Republic of Serbia (Figure 6). Sampling was performed in 2014 and 2015. A detailed description of all sampling locations is given in "Sampling locations" section and Table 9 in Results section, while below we give a short overview of main characteristics.

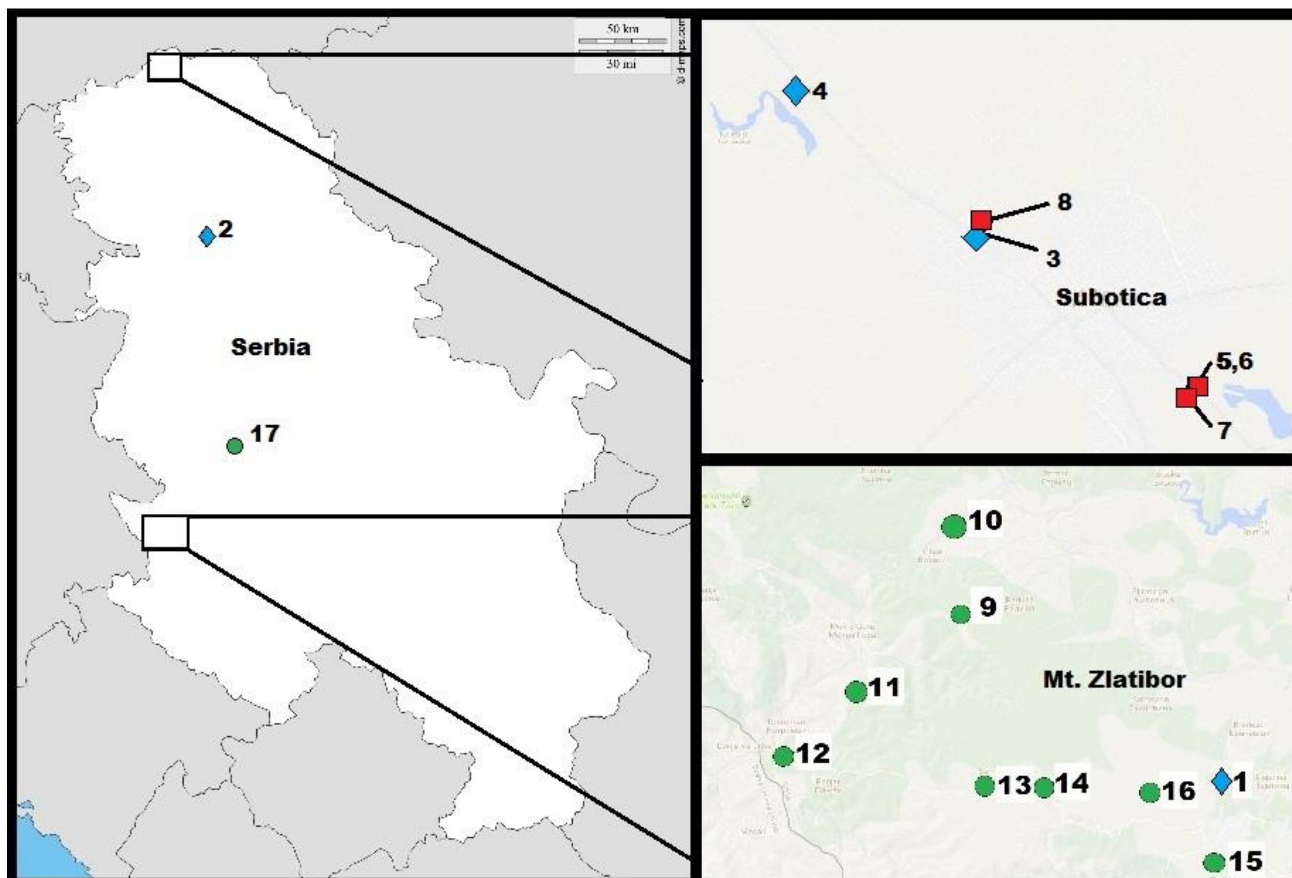

Figure 6 Locations of soil samples collected for the analyses of Cr influence on the cultivable soil bacterial community: ♦ (blue diamond) background Cr level (group LCr) - samples 1-4; ■ (red square) elevated Cr level of anthropogenic origin (group PCr) – samples 5-8; ●(green circles) elevated Cr level of natural origin (group NCr) – samples 9-17 (interactive KML map available on link: https://drive.google.com/open?id=12Lwi0S5yUbpeylGSSNZcWWsP6qQ&usp=sharing)

Serpentine soils with naturally high concentrations of chromium (**NCr**) were collected at Mt. Zlatibor and Mt. Maljen in western Serbia. The area includes serpentine soil formed on ultramafic parent rock, which is characterized by high levels of Cr and Ni (Vicic et al., 2014). No other contaminant was present, as there are no nearby pollution sources. Diverse sites including forest, meadow and agricultural soil were sampled.

Chromium polluted soils (**PCr**) were obtained from an industrially polluted sites of a former chemical factory and leather tannery located in the city of Subotica in the north of Serbia. More specifically, three samples (5, 6 and 7) were taken at the wastewater lagoon of the former leather tannery, now entirely dried up and covered with grass, while one sample (8) was taken in the yard of a chromic acid production facility near the waste piles.

Soils with low background Cr level (**LCr**) were selected to have overall similar characteristics to the samples of the other two groups, but with the Cr levels under 50 mg/kg and with low level of pollutants in general.

SAMPLING DESIGN FOR CHROMATE TOLERANT STRAINS ANALYSES

All samples collected for the cultivable soil bacterial community analyses were also used for the second research section (Investigation of chromate tolerant strains). In addition, water samples from salt marshes, industrial solid waste materials, and sediment samples from a canal receiving wastewaters were collected for the second research section. Environmental samples were collected from various sites exhibiting different characteristics in order to isolate chromate tolerant strains of diverse origin.

SAMPLING METHODOLOGY

All sampling was performed using sterile techniques. Samples were kept in a cold box until arrival at the laboratory, after which they were stored at 4°C until further analysis. Specific methodologies for the particular matrices are given in the following sections.

Soil sampling

After removal of vegetation, stones and visible soil fauna, a sample was collected from an area of 50x50 cm using a sterile shovel. Depth of soil collected was 0-10 cm, if not stated otherwise. After homogenization of a bulk sample in a large sterile polyethylene bag, subsample was collected in a smaller sterile polyethylene bag. Samples were sieved through a sterile 2 mm sieve before use. At certain locations additional samples of depth 10-20 cm and 20-30 cm were collected, as it was noticed they were clearly different from the surface 0-10 cm layer. In such case, samples were designated by subscripts: a – 0-10 cm, b – 10-20 cm, and c - 20-30 cm.

Figure 7 Soil sampling (preparation for soil collection - removal of surface vegetation, stones and fauna on sampling site 12)

Sediment sampling

Surface sediment samples (15 cm depth) were taken using the Van Veen grab sampler of 0.5 L capacity. In order to obtain samples reflecting the average chemical and

microbial composition of each location, composite samples were collected. Individual random samples from an area of 2x2 m were taken and homogenized at the site. Then, smaller samples were collected in sterile containers and taken to the laboratory.

Water sampling

Water samples were obtained from the surface water layer by submerging sterile glass bottle 10-15 cm under the surface or by collecting water by sterile spatula into bottle at locations where water was too shallow for bottle submergence (temporary shallow puddles of water on soil surface).

SAMPLING LOCATIONS

Sites with naturally elevated level of Cr (serpentine soils)

Serpentine soils are ubiquitous on the Earth, but have a patchy distribution. They cover less than 1% of the Earth's surface. Serpentine habitats are often extremely xeric, and prone to high temperatures and erosion, with typically shallow and rocky soil. Color of serpentine soils collected varied from reddish, brown to gray (Figure 8). Main characteristics are elevated Mg:Ca ratios, lack of N, P and K, high levels of Ni, Cr or Co which pose harsh conditions for plant growth. This has lead to formation of specific vegetation on such sites: specific plant community affiliated to serpentine soils is defined as low-productive, and comprises a high number of endemics and vegetation types distinct from neighboring areas (Vicic et al., 2014).

Plants growing on serpentine soil have developed specialized adaptations to high heavy metal content and for that reason have been employed in the phytoremediation of polluted soils (Morel et al., 2006). These characteristics derived from the parent rock are more expressed in forest habitats, while influence of parent rock on meadow and pasture habitats is much less pronounced (Vasić and Diklić, 2001). Forests are mostly made of different oak species (*Quercus* spp.), black pine (*Pinus nigra)*, Scots pine (*Pinus sylvestris)*, European hop-hornbeam (*Ostrya carpinifolia*) and cade (*Juniperus oxycedris*), silver fir (*Abies alba*) and European beech (*Fagus sylvatica*) (Vasić and Diklić, 2001).

Samples of serpentine soils for this research were collected at Mt. Zlatibor and Mt. Maljen. Mt. Zlatibor is located in the western Serbia. Parts of the mountain are under protection as a Nature Park Zlatibor since 2016 and 2017. It is an important tourist region with very limited industrial operations. Mt. Maljen is located in central Serbia, and, alike Mt. Zlatibor, has patches of serpentine soil.

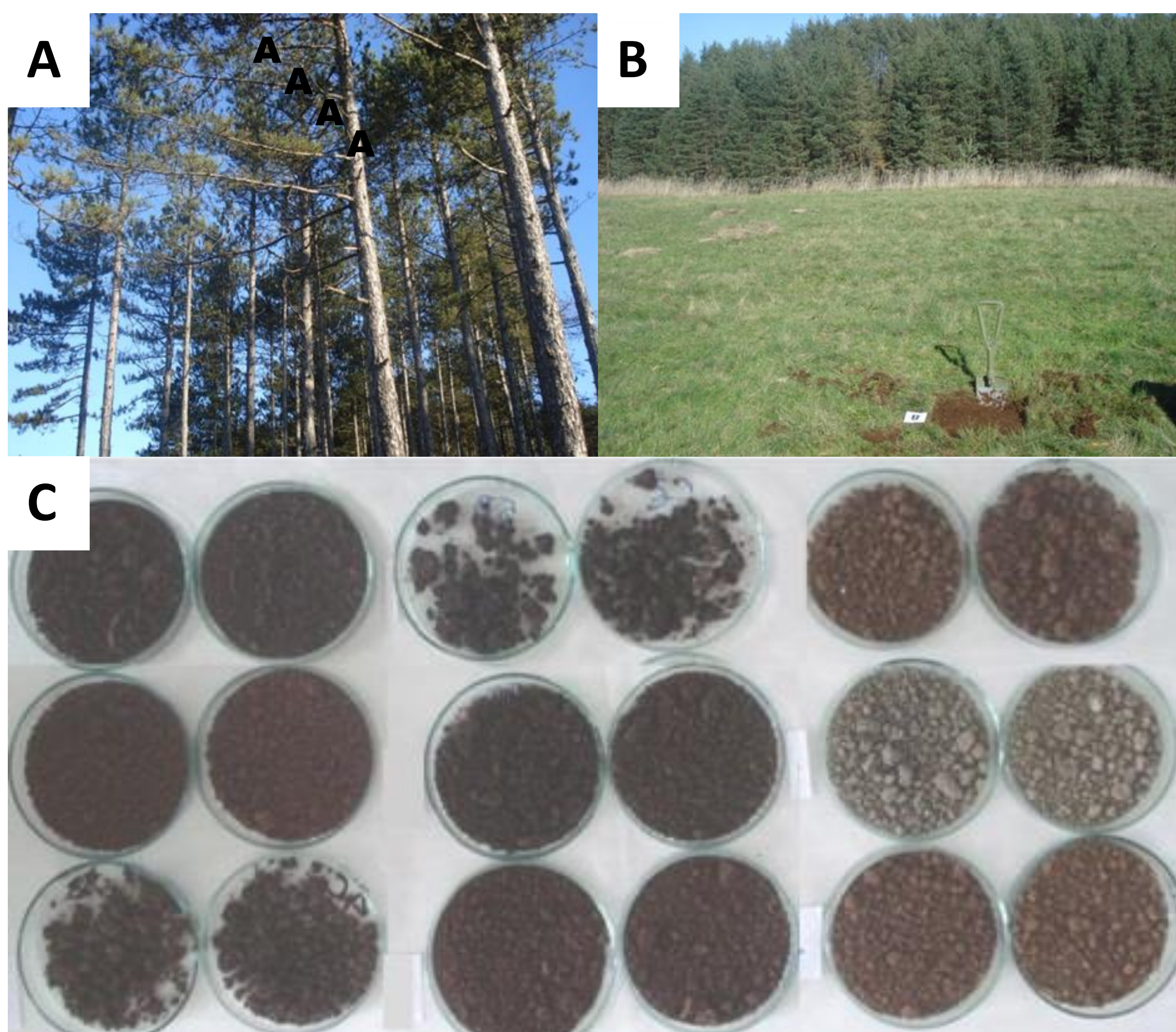

Figure 8 A and B - sites with naturally high level of chromium - serpentine soils, Mt. Zlatibor and Mt. Maljen; B – appearance of serpentine soil samples

Sites polluted with Cr

We collected samples from four locations in the north part of Serbia. Locations were affected by decades of industrial activity involving chromium compounds. Namely, sites of leather tanneries and chemical industry have been sampled.

Leather tannery "Panonija" in Subotica ceased working in 1990s after decades of industrial activity encompassing chemical processing, tanning and dyeing of leather. Due to the inadequate waste treatment and management, pollution to the nearby soil was extensive and persists to this day. According to the Soil monitoring data from 2013, Cr, Ni, Zn and Cu levels had been above maximum allowed concentration at this site (Kezić et al., 2013). Samples were collected on the site of the former wastewater lagoon (Figure 9). Lagoon is not currently in function and is covered with grass vegetation.

Chemical industry "Zorka" in Subotica began its activity in 1904. Its main products were chromic acid, mineral fertilizers, pesticides, herbicides (Mačković, 2004). Uncovered pyrite piles were left on site (Figure 10). Landscape is visibly affected from the intensive industrial activities. Soil layers of different depth at some locations

differed significantly in color and general appearance. At such locations, samples of different soil depths were recovered.

Figure 9 Leather tannery „Panonija" Subotica – site of the former wastewater lagoon (photo: D. Radnović)

Figure 10 Chemical factory "Zorka" Subotica (photo: D. Radnović)

Leather tanneries in Ruma and Zrenjanin. Tannery located in the City of Ruma discharges its wastewaters to irrigation canal Kudoš. We collected sediments from this

canal and soil which is irrigated by the canal's water. At Zrenjanin tannery we collected dried solid waste from a container for industrial waste material.

Sites with low background level of Cr

To determine influence of high Cr levels on bacterial community it was necessary to implement control soil group with similar properties, but with low background level of Cr. Such soils were collected nearby high Cr sites, had similar climate, vegetation, physical and chemical properties and differed only in their comparably much lower Cr level.

Beside such control sites used for bacterial community comparison, we also collected samples from halophilic habitats as high amount of sulfate salts could be connected to higher level of chromate tolerance. This is because similar transport pathways are employed for these anions. Namely, if high amount of sulfate is present, sulfate uptake channels (also used by chromate) will be less active which will result in diminished chromate uptake (Aguilar-Barajas et al., 2011; Viti et al., 2014).

Halophilic habitats – salt marshes Slano Kopovo and Medura are located in the northern flatland part of Serbia which is situated in southern part of Pannonian plain. Slano Kopovo is situated in the municipality of Novi Bečej (Figure 11).

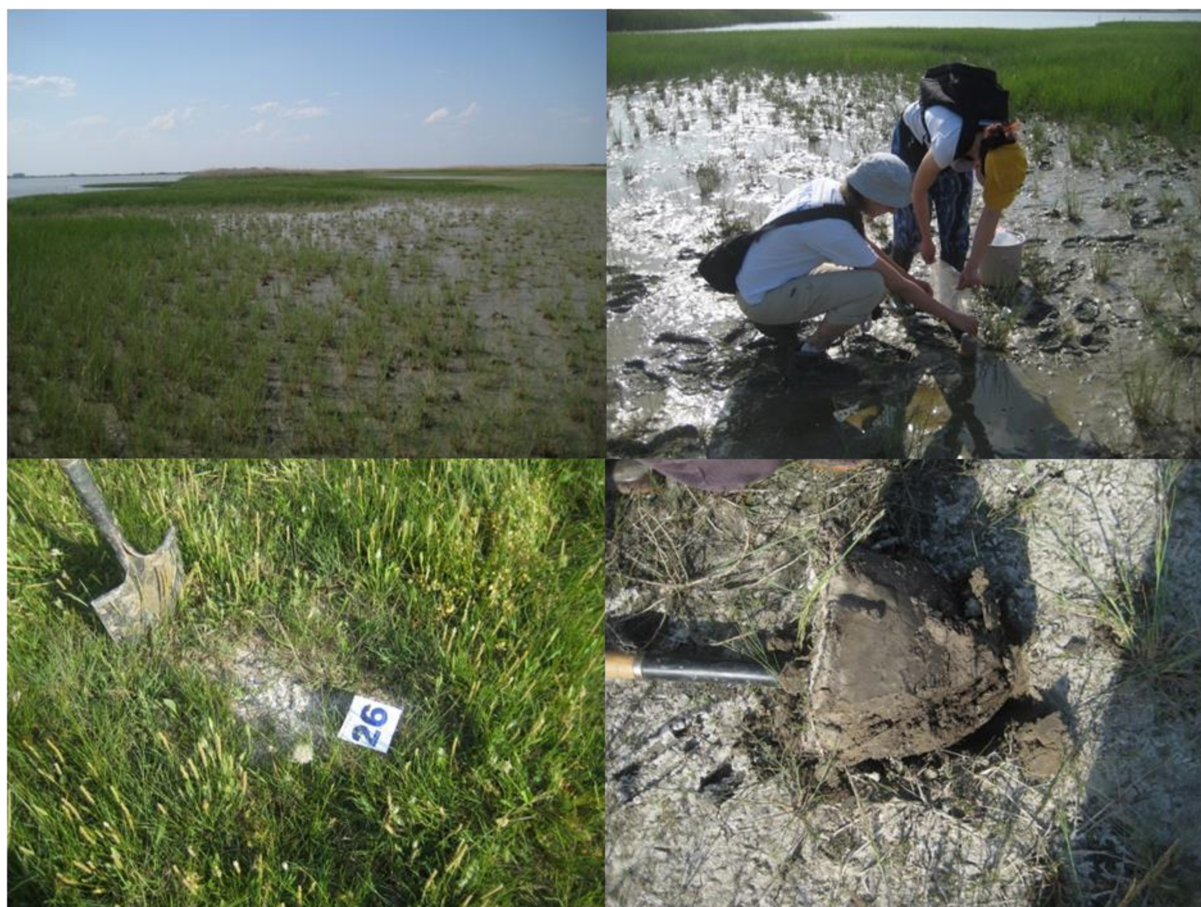

Figure 11 Salt marsh Slano Kopovo (photo: D. Radnović)

Slano Kopovo is protected as a Special nature reserve since 2001 and classified as IUCN category IV (habitats and species management area). It is an important bird and biodiversity area (IBA) since 1989, Ramsar site since 2000 and supports rich fauna. Slano Kopovo contains a fluvial lake of elongated shape, a backwater of Tisa River. Depth of the lake fluctuates seasonally and during lower water levels, large surfaces become dry (Knežev, 2013). At an average water level, the lake is 3 km long, maximum width is 625 m, surface 1.45 km^2, average depth 70 cm, while most of the lake is only 20 cm deep. Soil is of solonchak type with more than twofold concentration of chlorides and sulfates compared to the other Pannonian soloncak's. Water is highly alkaline with pH values over 9 (Knežev, 2011).

4.2. CHEMICAL ANALYSES OF SAMPLES

Chemical analyses performed on environmental samples are listed in the Table 5.
Total chromium content was determined by flame atomic absorption spectroscopy (PerkinElmer Aanalyst 700) according the USEPA 7000B method (USEPA, 2007). Soil pH values were determined in a 1:5 water suspension by International Organization for Standardization (ISO) method 10390:2005 (ISO, 2005) and clay content - fraction <2µm percentage by ISO 11277:2009 (ISO, 2009). Dry matter content of the soil was determined based on the soil weight after drying at 105°C by BS EN 15934:2012 (BSI group, 2012). Organic matter content was determined by ISO 12879:2000 (BSI group, 2000).

Table 5 Overview of methods used for physical and chemical characterization of samples

Parameter	Method	Reference
Total chromium	US EPA method 7000B: Flame Atomic Absorption Spectrophotometry.	USEPA, 2007
pH value	ISO method 10390:2005 Soil quality -- Determination of pH.	ISO, 2005
Clay content - fraction <2µm	ISO method 11277:2009 Soil quality -- Determination of particle size distribution in mineral soil material -- Method by sieving and sedimentation.	ISO, 2009
Dry matter (total solids)	BS EN method 15934:2012. Sludge, treated biowaste, soil and waste - Calculation of dry matter fraction after determination of dry residue or water content.	BSI group, 2012
Organic matter	BS EN method 12879:2000 - Characterization of sludges. Determination of the loss of ignition of dry mass.	BSI group, 2000

4.3. CULTIVABLE SOIL BACTERIA COMMUNITY ANALYSES

This section describes methods used for characterization of cultivable soil bacterial community counts, proportion of chromate tolerant bacteria and proportion of Gram-positive and Gram-negative bacteria. Analyses were conducted on 17 soil samples divided into three groups based on their Cr content and origin, as stated previously.

CULTIVABLE SOIL BACTERIA AND CHROMATE TOLERANT CULTIVABLE SOIL BACTERIA COUNT

Cultivable heterotrophic soil bacteria (in further text abbreviated as **CB or cultivable bacteria**) counts were determined by a spread plate method on R2A agar (Torlak, Serbia) without Cr(VI), after 7 days of incubation at 25°C. These counts are also known as HPC (heterotrophic plate counts).
Chromate tolerant cultivable heterotrophic soil bacteria (in further text abbreviated as **CrCB or chromate tolerant cultivable bacteria**) counts were determined on R2A agar supplemented with various concentrations of Cr(VI). Proportion of CrCB was calculated as ratio to counts of cultivable bacteria on medium without Cr(VI).
Filter sterilized 1M K_2CrO_4 stock solution was added to autoclaved R2A agar cooled to 50°C to a final concentrations of 0.25, 1, 2, 4 and 8 mM Cr(VI) (based on Mergeay, 1995; Viti *et al.*, 2006, modified). Nystatin (a fungal growth inhibitor) was also added to R2A at 30 mg/l because preliminary tests revealed fungal overgrowth that inhibited bacterial growth. Data from a minimum of three replicates were collected for each measurement. Bacterial counts were reported as CFU per gram of soil, calculated on a dry weight basis (CFU/g).

SELECTIVE ENRICHMENT OF CULTIVABLE BACTERIA IN MEDIUM WITH Cr(VI)

Soil samples were subjected to selective enrichment on acetate minimal medium (AMM) with increasing concentrations of Cr(VI). The composition of AMM was (g/l): 1.0 NH_4Cl, 0.2 $MgSO_4 \cdot 7H_2O$, 0.001 $FeSO_4.7H_2O$, 0.001 $CaCl_2.H_2O$, 5.0 CH_3COONa, 0.5 KH_2PO_4, and 0.5 yeast extract (Pattanapipitpaisal et al., 2001). Chromate was added to the sterile AMM as a filter sterilized 1M K_2CrO_4 stock solution to a desired final concentration (0.5, 1.5, 2 and 2.5 mM). A soil slurry was prepared by mixing 5 g of soil in 45 ml of sterile 0.1% sodium pyrophosphate solution on an orbital shaker for 10 min at 180 rpm. Soil slurry was used to inoculate tubes with 5 ml AMM in 1:10 v/v ratio. Tubes were incubated without shaking at 26°C for 24 to 72h and checked daily for evidence of

growth by visual inspection. After observation of growth, 0.5 ml of culture was transferred to fresh AMM with the next higher Cr(VI) concentration. Each sample was tested in duplicate. The maximum Cr(VI) concentration at which visible growth was evident was recorded as a general measure of the microbial community's tolerance to Cr(VI).

PROPORTION OF GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

The proportion of Gram-positive and Gram-negative bacteria in the cultivable bacterial soil population was determined by Gram staining of 120 single colonies randomly selected from the R2A plates used for cultivable bacteria enumeration. Colonies were re-streaked on R2A agar, incubated 24 h at 26°C and used for slide preparation. All slides were prepared in triplicates. Gram staining was performed by submerging staining rack with heat-fixed slides in crystal violet solution for 1 minute, Lugol's solution for 1 minute, 96 % ethanol for 30 seconds and safranin solution for 1 minute. Slides were rinsed with tap water after each solution, blot dried at the end and observed by light microscopy. *Escherichia coli* ATCC 25922, *Bacillus subtilis* PY79 and *Staphylococcus aureus* ATCC 25923 slides were used as controls for the staining procedure accuracy. Microscopy was performed on Olympus BX51 brightfield microscope (Olympus, Tokyo, Japan).

4.4. CHROMATE TOLERANT BACTERIAL STRAINS ANALYSES

Here we will describe the methods used for characterization of chromate tolerant strains isolated from a wide range of unpolluted and polluted environments, including soil, sediment, water and solid waste materials.

MEDIA COMPOSITION AND INCUBATION CONDITIONS

The composition of the acetate minimal medium (AMM) was (g/l): NH_4Cl 1.0; $MgSO_4 \cdot 7H_2O$ 0.2; CH_3COONa 3.02; KH_2PO_4 0.5 and yeast extract 0.5 (Pattanapipitpaisal et al., 2001). Composition of standard M9 medium (Atlas, 2010) was modified by addition of small quantity of yeast extract in order to supply all needed micronutrients and growth factors for diverse environmental isolates. Consequently, M9 medium composition was (g/l): Na_2HPO_4 6.0; KH_2PO_4 3.0; NH_4Cl 1.0; NaCl 0.5; glucose 2; $MgSO_4 \cdot 7H_2O$ 0.25, thiamine·HCl 0.001; $CaCl_2$ 0.0147 and yeast extract 0.5. Nutrient agar (Torlak, Serbia) supplemented with 0.03 g/l of $MnSO_4$ was used for the induction of sporulation and detection of endospore presence. Media with Cr(VI) were prepared by

adding filter sterilized 1M K_2CrO_4 stock solution (Sigma-Aldrich, Germany) to cooled autoclaved media. Hexavalent chromium containing media had light to strong yellow coloration, depending on the concentration of Cr(VI) (Figure 12). Media used for Cr(VI) tolerance testing were freshly prepared from chemicals of the same batch in order to minimize variation in composition. Incubations were performed at 26°C, if not stated otherwise. pH value of all media was 7.2±0.1. Liquid cultures were incubated on an orbital shaker at 150 rpm, if not stated otherwise.

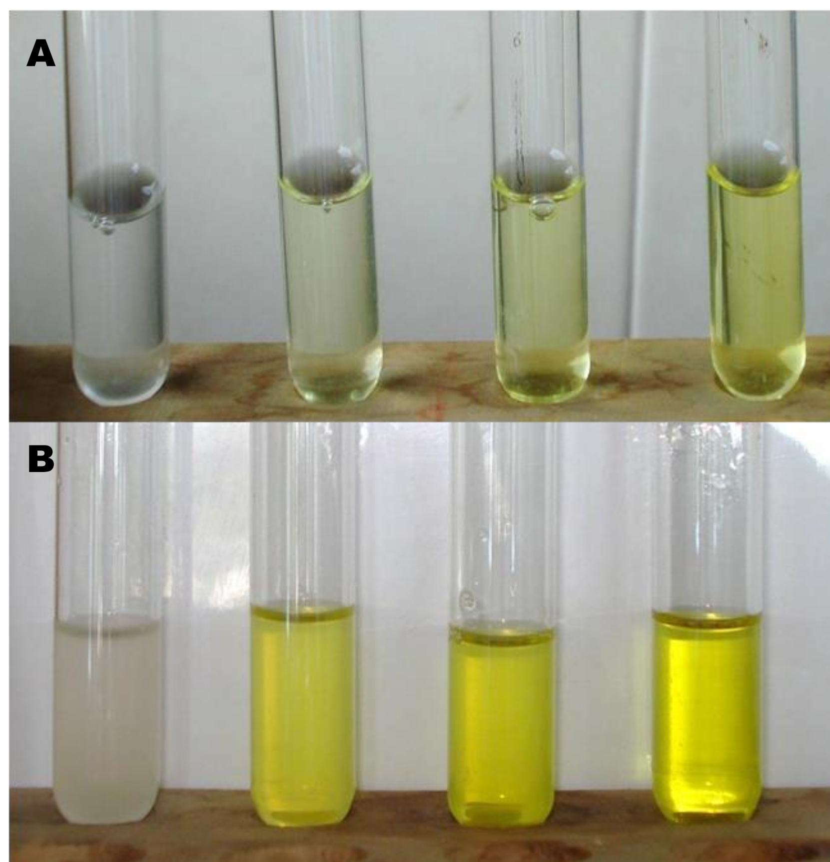

Figure 12 Appearance of the acetate minimal medium with: A - 0, 0.5, 1, 2 mM Cr(VI) before incubation; B - 0, 4, 6 and 8 mM Cr(VI) after incubation. Visible growth (cloudy, turbid appearance) is evident in the 0, 2 and 4 mM tubes, while no growth formed in 8 mM medium

Isolation of chromate tolerant strains

Chromate tolerant strains were isolated by selective enrichment on AMM with increasing Cr(VI) concentrations. Sample dilution was prepared by mixing 5 g of sample and 45 ml of saline solution for 10 minutes at 200 rpm. Next, 0.5 ml of this dilution was used to inoculate tubes with 5 ml of AMM in duplicates. Tubes were incubated for 24 to 72 h. When visible growth was observed, a small volume (0.5 ml) of the culture was transferred to a fresh AMM with the next higher Cr(VI) concentration. The concentrations of Cr(VI) tested were: 0.5, 1.5, 2, and 2.5 mM. If visible growth was

observed on 2 mM Cr(VI), an aliquot was spread plated on nutrient agar (Torlak, Serbia). Morphologically distinct single colonies were picked and repeatedly streaked on nutrient agar. As obtaining pure cultures proved to be difficult in some cases, additional steps of dispersion in 1 % sodium pyrophosphate solution and subsequent dilution plating were conducted. Pure cultures were rechecked for growth on AMM with 2 mM of Cr(VI) and preserved as glycerol stocks at -70°C until further analysis.

MALDI-TOF ANALYSIS

Isolates were grown overnight on LB agar at 28°C and analyzed using the standard Bruker's direct transfer sample preparation procedure for MALDI-TOF MS. Briefly, a small amount (tip of a toothpick) of bacterial colony was spotted directly onto a 96-spot MALDI polished steel sample target plate (Bruker Daltonics, Bremen, Germany), allowed to dry, and immediately overlaid with 1.0 μL of the matrix solution (Bruker Matrix HCCA; α-Cyano-4-hydroxycinnamic acid).

MALDI-TOF mass spectra were obtained with Microflex BioTyper spectrometer (Bruker Daltonics) with a nitrogen laser (λ= 337 nm) under control of Flexcontrol software ver. 3.1 (Bruker Daltonics). Automated spectra acquisitions in the mass range of 2 to 20 kDa were collected using the Auto Execute option by accumulating 240 laser shots.

MALDI TOF analysis was conducted at the "Institute of Public Health of Vojvodina" in Novi Sad, Serbia and "Institute of Biochemistry and Microbiology" in Bratislava, Slovakia. If results obtained by direct transfer procedure had a low reliability, than an additional step of total proteins extraction was conducted and resultant extract was tested on biotyper.

Extraction of total proteins for MALDI analysis

Extraction of total proteins for MALDI Biotyper using ethanol and formic acid was conducted according to the manufacturer's protocol as follows:

1. Add 300 μl of distilled water into clean eppi-tube.
2. Transfer the biological material (amount clearly visible on the tip of a toothpick: 5-10mg) into eppi-tube with water. Mix thoroughly by pipetting and subsequently by vortexing (vortex at least 1 minute).
3. Add 900 μl of 96% ethanol and mix thoroughly by vortexing.
4. Centrifuge at max speed (e.g. 13000 rpm) for 2 minutes, remove the supernatant, centrifuge once again and carefully remove remaining ethanol by pipetting, drying head down on a tissue (10 minutes), and air drying at 37°C (5 minutes).
5. In the meantime prepare 70% formic acid: mix 300 μl of distilled water and 700 μl of 100% formic acid and vortex.

6. Add 1 to 80 µl of 70% formic acid to the dried pellet and mix thoroughly by pipetting and vortexing; volume depends on the amount of used biomass:
    - 1 small colony 1-5 µl
    - 1 big colony 5-15 µl
    - Inoculating loop 1 µl 10-40 µl
    - Inoculating loop 10 µl 30-80 µl

   (5 µl of 70% formic acid was added in our analysis)
7. Add the same amount of 100% acetonitrile (the same amount as the amount of formic acid added in the previous step), and vortex thoroughly.
8. Centrifuge at max speed for 1 minute.
9. Load 1 µl of supernatant onto the MALDI sample target plate and let dry at room temperature.
10. Load 1 µl of MALDI matrix immediately.
11. Let dry at room temperature.
12. Sample is ready for analysis in MALDI biotyper.

CHROMATE TOLERANCE AND REDUCTION TESTING

Chromate tolerance and reduction testing was conducted by several different assays:

- Chromate tolerance and reduction testing of all the 33 isolated chromate tolerant strains - growth on 0, 0.5, 2 and 4mM and reduction of 0.5 mM Cr(VI) were tested;
- MIC (minimal inhibitory concentration) of Cr(VI) of selected *Bacillus cereus* group strains was additionally determined;
- Chromate tolerance and reduction testing, including growth and reduction dynamics at 0.2 and 1 mM Cr(VI), for selected *Bacillus cereus* group strains were additionally determined.

Each of the assays will be explained in the following sections.

<u>Chromate tolerance and reduction testing of all the chromate tolerant isolates</u>

All isolates were tested for growth on 0.5, 2 and 4 mM of Cr(VI) in M9 medium, while reduction was measured on a starting concentration of 0.5 mM Cr(VI). In short, washed cells suspension was used for inoculation of M9 medium with different concentrations of Cr(VI) to an uniform starting density. Concentration of Cr(VI) and OD600 (optical density measured at a wavelength of 600 nm) were measured at the start and after the incubation.

Inoculum preparation. Overnight culture on LB agar was used to inoculate tubes with 4ml M9 medium. Tubes were incubated 24h at 26°C, 150 rpm. Culture was then washed with saline solution three times. Specifically, culture was centrifuged 5 minutes at 2000

rpm, supernatant was discarded and 2 ml of saline was added. Cells were then resuspended by pipetting or vortexing. Washed suspension of cells in saline was used as an inoculum.

Inoculation of Cr(VI) medium. Tubes with 4ml of M9 with different concentrations of Cr(VI) (0, 0.5, 2 and 4 mM) (each concentration in duplicate for each strain) were prepared. Inoculum was added to the tubes in such amount that the uniform starting density of 0.5 McFarland units was obtained in each tube (Grant bio DEN-1B densitometer, Grant instruments). This density generally corresponds to 1-2x10^8 cells/ml. Volume of inoculum added depended on the density of inoculum. Tubes were incubated 24h at 26°C, 150rpm.

Determination of Cr(VI) concentration and OD600. Hexavalent chromium concentration and optical density were measured at the start and after the incubation. Hexavalent chromium concentration was measured by diphenylecarbazide (DPC) test (Urone, 1955) (described below). OD600 was measured spectrophotometrically by pipetting 300 µl of vortexed culture into microplate well. Reading was performed on microplate reader MultiSkan GO (Thermo Scientific, Unites States of America).

MIC (minimal inhibitory concentration) of Cr(VI) of selected Bacillus cereus group strains

MIC of Cr(VI) was determined on M9, AMM and LB medium for selected *Bacillus cereus* group stains. An overnight culture grown on LB was set to an optical density of 2 McFarland units by diluting it with fresh LB. Next, it was inoculated 1:100 in 25 ml of media in 100 ml flasks. The concentrations of Cr(VI) tested on LB medium were 0, 30, 60, 120 and 240 mM, while 0, 0.5, 1, 2, 4 and 8 mM were tested on M9 and AMM. Each concentration was tested in triplicate. The lowest Cr(VI) concentration on which no visible growth was observed, was recorded as MIC.

Chromate tolerance and chromate reduction testing of selected Bacillus cereus group stains

This testing included determination of growth and reduction dynamics. The ability of selected *Bacillus cereus* group strains to reduce hexavalent chromium to a trivalent state was tested on two starting concentrations of Cr(VI): 0.2 and 1 mM. An overnight culture grown on LB was set to an optical density of 2 McFarland units by diluting it with fresh LB. next, it was inoculated 1:100 in 25 ml of M9 medium with 0, 0.2 and 1 mM of Cr(VI), each in triplicate. An abiotic control was obtained by the addition of gentamycin to a final concentration of 16 µg/ml, which is double of the highest recorded gentamycin MIC for tested strains. MIC of gentamycin for all strains was previously determined by the standard microdilution method. Flasks were incubated for 72 h at 150 rpm. Measurements of OD600 and Cr(VI) concentration for an assay with a starting concentration of 0.2 mM Cr(VI) were performed every 12 hours. For an assay with a starting concentration of 1 mM Cr(VI), measurements were taken at 0 h and 72 h.

DPC TEST FOR Cr(VI) CONCENTRATION DETERMINATION

The Cr(VI) concentration was determined by the standard DPC (diphenylecarbazide) method (Urone, 1955). Method is based on a formation of a pink colored compound by reaction of DPC and Cr(VI). Cr(III) does not react with DPC to form colored compound. Intensity of pink color is proportional to the amount of Cr(VI) (Figure 13). Absorbance is measured spectrophotometrically and concentration of Cr(VI) is calculated from a formula based on a standard curve.

Reagents preparation:

10 mM Cr(VI) stock solution - add 0.19419 g of K_2CrO_4 (measured on analytical scale) to a volumetric flask and fill up to 100 ml with distilled water. Store at 4°C.

0.12 M H_2SO_4 – add ~50 ml of distilled water to a volumetric flask, slowly add 666 µl of concentrated H_2SO_4 (95-97 %), fill up to 100 ml with distilled water. Store at 4°C.

DPC reagent 2.5 mg/ml - measure DPC on analytical scale and dissolve in acetone to a final concentration of 2.5 mg/ml. Wrap in aluminum foil to protect light sensitive DPC. Make fresh solution for each measurement.

Standard Cr(VI) solutions used for construction of standard curve – standard solutions are prepared by mixing 10 mM Cr(VI) stock solution and distilled water according to the scheme in Table 6. Store at 4°C.

Table 6 Preparation of standard Cr(VI) solutions used for DPC test

Standard	Cr(VI) concentration (mM)	Preparation
S1	2	200 µl H_20 + 800 µl 10mM Cr(VI) stock solution
S2	1	500 µl H_20 + 500 µl S1
S3	0.5	500 µl H_20 + 500 µl S2
S4	0.25	500 µl H_20 + 500 µl S3
S5	0.125	500 µl H_20 + 500 µl S4
S6	0.0625	500 µl H_20 + 500 µl S5
S7	0.0313	500 µl H_20 + 500 µl S6
S8	0.0156	500 µl H_20 + 500 µl S7
S9	0	distilled water

Concentration of Cr(VI) in chromate reduction assays was measured in cell-free solution (supernatant).

Procedure was as follows:

1) Obtain cell-free supernatant by centrifugation of 300-1000 µl of culture for 5 min at 10000 g
2) Prepare reaction mixture in eppi-tubes by pipetting:

– 225 µl of 0.12 M H_2SO_4
– 50 µl of cell-free solution (supernatant)
– 125 µl of DPC reagent

3) Mix
4) Pipette 300 µl to microplate
5) Measure absorbance at 540 nm

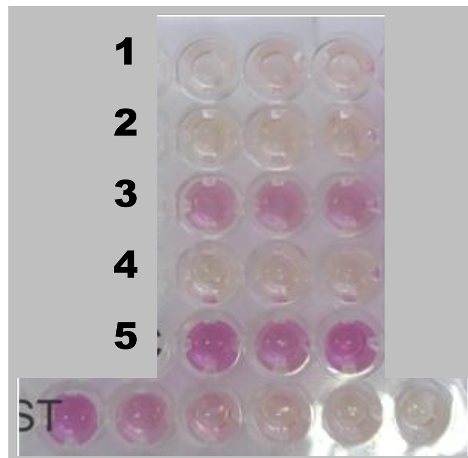

Figure 13 Determination of Cr(VI) concentration by DPC test – microplate ready for absorbance measurement: 1-4 – four samples with different Cr(VI) concentration as visible from variable intensity of pink color (three replicates for each sample), 5 - abiotic control, ST – standard solutions

Standard solutions of Cr(VI) were measured in the same way as samples. Absorbance of standard solutions and their known Cr(VI) concentration were used for making the standard curve on each measurement. Linearity of standard curve was determined by coefficient of determination R^2. If R^2 was below 0.95 testing was repeated. R^2 was usually above 0.98. Linear formula of the standard curve was used to calculate Cr(VI) concentration of samples based on their absorbance.

BIOCHEMICAL AND MORPHOLOGICAL CHARACTERIZATION

Biochemical tests were performed by the standard microbiological techniques, as recommended by Dworkin et al. (2006) and Logan and De Vos (2009).
Microscopic description of isolates was based on light microscopy of Gram stained and Schaefer-Fulton stained slides of 1 to 7 day-old cultures. Stained slides were viewed on Olympus BX51 microscope (Olympus, Japan). Native slides were observed on Olympus BX61 microscope equipped with an Olympus DP30BW camera (Olympus, Japan) at the "Institute of molecular biology" in Bratislava, Slovakia.
To record colony morphology of isolate NCr1a cell suspension in saline solution was spot inoculated at the center of the LB agar plate. Plate was incubated at 26°C. Colony morphology was documented daily by photographing the plate from above and below.

16S rRNA AND PYCA GENE SEQUENCE ANALYSIS AND DETECTION OF B. ANTHRACIS VIRULENCE GENES

Genomic DNA was isolated by phenol extraction protocol as described previously by Sambrook and Russell (2001).

16S rRNA gene sequence was obtained by PCR using the universal bacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 685R (5'-TCTACGCATTTCACCGCTAC-3'). The final PCR mixture (50µl) consisted of 0.4 µM of each primer, 250 µM of each of dNTP, 1U Dynazyme Taq DNA polymerase (Finnzymes Reagents, Thermo Fisher Scientific, Inc. Finnland), 1 x PCR Dynazyme buffer with final $MgCl_2$ concentration of 1.5 mM, and 1µl of the template DNA solution. The temperature program consisted of an initial denaturation at 94º C for 1 min, 30 cycles (94°C for 30 s, 60°C for 30 s, 72°C for 1 min) and a final extension step at 72°C for 8 min. PCR products were purified by QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) or by 0.8 % agarose gel electrophoresis followed by extraction with QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Purified PCR products were sequenced (GATC-Biotech, Konstanz, Germany) using PCR primers and analyzed using BLASTn search (Zhang et al. 2000).

Primers used for ***pycA*** gene were F: 5'-TTAGCAGATGAAGAGGGCAATG-'3 and R: 5'-CACTTCCTCTTGCTTTGGAACAC-'3 (Microsynth AG, Balgach, Switzerland). PCR mixture, conditions, product purification and sequencing were conducted as above, except for the PCR annealing temperature which was 52°C.

Sequence alignment and phylogenetic tree building were performed in MEGA 7 software (Kumar et al., 2016).

In order to eliminate the possibility of strains being *B. anthracis* we tested the presence of **virulence factor genes** *pagA* and *capA* located on plasmids pXO1 and pXO2, respectively, as described elsewhere (Ramisse et al., 1996).

BIOFILM FORMATION ASSAY

Biofilm formation ability in media with different concentrations of Cr(VI) was tested by the standard microplate procedure (O'Toole, 2011).

Plates were filled with 100 µl of medium (LB or M9) with varying concentrations of Cr(VI). In LB medium Cr(VI) concentrations tested were 0, 0.1, 1 and 16 mM Cr(VI), while 0, 0.1 and 1 mM were tested in M9 medium, each in triplicate.

Strains were grown overnight in LB at 28°C and 150rpm and set to an uniform optical density as measured by McFarland densitometer (MF 4.8-5.0). Then, 10 µl of culture was added to the wells. Plates were incubated at 28°C for 24 h or 7 days. After incubation, biofilm formation was assessed by following procedure:

1. Dispense the culture from the plate by inverting plate and shaking it gently
2. Rinse (submerge in water and dispense by inverting, repeat twice)

3. Remove excess water by tapping the plate on filter paper
4. Add 125 µl 0.1 % crystal violet solution, leave for 10 minutes
5. Dispense
6. Rinse
7. Remove excess water
8. Dry completely (leave for 30 minutes at 44°C or leave at room temperature overnight)
9. Add 150 µl 30 % acetic acid, leave for 10 minutes
10. Resuspend biofilm by up and down pipetting
11. Transfer 125 µl to optically clear flat bottom plate
12. Measure absorbance at 550 nm

Polystirene flat bottom plates (Biolite, Thermo Scientific) were used in all the assays. *Pseudomonas aeruginosa* ATCC 15692 was used as a control strain.

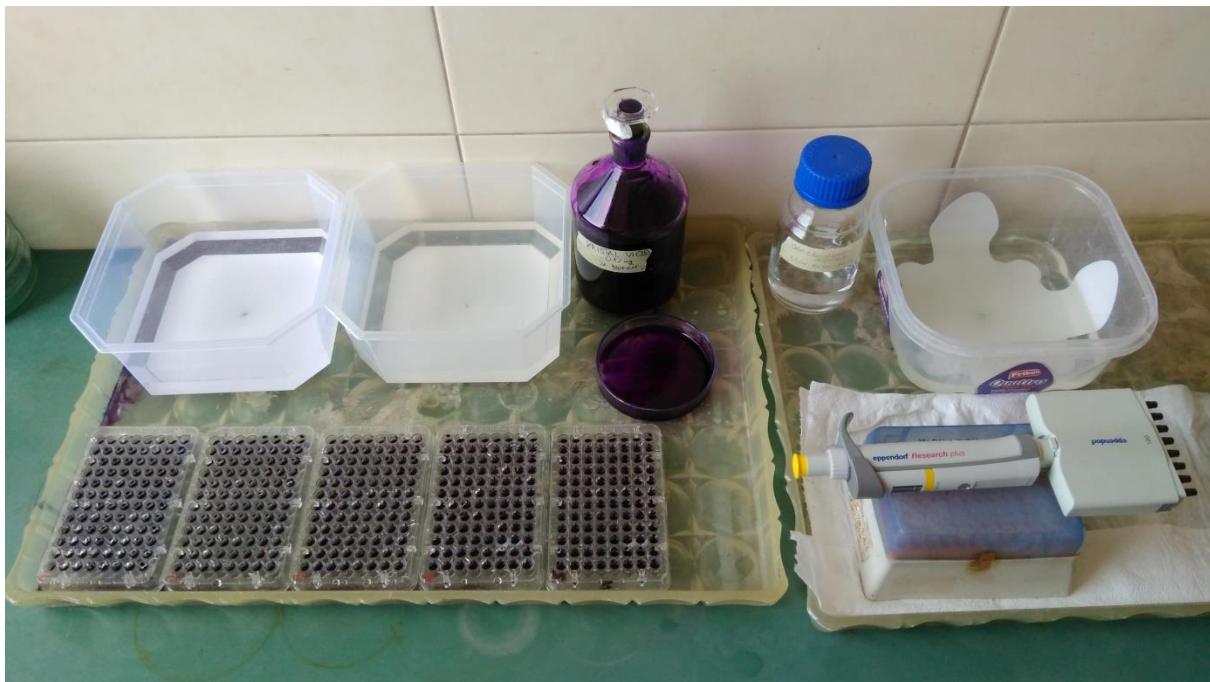

Figure 14 Biofilm formation assay experimental setup

HEAVY METAL TOLERANCE DETERMINATION

M9 agar plates with various concentrations of heavy metals were prepared by adding filter-sterilized stock solutions of heavy metal to an autoclaved medium cooled to 45°C. Overnight culture on LB agar was used to make a suspension of 2 McFarland units in sterile saline solution. Suspension was spot inoculated on M9 agar plates with various concentrations of heavy metals (10 µl per spot). Each strain was tested on three separate plates.

Table 7 Testing of heavy metal tolerance of chromate resistant strains: overview of metal forms and concentrations tested

Metal	Concentrations tested (mM)			
Cu^{2+}	10	1	0.5	0.1
Co^{2+}	10	1	0.5	0.1
Ni^{2+}	10	1	0.5	0.1
Ag^{+}	0.5	0.1	0.01	0.001
Hg^{2+}	0.1	0.01	0.001	0.0001
Zn^{2+}	10	1	0.5	0.1
Cr^{6+} (CrO_4^{2-})	30	10	2	0.2
Cr^{3+}	30	10	2	0.2
Cd^{2+}	4	2	0.5	0.1
W (WO_4^{2-})	30	10	2	0.2
Mo (MoO_4^{2-})	30	10	2	0.2

After 7 days of incubation, absence/presence of growth was detected by visual inspection (example given in Figure 15). The lowest metal concentration on which growth was completely inhibited was recorded as MIC. Forms and concentrations of each metal tested are given in Table 7.

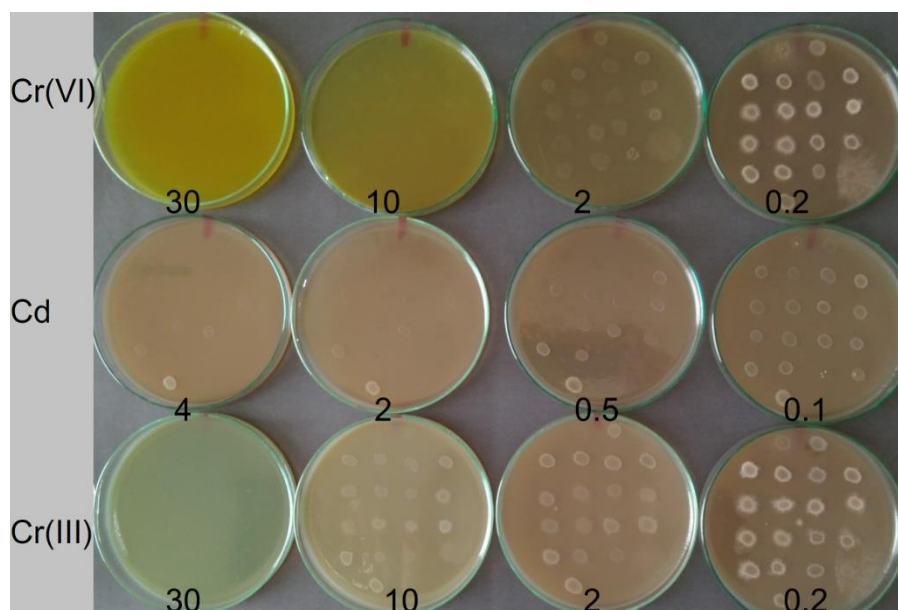

Figure 15 Heavy metal tolerance testing by agar spot inoculation

DETECTION OF CHROMATE TRANSPORTER GENE

Strain NCr1a was subjected to detection and sequencing of *chrA* chromate transporter gene, known for its role in high chromate tolerance (Cervantes et al., 1990; Díaz-Pérez et al., 2007; Nies et al., 1990).

The final PCR mixture (50µl) consisted of 0.4 µM of each primer, 250 µM of each of dNTP, 1U Dynazyme Taq DNA polymerase (Finnzymes Reagents, Thermo Fisher Scientific, Inc. Finnland), 1 x PCR Dynazyme buffer with final $MgCl_2$ concentration of 1.5 mM, and 1µl of the template DNA solution. The temperature program consisted of an initial denaturation at 94º C for 1 min, 30 cycles (94°C for 30 s, 51°C for 30 s, 72°C for 1min 30 sec) and a final extension step at 72°C for 8 min. PCR products were purified by QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) or by 0.8 % agarose gel electrophoresis followed by extraction with QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Purified PCR products were sequenced (GATC-Biotech, Konstanz, Germany) using PCR primers and analyzed using BLASTn search (Altschul et al. 1990; Zhang et al. 2000).

Primers used were:

- ChrABP_AccF: 5' atcatcatcGGTACCTTGAAAACAATAATAGAAATCTTTCTCG 3'

(characteristics: 28 bp; without tail 25% GC, Tm 53.4 / with tail 32.6% GC, Tm 68.5)

- ChrABP_BamR2: 5' atcatcatcGGATCCCTAGATAATAGAAAGAATATAACCGCTTAAAG 3'

(characteristics: 32bp; without tail: 28.1% GC, Tm 53.5 / with tail: 34% GC, Tm 69).

STEM EDS ANALYSIS OF CELLS AND ENDOSPORES EXPOSED TO Cr

Based on its highest chromate tolerance, strain NCr1a was selected for analysis of Cr content and localization in cells and endospores by STEM-EDS (scanning transmission electron microscopy coupled to energy dispersive X-ray spectroscopy). Reference strain *B. subtilis* PY79 (shortened as BS) was tested in parallel as a chromate sensitive control. Growth comparison of NCr1a and *B. subtilis* PY79 were conducted on M9 and DSM media with different Cr(VI) concentrations. Cells and endospores were prepared by different treatments for STEM-EDS analysis, as will be detailed in the following sections.

Cell preparations

Exposure from start of growth. Single colony from overnight LBA plate was inoculated to LB and incubated overnight at 150 rpm. Next, 10 ml of M9 medium with different concentrations of Cr(VI) was inoculated with LB overnight culture to an 0.1 starting OD600. Tested concentrations were 0, 10, 100 and 1000 µM Cr(VI), each in triplicate. Flasks were incubated at 150 rpm at 37°C for *B. subtilis* PY79 and 28°C for NCr1a. OD600 was measured each hour in exponential phase, and every 6 hours at later stages of growth. Middle exponential phase (4 hours of growth) culture was fixed with 2.5 % glutaraldehyde buffer for 30 minutes, washed and stored in PBS. Prior to STEM analysis suspension was washed 3 times in distilled water in order to remove salt residues from PBS which could affect analysis. *B. subtilis* PY79 cultures grown with Cr(VI) had to be concentrated because growth was very limited.

Induction experiments. Cultures grown with 0 and 10 μM Cr(VI) were collected in exponential phase, washed to remove residual Cr(VI), set to uniform OD600 and exposed to 1 mM Cr(VI) in PBS for 1h. After that, fixation and preparation for STEM were performed as stated above.

Endospore preparations

Sporulation in the presence of Cr(VI). DSM medium with 0 and 1 mM Cr(VI) was inoculated with overnight LB culture to an 0.1 starting OD600 and incubated for 6 days at 150 rpm. After 6 days sporulation was confirmed by brightfield microscopy, culture was collected, fixed and resuspended in water until STEM analysis.

As *B. subtilis* PY79 failed to grow in medium with 1000 μl Cr(VI), experiments with higher starting OD600 were also performed.

Exposure of mature spores to Cr(VI). Strains were sporulated in DSM without Cr(VI), endospores were washed, set to same OD600 and exposed to 1 mM Cr(VI) in distilled water to prevent germination. After 1 hour and 6 days of exposure, endospores were fixed and resuspended in water until STEM analysis.

STEM-EDS conditions

Scanning Transmission Electron Microscopy with energy dispersive spectroscopy (STEM-EDS) was used to obtain elemental composition maps and to compare elemental content in the two examined bacterial strains' cells and endospores. Elemental content was reported in atomic percentages (at%), which is the percentage of one kind of atom relative to the total number of atoms.

STEM EDS was performed at the Interdisciplinary centre for electron microscopy (CIME) at the Ecole polytechnique federale de Laussane (EPFL) in Lausanne, Switzerland. Data were acquired at following conditions: Tecnai Osiris, 200 kV, 1.2 nA beam current as was described previously (Jamroskovic et al., 2016). Namely, an X-ray EDS system (Esprit/Quantax Bruker) in STEM mode in a microscope FEI Tecnai Osiris [200 kV X-FEG field emission gun, X-ray detector (Super-X) with 4 mm × 30 mm windowless SDD diodes and 0.9 sr collection angle] was used. Quantitative EDS analysis was carried out using the Cliff–Lorimer standard- less method with thickness correction using K-series. The physical Bremsstrahlung background was calculated based on the sample composition. Some elements such as Cu originating from the Cu grid were removed from quantification after the deconvolution procedure in the quantification process. Elemental concentrations in atomic % and net counts (signal above background) were derived from deconvoluted line intensities within a 95% confidence level. The process time and acquisition rates were adapted to get the most accurate data for specific element such as Ca, P, Cr and Mn. The experimental spectra were collected with no pile-up artifacts. A correction for specimen drift was applied during acquisition to improve elemental mapping accuracy.

4.5. STATISTICAL ANALYSIS

Presence of linear correlation between chemical, physical and bacterial soil properties (CB counts, percentage of CrCB and percentage of Gram-positive bacteria) was tested by Pearson correlation coefficient (r). Comparison among different soil groups was performed by one-way analysis of variance (ANOVA) and Unequal N HSD Post-hoc test. All tests were done in Statistica 12.0 software at $p<0.05$ (Dell Inc., 2015). Graphic data representations were made in Microsoft Office Excel 2007, Statistica 12.0 or Origin Pro 9 (Dell Inc., 2015; OriginLab, 2007).

5. RESULTS

Results of this thesis will be presented in 4 main sections (Figure 16):

1) Influence of chromium on the cultivable soil bacterial community
2) Isolation of chromate tolerant bacterial strains
3) Characterization of selected chromate tolerant *Bacillus cereus* group strains
4) STEM EDS analysis of chromium content and localization in cells and endospores of strains NCr1a and *B. subtilis* PY79.

First section covers examination of influence of soil Cr level and Cr origin on the characteristics of cultivable bacterial soil community.

Second section deals with isolation and characterization of chromate tolerant strains from diverse habitats including soil, water, sediments and solid waste materials.

Third section is concerned with detailed characterization of selected *Bacillus cereus* group strains. Their cultivable, morphological and genetic properties as well as chromate tolerance and chromate reduction ability is reported.

In forth section, response to chromate of highly chromate tolerant strain NCr1a and chromate sensitive strain *B. subtilis* PY79 is probed. Elemental mapping by STEM EDS gives insight into differences in Cr content and localization in cells and endospores of these two strains.

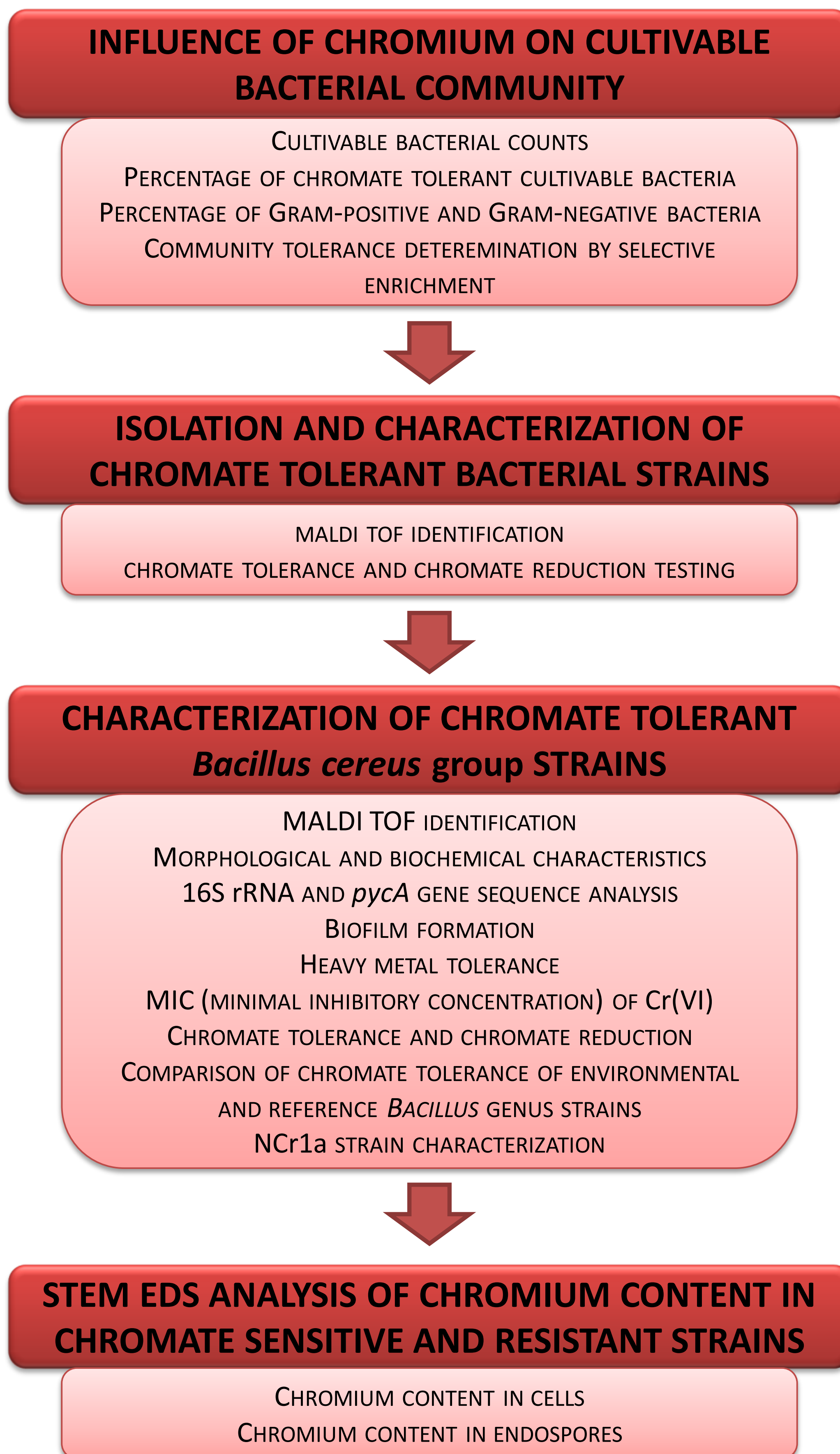

Figure 16 Outline of the research plan: four main research sections and analyses conducted in each section

5.1. Influence of chromium on the cultivable soil bacterial community

To better understand the impact of Cr on bacterial communities, we compared the cultivable bacterial community of soils with elevated Cr level of natural and anthropogenic origin to that of soils with low background Cr level.
A total of 17 soil samples were divided in three groups based on content of chromium and general characteristics:

- LCr - background low Cr level,
- PCr - elevated Cr level of anthropogenic origin, and
- NCr - elevated Cr level of natural origin.

By comparing results of microbial and chemical analyses of these sample groups, we could assess whether high soil Cr level of natural origin and of anthropogenic origin affects cultivable bacterial soil community in a significant manner.
Experimental approach consisted of following sections:

- Chemical and physical analysis of soil samples
- Determination of cultivable bacteria counts
- Determination of proportion of chromate tolerant cultivable bacteria
- Determination of proportion of Gram-positive and Gram-negative bacteria.

Statistical analysis of the obtained data was performed in order to determine whether Cr soil content or origin made an impact upon the abundance, composition and chromate resistance of the bacterial community.

Chemical characterization of the soil samples

As mentioned before, three groups of soil samples according to their different Cr levels and Cr origin were sampled at several locations in the Republic of Serbia (Table 9, Figure 6).
Chemical characterization of the soil samples included determination of Cr content, granulometry, pH and dry matter content (Table 8).
The soil samples with low background Cr level below 50 mg/kg (group **LCr**) were collected in the vicinity of the sites with elevated Cr concentrations, and their soil properties were largely comparable to those of soils with high Cr levels. They differed only in low Cr content.

Samples with Cr content elevated due to anthropogenic activities (group **PCr**) were collected at contaminated sites and their chromium content ranged from 112 to 818 mg/kg.

Samples with naturally elevated Cr level (group **NCr**) had chromium concentrations ranging from 342 to 1287 mg/kg, while almost all other metals were below the limit value for soils according to the proposed European commission threshold values (Gawlik and Bidoglio, 2006). Exceptions were elevated nickel levels due to the ultramafic origin of the soils and Hg level which was above the limit only in sample 10 (Table 10). Samples of NCr group exhibited slightly acidic pH values in the range of 5.1 to 5.8.

Low Cr group had significantly lesser content of Cr compared to PCr and NCr groups, while PCr and NCr did not significantly differ in Cr level (t-test for independent samples, $p<0.05$).

Table 8 Chemical characteristics of soil samples used in the cultivable soil bacterial community analyses

Sample group	Sample	Cr (mg/kg)	Granulometry (% < 2μm)	pH	Dry matter (%)
LCr[a]	1	39.0	37.1	4.1	69.8
	2	25.3	21.9	8.8	70.0
	3	47.2	2.4	7.6	88.0
	4	40.8	21.6	8.4	67.9
PCr[b]	5	496.0	17.2	7.9	45.6
	6	419.0	32.0	6.8	48.5
	7	112.1	17.9	5.6	42.8
	8	818.0	14.5	8.3	81.3
NCr[c]	9	537.0	27.6	5.5	73.3
	10	470.0	38.4	5.7	49.6
	11	1287.0	31.9	5.1	80.9
	12	496.0	44.7	5.4	71.4
	13	395.0	33.9	5.8	73.0
	14	342.0	25.8	5.3	78.1
	15	420.0	24.2	5.5	72.0
	16	835.0	22.9	6.1	66.7
	17	663.0	30.2	5.2	69.8
EU limit[d]	-	50-100	-	-	-

[a] LCr – samples with low background Cr; [b]PCr – samples polluted with Cr; [c]NCr – samples with natural geogenic Cr; [d]Proposed EU threshold for soils (Gawlik and Bidoglio, 2006)

Table 9 Description of sampling locations: LCr – low background Cr level, PCr - elevated Cr level of anthropogenic origin, NCr - elevated Cr level of natural origin

	Sample No.	Location	Location description	Latitude	Longitude	Sampling date
LCr	1	Branešci	Mt. Zlatibor, beech and oak forest	43.75083	19.68977	19.10.2014
	2	Fruška gora	Mt. Fruška gora, lime and oak forest	45.15762	19.87740	19.11.2014
	3	Subotica, chemical factory	Yard of the closed chemical factory, far from pollution sources	46.115725	19.635603	20.3.2015
	4	Subotica	Mesophile meadow 4 km away from the chemical factory circle, near Subotica city water plant	46.14202	19.58664	20.3.2015
PCr	5	Subotica, leather tannery	Wastewater lagoon 2 of the closed leather tannery; soft soil covered with grass, presence of annelidae and snails	46.08203	19.69117	11.3.2015
	6	Subotica, leather tannery	Wastewater lagoon 2 of the closed leather tannery; soft soil covered with grass, presence of annelidae and snails	46.08203	19.69117	11.3.2015
	7	Subotica, leather tannery	Wastewater lagoon 1 of the closed leather tannery; soft soil covered with grass	46.08256	19.69183	11.3.2015
	8	Subotica, chemical factory	Yard of the closed chromic acid production facility, near chemical waste piles	46.116355	19.635592	20.3.2015
NCr	9	Kremna	Mt. Zlatibor, coniferous forest	43.80964	19.56332	18.10.2014
	10	Vitasi	Mt. Zlatibor, agricultural soil	43.84005	19.56139	18.10.2014
	11	Mokra Gora	Mt. Zlatibor, by the dirt road, near oak forest	43.78237	19.51317	18.10.2014
	12	Kotroman	Mt. Zlatibor, meadow by the dirt road	43.76023	19.47733	18.10.2014
	13	Semegnjevo 1	Mt. Zlatibor, meadow by the road	43.74932	19.576	18.10.2014
	14	Semegnjevo 2	Mt. Zlatibor, clearing in coniferous forest, by the spring	43.74942	19.60244	19.10.2014
	15	Obudojevica	Mt. Zlatibor, meadow by the coniferous forest	43.72284	19.68799	19.10.2014
	16	Čajetina	Mt. Zlatibor, grazing meadow	43.74715	19.65551	19.10.2014
	17	Maljen	Mt. Maljen, clearing in the coniferous forest	44.11751	19.97322	19.10.2014

Table 10 Results of the additional chemical analyses of soil samples with elevated Cr level of natural origin (mg/kg)

Sample	Cu	Zn	Ni	Pb	Cd	Hg	TOC
9	26.6	60.7	1048.0	35.2	0.1	0.6	112.0
10	24.5	55.4	938.0	23.8	0.1	1.5	110.0
11	22.7	40.4	2328.0	6.4	0.0	0.8	15.6
12	30.0	62.5	1962.0	8.0	0.0	0.6	51.5
13	21.7	67.1	2199.0	14.8	0.1	0.5	48.4
14	18.1	43.2	1808.0	9.8	0.0	0.5	51.1
15	19.8	66.6	740.0	30.8	0.1	0.4	121.0
16	25.6	79.1	905.0	13.8	0.1	0.6	87.7
17	25.4	175.5	748.0	31.0	0.2	0.8	89.1
EU limits[a]	40-100	100-200	30-70	50-100	0.5-1.5	0.2-1	-

[a]Proposed EU thresholds for soils (Gawlik and Bidoglio, 2006)

CULTIVABLE COMMUNITY TOLERANCE TO CHROMATE DETERMINED BY A SELECTIVE ENRICHMENT METHOD

Soil samples were tested for growth in Cr(VI) containing media by a selective enrichment method in order to determine the overall Cr(VI) tolerance of the cultivable microbial community.

As it can be seen from the results of the selective enrichment (Table 11, Figure 17) all soil samples contain microorganisms capable to tolerate elevated concentrations of Cr(VI) during cultivation. More than half of the samples (64.7 %) exhibited visible growth on the maximal Cr(VI) concentration tested (2.5 mM). Furthermore, all samples showed growth on 1.5 mM Cr(VI).

Table 11 Chromate tolerance of cultivable soil microbial communities determined as the ability to grow upon successive transfers in medium containing increasing Cr(VI) concentrations

Cr(VI) (mM)	Number of soil samples with visible growth			Total
	LCr[a]	PCr[b]	NCr[c]	
0	4	4	9	17
0.5	4	4	9	17
1.5	4	4	9	17
2	3	4	5	12
2.5	3	4	4	11

[a] LCr – samples with low background Cr; [b]PCr – samples polluted with Cr; [c]NCr – samples with natural geogenic Cr

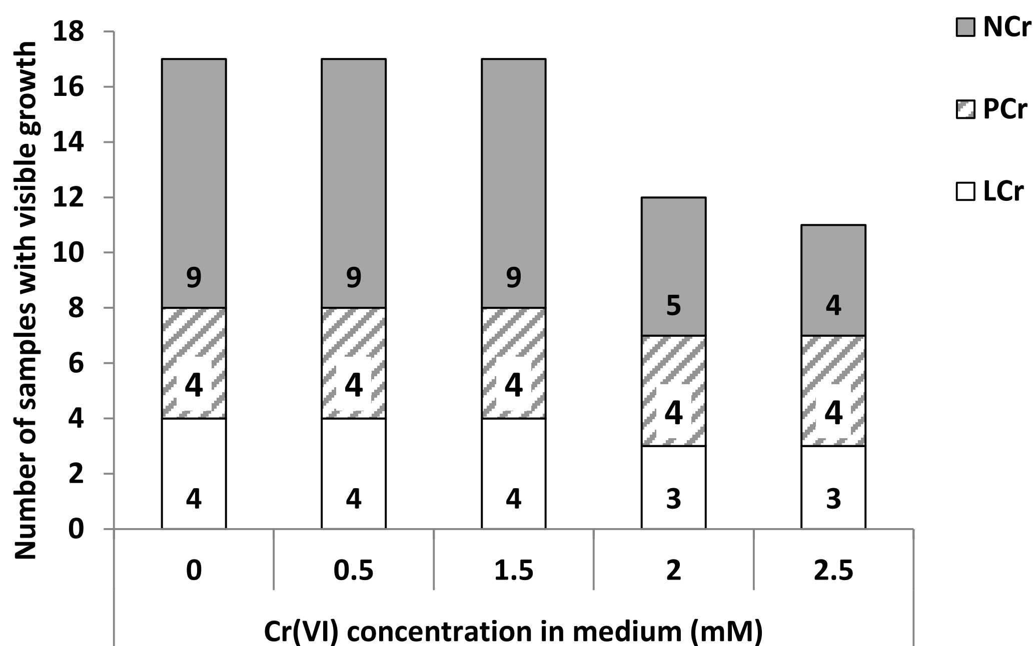

Figure 17 Selective enrichment of soil microbial community on media with increasing Cr(VI) concentrations – number of samples in each sample group showing growth on medium with different Cr(VI) level: NCr – samples with natural geogenic Cr; PCr – samples polluted with Cr; LCr – samples with low background Cr

When comparing chromate resistance among soil sample groups, 75 % of soil samples with low background Cr concentration, 100 % of samples with anthropogenic Cr and 44.4 % of samples with naturally occurring Cr exhibited bacterial growth on 2.5 mM of Cr(VI) in minimal media. Therefore, the highest chromate tolerance at the microbial community level, as determined by the selective enrichment method, could be noted in samples with anthropogenic Cr.

ENUMERATION OF CULTIVABLE BACTERIA AND CHROMATE TOLERANT CULTIVABLE BACTERIA

In order to assess influence of high Cr level on cultivable bacteria we compared their counts in the samples of the three different soil groups. Counts of cultivable soil bacteria (CB) were determined by the spread plate method on R2A agar as described in Materials and methods. Bacterial counts on R2A agar supplemented with increasing concentrations of Cr(VI) were compared to counts on R2A without Cr(VI) and the percentage of chromate tolerant cultivable bacteria (CrCB) was calculated. Thus, we could determine which proportion of the total cultivable heterotrophic soil bacteria expresses elevated level of chromate tolerance. Also, we could determine if high Cr levels select for the presence of more chromate tolerant bacteria compared to low Cr soils.

Cultivable bacteria counts or heterotrophic plate counts (HPC) of soils with background Cr and naturally high Cr level ranged from 10^6 to 10^7 CFU/g, while counts in soils with anthropogenic Cr were 10^6 to 10^8 CFU/g (Table 12, Figure 18).

Table 12 Cultivable bacteria counts in different soil groups: LCr **-** background Cr level, PCr -elevated Cr level of anthropogenic origin**,** NCr **-** elevated Cr level of natural origin

Sample group	Sample	$\cdot 10^6$ CFU/g ± SD
LCr	1	3.9±0.5
	2	12.4±4.5
	3	14.4±0.1
	4	15.9±0.9
PCr	5	188.2±1.5
	6	191.8±25.7
	7	359.0±55.3
	8	7.2±0.8
NCr	9	8.0±0.9
	10	43.9±6.1
	11	13.2±0.7
	12	18.4±2.9
	13	68.9±7.0
	14	14.2±3.1
	15	15.4±3.8
	16	86.4±22.1
	17	11.2±2.9

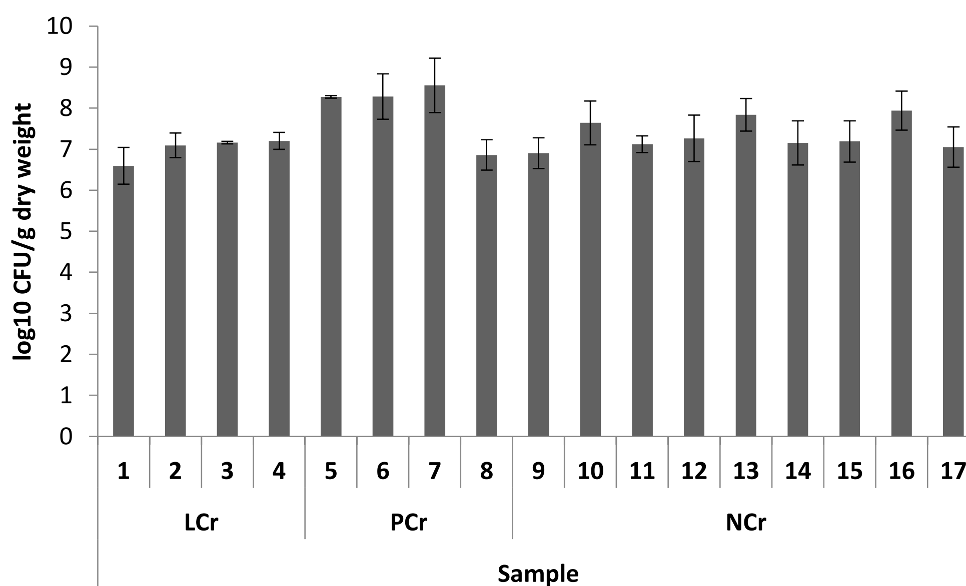

Figure 18 Count of cultivable bacteria in different soil groups: LCr **-** background Cr level, PCr - elevated Cr level of anthropogenic origin**,** NCr **-** elevated Cr level of natural origin

Table 13 Linear correlation of cultivable bacteria counts (CFU/g), percentage of chromate tolerant cultivable bacteria and Gram-positive bacteria and soil chemical and physical properties determined by Pearson correlation coefficients (r)

Variables	Cultivable bacterial counts (CFU/g)	Gram-positive bacteria (%)	Chromate tolerant bacteria (%)
Cultivable bacterial counts (CFU/g)	1		
Gram-positive bacteria (%)	0.007	1	
Chromate tolerant bacteria (%)	-0.547*	0.416	1
Cr (mg/kg)	-0.150	-0.200	0.224
Granulometry (particles <2μm)	-0.177	-0.331	-0.161
pH	0.037	0.686**	0.170
Dry matter (%)	-0.793**	-0.069	0.448

*Significant at the 0.05 probability level. **Significant at the 0.01 probability level.

There was no statistically significant correlation between cultivable bacteria counts and Cr concentration in soil for all the samples considered (Table 13). Coefficient of determination R^2 was very low (R^2=0.0224) indicating there is no linear dependence between these two variables (Figure 19). In fact, soils with anthropogenic Cr exhibited significantly higher bacterial counts compared to the other soil groups (one-way ANOVA, $p<0.05$). Correlation between cultivable bacteria count and soil granulometric properties and pH value was also non-significant, while correlation with dry matter percentage was moderate ($r=-0.793$, $p<0.05$). When considering pH value as a possible influence on bacterial counts, it should be noted that the relatively low pH value of soil sample 1 (4.1) could have contributed to its low bacterial count.

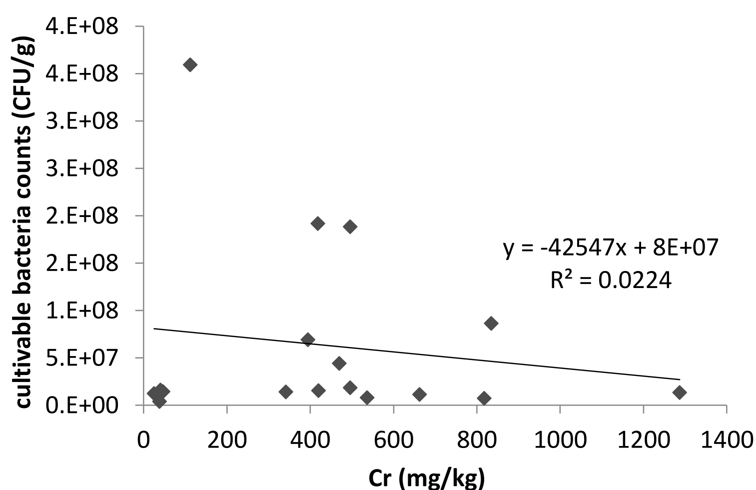

Figure 19 Linear correlation of cultivable bacteria counts and Cr content in soil samples with linear trendline and coefficient of determination R^2

Considering the impact of increasing Cr(VI) concentrations in R2A agar on bacterial counts, it was noted that even a relatively low concentration (0.25 mM) of Cr(VI) led to a large (47.5±21.6 %) drop compared to no Cr(VI) count. Cultivable counts decreased exponentially with the increase of Cr(VI) concentration in the medium in samples of all groups, regardless of Cr content (Figure 20).

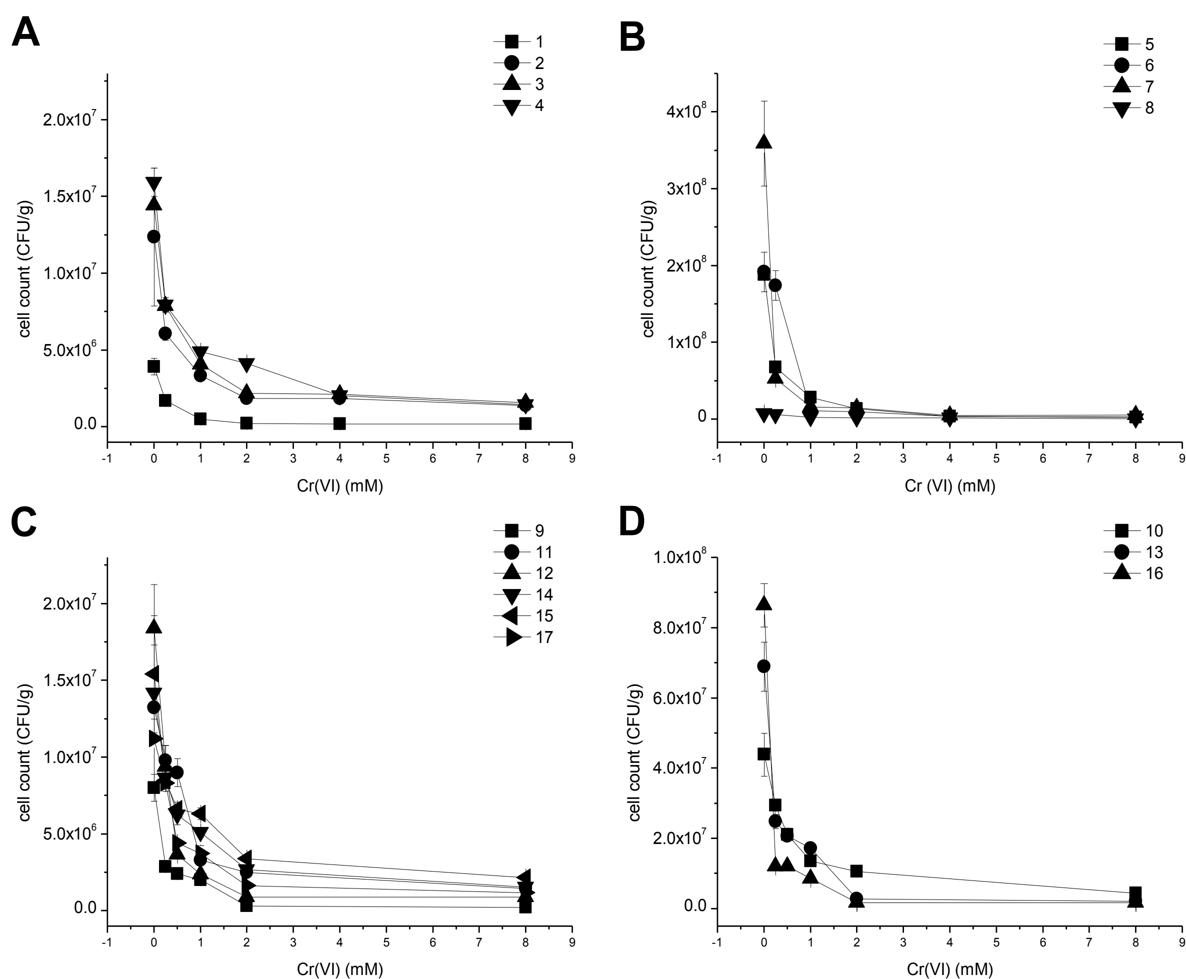

Figure 20 Count of cultivable bacteria obtained by spread plate method on R2A agar with increasing Cr(VI) concentration: A - background Cr level, B - elevated Cr level of anthropogenic origin**,** C and D - elevated Cr level of natural origin

On average, 86.6±9.6 % of the cultivable bacterial population was inhibited by a concentration of 2 mM Cr(VI) in R2A agar, and a further increase of concentration to 4 and 8 mM did not cause substantial additional inhibition (Table 14). Presumably, at concentrations of 2 mM and higher, similar bacterial counts remain because the critical toxicity level that eliminates the majority of sensitive strains is already reached. In a few samples (samples 6, 7 and 16), lower concentration of Cr(VI) (1 mM) inhibited growth to a similar extent as 2 mM Cr(VI). In eight soil samples, the decrease in cultivable counts was less pronounced, as even at the highest tested Cr(VI) concentration of 8 mM, more than 10 % of counts remained (samples 2, 3, 8, 10, 11, 14, 15 and 17). However, it should be noted these particular samples belong to all the three soil groups, thus soil Cr

content could not be considered as a reason for their exceptional percentage of chromate tolerant bacteria. Other chemical and physical properties also could not be linked to their higher percentage of chromate tolerant bacteria. The size of colonies was noticeably smaller on R2A medium with Cr(VI) concentrations above 1 mM suggesting a toxic effect.

Table 14 Percentage of cultivable soil bacteria tolerant to various Cr(VI) medium concentrations in soil samples of different groups

Sample group	Sample	Concentration of Cr(VI) in medium (mM)				
		0.25	1	2	4	8
LCr[a]	1	43±6	13±2	6±3	5±2	5±1
	2	49±22	27±6	15±0	15±0	11±0
	3	55±5	28±4	15±2	15±1	11±1
	4	50±6	31±1	26±2	13±3	9±1
PCr[b]	5	36±1	15±0	7±1	2±0	1±0
	6	91±11	6±0	5±1	2±0	2±0
	7	15±1	4±0	4±0	1±0	2±0
	8	84±3	35±1	34±3	30±2	13±3
NCr[c]	9	36±8	25±2	4±0	3±1	3±0
	10	67±1	31±6	24±2	11±2	10±2
	11	74±10	25±3	19±4	18±0	11±3
	12	51±15	13±2	5±2	5±2	5±1
	13	36±8	25±2	4±0	3±1	3±0
	14	61±10	36±6	19±3	14±1	11±2
	15	55±6	41±6	22±3	21±3	14±3
	16	14±1	10±2	2±1	2±0	2±1
	17	74±4	33±14	15±3	16±3	11±2
% tolerant	average	52.5±21.6	23.4±11.3	13.4±9.6	10.5±8.2	7.3±4.5
% inhibited	average	47.5±21.6	76.6±11.3	86.6±9.6	89.5±8.2	92.7±4.5

[a] LCr – samples with low background Cr; [b]PCr – samples polluted with Cr; [c]NCr – samples with natural geogenic Cr; higher percentage values compared to other samples are highlighted

In general, a similar percentage of cultivable bacteria capable of growth on media with increasing Cr(VI) concentrations was observed in all the three soil groups (Figure 21). No statistically significant difference between percentage of chromate tolerant bacteria among the three soil groups was observed (one-way ANOVA, $p>0.05$). Moderate correlation of chromate tolerant bacteria percentage and cultivable bacteria count was found ($r=-0.547$, $p<0.05$). However, there was no correlation with Cr soil concentration, pH value, granulometry and dry matter content of the samples (Table 13).

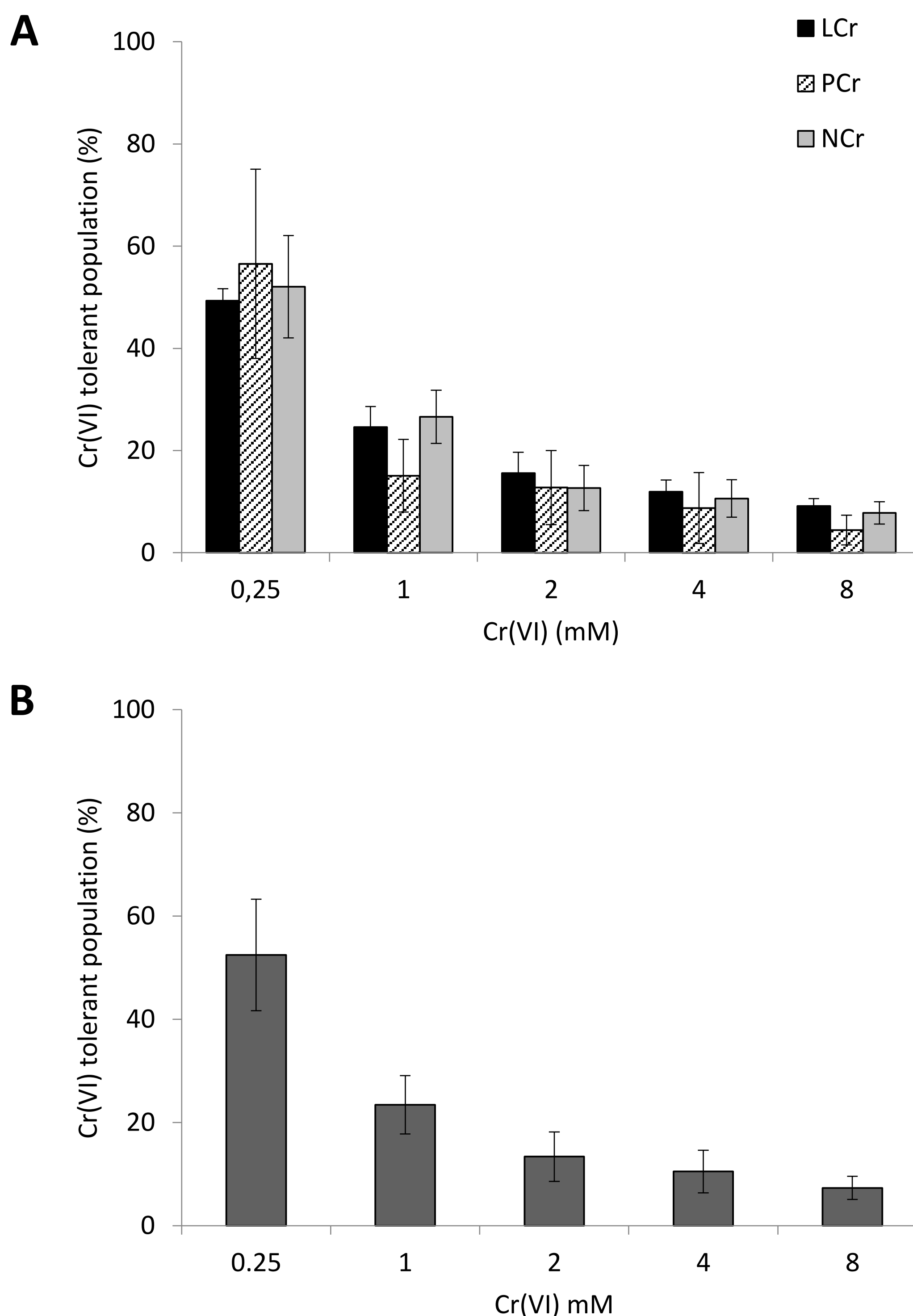

Figure 21 Percentage of chromate tolerant cultivable bacteria (percentage of cell count on medium with Cr(VI) relative to cell count on medium without Cr(VI) which represents 100%): A - average for each sample group: LCr - background Cr level, PCr - elevated Cr level of anthropogenic origin, NCr - elevated Cr level of natural origin; B – average for all samples

Taking everything into account, obtained results indicate that cultivable bacteria abundance and chromate tolerant bacteria proportion are not clearly affected by the total soil Cr level nor its origin.

Proportion of Gram-positive and Gram-negative bacteria within the cultivable bacteria soil population

In some previous work it was suggested Gram-positive bacteria exhibit higher chromate tolerance and that they are more dominantly present in Cr(VI) contaminated soils. Therefore, we tested their proportion in soils of the three different groups.

The ratio of Gram-positive and Gram-negative bacteria varied considerably in all the sample groups (Figure 22).

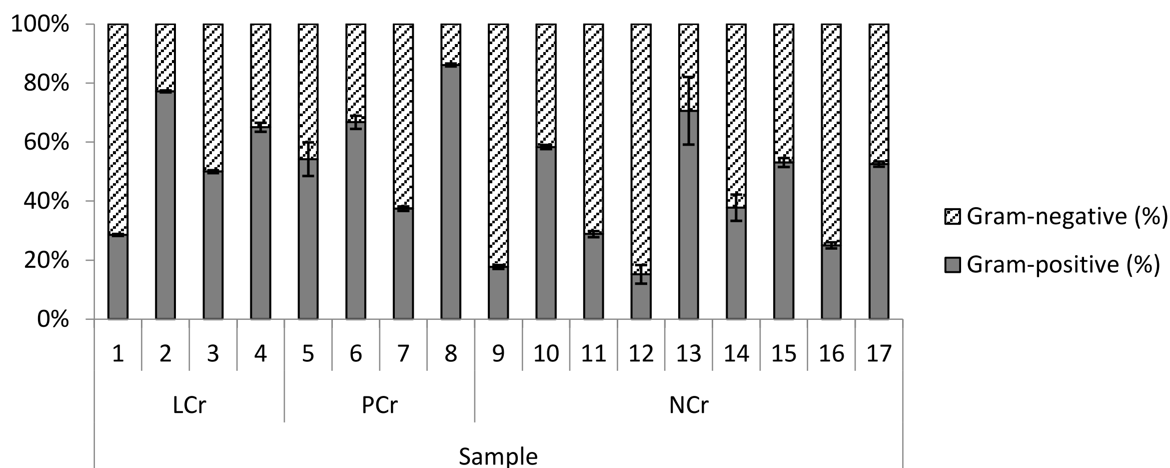

Figure 22 Percentage of Gram-positive and Gram-negative bacteria in different soil groups: LCr - background Cr level, PCr - elevated Cr level of anthropogenic origin, NCr - elevated Cr level of natural origin.

There was no statistically significant linear correlation between the proportion of Gram-positive bacteria and the soil Cr concentration (Pearson product moment correlation coefficient r=-0.200, coefficient of determination R^2=0.0399) (Figure 23, Table 13).

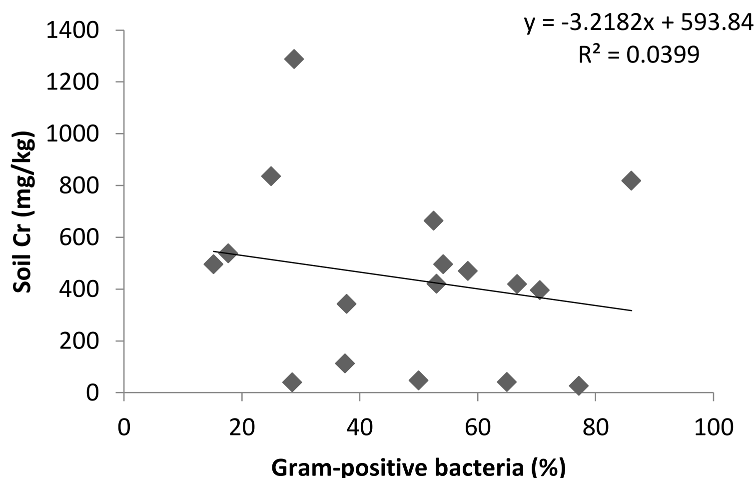

Figure 23 Linear correlation of Gram-positive bacteria percentage and Cr content in soil samples with linear trendline and coefficient of determination R^2

No statistically significant difference in Gram-positive bacteria percentage among the three sample groups was observed (one-way ANOVA, $p=0.195$). Moderate correlation to pH value was found ($r=0.686$, $p<0.01$), while there was no correlation to dry matter content and granulometry.
Hence, chromium soil concentration nor its origin could be linked to the proportion of Gram-positive to total cultivable soil bacteria in the present study.

5.2. ISOLATION OF CHROMATE TOLERANT BACTERIAL STRAINS

Diverse environmental samples with low and elevated chromium concentrations (soil, sediment, water and solid waste samples) were used for the isolation of chromate tolerant bacterial strains. By attempting isolation from various habitat types, we aimed to isolate diverse strains and to determine whether chromate resistant strains are present both in environments with high and low chromium levels.

In total, 26 environmental samples were collected and used as a source to isolate chromate tolerant bacterial strains. Based on the concentration and the origin of chromium, samples were divided in three groups, as can be seen in Table 15.

Table 15 Overview of environmental sample groups collected for the purpose of the isolation of chromate tolerant bacterial strains

Sample group mark	Cr level (qualitative)	Description of sample group	Sample markings	No of samples	Range of Cr concentration (mg/kg)
LCr	Low background	Background Cr concentration	LCr1 to LCr9	9	<50
PCr	High	Elevated Cr concentration caused by pollution	PCr1 to PCr12	12	84 -496
NCr	High	Elevated Cr concentration of natural origin	NCr1 to NCr5	5	395-1287

Detailed properties of each analyzed sample are given in the Appendix (Section 9.1). The sample group with low Cr content (group marked as LCr) contained 9 samples with low background Cr concentration below 50 mg/kg, marked as LCr1 to LCr9. Sample group with high Cr content caused by pollution (group marked as PCr) contained 12 samples. Sample group with high naturally high Cr content (group marked as NCr) encompassed 5 samples). High Cr samples (PCr and NCr) had Cr concentration above the European Union proposed threshold of 100 mg/kg soil (Gawlik and Bidoglio, 2006). Exceptions were two samples (PCr5 and PCr12), which had a concentration slightly below the threshold value (92.4 and 85 mg/kg, respectively). However, they also were regarded as high Cr samples, because they were collected from industrially polluted sites and had higher Cr compared to corresponding nearby unpolluted low Cr controls.

Environmental samples were subjected to the isolation of chromate tolerant bacteria by selective enrichment method as described in Materials and methods. Isolated strains were identified by MALDI TOF. Further, their chromate tolerance as the ability to grow on media with high levels of Cr(VI) was determined. Finally, their bioremediation potential as the ability to reduce toxic Cr(VI) to innocuous Cr(III) form was measured.

MALDI TOF IDENTIFICATION

We isolated a total of 33 chromate tolerant bacterial strains with the ability to grow on 2 mM Cr(VI) in minimal media (Table 16). According to MALDI TOF identification, the isolated strains belong to the 12 species and 8 different genera. Strains belonging to the *Bacillus* genus were predominant among the isolated chromate tolerant strains (21 out of 33 isolates). Among them, one strain (PCr7) was classified as *B. subtilis*, while the rest were from the *B. cereus* species group.

Table 16 Chromate tolerant bacterial strains isolated from environmental samples with high and low Cr level: overview of isolated strains with the general characteristics of the source of isolation

Sample group	Sample type	Sample	Total Cr (mg/kg)[a]	Strain mark	Isolated strains MALDI TOF identification
High Cr[b]	NCr[c] soil	NCr1	470	NCr1a	*Bacillus cereus* group
				NCr1b	*Bacillus cereus* group
				NCr1c	*Bacillus cereus* group
				NCr1d	*Serratia fonticola*
		NCr2	1287	NCr2	*Bacillus cereus* group
		NCr3	395	NCr3	*Bacillus cereus* group
		NCr4	835	NCr4	*Bacillus cereus* group
		NCr5	663	NCr5	*Ochrobactrum anthropi*
	PCr[d] soil	PCr1	419	PCr1	*Bacillus cereus* group
		PCr2	84	PCr2a	*Bacillus cereus* group
				PCr2b	*Ochrobactrum grignonense*
		PCr3	496	PCr3a	*Bacillus cereus* group
				PCr3b	*Cellulosimicrobium cellulans*
		PCr4	797	PCr4a	*Arthrobacter woluwensis*
				PCr4b	*Rhodococcus erythropolis*
		PCr5	112.1	PCr5	*Bacillus cereus* group
		PCr6	92.4[e]	PCr6	*Ochrobactrum tritici*
		PCr7	119	PCr7	*Bacillus subtilis*
		PCr8	534	PCr8	*Staphylococcus haemolyticus*
		PCr9	117	PCr9	*Bacillus cereus* group
		PCr10	175	PCr10	*Ochrobactrum grignonense*
	PCr sediment	PCr11	334	PCr11	*Microbacterium maritypicum*
	PCr solid waste	PCr12	85[e]	PCr12	*Bacillus cereus* group
Low Cr	soil	LCr1	40.8	LCr1	*Staphylococcus warneri*
	soil	LCr2	25	LCr2	*Bacillus cereus* group
	soil	LCr3	47.2	LCr3	*Bacillus cereus* group
	Salt march, soil	LCr4	9.25	LCr4	*Bacillus cereus* group
	Salt march, soil	LCr5	12.13	LCr5	*Bacillus cereus* group
	Salt march, soil	LCr6	7.7	LCr6	*Bacillus cereus* group
	Salt march, water	LCr7	1.46	LCr7	*Bacillus cereus* group
	Salt march, water	LCr8	1.33	LCr8	*Bacillus cereus* group
	Salt march, water	LCr9	1.52	LCr9a	*Bacillus cereus* group
				LCr9b	*Staphylococcus haemolyticus*

[a] unit for water samples is µg/l, [b] samples with >100 mg/kg Cr, [c] NCr – samples with natural geogenic Cr, [d] PCr – samples polluted with Cr; [e] sample was collected from Cr polluted site and has elevated Cr compared to background Cr level in the same region, thus it is considered as high Cr sample

Samples with elevated Cr levels exhibited higher diversity of chromate tolerant strains - 11 different species were isolated from a total of 17 samples (Table 17). An especially high number of distinct species was noted among PCr samples (9 species from 12 samples), while NCr soils yielded strains of three different species from 5 samples. Conversely, LCr samples showed a lower diversity of chromate tolerant isolates as only three different species were recovered from 9 samples. Thus, PCr samples seem to harbor a higher diversity of chromate tolerant strains, even though the proportion of chromate tolerant cultivable bacteria in them is not necessarily higher.

Table 17 Summary of chromate tolerant strains isolated from the environmental samples with different concentration of Cr

Genera	Species	Number of isolated strains			
		High Cr samples		Low Cr samples	Total
		PCr[a] (n=12)	NCr[b] (n=5)	LCr[c] (n=9)	
Arthrobacter	*Arthrobacter woluwensis*	1			1
Bacillus	*Bacillus cereus group*	6	6	8	20
	Bacillus subtilis	1			1
Cellulosimicrobium	*Cellulosimicrobium cellulans*	1			1
Microbacterium	*Microbacterium maritypicum*	1			1
Ochrobactrum	*Ochrobactrum anthropi*		1		1
	Ochrobactrum grignonense	2			2
	Ochrobactrum tritici	1			1
Rhodococcus	*Rhodococcus erythropolis*	1			1
Serratia	*Serratia fonticola*		1		1
Staphylococcus	*Staphylococcus haemolyticus*	1		1	2
	Staphylococcus warneri			1	1
Total		15	8	10	33

[a] PCr – samples polluted with Cr; [b] NCr – samples with natural geogenic Cr; LCr – samples with low (background) concentration of Cr

Majority of isolates were Gram-positive (28 out of 33), while only 5 were Gram-negative. In the majority of samples (21 out of 26), only one chromate tolerant strain was isolated per sample. This is probably a consequence of the strain's ability to outgrow other present chromate tolerant strains during selective enrichment, as it is highly unlikely that only one strain in the sample is chromate tolerant. It should be noted that the isolation methodology could limit the diversity of isolated strains. One

possible explanation as to why *Bacillus* sp. strains were dominantly isolated is their colony morphology. Possibly, when strains were checked for growth on 2 mM Cr(VI) on agar medium, *Bacillus* strains were more easily noticeable, due to their large spreading colonies. As it was stated previously, concentration of ≥1 mM of Cr(VI) in medium caused notable decrease in colony size. Therefore, colonies of strains with naturally small size could have been even more diminished, to the point of them not being noticed during this step of the isolation procedure.

CHROMATE TOLERANCE AND REDUCTION

Chromate tolerance and chromate reduction were tested for all the isolated strains. Chromate tolerance was measured as a percentage of growth on Cr(VI) containing medium compared to no Cr(VI) medium (maximum relative growth). Tested concentrations were 0.5, 2 and 4 mM Cr(VI). Chromate reduction was measured after 24 h of incubation on a starting Cr(VI) level of 0.5 mM (Figure 24). Minimal chemically defined M9 medium was used as nutrient rich media can give overestimated or nonreplicable results of metal tolerance and reduction testing.

Chromate reduction testing results are given in Figure 24 and Table 18.
Among the strains isolated from LCr samples, the highest chromate reduction was noted in *B. cereus* group strain LCr7 (42.5 %), while others had reduction below 33.5 %. Among the NCr strains, highest reduction of 58.5 % was exhibited by *B. cereus* group strain NCr2, while *Serratia fonticola* NCr1d reduced the least Cr(VI) (5.5 %). High reduction of strain NCr2 was accompanied with high growth percentage (>75 %) on all Cr(VI) concentrations tested (0.5, 2 and 4 mM). The most efficient chromate reducers in PCr group were *Bacillus cereus* group strains PCr2a (55.0%) and PCr3a (56.0%) and *Cellulosimicrobium cellulans* strain PCr3b (55.0%). Almost every strain from PCr group had high growth percentage of more than 80% on 0.5 mM Cr(VI).
In summary, the highest Cr(VI) reduction (above 50 %) was noted in *Bacillus cereus* group strains NCr2, PCr2a and PCr3a and *Cellulosimicrobium cellulans* PCr3b (Figure 24, Table 18). In general, lower reduction was noted among LCr strains. However, certain PCr and NCr strains also had lower reduction, so no direct link between strains' origin and its chromate reduction ability could be established. Similarly, there was no clear distinction between genera or species. Some *Bacillus cereus* group strains had high, while others had low reduction. Two *Staphylococcus haemolyticus* strains had identical low reduction, however *S. warneri* strain had moderate level of reduction. *Ochrobactrum* strains were characterized by moderate to low reduction.

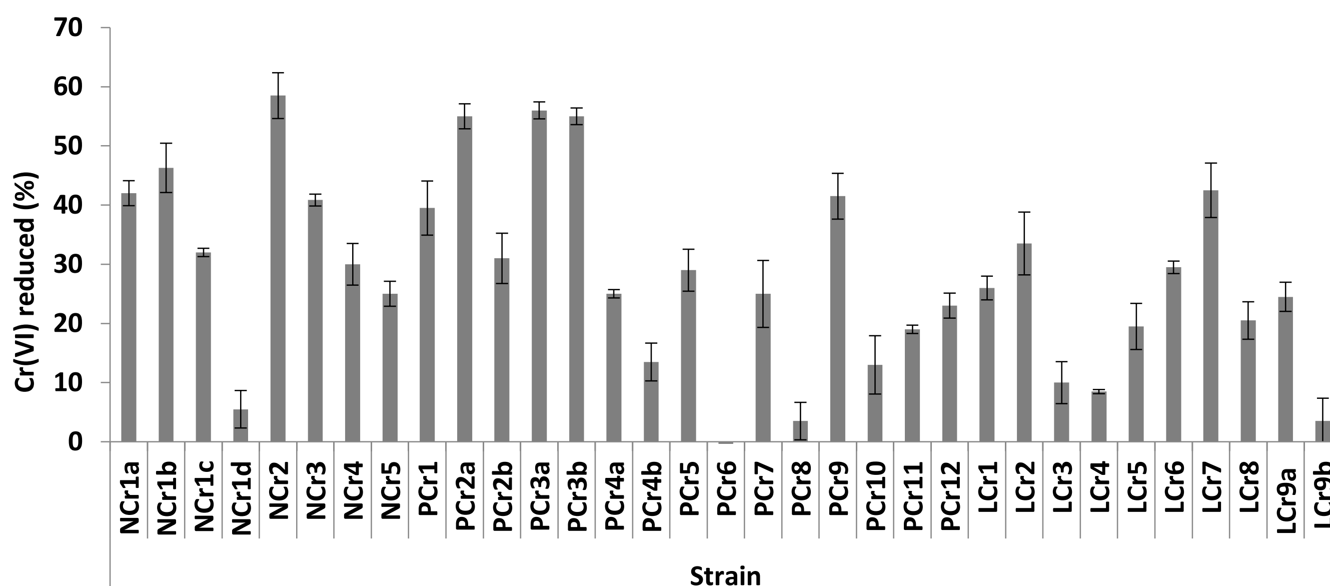

Figure 24 Chromate reduction of 33 environmental isolates - percentage of Cr(VI) reduced in M9 medium with a starting concentration of 0.5mM Cr(VI) after 24 hours of incubation

Chromate tolerance was measured as a percentage of growth on 0.5, 2 and 4 mM Cr(VI) media compared to no Cr(VI) media.

Growth percent on medium with **0.5 mM Cr(VI)** was above 50% in all the strains (Figure 25A). Eight strains had growth of over 95%, namely, *Bacillus cereus* group strains (NCr1a, NCr2, PCr2a, PCr5, PCr9), *Cellulosimicrobium cellulans* PCr3b, *Microbacterium maritypicum* PCr11 and *Ochrobactrum grignonense* PCr2b. The lowest growth (<70%) was noted for a number of *Bacillus cereus* group strains (LCr2, LCr3, LCr4, LCr5, LCr9a, NCr1b, NCr3) and *O. anthropi* NCr5. It should be noted that in some instances growth on 0.5 mM Cr(VI) was higher than in medium without Cr(VI), which resulted in growth percentages above 100 (strains NCr1a, NCr2, PCr2b and PCr3b).

At the more toxic **2 mM Cr(VI)** concentration, the most tolerant strains were *Bacillus cereus* NCr2 and *Cellulosimicrobium cellulans* PCr3b (growth >80%) (Figure 25B). Growth of 70-80% was noted for strains of *Bacillus cereus* group (NCr1a, NCr4, PCr3a and PCr5), *Microbacterium maritypicum* PCr11, *Ochrobactrum grignonense* PCr2b and *Serratia fonticola* NCr1d. Generally, growth percentage of all the isolates at 2 mM Cr(VI) was ≥36.2%. Similarly to 0.5 mM concentration, the lowest growth was noted for a number of *Bacillus cereus* group strains and *O. anthropi* NCr5.

At the highest tested concentration of **4 mM Cr(VI)** only three strains were able to grow to over 70% - *Bacillus cereus* group strain NCr2, *Ochrobactrum grignonense* PCr2b, and *Microbacterium maritypicum* PCr11 (Figure 25C). Beside them, notable chromate tolerance was found in strains with >60% growth: *Bacillus cereus* group strains (NCr4, PCr3a PCr5, LCr6), *Cellulosimicrobium cellulans* PCr3b and *Serratia fonticola* NCr1d.

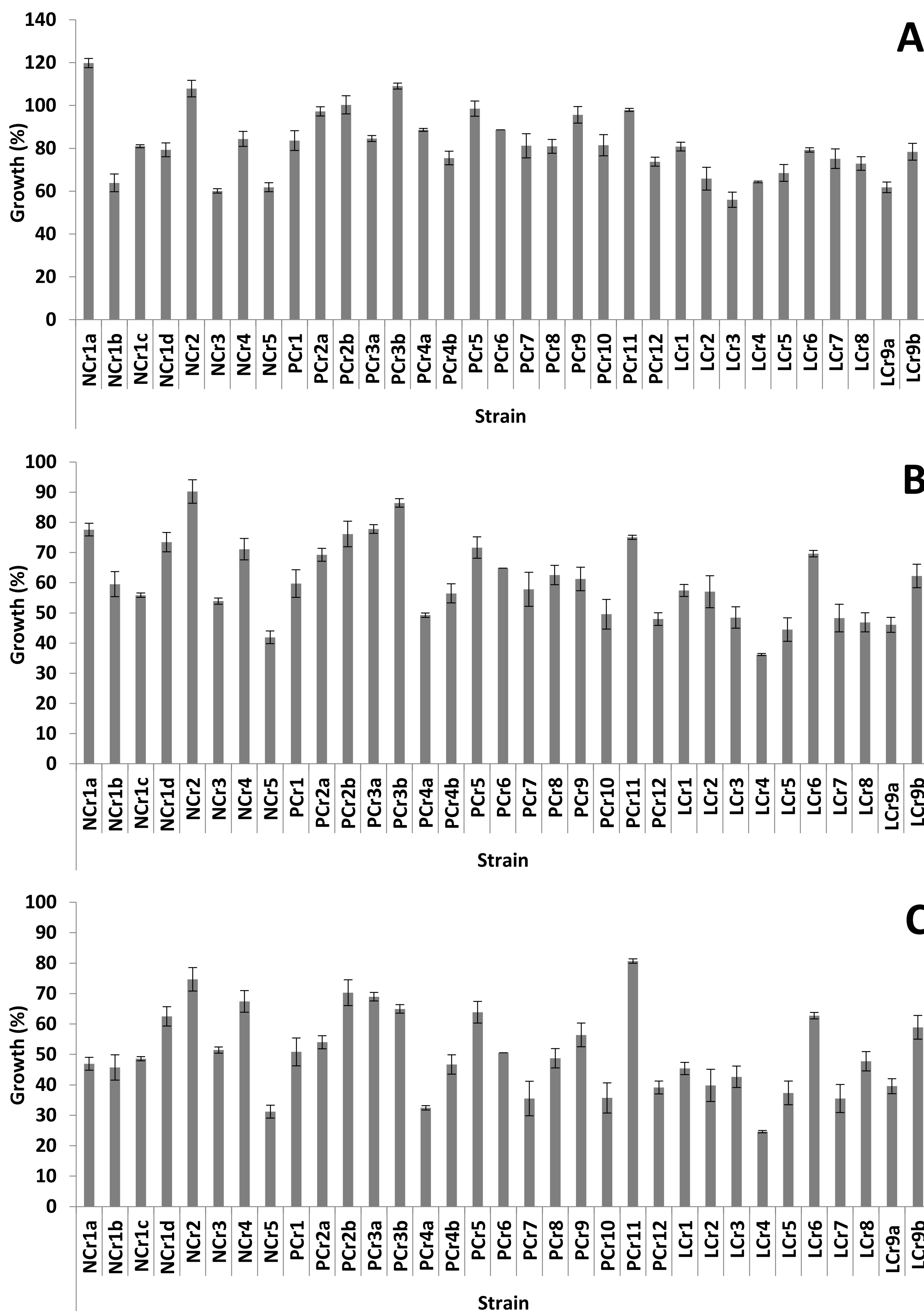

Figure 25 Chromate tolerance of 33 environmental isolates – percentage of growth on M9 medium with: A – 0.5 mM, B – 2 mM and C - 4 mM Cr(VI) compared to no Cr(VI) control

Table 18 Chromate tolerance and reduction of the 33 environmental bacterial isolates

Strain		Cr(VI) reduced (%)[a]	Growth on M9 medium with Cr(VI) (%)[b]		
			0.5 mM	2 mM	4 mM
Bacillus cereus group	NCr2	58.5	107.9	90.3	74.7
Bacillus cereus group	PCr3a	56.0	84.6	77.8	69.0
Cellulosimicrobium cellulans	PCr3b	55.0	109.0	86.4	64.9
Bacillus cereus group	PCr2a	55.0	97.2	69.3	54.0
Bacillus cereus group	NCr1b	46.3	63.9	59.5	45.7
Bacillus cereus group	LCr7	42.5	75.2	48.3	35.5
Bacillus cereus group	NCr1a	42.0	119.8	77.6	46.9
Bacillus cereus group	PCr9	41.5	95.7	61.2	56.4
Bacillus cereus group	NCr3	40.9	60.1	53.9	51.4
Bacillus cereus group	PCr1	39.5	83.6	59.7	50.8
Bacillus cereus group	LCr2	33.5	65.8	57.0	39.8
Bacillus cereus group	NCr1c	32.0	81.0	55.9	48.6
Ochrobactrum grignonense	PCr2b	31.0	100.3	76.1	70.3
Bacillus cereus group	NCr4	30.0	84.4	71.1	67.4
Bacillus cereus group	LCr6	29.5	79.3	69.6	62.7
Bacillus cereus group	PCr5	29.0	98.5	71.7	63.9
Staphylococcus warneri	LCr1	26.0	80.8	57.4	45.4
Bacillus subtilis	PCr7	25.0	81.2	57.8	35.5
Arthrobacter woluwensis	PCr4a	25.0	88.6	49.2	32.4
Ochrobactrum anthropi	NCr5	25.0	61.8	41.9	31.2
Bacillus cereus group	LCr9a	24.5	61.8	46.1	39.6
Bacillus cereus group	PCr12	23.0	73.8	48.0	39.1
Bacillus cereus group	LCr8	20.5	72.9	46.9	47.7
Bacillus cereus group	LCr5	19.5	68.5	44.5	37.3
Microbacterium maritypicum	PCr11	19.0	97.9	75.1	80.7
Rhodococcus erythropolis	PCr4b	13.5	75.5	56.5	46.7
Ochrobactrum grignonense	PCr10	13.0	81.4	49.5	35.7
Bacillus cereus group	LCr3	10.0	56.0	48.5	42.6
Bacillus cereus group	LCr4	8.5	64.3	36.2	24.6
Serratia fonticola	NCr1d	5.5	79.3	73.5	62.5
Staphylococcus haemolyticus	LCr9b	3.5	78.4	62.2	58.9
Staphylococcus haemolyticus	PCr8	3.5	80.9	62.5	48.7
Ochrobactrum tritici	PCr6	0.0	88.6	64.9	50.6

color scale indicates reduction/growth percentage (low – red to high – green), data are in descending order according to the value of reduction percentage; [a] percentage of starting 0.5 mM reduced after 24h of growth on M9 medium; [b] growth in Cr(VI) containing media compared to no Cr(VI) media

There was no clear correlation between the growth percentage and the chromate reduction as determined by Pearson correlation coefficient ($r<0.40$) or coefficient of determination ($R^2=0.1222$) (Figure 26).

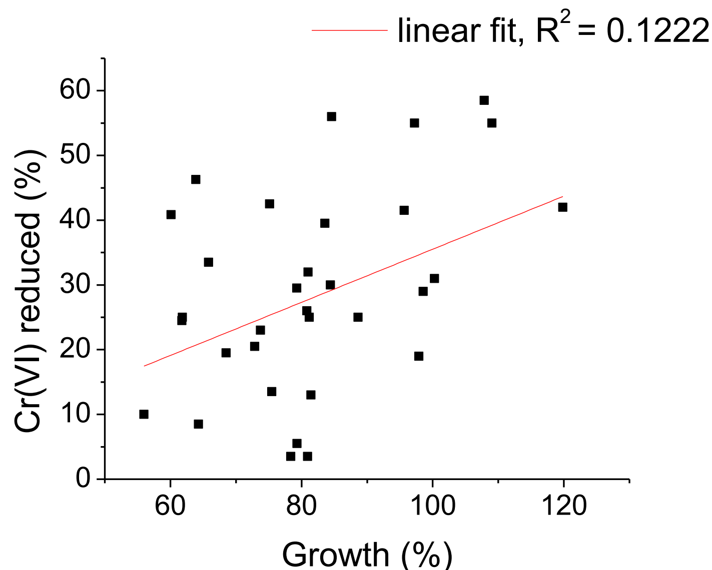

Figure 26 Linear correlation between percentage of Cr(VI) reduction and percentage of growth on Cr(VI) contacting media for environmental chromate tolerant isolates

According to the acquired data, strains which are the most interesting in the light of their bioreduction potential are *Bacillus cereus* group strains NCr1a, NCr2, PCr2a and PCr3a and *Cellulosimicrobium cellulans* PCr3b. These strains exhibit high reduction accompanied by high chromate tolerance, which would enable them to successfully grow and reduce Cr(VI) in highly toxic Cr(VI) polluted environments.

From the point of view of examining alternative chromate resistance mechanisms, such as chromate efflux, strains with low reduction, but with high growth would be of interest. Namely, *Serratia fonticola* NCr1d, *Staphylococcus haemolyticus* LCr9b and PCr8, *Ochrobactrum tritici PCr6 and Microbacterium maritypicum* PCr11.

5.3. CHARACTERIZATION OF SELECTED CHROMATE TOLERANT *BACILLUS CEREUS* GROUP STRAINS

Diverse environmental samples with low background and highly elevated chromium concentration were subjected to isolation of chromate tolerant strains, as stated in previous section. A number of Gram-positive and Gram-negative strains with the ability to grow on 2 mM Cr(VI) in minimal media were isolated. The majority of isolated strains belonged to the *Bacillus cereus* group, which is environmentally important group but less studied in respect of its chromate tolerance. Thus, we focused further investigations on the 13 selected *B. cereus* group strains (out of 20 isolated) (Table 19). The isolates were chosen so that the various sample types were represented to an approximately proportionate degree. Four selected strains (LCr6, LCr7, LCr8 and LCr9a) were isolated from soil and water samples obtained from the unpolluted salt marshes. Six strains (NCr1a, NCr1b, NCr1c, NCr2, NCr3 and NCr4) originated from serpentine soils with elevated concentrations of chromium of natural geological origin. Finally, three strains (PCr1, PCr2a and PCr12) isolated from locations impacted by industrial Cr pollution were chosen.

Table 19 List of chromate tolerant *Bacillus cereus* group strains selected for detailed analysis, with the characteristics of the source of isolation

Strain	Sample type	Description of sample	Total Cr (mg/kg)[a]
NCr1a	Natural Cr	Serpentine soil	470
NCr1b	Natural Cr	Serpentine soil	470
NCr1c	Natural Cr	Serpentine soil	470
NCr2	Natural Cr	Serpentine soil	709
NCr3	Natural Cr	Serpentine soil	395
NCr4	Natural Cr	Serpentine soil	835
PCr1	Polluted with Cr	Leather tannery in Subotica – soil from former wastewater lagoon	496
PCr12	Polluted with Cr	Leather tannery in Zrenjanin – sample from solid waste container	85
PCr2a	Polluted with Cr	Leather tannery in Subotica – soil in front of tannery	84
LCr6	Low Cr – salt march	Salt marsh Medura – soil	<50
LCr7	Low Cr – salt march	Salt marsh Slano Kopovo – water sample	<50
LCr8	Low Cr – salt march	Salt marsh Medura – water sample	<50
LCr9a	Low Cr – salt march	Salt marsh Slano Kopovo – water sample	<50

[a] unit for water samples is µg/l

Selected strains were subjected to detailed analysis. Namely, MALDI TOF identification, cell and colony morphology, biochemical properties, 16S rRNA and *pycA* gene sequence analysis, biofilm formation potential, tolerance to Cr and heavy metals and Cr(VI) reduction were determined, as will be reported in the following sections. To clarify, MALDI TOF identification and Cr(VI) tolerance and reduction for these strains were also reported in the previous section, but more detailed account on this will be given bellow.

MALDI TOF IDENTIFICATION

Based on MALDI TOF analysis, all the 13 strains considered, belonged to the *B. cereus* group. This species group until recently contained six species: *B. cereus, B. anthracis, B. thuringiensis, B. mycoides, B. pseudomycoides* and *B. weihenstephanensis* (Fritze, 2004). In the past few years, more species have been proposed, adding up to 11 described species (Jiménez et al., 2013; Jung et al., 2011; Liu et al., 2014) and 19-20 species which are awaiting further characterization (Liu et al. 2015). For the purposes of this study, we will limit comparison of isolates to the six well known species, for which detailed characterization of taxonomically important properties exists. All the six species are highly similar in morphological, biochemical and genetic characteristics and are thus difficult to distinguish.

Principle of MALDI TOF analysis is based on acquisition of mass spectra for each tested isolate and its comparison to a database. As a result, a list of the ten most similar matches in the database is given, together with the parameters of analysis reliability for each match (score value and consistency category) (Table 20). For instance, analysis is highly reliable at a species level if score value is above 2.3 and consistency category is A. If score value is 2.2-2.299 species identification is probable, while genus identification is secure, etc.

Results of the MALDI TOF analysis for the 13 examined strains are given in Table 21.

For certain strains MALDI TOF classification gave ambiguous results. Specifically, for strains NCr1a, NCr1b, NCr1c, NCr2, NCr3, PCr2a, PCr12 and LCr8, the best match and the second best match did not belong to the same species or the second best match was not reliable (Table 21). For example, for strain PCr2a, the best match was *B. cereus* and the second best match was *B. mycoides*. For the remaining strains, the best and the second best match belonged to the same species (*B. cereus*), but the score value and consistency of the results indicated reliable identification at the genus level, but only probable rather than definite species identification.

Thus, the MALDI TOF analysis could only classify all strains in the *B. cereus* group but could not make reliable species identification, due to the high degree of similarity amongst species belonging to this group.

Table 20 Interpretation of reliability of MALDI TOF analysis results (Bruker Daltonics Inc., 2018)

Meaning of Score Values			
Range	Description	Symbols	Color
2.300 ... 3.000	highly probable species identification	(+++)	green
2.000 ... 2.299	secure genus identification, probable species identification	(++)	green
1.700 ... 1.999	probable genus identification	(+)	yellow
0.000 ... 1.699	not reliable identification	(-)	red

Meaning of Consistency Categories (A - C)	
Category	Description
A	Species Consistency: The best match was classified as 'green' (see above). Further 'green' matches are of the same species as the first one. Further 'yellow' matches are at least of the same genus as the first one.
B	Genus Consistency: The best match was classified as 'green' or 'yellow' (see above). Further 'green' or 'yellow' matches have at least the same genus as the first one. The conditions of species consistency are not fulfilled.
C	No Consistency: Neither species nor genus consistency (Please check for synonyms of names or microbial mixture).

Table 21 Results of MALDI TOF analysis of chromate tolerant *Bacillus cereus* group isolates and legend for the interpretation of the results according to the manufacturer instructions

Strain	**The best match**	**The second best match**	**Score Values and Consistency Category**
NCr1a	*Bacillus cereus*	*Bacillus pseudomycoides*	(++) (A)
NCr1b	*Bacillus mycoides*	*Bacillus thuringiensis*	(+) (B)
NCr1c	*Bacillus mycoides*	*Bacillus weihenstephanensis*	(+++) (B)
NCr2	*Bacillus mycoides*	*Bacillus weihenstephanensis*	(+++) (B)
NCr3	*Bacillus mycoides*	*Bacillus cereus*	(++) (A)
NCr4	*Bacillus cereus*	*Bacillus cereus*	(+) (B)
PCr1	*Bacillus cereus*	*Bacillus cereus*	(+) (B)
PCr2a	*Bacillus cereus*	*Bacillus mycoides*	(++) (A)
PCr12	*Bacillus cereus*	not reliable identification	(+) (B)
LCr6	*Bacillus cereus*	*Bacillus cereus*	(++) (A)
LCr7	*Bacillus cereus*	*Bacillus cereus*	(+) (B)
LCr8	*Bacillus cereus*	not reliable identification	(+) (B)
LCr9a	*Bacillus cereus*	*Bacillus cereus*	(++) (A)

To further characterize these strains at the species level, we tested morphological and biochemical characteristics as well as 16S rRNA and *pycA* gene sequences, which could offer more precise species delineation.

MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS

Biochemical and morphological properties suggested for the identification of *B. cereus* group species include haemolysis, penicillin (ampicillin) resistance, motility, colonial morphology and the presence of parasporal crystals (Dworkin et al., 2006; Logan and De Vos, 2009; Luna et al., 2007; Turnbull et al., 2004). To confirm *B. anthracis*, detection of virulence genes *pagA* and *capA* is performed (Papaparaskevas et al., 2004; Ramisse et al., 1996). Inability to produce acid from mannitol is used to distinguish *B. cereus* group species from other *Bacillus* species (Public Health England, 2015).
Results of these analyses for the 13 tested isolates, together with the typical results for the *B. cereus* group species are given in the Table 22.
None of the strains grew at 5°C, which eliminated the possibility that strains belong to *B. weihenstephanensis* species. *B. anthracis* contains virulence genes, does not produce haemolysis, is nonmotile, and the majority of strains are ampicillin sensitive. Of the 13 strains, all were negative for the presence of *B. anthracis* virulence genes, were beta-haemolytic, motile (except strain NCr1a which was nonmotile) and resistant to ampicillin. Hence, it was confirmed that none of the strains is *B. anthracis*. Isolate NCr1a exhibited rhizoidal colony morphology and lack of motility characteristic of *B. mycoides* or *B. pseudomycoides*. Other strains exhibited the typical *B. cereus* colony morphology (large, irregular, rough, slightly raised, opaque, whitish to cream) and were motile (Figure 27).

Figure 27 Colony morphology of chromate tolerant environmental *Bacillus cereus* group isolates: A - strain NCr1a, B - representative image of the colony morphology shared by the rest of the isolates. The scale bar represents 5 mm.

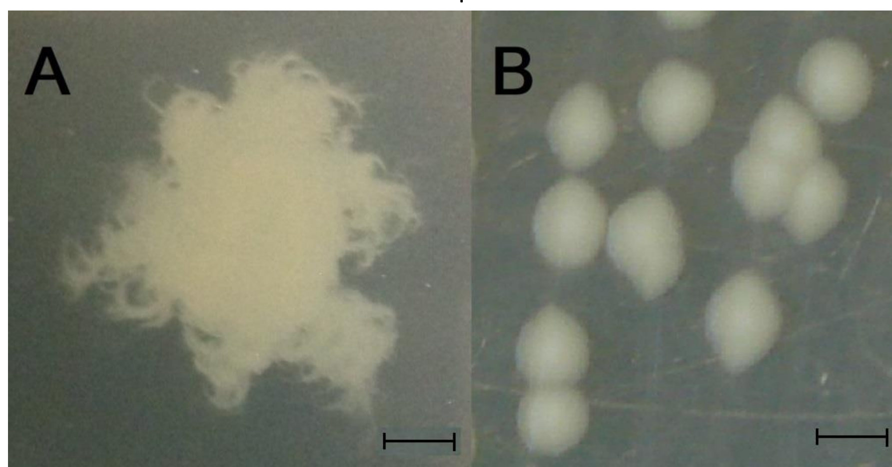

Table 22 Biochemical and cultural characteristics of chromate tolerant *Bacillus cereus* group isolates

Strain	Motility	Hemolysis	Acid from mannitol	Rhizoidal colonies	Ampicillin resistance	Growth on 5°C	Virulence genes[d]
NCr1a	-[a]	β[c]	-	+	+	-	-
NCr1b	+[b]	β	-	-	+	-	-
NCr1c	+	β	-	-	+	-	-
NCr2	+	β	-	-	+	-	-
NCr3	+	β	-	-	+	-	-
NCr4	+	β	-	-	+	-	-
PCr1	+	β	-	-	+	-	-
PCr2a	+	β	-	-	+	-	-
PCr12	+	β	-	-	+	-	-
LCr6	+	β	-	-	+	-	-
LCr7	+	β	-	-	+	-	-
LCr8	+	β	-	-	+	-	-
LCr9a	+	β	-	-	+	-	-
B. cereus[e]	+	β	-	-	+	-	-
B. thuringiensis[e]	+	β	-	-	+	-	-
B. mycoides[e]	-	β	-	+	+	-	-
B. pseudomycoides[e]	-	β	-	+	+	-	-
B. weichenstephanensis[e]	+	β	-	-	+	+	-
B. anthracis[e]	-	-	-	-	-	-	+

[a] + positive result, [b]- negative result, [c] β – beta haemolysis, [d] presence of *B. anthracis* virulence genes *pagA* and *capA*, [e] expected results for >85% strains of the species based on literature data (Dworkin et al., 2006; Logan and De Vos, 2009)

Thus, when morphological and biochemical properties are taken into account, strains could belong to *B. cereus, B. thuringiensis, B. mycoides* or *B. pseudomycoides*, while *B. anthracis* and *B. weichenstephanensis* are eliminated. It should be noted that classification is based on results exhibited by the majority of the strains of the species (>85 %) and that strains with atypical characteristics exist in each species (Dworkin et al., 2006; Logan and De Vos, 2009).

16S rRNA AND *PYCA* GENE SEQUENCE ANALYSIS

Analysis of the 16S rRNA gene sequence confirmed that all the chromate tolerant isolates were closely related to the *B. cereus* group species (Figure 28). The 27F and 685R universal bacterial primers used in the present study allowed sequencing of the V1-V3 variable region, which was proposed as the most applicable for *Bacillus* species

identification (Blackwood et al., 2004). However, BLASTn search resulted in multiple *B. cereus* group and *Bacillus* sp. strain hits with the identical max score, total score, coverage, E-value and identity, precluding precise identification. Thus, 16S rRNA gene amplicon sequence analysis revealed only the closest relative of isolates within the *B. cereus* group, but did not allow identification at the species level.

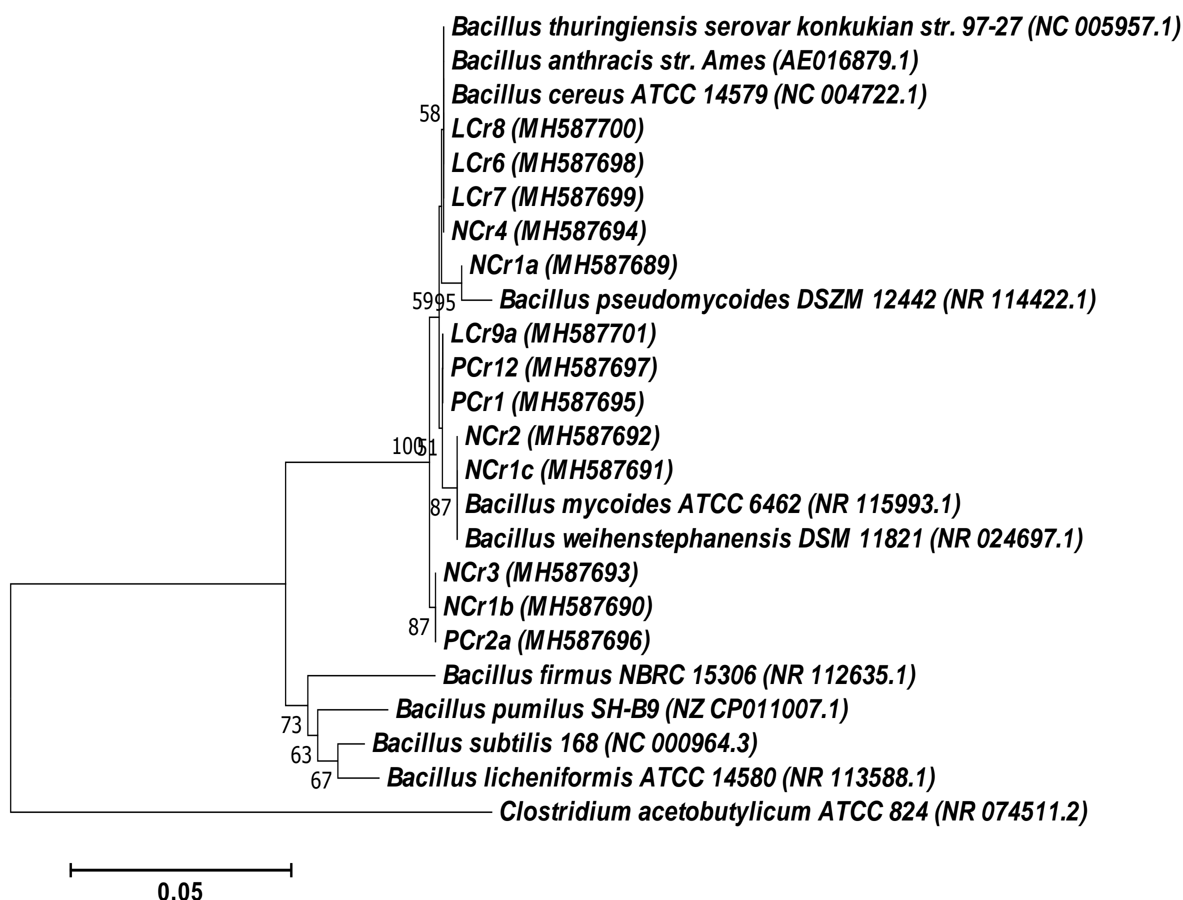

Figure 28 Phylogenetic tree based on 16S rRNA partial gene sequence, 588 positions showing the relationship of 13 chromate tolerant isolates to closely related *Bacillus cereus* group strains and other *Bacillus* reference strains. *Clostridium acetobutylicum* was used as an out-group. Constructed using Neighbor-Joining method, evolutionary distances were computed using the p-distance method, the bootstrap probabilities calculated from 1000 replications with values greater than 50% indicated at the nodes, the bar represents distance value calculated in MEGA7, GenBank accession no. indicated in brackets

We examined the sequence of the *pycA* gene, which was reported to aid in the identification of *B. cereus* group (Liu et al. 2015). BLASTn analysis of the *pycA* gene sequence proved to be more applicable and restricted hits only to members of *B. cereus* group, but still did not allow unambiguous species assignment (Table 23).

Phylogenetic trees based on 16S rRNA and *pycA* gene sequence were constructed (Figure 28, Figure 29). *Clostridium acetobutylicum* was used as an out group, while reference sequences of different *Bacillus* sp. were also included. *Bacillus* genus species

not belonging to the *B. cereus* group (*B. subtilis, B. pumilus, B. licheniformis, B. firmus*) were clearly separated from our isolates on both trees, while *B. cereus* group reference strains grouped together with our isolates.

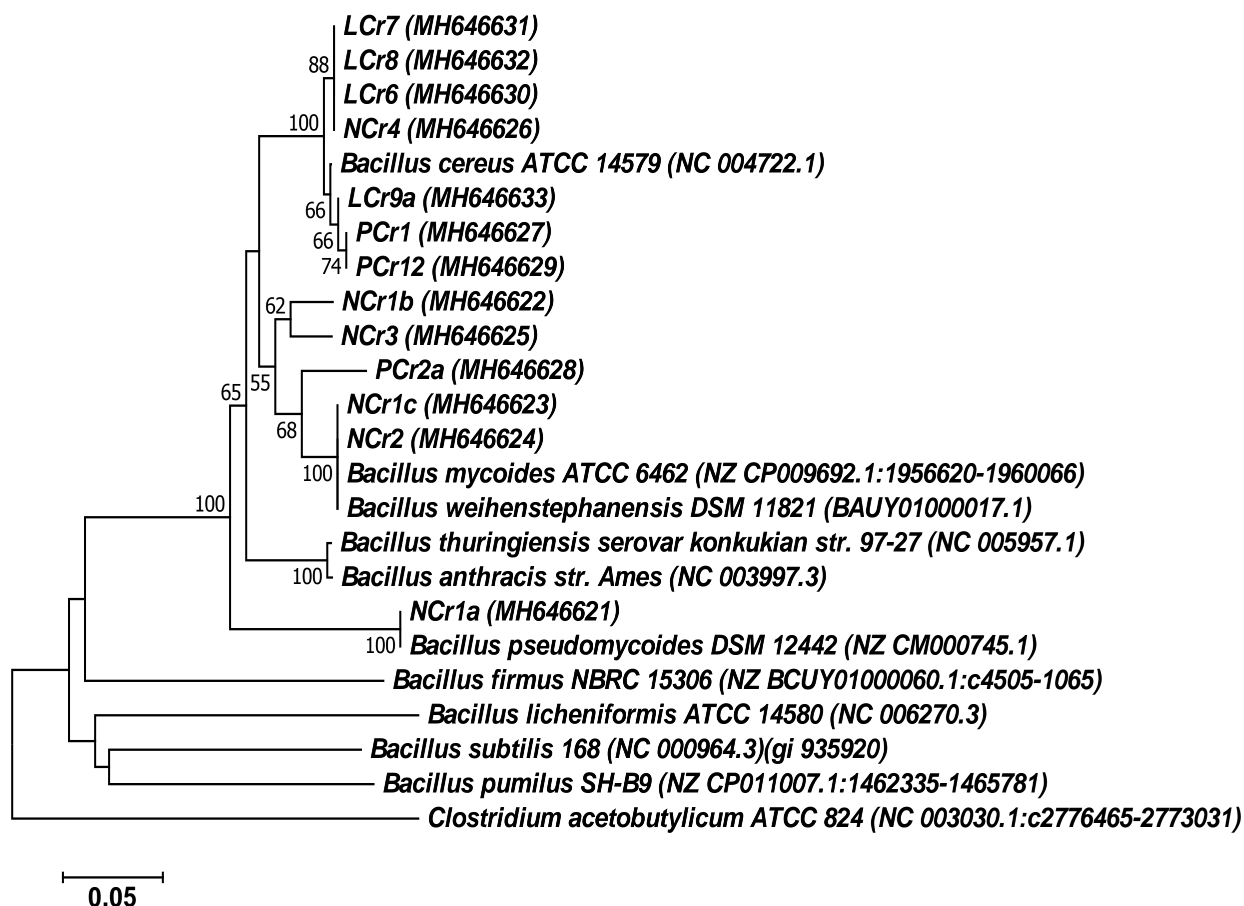

Figure 29 Phylogenetic tree based on *pycA* gene partial sequence, 240 positions showing the relationship of 14 chromate tolerant isolates to closely related *Bacillus cereus* group strains and other *Bacillus* reference strains. *Clostridium acetobutylicum* was used as an out-group. Constructed using Neighbor-Joining method, the bootstrap probabilities calculated from 1000 replications with values greater than 50% indicated at the nodes, the bar represents distance value calculated in MEGA7, GenBank accession no. indicated in brackets.

A few clear findings emerge from comparison of the 16S rRNA and *pycA* phylogenetic trees. First, it is evident that strains NCr1c and NCr2 are closely related to *B. mycoides* and *B. weihenstephanensis* (based on both trees). Second, strain NCr1a is closely related to *B. pseudomycoides*. Third, strains PCr1, PCr12, and LCr9a are closely related. Finally, strains LCr6, LCr7, LCr8, and NCr4 are closely related. Nonetheless, in most cases, definitive species assignment is not possible.

Table 23 Selected highest hits recorded on BLASTn search of *pycA* gene sequence of environmental chromate tolerant *Bacillus cereus* group strains (hits with the highest score value shown) (database nucleotide collection nr/nt)

Strain	Species	GenBank Accession Numbers	Max score	E value	Seq. length	Identity (%)
NCr1a	*Bacillus mycoides* strain BTZ *B. mycoides* strain 219298 *B. mycoides* strain BGSC 6A19	CP009651.1 CP007626.1 KC196989.1	508	5e-140	281	100
NCr1b	*B. cereus* strain TG1-6 *B. thuringiensis* strain L-7601 *B. thuringiensis* serovar israelensis strain AM65-52*	CP026678.1 CP020002.1 CP013275.1	444	5e-121	281	95
NCr1c	*B. mycoides* strain Gnyt1 *B. mycoides* strain ATCC 6462 *B. weihenstephanensis* strain WSBC 10296**	CP020743.1 CP009692.1 KC197001.1	502	2e-138	281	99
NCr2	*B. mycoides* strain Gnyt1 *B. mycoides* strain ATCC 6462 *B. weihenstephanensis* strain WSBC 10296**	CP020743.1 CP009692.1 KC197001.1	502	2e-138	281	99
NCr3	*B. thuringiensis* serovar sylvestriensis strain IEBC-T61 001 *B. thuringiensis* serovar balearica strain IEBC-T48 001 *B. thuringiensis* serovar toguchini strain IEBC-T31 001 *B. cereus* strain IMSNU 13043	KC196968.1 KC196958.1 KC196944.1 AY265533.1	502	2e-138	281	99
NCr4	*B. bombysepticus* str. Wang *B. thuringiensis* serovar londrina strain IEBC-T10A001	CP007512.1 KC196923.1	470	1e-128	263	99
PCr1	B. *thuringiensis* strain c25 *B. thuringiensis* strain YGd22-03*** *B. cereus* strain IMSNU 10013	CP022345.1 CP019230.1 AY265535.1	502	2e-138	281	99
PCr2a	*Bacillus* sp. FDAARGOS_235 B. toyonensis BCT-7112 *B. thuringiensis* ser. wratislaviensis str IEBC-T47 001****	CP020437.2 CP006863.1 KC196957.1	499	3e-137	281	99
PCr12	B. *thuringiensis* strain c25 *B. thuringiensis* strain YGd22-03*** *B. cereus* strain IMSNU 10013	CP022345.1 CP019230.1 AY265535.1	502	2e-138	281	99
LCr6	*B. bombysepticus* str. Wang *B. thuringiensis* serovar londrina strain IEBC-T10A001	CP007512.1 KC196923.1	502	2e-138	281	99
LCr7	*B. bombysepticus* str. Wang *B. thuringiensis* serovar londrina strain IEBC-T10A001	CP007512.1 KC196923.1	502	2e-138	281	99
LCr8	*B. bombysepticus* str. Wang *B. thuringiensis* serovar londrina strain IEBC-T10A001	CP007512.1 KC196923.1	466	2e-127	258	100
LCr9a	*B. cereus* strain K8 *B. cereus* strain A1 *B. thuringiensis* serovar indiana strain HD521	CP016595.1 CP015727.1 CP010106.1	502	2e-138	281	99

*13 more *B. thuringiensis* strains and one more *B. cereus* strain with the same score, ** one more *B. weihenstephanensis*, 5 more *B. mycoides*, two more *B. thuringiensis* strains with the same score, *** 18 more *B. thuringiensis* strains with the same score, **** two more *B. thuringiensis* strains with the same score

BIOFILM FORMATION

Living in the form of a biofilm provides microorganisms with increased protection from many environmental factors, including heavy metal toxicity. Therefore, biofilm formation could be a useful trait for practical use of microorganisms in bioremediation.
For that reason, we tested the ability of the 13 chromate tolerant *Bacillus cereus* group isolates to form biofilm in two media (nutrient rich LB and minimal M9 medium) after 1 and 7 days of incubation. Influence of different Cr(VI) concentrations on biofilm formation potential was also examined. Biofilm formation potential was categorized in 4 groups: 0 - unadherent; 1 - weakly adherent; 2 - moderately adherent; 3 - very adherent according to Stepanovic et al. (2000). Results are presented in Table 24 and Figure 30.

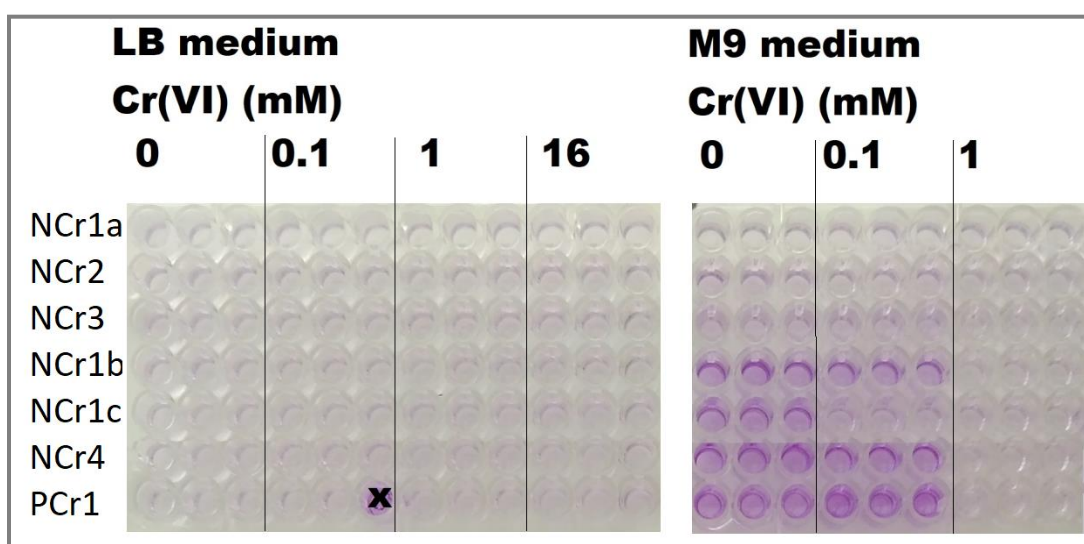

Figure 30 Illustration of biofilm formation potential testing on LB and M9 medium with different concentrations of Cr(VI) after 1 day (X marks faulty well).

Biofilm formation potential was lower on nutrient rich LB medium compared to minimal M9 medium. Longer incubation did not promote biofilm formation. In LB medium strains were generally unadherent or weakly adherent.
Biofilm formation potential was the highest in M9 medium without Cr(VI) after 1 day of incubation. In stated experimental conditions, strains NCr4, PCr12, LCr7, LCr8 and LCr9a were moderately adherent, while LCr6 was very adherent. Concentration of 0.1 mM Cr(VI) did not influence biofilm formation in most strains, while 1 mM concentration caused decrease in bacterial growth accompanied with decreased biofilm formation potential.
Reference strains of *B. cereus* group (*B. cereus* ATCC 14579 and *B. pseudomycoides* DSM 12442) showed similar biofilm formation potential as our isolates. They were unadherent to moderately adherent in most of the experimental conditions. Reference strain *B. subtilis* PY79 exhibited higher biofilm formation, especially in LB medium after 24h, when it was very adherent. Reference strain *P. aeruginosa* ATCC 15692 was more adherent in LB compared to M9 medium.

Table 24 Biofilm formation potential of chromate tolerant *Bacillus cereus* group strains in various concentrations of Cr(VI) (mM) in LB and M9 medium

Incubation duration	**1 day**							**7 days**						
Medium	**LB**				**M9**			**LB**				**M9**		
Cr(VI) concentration (mM)	**0**	**0.1**	**1**	**16**	**0**	**0.1**	**1**	**0**	**0.1**	**1**	**16**	**0**	**0.1**	**1**
NCr1a	0	0	0	0	0	1	1	0	0	0	0	1	1	1
NCr1b	0	0	0	0	1	1	1	1	0	0	0	0	1	1
NCr1c	0	0	0	1	1	1	1	1	0	0	0	1	1	1
NCr2	0	0	0	0	1	1	1	1	0	0	0	0	1	1
NCr3	0	0	0	0	0	1	1	0	0	0	0	1	1	1
NCr4	0	0	1	1	2	2	1	1	0	0	0	0	1	1
PCr1	0	1	1	1	1	1	1	1	1	0	0	1	1	1
PCr2a	1	1	1	1	1	1	0	0	0	0	0	0	1	1
PCr12	0	0	1	1	2	2	1	0	0	0	0	0	0	1
LCr6	0	0	0	1	3	3	0	0	0	0	0	0	1	1
LCr7	0	0	0	0	2	2	0	0	0	0	0	0	0	1
LCr8	0	0	0	1	2	2	0	0	0	0	0	1	1	1
LCr9a	0	0	0	1	2	1	1	0	0	0	0	0	1	1
B. cereus ATCC 11775	0	0	1	1	2	1	1	0	0	0	0	1	1	1
B. pseudomycoides DSZM 12442	0	0	0	1	1	1	1	0	0	0	0	1	1	1
B. subtilis PY79	1	2	1	0	1	1	1	3	3	1	0	1	1	1
P. aeruginosa ATCC 15692	3	3	3	2	1	1	1	2	2	2	2	1	1	1

0 (red) - unadherent; 1 (yellow) - weakly adherent; 2 (green) - moderately adherent; 3 (blue) - very adherent

HEAVY METAL TOLERANCE

As pollution is rarely comprised of only one particular pollutant, multiple heavy metal tolerance would be an advantage for a strain in prospective bioremediation application. Thus, we tested heavy metal tolerance of selected chromate tolerant *Bacillus cereus* group isolates (Table 25).

MIC values of Hg, Cd and Zn were out of the scope of the testing and were reported as higher than the highest tested concentration. Thus, their MIC could possibly be much higher. For that reason, their toxicity was not discussed in detail.

Majority of strains exhibited similar pattern of metal tolerance, while some strains had different response, as will be discussed.

Wolfram (W) and molybdenum (Mo) were the least toxic heavy metals among those tested, with MIC values of >30 mM for all the tested strains.

Ag was the most toxic with MIC value of 0.5 mM for all the strains (the lowest precisely determined MIC among all the metals tested). Strains NCr1a, *B. subtilis* PY79 and *B. pseudomycoides* DSM 12442 were more sensitive to Cd than remaining strains – their

MIC was 0.5 mM compared to 4mM for the rest of the strains. Generally, *B. subtilis* PY79 was the most sensitive strain, with comparably lower tolerance to Hg, Cd, Cu, Co, Ni and Zn. Hexavalent chromium was more toxic than trivalent (MIC of Cr(VI) was 10 mM, while MIC of Cr(III) was 30 mM). *E. coli* ATCC 25922 was more sensitive to Cr(VI), compared to other strains with MIC of 2 mM.

General order of toxicity of heavy metals to *Bacillus cereus* group chromate tolerant isolates, in terms of MIC values could be (most toxic to least toxic from left to right):

$$Ag^{+} > Cu^{2+}, Cr^{6+} > Co^{2+}, Ni^{2+} > Cr^{3+} > W^{6+}, Mo^{6+}.$$

Table 25 Heavy metal resistance of chromate tolerant *Bacillus cereus* group isolates

Strain	MIC (mM)										
	Hg^{2+}	Ag^{+}	Cd^{2+}	Cu^{2+}	Co^{2+}	Ni^{2+}	Zn^{2+}	Cr^{6+}	Cr^{3+}	W^{6+}	Mo^{6+}
NCr1a	>0.1	0.5	0.5	10	10	10	>10	10	30	>30	>30
NCr1b	>0.1	0.5	>4	10	10	10	>10	10	30	>30	>30
NCr1c	>0.1	0.5	>4	10	>10	>10	>10	10	30	>30	>30
NCr2	>0.1	0.5	>4	10	>10	10	>10	10	30	>30	>30
NCr3	>0.1	0.5	>4	10	10	>10	>10	10	30	>30	>30
NCr4	>0.1	0.5	>4	10	10	10	>10	10	30	>30	>30
PCr1	>0.1	0.5	>4	10	10	10	>10	10	30	>30	>30
PCr2a	>0.1	0.5	>4	10	10	10	>10	10	30	>30	>30
PCr12	>0.1	0.5	>4	10	10	10	>10	10	30	>30	>30
LCr6	>0.1	0.5	>4	10	10	10	>10	10	30	>30	>30
LCr7	>0.1	0.5	>4	10	10	10	>10	10	30	>30	>30
LCr8	>0.1	0.5	>4	10	10	10	>10	10	30	>30	>30
LCr9a	>0.1	0.5	>4	10	>10	10	>10	10	30	>30	>30
BC	>0.1	0.5	>4	10	>10	10	>10	10	30	>30	>30
BS	0.1	0.5	0.5	<0.1	0.5	1	10	10	30	>30	>30
BP	>0.1	0.5	0.5	10	>10	10	>10	10	30	>30	>30
EC	>0.1	0.5	>4	>10	>10	10	>10	2	30	>30	>30

BC – *Bacillus cereus* ATCC 14579, BS – *B. subtilis* PY79 , BP – *B. pseudomycoides* DSM 12442, EC - *Escherichia coli* ATCC 25922, green color indicated the highest and orange color the lowest metal tolerance among the tested strains

It should be noted that only a limited range of concentrations with large gaps in between was tested. Therefore, the results obtained give only a rough preliminary assessment of heavy metal tolerance.

MIC (MINIMAL INHIBITORY CONCENTRATION) OF Cr(VI) OF SELECTED BACILLUS CEREUS GROUP STRAINS

The level of chromate tolerance was described by minimal inhibitory concentration (MIC) of Cr(VI) determined on different types of media: minimal defined media (M9 and AMM) and nutrient rich media (LB) (Table 26). Multiple media were used in order to compare differences between minimal and nutrient rich media.

MIC on nutrient rich LB medium was at least 15 times higher, compared to minimal defined media. Minimal media gave similar or identical MIC values for all of the strains. The majority of strains had similar level of chromate tolerance (MIC of 4 mM).

Table 26 Minimal inhibitory concentration of Cr(VI) (mM) for selected chromate resistant *Bacillus cereus* group strains determined on complex nutrient-rich (LB broth) and defined minimal (M9 and acetate minimal medium (AMM)) media

Strain	MIC [mM Cr(VI)]		
	LB	M9	AMM
NCr1a	120	8	4
NCr1b	120	4	4
NCr1c	120	4	4
NCr2	120	4	4
NCr3	120	4	4
NCr4	240	4	4
PCr1	120	4	4
PCr2a	120	4	4
PCr12	120	4	4
LCr6	120	4	4
LCr7	120	4	4
LCr8	120	4	4
LCr9a	120	4	4

Only strain NCr1a stood out with a MIC value of 8 mM Cr(VI) on M9 medium. Strain NCr1a forms clumps in liquid media, which may protect cells inside the clumps from Cr(VI), if the matrix provides diffusional limitations. In addition, formation of clumps may result in more visible growth, thus increasing determined MIC value. The twelve other strains had the same level of chromate tolerance (MIC of 4 mM) despite their distinct origins.

CHROMATE TOLERANCE AND CHROMATE REDUCTION TESTING OF SELECTED BACILLUS CEREUS GROUP STAINS

Preliminary determination of chromate tolerance and chromate reduction ability of the 13 selected *Bacillus cereus* group strains was already reported in section regarding all the isolated chromate tolerant strains (section “Chromate tolerance and reduction”, page 67). However, here we will report on more detailed measurements of growth in Cr(VI) containing media and Cr(VI) reduction, including time dynamics. The ability of the strains to reduce chromate was tested on M9 minimal medium with a starting Cr(VI) concentration of 0.2 mM. OD600 and Cr(VI) concentration was measured at different time points during 72 hours of incubation. Thus, compared to previous testing, longer incubation time and lower starting Cr(VI) concentration was used in this experiment. Also, inoculum was prepared differently - it consisted of non-washed preculture in LB

medium. Thus, carryover of spent medium occurred. This way we examined the full potential of bacterial biomass to reduce Cr(VI) including excreted metabolites from spent medium, as well as cellular debris. The maximum relative growth was calculated as a percentage of maximum OD600 in media with Cr(VI) compared to no Cr(VI) media. Reduction ability was expressed as a percentage of Cr(VI) reduced compared to the starting 0.2 mM concentration.

Strains exhibited varying chromate reduction ability (Figure 31A and B, Table 27). The percentage of Cr(VI) reduced varied from 22.9 % (strain PCr2a) to 98.5 % (strain NCr4). The maximum relative growth varied from 29.5 % (strain NCr3) to 65.3 % (strain NCr1a) depending on the strain (Figure 31C). In most cases, the presence of chromate halted bacterial growth after 12 h or 24 h of incubation, after which stationary phase was established (Figure 32). In the growth control without Cr(VI), the increase in OD600 continued until 36, 48 or 72 hours, depending on the strain. Reduction continued throughout the stationary phase, resulting in a linear decrease in Cr(VI) concentration over time for all strains (r>0.912).

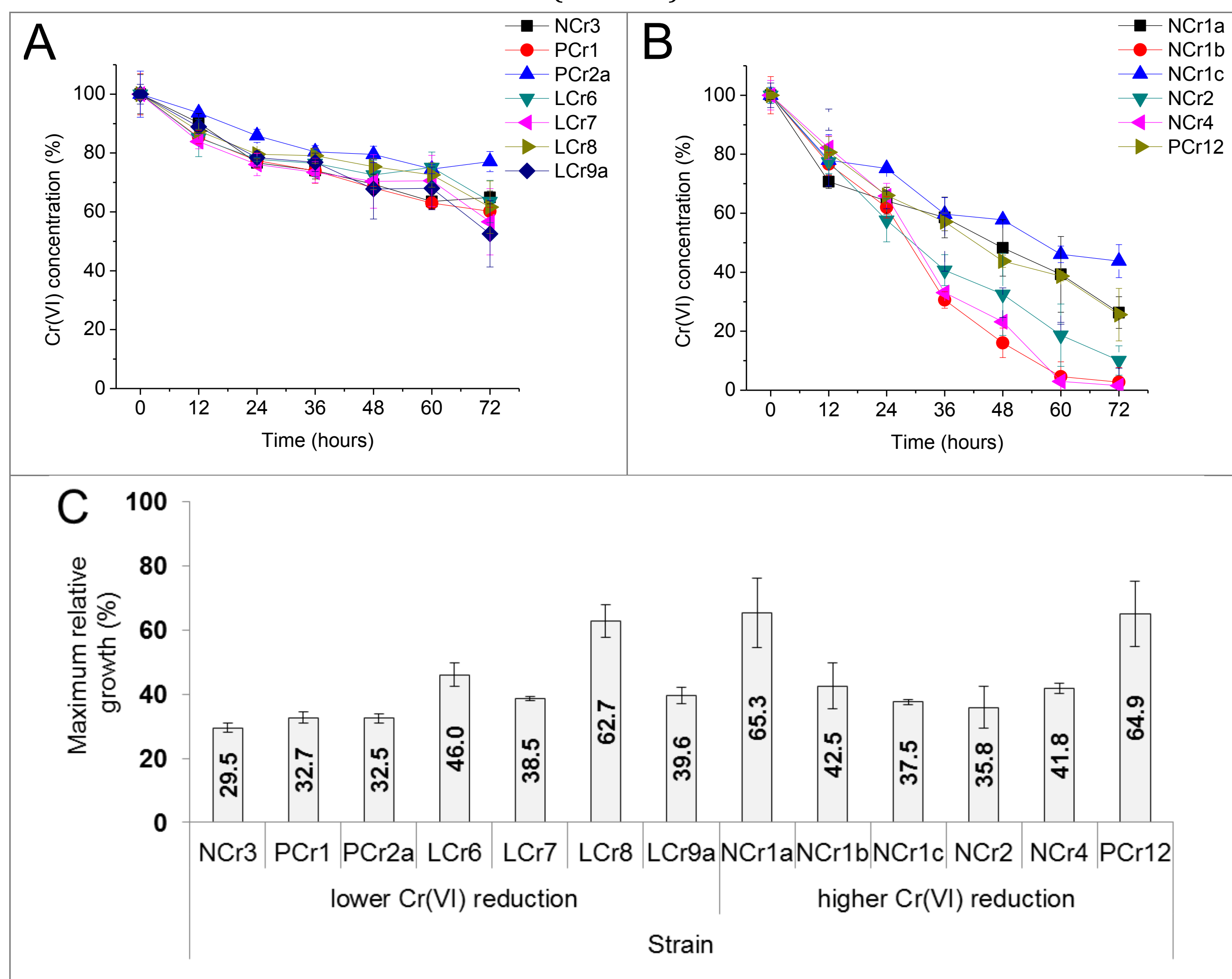

Figure 31 Hexavalent chromium reduction of environmental chromate tolerant *Bacillus cereus* group strains in M9 medium with a starting concentration of 0.2 mM Cr(VI): A – reduction curve for strains with a lower reduction activity (<50 % reduced after 72h), B – reduction curve for strains with a higher reduction activity (>50% reduced after 72h), C – maximum relative growth - percentage of maximum OD600 in medium with Cr(VI) compared to no Cr(VI) medium

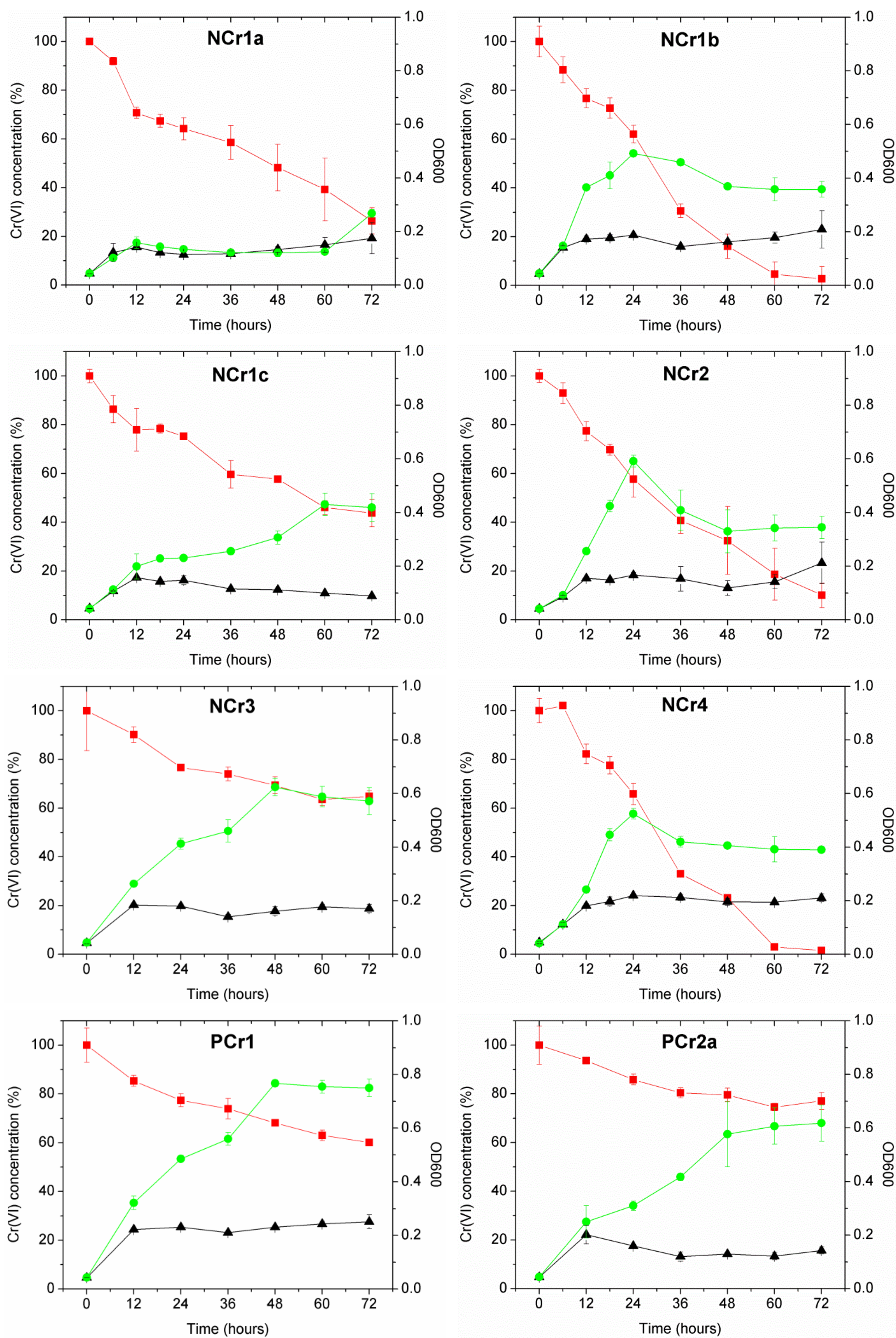

Figure 32 (continued on the next page)

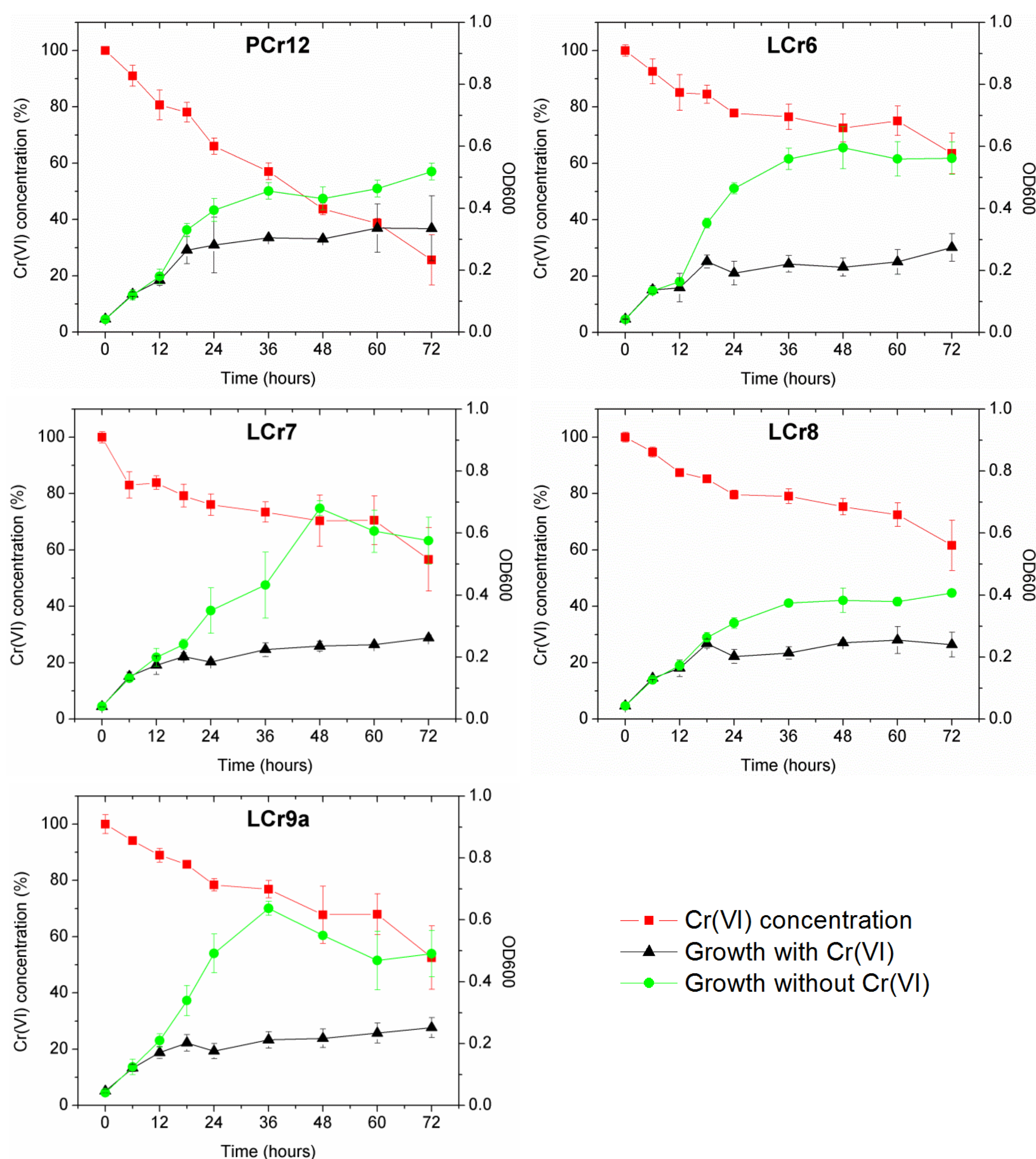

Figure 32 Hexavalent chromium reduction and growth curves with and without Cr(VI) during 72 h of incubation in M9 medium with starting concentration of 0.2 mM Cr(VI); left y axis – Cr(VI) concentration during the experiment expressed as the percentage of the starting concentration (red squares); right y axis – growth curve in medium with Cr(VI) (black triangles) and without Cr(VI) (green circles) measured as OD600

A number of isolated strains exhibited a high hexavalent Cr(VI) reduction capacity. Three strains (NCr1b, NCr2, and NCr4) displayed the highest Cr(VI) reduction of over 90 % in 72 hours. Interestingly, the greatest extent of reduction was not necessarily accompanied by the most substantial growth, as their maximum relative growth was not among the highest (42.5 %, 35.8 %, and 41.8 %, relative to the no Cr control, respectively). This lack of correlation between growth and reduction is evident from a

very low correlation coefficient R^2 of -0.07 (Figure 33). Furthermore, these strains did not exhibit as high an OD600 as other strains (e.g., NCr3 or PCr1) even in the absence of Cr(VI).

Cr(VI) had the least impact on growth of strains NCr1a and PCr12 (65.3 % and 64.9 % maximum relative growth, respectively) accompanied with high Cr(VI) reduction activity (73.7 % and 74.4 % reduced, respectively). The rest of the strains (NCr3, PCr1, PCr2a, LCr6, LCr7, LCr8 and LCr9a) exhibited lower reduction activity (<50 %). Possibly some other chromate resistance mechanisms, such as reduced chromate uptake or enhanced chromate efflux, also play a part in their chromate tolerance.

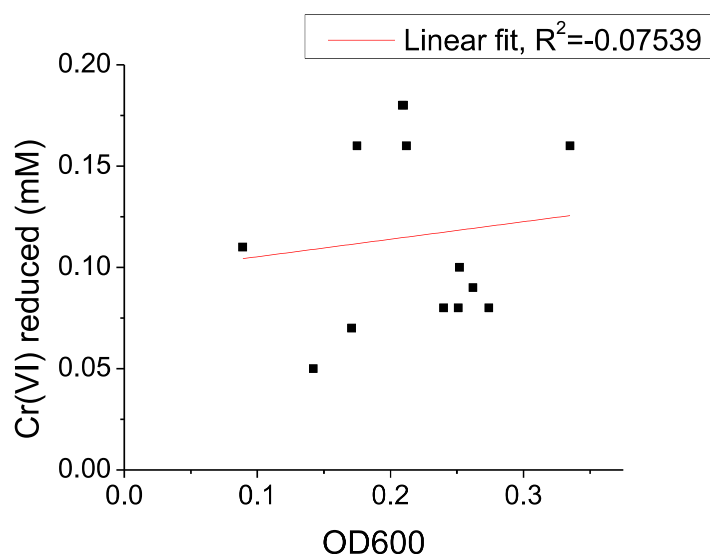

Figure 33 Linear correlation of Cr(VI) reduction and growth on Cr(VI) containing media for the *B. cereus* group isolates

Table 27 Comparison of chromate reduction in different assays with starting Cr(VI) concentrations of 0.2 and 0.5 mM

Strain	Cr(VI) reduced in percentages		Cr(VI) reduced in mM	
	0.5 mM	0.2 mM	0.5 mM	0.2 mM
NCr1a	42.0	73.7	0.21	0.16
NCr1b	46.3	97.3	0.23	0.18
NCr1c	32.0	56.2	0.16	0.11
NCr2	58.5	89.9	0.29	0.16
NCr3	40.9	35.1	0.20	0.07
NCr4	30.0	98.5	0.15	0.18
PCr1	39.5	39.8	0.20	0.08
PCr2a	55.0	22.9	0.28	0.05
PCr12	23.0	74.4	0.12	0.16
LCr6	29.5	36.5	0.15	0.08
LCr7	42.5	43.3	0.21	0.09
LCr8	20.5	38.4	0.10	0.08
LCr9a	24.5	47.4	0.12	0.10

On higher starting Cr(VI) concentration of 1 mM, reduction ability and maximum relative growth of strains were significantly diminished (Figure 34). Reduction ability was below 10 % for all the isolates. In most instances, a difference in the reduction between strains was in the range of standard deviation. Growth was also decreased to a level below 18.3 % for all the strains, except for NCr1a which recorded 34.3 % of growth.

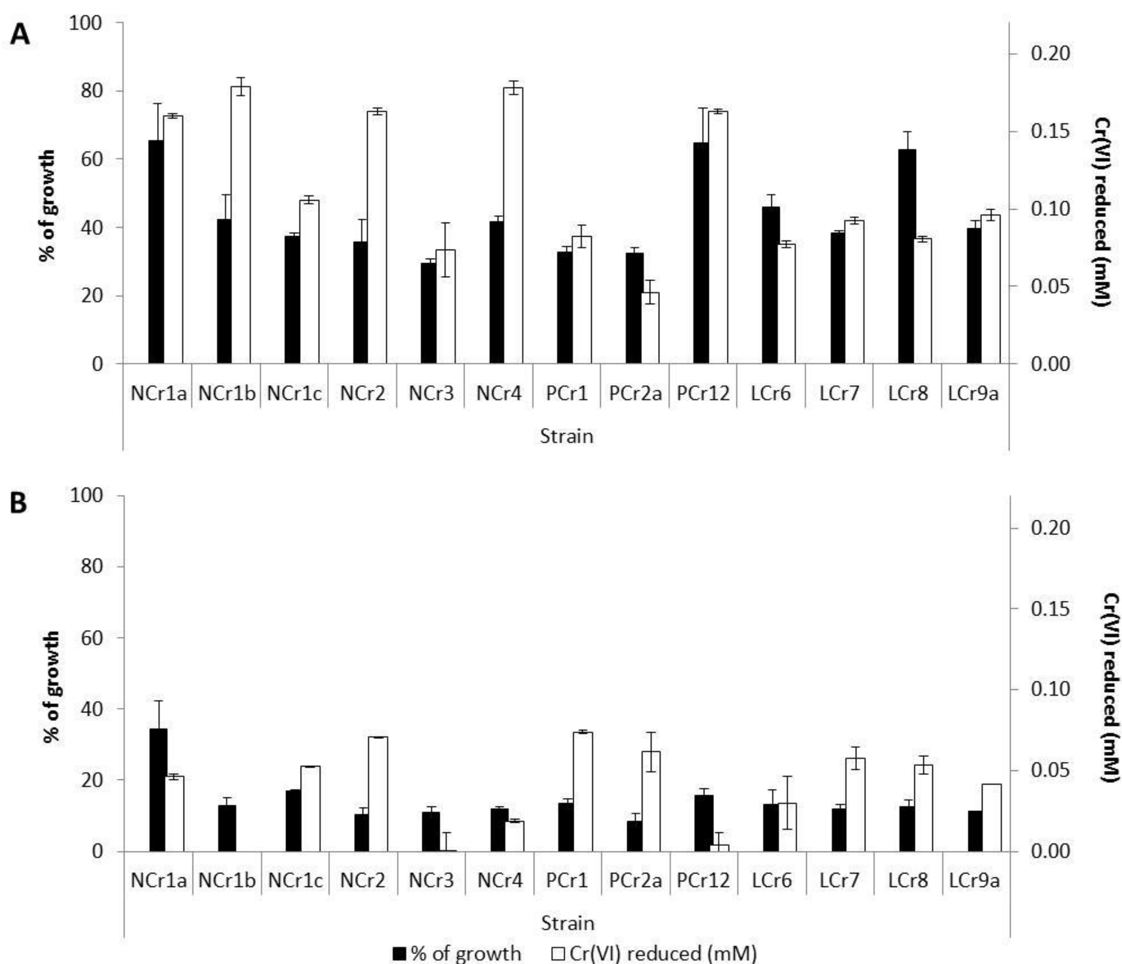

Figure 34 Hexavalent chromium reduction ability and growth (relative to no Cr(VI) cultures) after 72 h of incubation in M9 medium with different starting Cr(VI) concentrations: A - 0.2 mM; B - 1 mM

If we compare reduction on the 0.5 mM test (results given in Table 18) and 0.2 mM test we can notice percentage of reduction was much higher with the lower tested concentration and that there were some discrepancies among the two assays (Table 27). Strains with the most pronounced chromate reduction activity in both assays with 0.2 and 0.5 mM Cr(VI) starting level were NCr1a, NCr1b and NCr2. However, chromate tolerance of strains NC1b and NCr2 was not among the highest in both assays, as measured by growth percentage.

Strain NCr1a had consistently high chromate reduction activity coupled with robust growth in the presence of Cr(VI) on all assays (0.2, 0.5 and 1 mM starting Cr(VI) concentration), and is therefore the best candidate for further studies.

COMPARISON OF CHROMATE TOLERANCE OF ENVIRONMENTAL AND REFERENCE BACILLUS GENUS STRAINS

Chromate tolerance of selected isolates and reference laboratory strains of the *Bacillus* genus was compared by measurement of OD600 after growth in minimal M9 medium with 0.5, 1 and 2 mM of Cr(VI) (Figure 35). For improved growth of the *B. subtilis* PY79 reference strain, phenylalanine, tryptophan and increased amount of glucose (5 g/l) were added to M9 medium in this experiment, which resulted in higher growth compared to chromate reduction assay results (Figure 31). Strains NCr1a and PCr12 were selected as they had the highest maximum relative growth in the presence of Cr(VI) accompanied with moderately high level of Cr(VI) reduction. Isolate NCr1a originated from a serpentine soil with naturally elevated level of Cr, while PCr12 was isolated from tannery industry solid waste material with high levels of Cr.

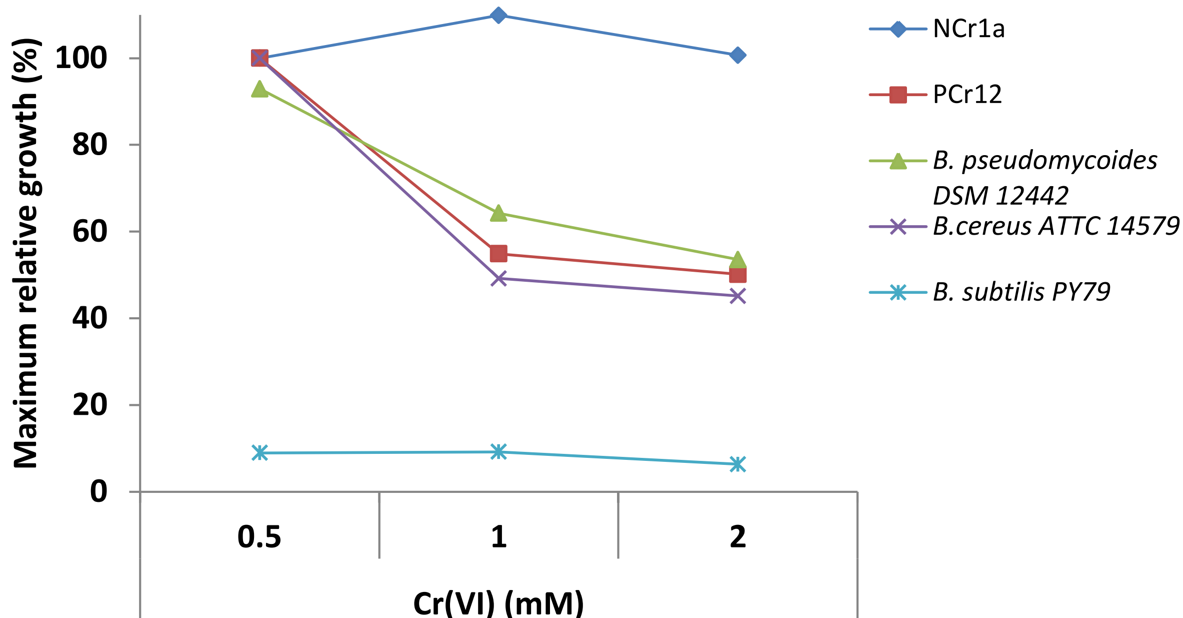

Figure 35 Growth of chromate tolerant isolates NCr1a and PCr12 and reference *Bacillus* sp. strains measured as OD600 after 24h of incubation in M9 minimal medium with different concentrations of Cr(VI) (maximum relative growth - percentage of maximum OD600 in medium with Cr(VI) compared to no Cr(VI) medium)

Serpentine soil isolate NCr1a proved to be the most tolerant, with no reduction in growth even in 2 mM Cr(VI). Isolate PCr12 and reference strains of *B. cereus* group (*B. pseudomycoides* DSM 12442 and *B. cereus* ATCC 14579) were not affected by 0.5 mM Cr(VI), while growth on 1 and 2 mM was similar and moderately decreased to around 60 %. Conversely, *B. subtilis* PY79 was highly sensitive to Cr(VI) and even the lowest concentration (0.5 mM Cr(VI)) strongly inhibited its growth.

This indicates *B. cereus* group strains in general (both environmental and reference strains) have higher chromate tolerance compared to *B. subtilis*.

As mentioned, growth of NCr1a was not affected by 2 mM Cr(VI) in medium in this testing (growth of nearly 100 % compared to no Cr(VI) control). However, in MIC determination testing and chromate reduction assays, very different results were obtained. Specifically, at chromate reduction assay with 0.2 mM starting Cr(VI) concentration percentage of growth was around 40 %, while on 0.5 mM test it was more than 100 %. These results highlight how slight differences in experimental set-up (e.g. preculture conditions, inoculum size, and medium composition) can have strong influence on the measurement of chromate tolerance level. However, in majority of these tests relation among the strains has been similar - meaning that the order of strains' chromate tolerance and chromate reduction activity were similar in all the tests.

NCr1a STRAIN CHARACTERIZATION

Strain NCr1a had the highest chromate tolerance of all the tested *B. cereus* group strains. It exhibited sturdy growth in Cr(VI) containing media and high to moderately high chromate reduction, depending on the test conditions. Thus, it was chosen for more detailed characterization of cultural characteristics and cellular morphology, as it will be reported in following section.

Cultural characteristics of strain NCr1a

To observe colony morphology of the NCr1a isolate, cell suspension in saline solution was spot inoculated on LB agar in Petri dish (∅ 90mm) and incubated at 28°C. Each day plates were photographed from bellow and above.

Strain exhibits characteristic rhizoidal colony morphology typical for *B. pseudomycoides* and *B. mycoides* strains. Direction of filament formation as observed from bellow the plate is counter-clockwise (DX morphotype according to Di Franco et al. (2002)). Direction of filament formation stayed unchanged on all media and incubation conditions. Colony morphology starts off with uniform surface appearance (day 1 of incubation), however, prolonged incubation leads to formation of heterogeneous color, texture and general appearance of colony (Figure 36). Central zone of colony, formed in the first few days of incubation, is thicker and 1 to 2 cm in diameter. Growth zones formed afterwards spread out from the center and cover almost the entire plate after 14 days. After prolonged incubation, parts of the colony become slightly transparent indicating cell lysis in this area. Released metabolites from the dead cells possibly cause new centers of regrowth, as evident from thicker zones of growth on the edges. Same colony morphology dynamics are seen after streak inoculation (Figure 37).

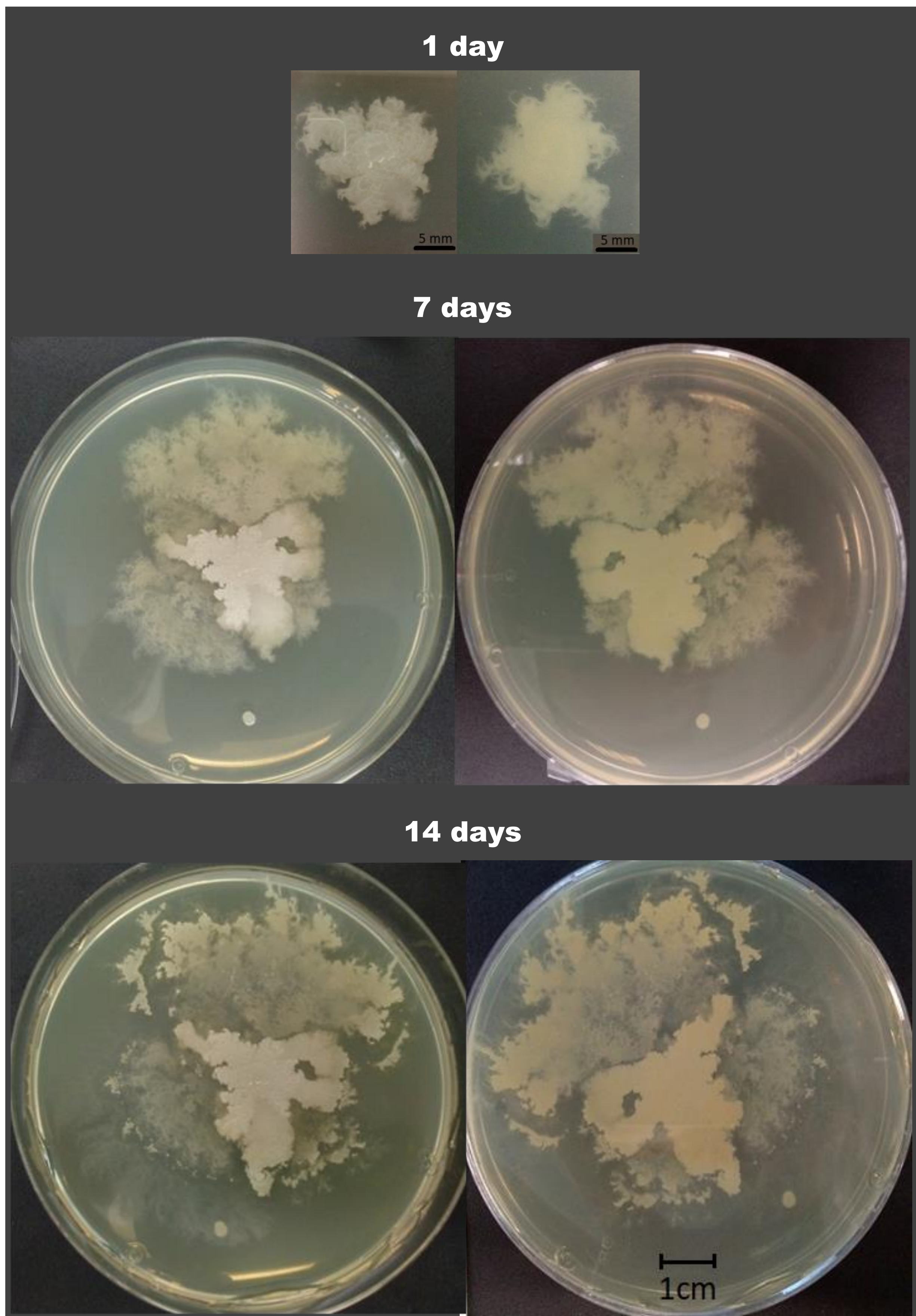

Figure 36 Colony morphology of strain NCr1a on LB agar plates after different periods of incubation at 28°C (photographs on left are captured from above, photographs on right from below the plate, plate diameter is 90mm)

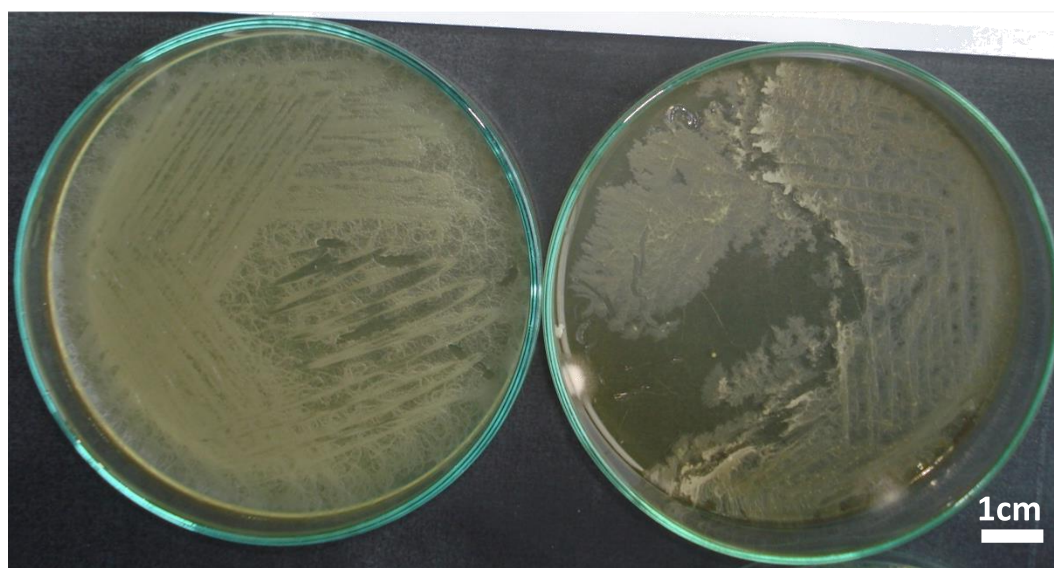

Figure 37 Colony morphology of strain NCr1a on LB agar plates after 2 (left) and 20 days of growth (right) on 28°C, streak inoculation (plate diameter is 90mm)

Formation of filaments is more intense and spreading on nutrient limited (oligotrophic) media such as R2A (Figure 38). Filaments are thinner and spread out on agar surface more intensely. R2A contains fewer nutrients than LB agar, thus the need to obtain limited nutrients on R2A would drive outward spreading of filaments to the fresh areas of agar.

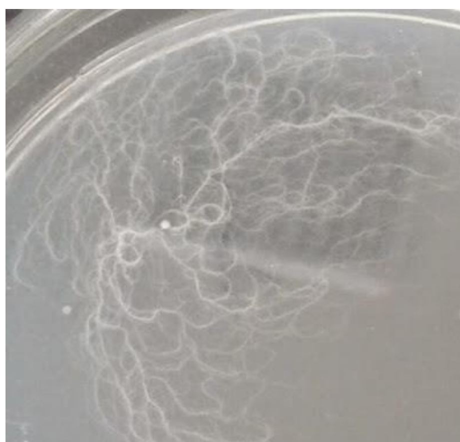

Figure 38 Colony morphology of strain NCr1a on oligotrophic medium R2A – formation of finer, thinner, more widely spreading filaments compared to those formed on nutrient rich LB agar

When grown in liquid media, strain NCr1a forms patches and flakes of different size depending on the medium and mixing conditions. Generally, nutrient rich media lead to large flakes which are very difficult to disperse, while minimal media such as M9 give more uniform and smaller sized flakes and are more easily dispersed (Figure 39). Biomass in liquid culture precipitates on the bottom of the tube, once more proving strain is nonmotile. No tendency to form pellicle was noticed.

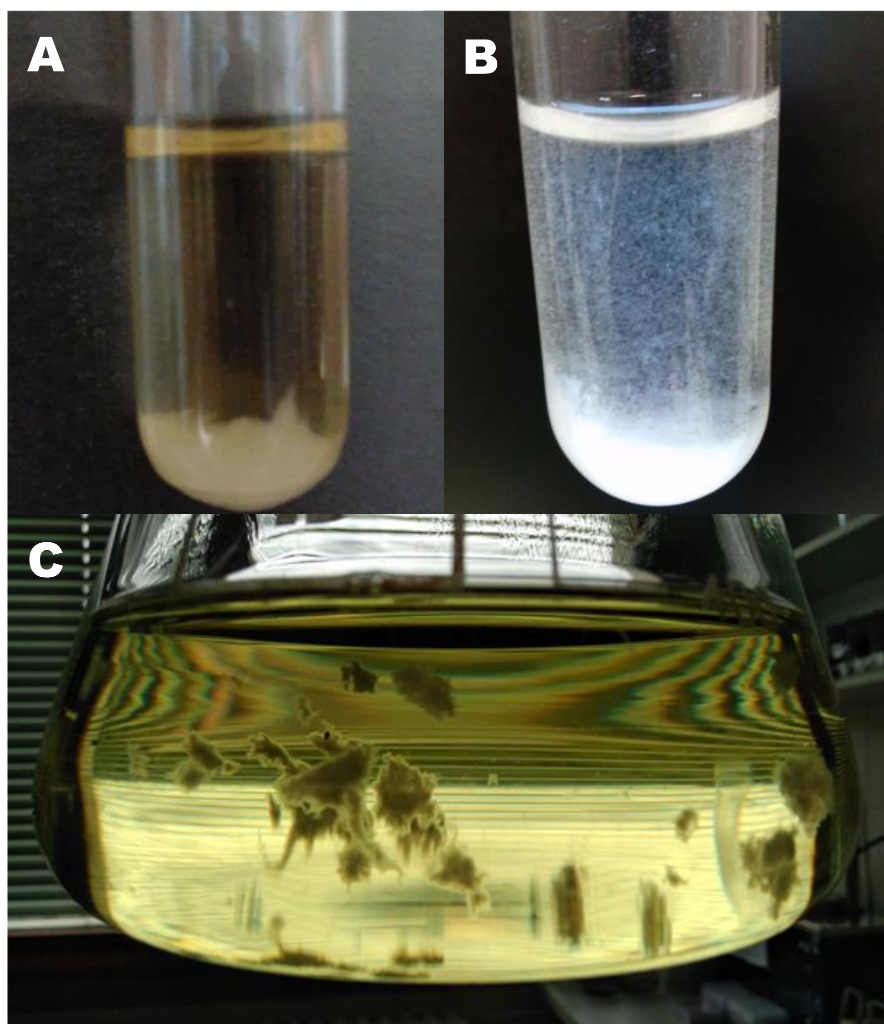

Figure 39 Growth of strain NCr1a in liquid culture - flakey growth with sedimentation indicating lack of motility: A – nutrient rich LB broth, B – minimal M9 medium without Cr(VI), C – minimal M9 medium with 0.2 mM Cr(VI)

SEM micrographs (Figure 40) illustrate growth in long chains, which tend to group together and form bundles. Formation of such bundles is the basis for strain's patchy growth visible in liquid culture.

Cell morphology of strain NCr1a

NCr1a is a Gram-positive rod arranged in short or long chains (Figure 40, Figure 41). Endospores are subterminal and elliptical. Cytoplasm often appears grainy of vacuolated, due to the accumulation of polyhydroxybutyrate granules, especially if grown on media with higher C:N ratio (e.g. media with high carbohydrate content) (Narayanan and Ramana, 2012).

NCr1a has lower sporulation efficiency compared to the *B. pseudomycoides* reference strain DSM 12242 as determined by microscopy of overnight cultures in sporulation medium (Figure 41). Cellular and endospore morphology is similar in both strains.

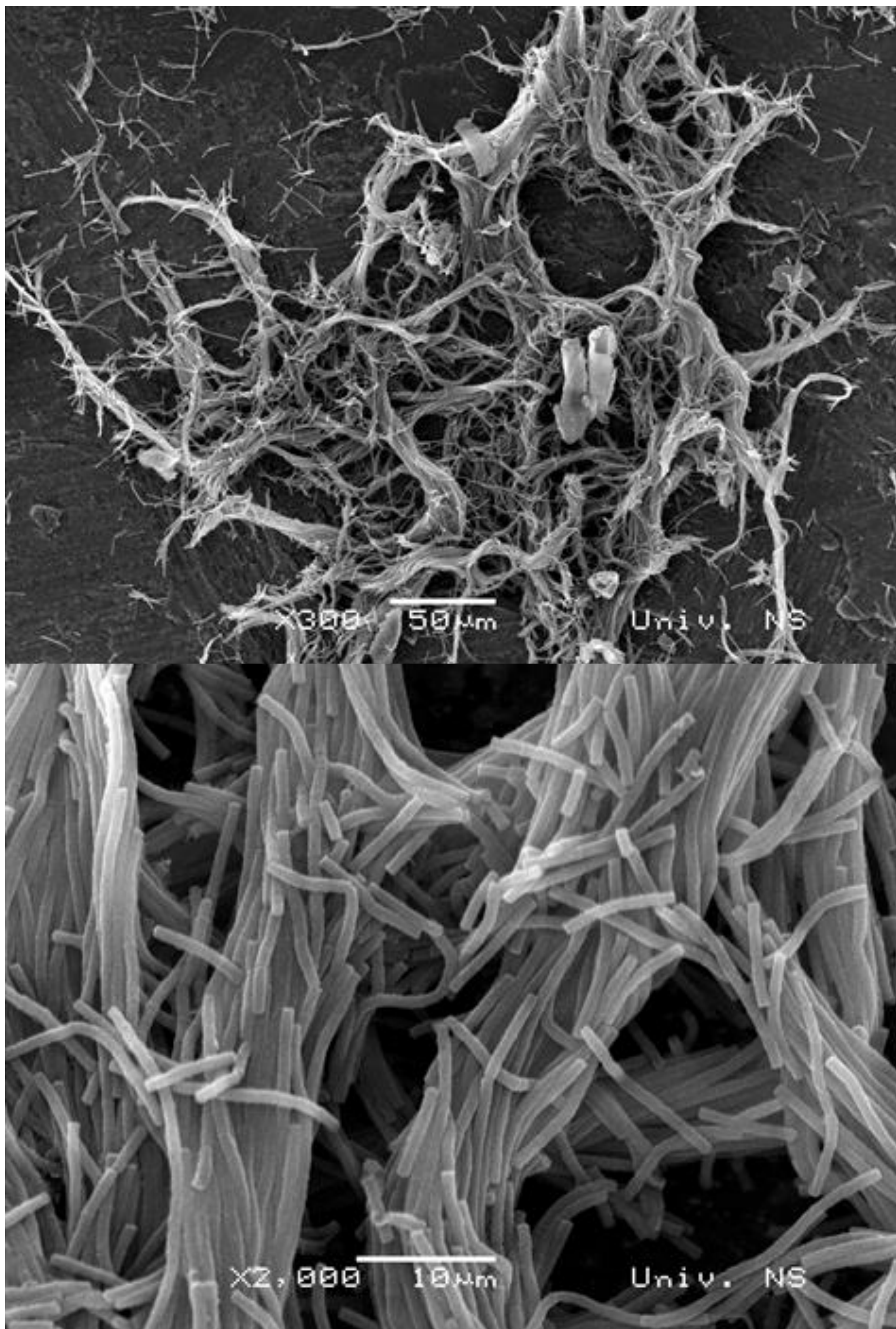

Figure 40 Scanning electron micrographies of strain NCr1a (Photo: M. Bokorov)

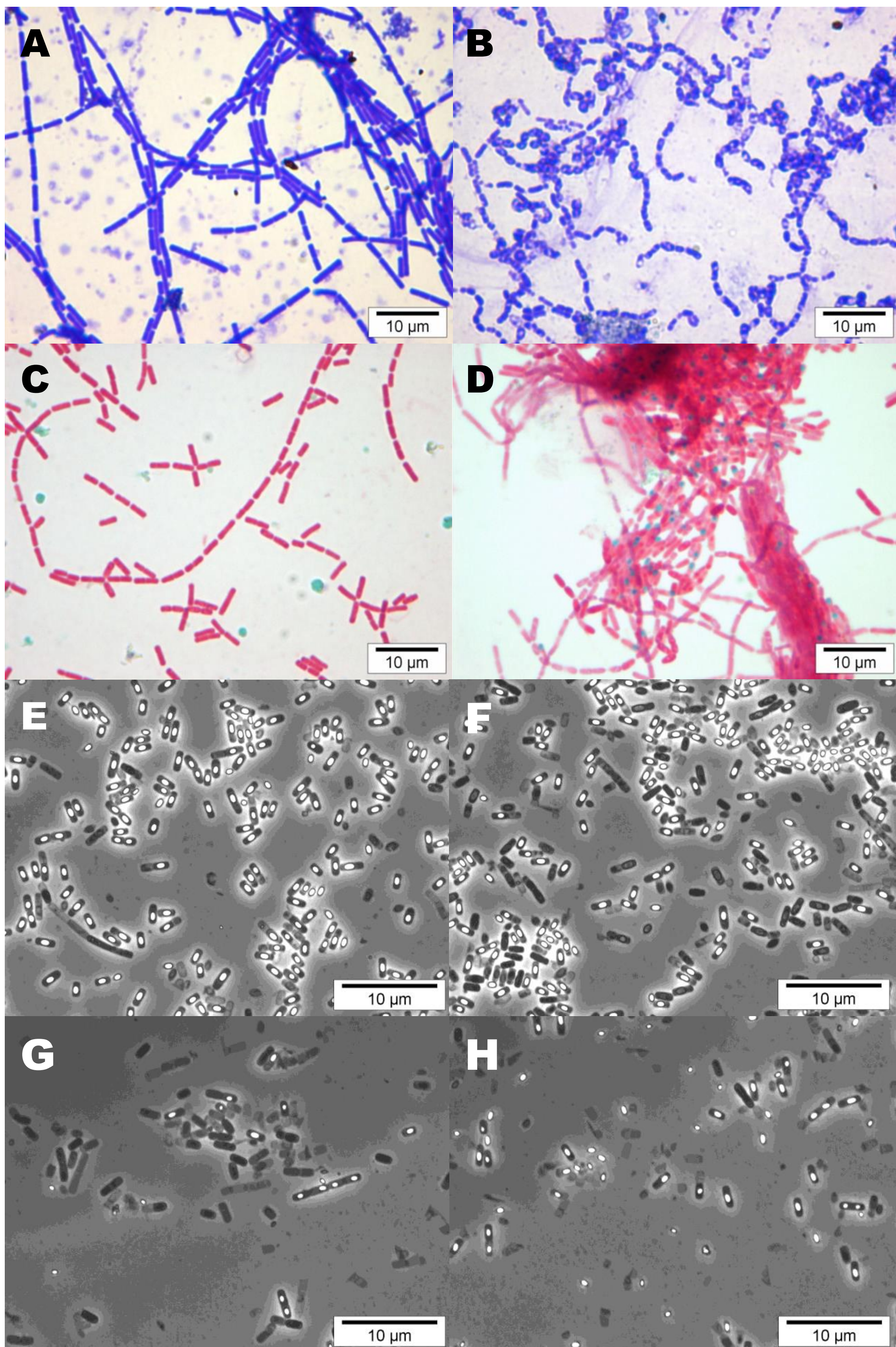

Figure 41 Cell and endospore morphology of isolate NCr1a and reference strain *B. pseudomycoides* DSM 12442 observed by brightfield microscopy: A and B - Gram stained; C and D - endospore stained slides (Schaeffer-Fulton staining) (24 h culture on A and C, and 6 day culture on B and D); native slides of culture grown in sporulation medium DSM for 24h: E and F - *B. pseudomycoides* DSM 12442; G and H - isolate NCr1a (Photo E-H: I. Barák)

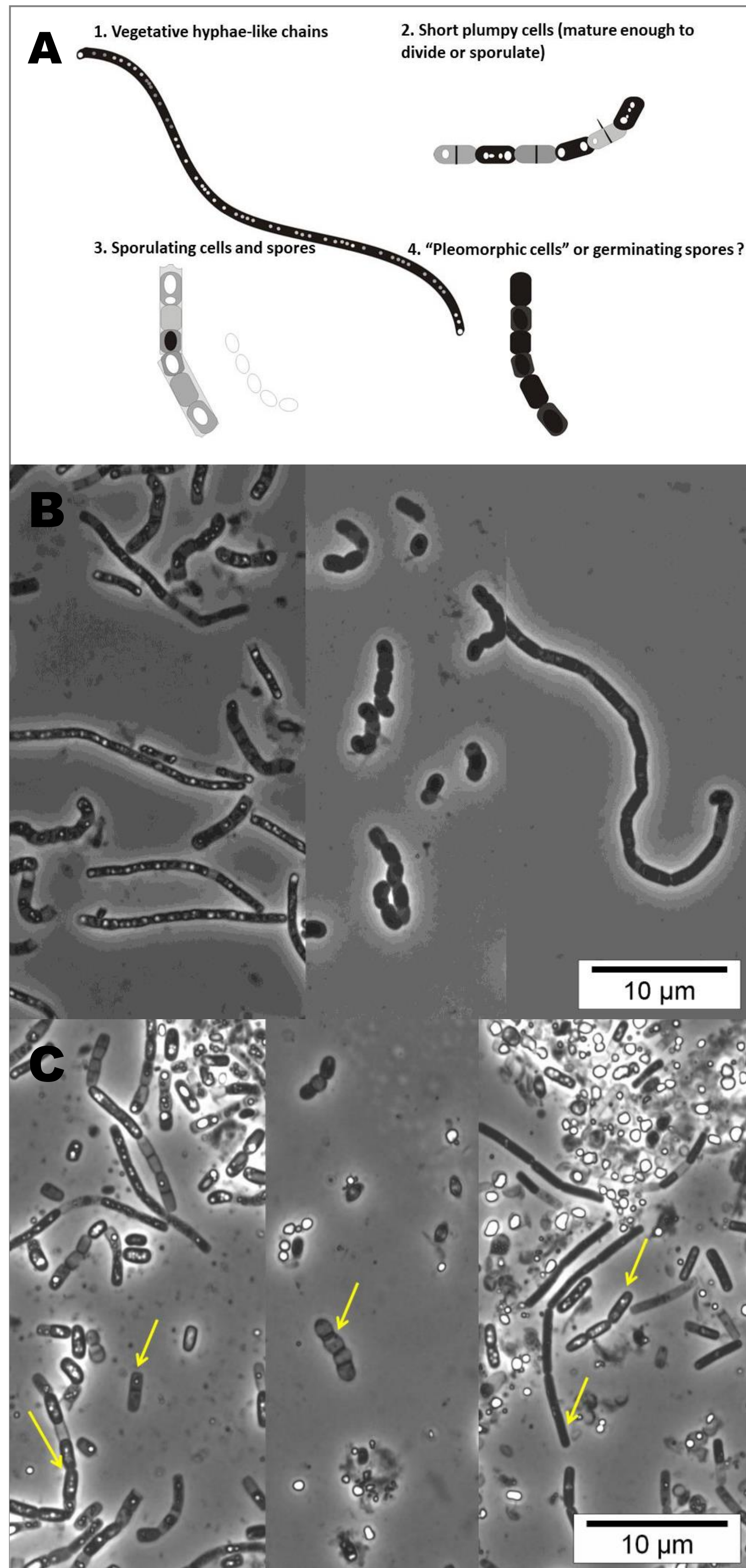

Figure 42 Morphological types of NCr1a cells: A - graphical illustration, B and C - brightfield microscopy micrographs (Photos and graphics: I. Barák and Z. Chromiková)

Cellular morphology exhibits variability during ageing (Figure 42), which is long known about *B. pseudomycoides* and *B. mycoides* species (Lewis, 1932). Cells in young culture are usually in very long chains, cytoplasm is not grainy, and cells are without or with prospore. Cells in chains are of uniform diameter and separation between them is not clearly noticeable. After longer incubation, cells become wider and shorter and chains appear more “sausage like”.

Detection of chromate transporter gene of strain NCr1a

Strain NCr1a was subjected to detection and sequencing of *chrA* chromate transporter gene, known for its role in high chromate tolerance (Cervantes et al. 1990; Díaz-Pérez et al. 2007; Nies et al. 1990). Product obtained was 1373 base pairs long. Sequence is given in the appendix (section 9.4; page 152).

Sequence was analyzed using BLASTn search. It had 97 % identity with the two *B. pseudomycoides* genome sequences, namely with strain BTZ (Acc. No. CP009651.1) and strain 219298 (CP007626.1). Further hits belonged to other members of the *B. cereus* group and had identity of 75 % or less. Distance tree of results is given in Figure 43.

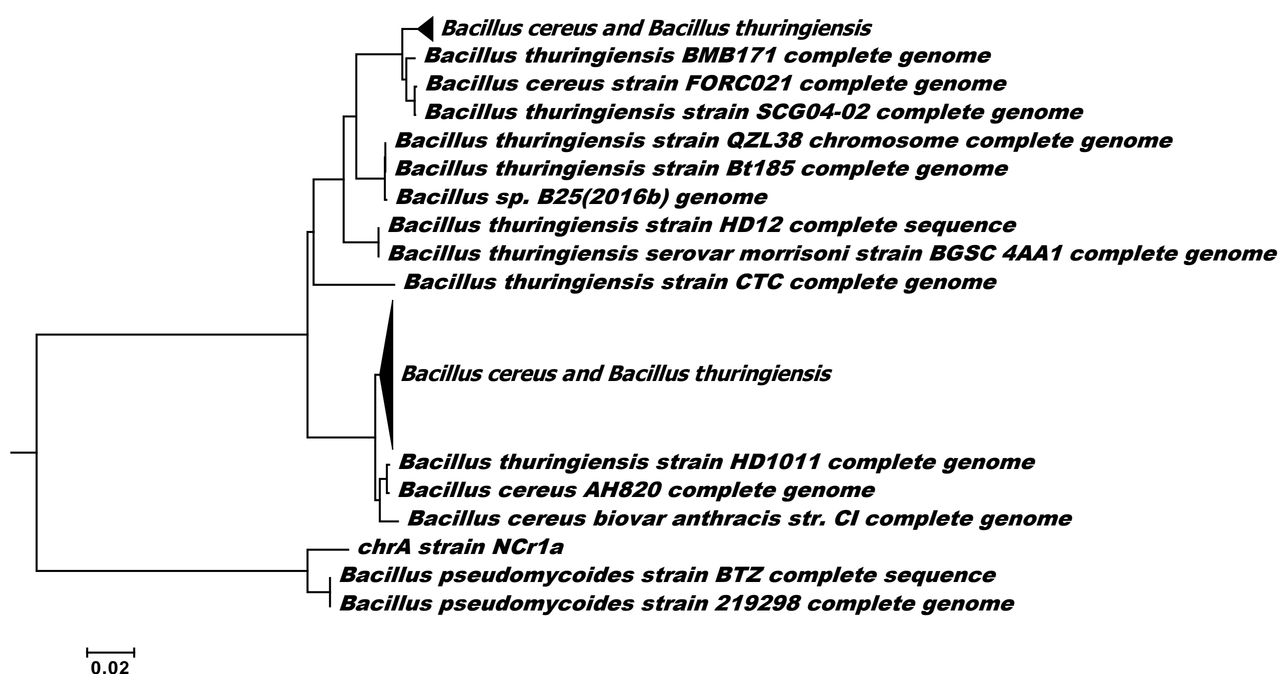

Figure 43 Distance tree of BLASTn search results for chromate transporter *chrA* gene sequence of strain NCr1a (constructed using Fast minimum evolution method after BLAST pairwise alignment)

Chromate transporter of strain NCr1a belongs to the long chain chromate transporter family of chromate ion transporter superfamily (Díaz-Pérez et al., 2007; Nies et al., 1998).

5.4. STEM EDS ANALYSIS OF CHROMIUM CONTENT AND LOCALIZATION IN CELLS AND ENDOSPORES OF STRAINS NCr1a AND *B. SUBTILIS* PY79

In previous tests it was determined that strain NCr1a, tentatively identified as *B. pseudomycoides,* expressed the highest Cr(VI) tolerance level compared to all the tested environmental *B. cereus* group isolates. Reference laboratory strain *B. subtilis* PY79 (BS) proved to be much more sensitive to Cr(VI). Hence, these two strains were chosen for further testing of chromium content and localization by elemental mapping using scanning transmission electron microscopy with energy dispersive X-ray spectroscopy (STEM-EDS).

Chromium content and localization was determined in **cells** grown in the presence of Cr(VI) for different periods of time. Also, influence of induction (exposure to low subinhibitory concentration of Cr(VI) prior to exposure to high Cr(VI) concentration) on Cr cellular content was analyzed. To summarize, chromium content was determined in:

- Cells grown in Cr(VI)-containing medium for 4 hours
- Cells grown in Cr(VI)-containing medium for 18 hours
- Induced cells exposed to Cr(VI) for 1 hour
- Uninduced cells exposed to Cr(VI) for 1 hour.

Chromium content and localization was tested in **endospores** formed in the Cr(VI) containing medium. We also tested endospores formed without the presence of Cr(VI), which were subsequently exposed to Cr(VI) for different periods of time (exposure of mature endospores). To summarize, chromium content was determined in:

- endospores formed in Cr(VI)-containing medium (6 days of exposure)
- mature endospores exposure to Cr(VI) for 1 hour
- mature endospores exposure to Cr(VI) for 6 days

CHROMIUM CONTENT IN CELLS

First, growth of the two strains on different Cr(VI) concentrations in minimal medium was examined (Figure 44).

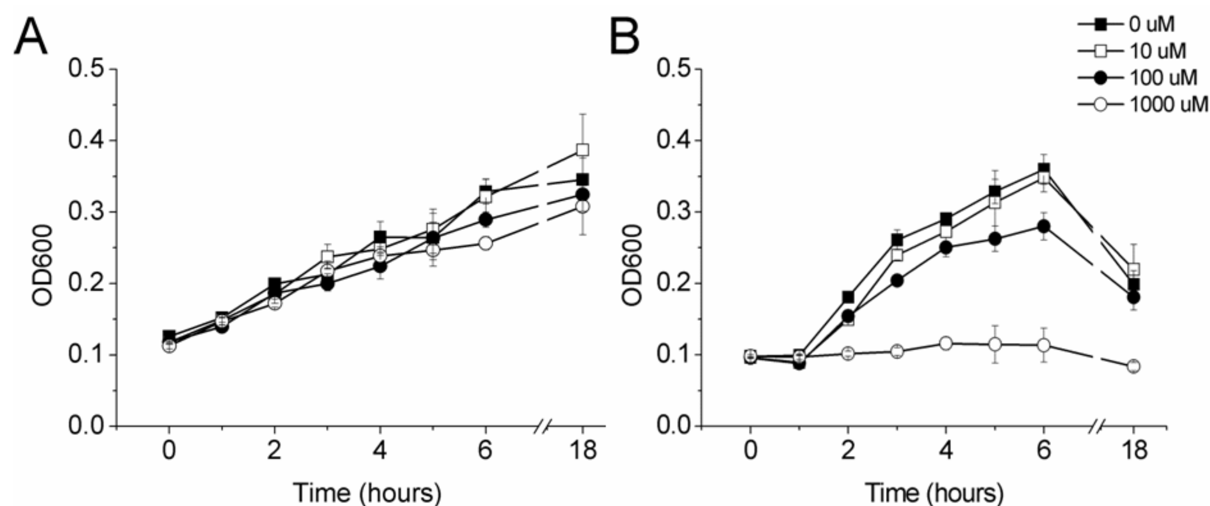

Figure 44 Comparison of growth of strains in minimal M9 medium with different concentrations of Cr(VI): A – chromate resistant environmental isolate NCr1a; B – chromate sensitive laboratory strain *B. subtilis* PY79

Growth of *B. subtilis* PY79 was strongly inhibited by 1 mM Cr(VI). Contrary, isolate NCr1a was much more resistant to high Cr(VI) level and grew to 80 % compared to no Cr(VI) control in exponential phase, and up to 60 % in stationary phase. Concentration of 100 μM caused significantly reduced growth of *B. subtilis* PY79 compared to NCr1a. Concentration of 10 μM Cr(VI) did not significantly influence growth of both isolates and therefore was chosen as a concentration for induction experiments.

Chromium content in cells grown in Cr(VI)-containing medium

The two strains were analyzed for chromium content in cells during cultivation in M9 minimal medium with 1 mM Cr(VI). Cells were collected at two time points: 4h of growth (exponential phase) and 18h of growth (stationary phase).

The accumulation of chromium inside of **NCr1a** cells collected in exponential phase was very low and with small standard deviation close to the instrument's level of detection - 0.03±0.01 at% (atomic percentages) (Figure 45A**,** Table 28). After prolonged incubation in Cr(VI) containing medium up to 18h, chromium content in NCr1a cells increased to 0.09±0.03 at%.

Cells of ***B. subtilis*** **PY79** showed large variation in chromium content, with some taking up more Cr, while others took up only to a level similar to that of NCr1a cells. Chromium content ranged from 0.04 to 0.70 at%. Variability of Cr uptake of *B. subtilis* PY79 can be easily seen on variability plot of raw data (Figure 45B). There was no significant difference among Cr content in *B. subtilis* PY79 cells after 4h and 18h of incubation in Cr(VI) medium.

Cells of NCr1a had significantly lower chromium content compared to cells of *B. subtilis* PY79, both after 4h and 18h of incubation.

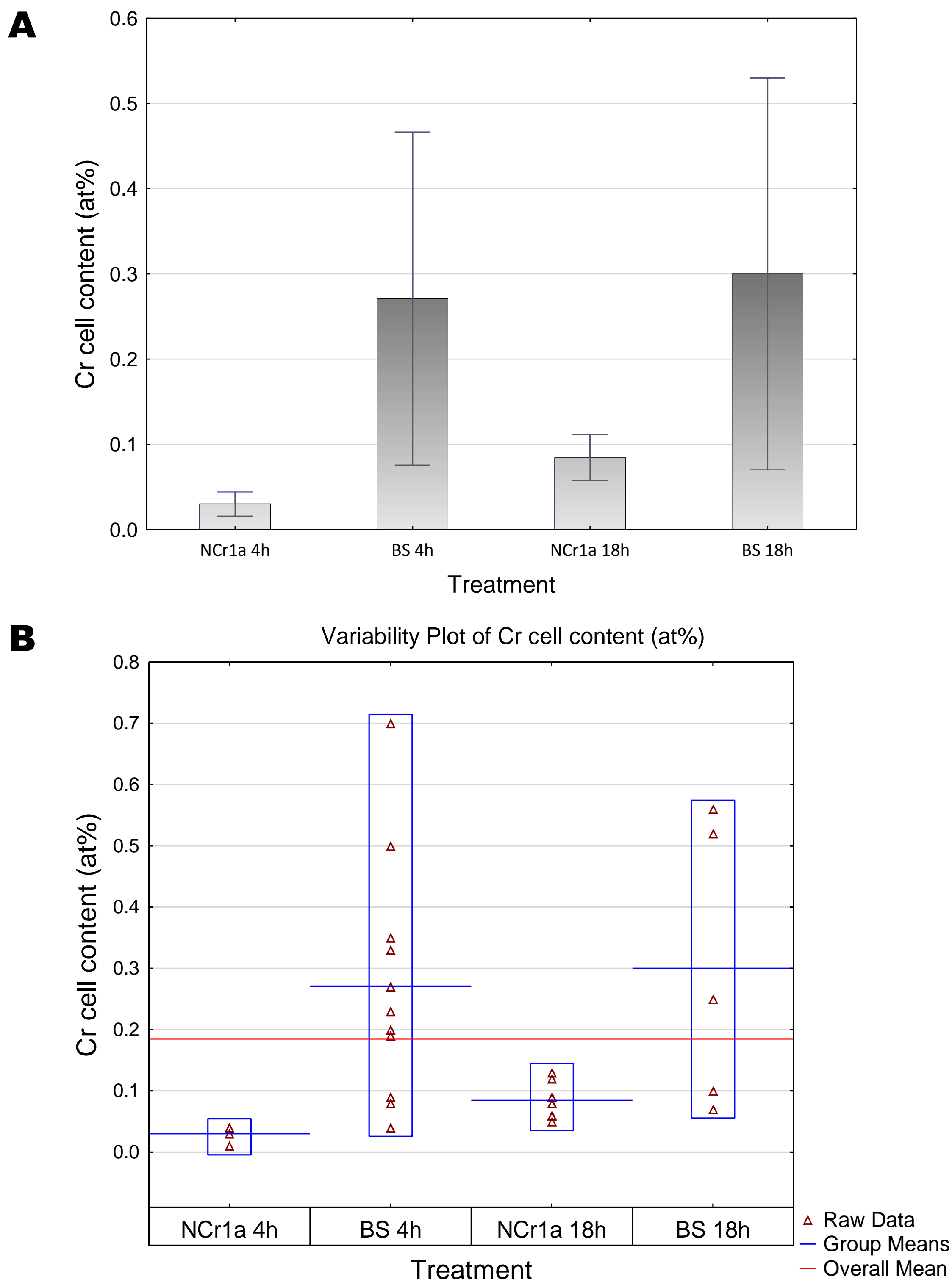

Figure 45 Cellular chromium content of chromate resistant isolate NCr1a and control laboratory strain *B. subtilis* PY79 (BS) after 4h and 18h of growth in M9 minimal medium with 1 mM Cr(VI) as measured by scanning transmission electron microscopy coupled with energy dispersive spectroscopy (STEM-EDS): A – mean values with standard deviation, B – variability plot with raw data

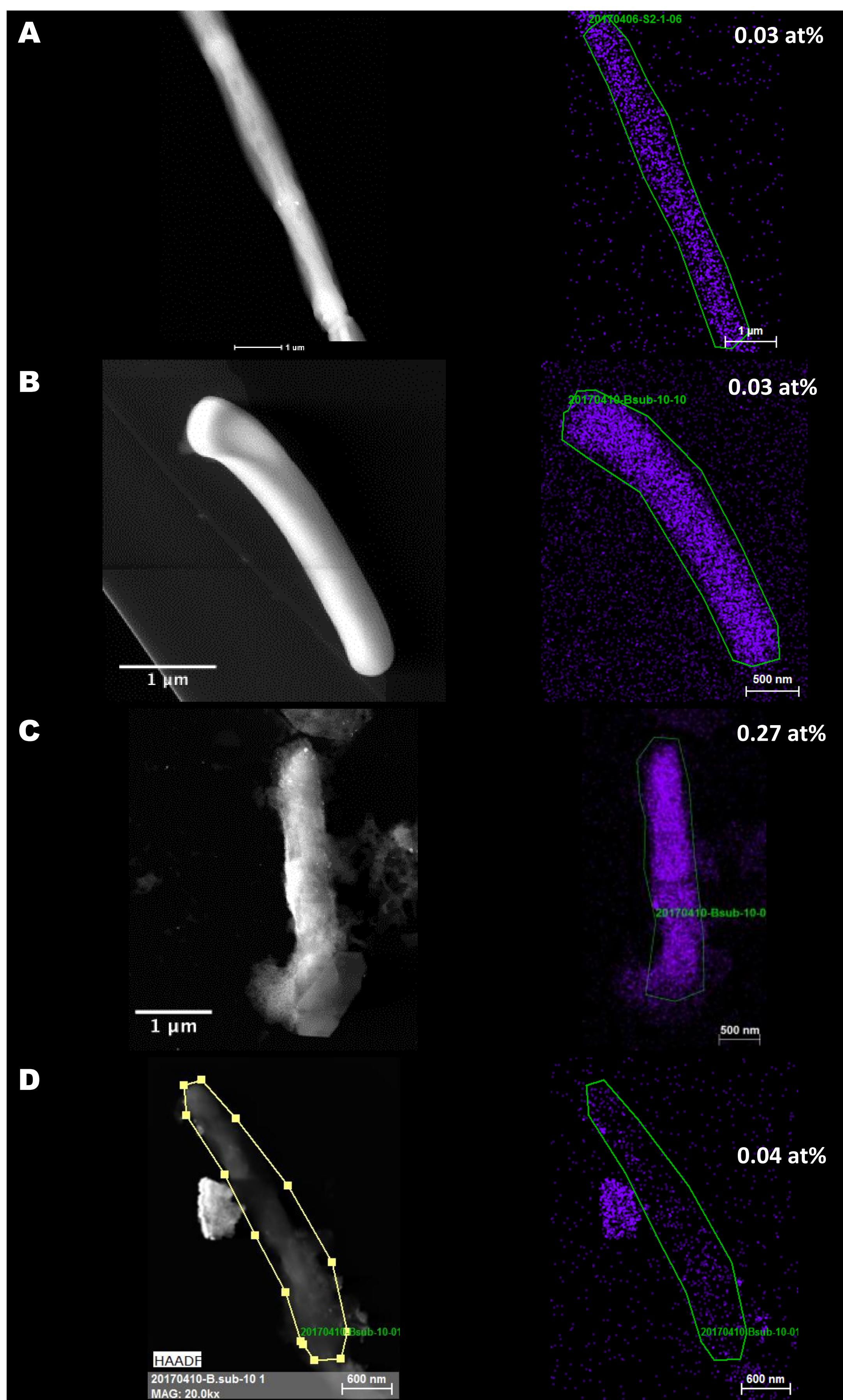

Figure 46 STEM EDS images of cells grown for 4h in M9 medium with 1 mM Cr(VI): A – NCr1a, B – *B. subtilis* PY79, C and D –damaged/lysed cells of *B. subtilis* PY79 (HAADF images on the left, elemental maps on the right, Cr colored in purple)

Many cells of *B. subtilis* PY79 appeared damaged/lysed in the presence of Cr(VI), which was not seen in no Cr(VI) control of *B. subtilis* PY79. Contrary, cells of NCr1a did not show any sign of damage, lysis or unusual appearance neither with nor without Cr (Figure 46). This additionally indicates that *B. subtilis* PY79 suffers more toxicity compared to NCr1a who's morphology is not disturbed by the presence of high Cr(VI) levels.
Probable reason for the high variation of chromium content in cells of *B. subtilis* PY79 is that STEM EDS measurements indiscriminately included live and dead cells (Figure 46). Cellular content, including Cr, exits from the dead lysed cells. For that reason, dead cells could have much lower Cr content. It is difficult to distinguish live and dead cells in STEM EDS images. Some prediction of the viability of the cell can be assumed by comparing density of the cell in HAADF (high-angle annular dark-field) images. However, this is only assumption of viability state. We tried to distinguish live from dead cells by staining cells with propidium iodide. Propidium iodide enters only dead cells, and therefore, content of iodine in dead cell should have been higher compared to live cells. However, we were not able to discern live and dead cells based on concentration of iodine measured by STEM EDS, because of two reasons: a) iodine content in cells was too small (STEM EDS detection limit is 200 ppm at the very best), b) iodine peak overlaps with the calcium peak, thus masking it.

Influence of induction on chromium cellular content

Next, we examined the influence of induction with low levels of Cr(VI) prior to exposure of cells to high Cr(VI) level. Strains were grown without Cr(VI) (uninduced cells) and with low subinhibitory Cr(VI) concentration (induced cells) in M9 medium. Cells were collected in exponential phase and washed. Subsequently, they were exposed to 1 mM Cr(VI) in PBS for 1h, fixed and analyzed by STEM-EDS. Induction concentration of 10 µM was chosen as it was previously determined it did not significantly affect growth of the two strains during exponential growth when compared to no Cr(VI) control (Figure 44).

Induction had no effect on chromium intake in cells of **NCr1a** (Figure 47, Table 28). Chromium content, both in uninduced and induced cells, was equally low and stable - 0.02±0.01 at%.
Contrary, induced cells of ***B. subtilis*** **PY79** had significantly lower chromium content compared to uninduced cells of *B. subtilis* PY79. Induced cells of *B. subtilis* PY79 contained 0.03±0.02 at% Cr, which is 5.7 times lower compared to uninduced cells (0.17±0.05 at%). To that, induced *B. subtilis* PY79 cells had similar cellular content as cells of NCr1a. Thus, it appears that *B. subtilis* PY79 cell are capable of maintaining low cellular Cr content, but only if they are induced by low Cr(VI) concentration prior to exposure to high Cr(VI) level.

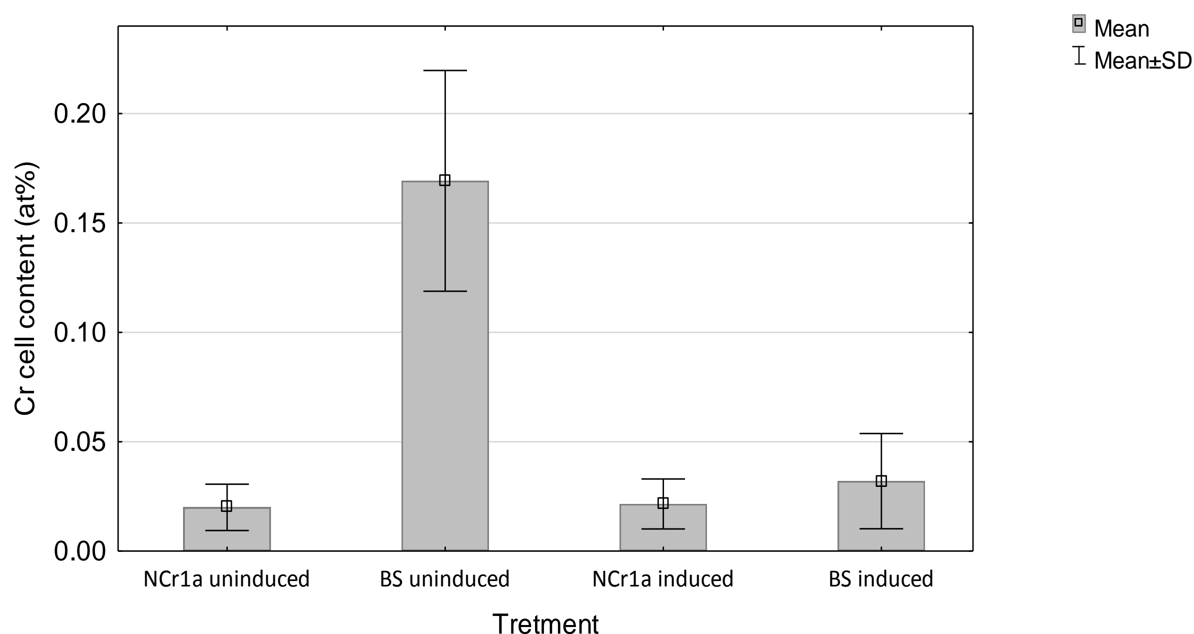

Figure 47 Influence of induction by subihibitory Cr(VI) concentration on Cr cell content of chromate resistant isolate NCr1a and laboratory strain *B. subtilis* PY79. Induced and uninduced cells were collected in exponential phase and exposed to 1 mM Cr(VI) for 1h in PBS

Table 28 Chromium cell content in environmental chromate resistant isolate NCr1a and control laboratory strain *B. subtilis* PY79 (BS): A - cells grown in M9 medium with 1 mM Cr(VI) for 4 or 18h (until exponential or stationary phase); B - uninduced or induced cells exposed to 1 mM Cr(VI) for 1h

Treatment			**Cr content in cells (at%)**	
			NCr1a	**BS**
A	Cells grown in medium with 1 mM Cr(VI)	4h	0.03±0.01 [a]	0.27±0.20 [b]
		18h	0.09±0.03 [a]	0.30±0.23 [b]
B	Cell exposure to 1 mM Cr(VI)†	Uninduced	0.02±0.01 [a]	0.17±0.05 [b]
		Induced††	0.02±0.01 [a]	0.03±0.02 [a]

† cells collected in exponential stage, washed and exposed to 1 mM Cr(VI) in PBS for 1h; †† preculture was induced with 10 μM of Cr(VI); values in rows with the same letter in superscript are not significantly different at $p<0.05$, t-test

CHROMIUM CONTENT IN ENDOSPORES

We examined chromium content and localization in endospores of NCr1a and *B. subtilis* PY79. First, we examined endospores formed in the presence of Cr(VI) in sporulation medium from the start of the growth. Second, we tested spores formed without presence of Cr(VI) and subsequently exposed to 1 mM Cr(VI) for different periods of time.

Strain *B. subtilis* PY79 failed to grow and form spores in Difco sporulation medium (DSM) with 1 mM of Cr(VI) (Figure 48). Strain NCr1a grew to a 47.5±0.1 % after 24h and 58.0±7.6 % after 48h of incubation, compared to no Cr(VI) control.

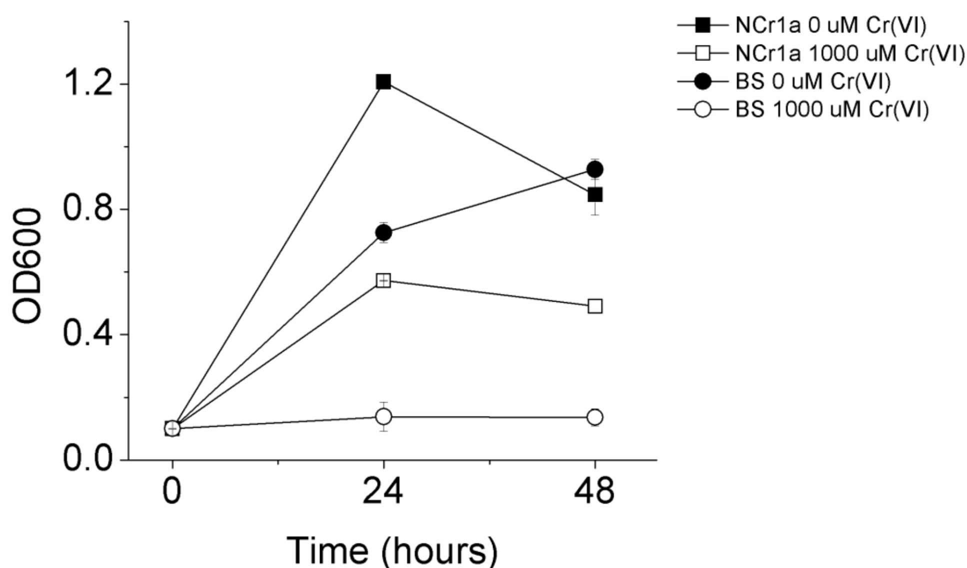

Figure 48 Growth of environmental chromate resistant isolate NCr1a and chromate sensitive laboratory strain *B. subtilis* PY79 (BS) in sporulation medium (DSM) with different concentrations of Cr(VI)

It should be noted that on preliminary testing, endospores of *B. subtilis* PY79 were found in small numbers. In preliminary testing, preculture in no Cr(VI) medium used for inoculation of final culture was older. Thus, there could have been a carryover of spores from preculture to final culture. This would result in observation of spores even though there was no sporulation in the presence of Cr(VI) in medium. Also, sample for STEM analysis was more concentrated in preliminary testing. Another reason for presence of spores in the preliminary STEM could be the higher starting density of *B. subtilis* PY79. It is known *B. subtilis* PY79 fails to grow in culture if it is inoculated in a too low starting density. Low starting density could thus result in a strain's failure to grow in Cr(VI) media. However, spores were successfully produced in no Cr(VI) medium inoculated with same starting density, indicating this is not a probable reason.

Endospores formed in Cr(VI)-containing medium

We tested chromium content in endospores formed after 6 days of incubation in sporulation medium with 1 mM Cr(VI).

Endospores of **NCr1a** formed in medium with 1 mM Cr(VI) did not appear morphologically different to those formed in medium without Cr(VI). They take up the largest amount of Cr (0.50±0.28 at%), which is significantly more compared to all the tested spore preparations ($p<0.05$, One-way ANOVA) (Figure 49, Table 29).

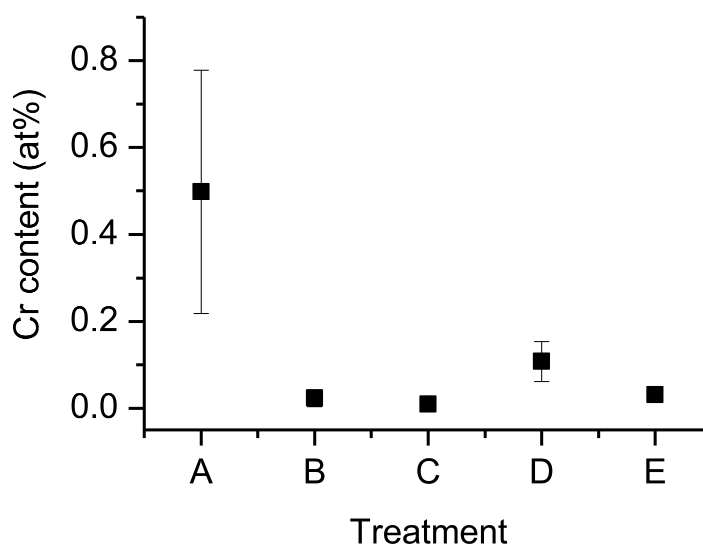

Figure 49 Chromium content in endospores after different treatments: A - NCr1a, exposure from start of growth, 6 days; B - NCr1a, exposure of mature spores, 1h; C – *B. subtilis* PY79, exposure of mature spores, 1h; D - NCr1a, exposure of mature spores, 6 days; E – *B. subtilis* PY79, exposure of mature spores, 6 days

Table 29 Chromium content (at %) in endospores of NCr1a and *B. subtilis* PY79

Treatment		Cr content in endospores (at %)	
		NCr1a	***B.subtilis* PY79**
Sporulation in 1 mM Cr(VI) medium	6 days	0.50±0.28	No spores observed
Exposure of mature spores formed in medium without Cr(VI)	1h	0.02±0.02 [a]	0.01±0.00 [a]
	6 days	0.11±0.04 [a]	0.03±0.01 [b]

Values in rows with the same letter in superscript are not significantly different at $p<0.05$, t-test

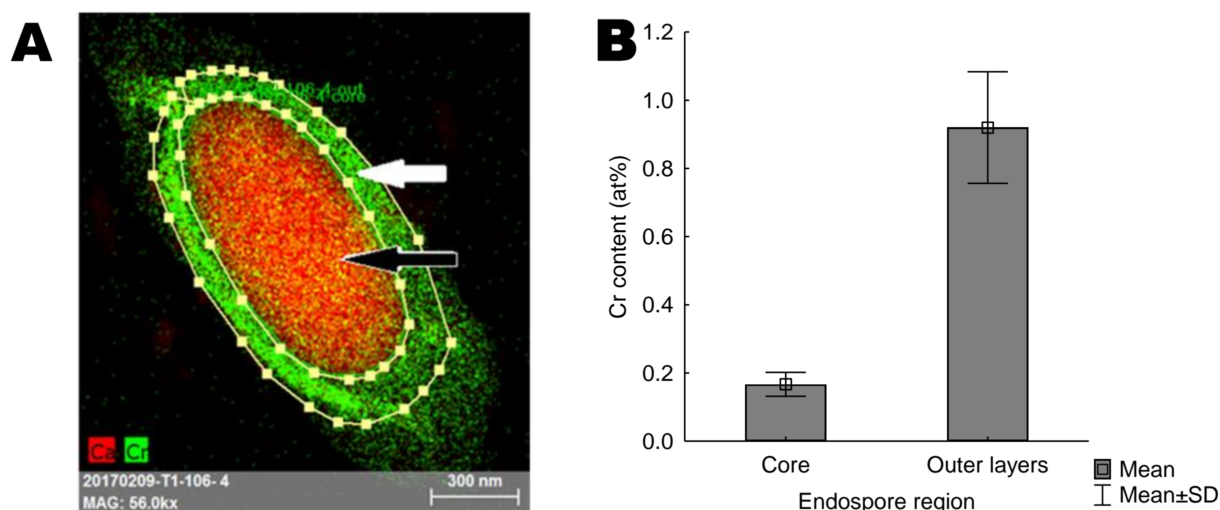

Figure 50 Localization of Ca and Cr in NCr1a endospore: A - STEM-EDS image showing Cr falsely colored in green, Ca in red, outer layers marked by a white arrow and core marked by a black arrow; B –Cr content measured in outer layer and core

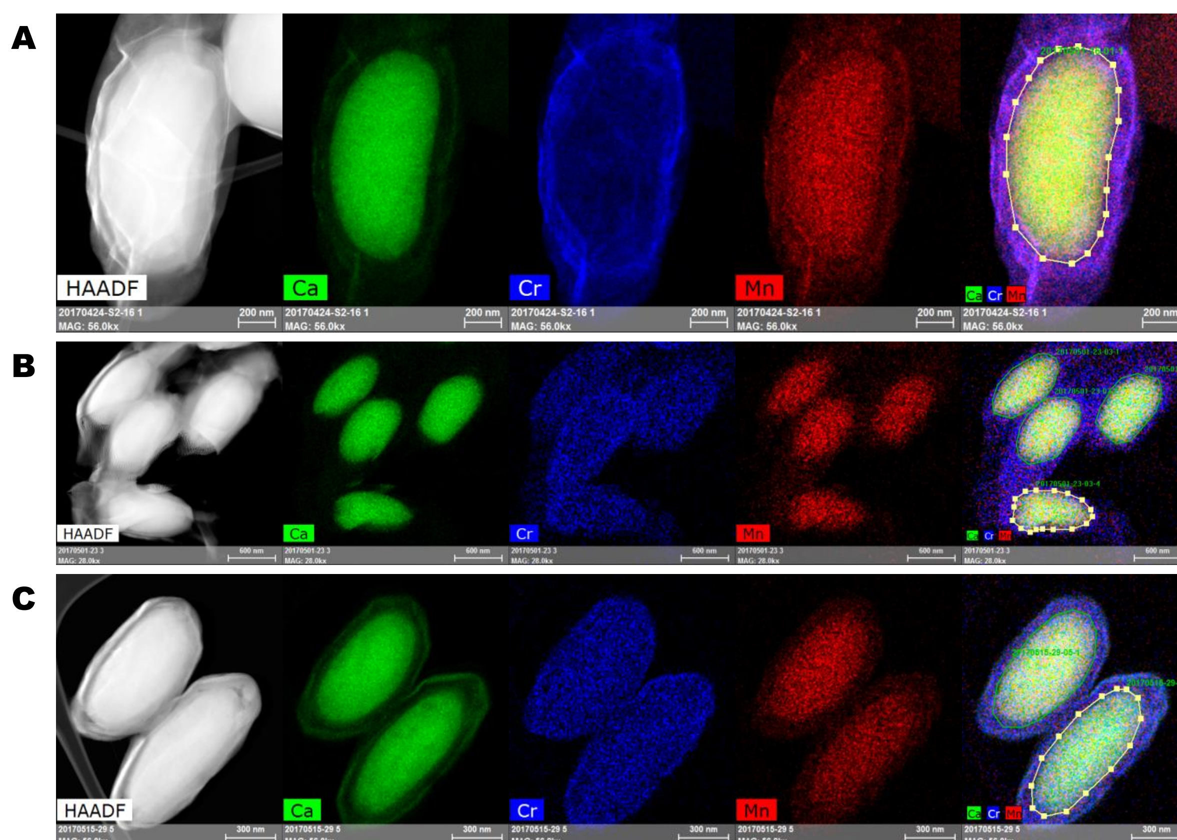

Figure 51 Scanning transmission electron microscopy coupled to energy dispersive spectroscopy (STEM-EDS) images of endospores: A – NCr1a endospores formed in DSM with 1 mM Cr(VI), 6 days of incubation; B – NCr1a endospores formed without Cr(VI) and exposed to 1 mM Cr(VI) for 6 days; C – *B. subtilis* PY79 (BS) endospores formed without Cr(VI) and exposed to 1 mM Cr(VI) for 6 days. Elements false colored in EDS image: Ca green, Cr blue, Mn red

From EDS elemental mapping it can be putatively concluded chromium is mostly located in the outer layers of endospore (coat and exosporium) (Figure 51). This could also be inferred from separate measurements made in area of spore coat and outer layers (Figure 50). Differences in Cr content in core and outer region were significant at $p>0.01$ (t-test). However, to precisely map out Cr localization, imaging of microtome sections with uniform thickness would be warranted.
Endospores of ***B. subtilis* PY79** formed in medium with 1 mM Cr(VI) could not be tested as there was no growth nor sporulation of this strain in medium with 1 mM Cr(VI).

Exposure of mature endospores

We determined chromium content in mature endospores which were formed in the absence of chromium and were subsequently exposed to 1 mM of Cr(VI) for different periods of time (1 hour and 6 days) (Figure 49, Table 29).
Mature spores of **NCr1a** exposed to Cr(VI) for 1 hour took up significantly less chromium (0.02±0.02 at%), compared to those exposed for 6 days (0.11±0.04 at%). Mature spores of NCr1a exposed to Cr(VI) for 6 days, take up 4.5 times less chromium, compared to spores formed in the presence of Cr(VI).
Mature spores of ***B. subtilis* PY79** took up similar amounts of chromium both after 1 hour and 6 days of exposure (0.01±0.00 and 0.03±0.01 at%, respectively).
After 1 hour of exposure, mature spores of both strains contain similar amounts of chromium. However, after longer 6 day-exposure, mature spores of NCr1a take up 3.7 times more chromium than *B. subtilis* PY79 spores (t-test, significant at $p<0.05$). Probable reason is that NCr1a spores possess exosporium, which binds more chromium. Contrary, *B. subtilis* PY79 spores do not possess exosporium .

To summarize, sporulation of NCr1a in the presence of Cr(VI) is unhindered, spores maintain normal morphology and take up significantly higher amount of chromium compared to all other spore treatments. Chromium is located mainly in the outer spore layers (coat and exosporium). Contrary, *B. subtilis* PY79 fails to efficiently sporulate in the presence of Cr(VI). If mature spores of *B. subtilis* PY79 formed without Cr(VI) are exposed to Cr(VI), they take up significantly less Cr compared to NCr1a spores, probably due to their lack of exosporium.

6. DISCUSSION

6.1. CHROMIUM INFLUENCE ON CULTIVABLE BACTERIAL SOIL COMMUNITY

The influence of any particular metal on soil microbial community is a complex and intricate phenomenon that is difficult to elucidate. One important cause for this is the high complexity of soil as a habitat. The vast diversity of soil types and contamination combinations lead to a large number of variables and preclude inference of any robust or general conclusions.

Cultivable bacteria represent only a fraction of the total microbial diversity (Torsvik et al., 1990), however they are still highly important in studies of metal tolerance. Only cultivated strains and consortia can be probed for mechanism of resistance to pollutants. Thus, cultivable bacteria are of great importance for the practical aspects of Cr(VI) tolerance and for bioremediation. Knowledge on which environments contain more Cr(VI) tolerant bacteria could indicate where to search for strains with high potential for practical use in chromate bioreduction.

To better understand how the Cr level in soil affects cultivable bacteria and in which environments the highest proportion of chromate tolerant cultivable bacteria might be expected, we compared the cultivable bacteri (CB) counts and percentage of chromate tolerant cultivable bacteria (CrCB) in soil samples with low background Cr level and elevated Cr level of natural and anthropogenic origin. A total of 17 soil samples were divided in three groups based on the content and origin of chromium: (A) background Cr level, (B) elevated Cr level of anthropogenic origin and (C) elevated Cr level of natural origin. By comparing results of microbial and chemical analyses of these sample groups we could assess the effects of high Cr level of natural origin and of anthropogenic origin.

No significant correlation was observed between CB count and total Cr concentration in soil in the present study. This finding suggests that bulk Cr is a poor determinant of microbial population and that other factors surely play an important role. This is not surprising as it was also observed by Viti and Giovannetti (2001) who did not find a statistically significant relationship between total Cr soil concentration and CB counts. However, their study examined only one Cr polluted soil sample and two unpolluted soils. In comparison to our study, they recorded relatively low cultivable bacteria counts - from $6.4 \cdot 10^5$ CFU/g in Cr contaminated soil to $1.0 \cdot 10^6$ CFU/g in uncontaminated soil (Viti and Giovannetti, 2001). This discrepancy could be due to the use of nutrient rich medium (Luria agar) in their case rather than the oligotrophic medium (R2A agar), in our case, as the former is known to give 3-5 times lower bacterial counts (Olsen and Bakken, 1987). A separate study that examined sites polluted with Cr, As and Cu and unpolluted control soils reported similar counts to those in the present study, in the

range of 10^6 to 10^7 CFU/g and also concluded that metal contamination did not affect CB counts (Turpeinen et al., 2004).

Furthermore, the percentage of chromate tolerant cultivable bacteria did not differ notably in the three soil groups. CrCB proportion was not linked to soil Cr level or origin and other soil properties. For instance, the samples with the highest Cr (sample 11) and with the lowest Cr content (sample 2) had identical percentage of cultivable bacteria tolerant to 8 mM Cr(VI).

Regardless of the history of their exposure to Cr, all soil samples exhibited a similar percentage of cultivable cell numbers capable of withstanding 2 mM Cr(VI). Similar conclusions were found in the previous study, where no significant correlation between Cr(VI) concentrations in polluted river sediments and cultivable counts, diversity at the genus level, nor the ability of the microbial community to withstand or to reduce high Cr(VI) concentrations was observed (Branco et al., 2008). Contrary to our results, (Viti and Giovannetti 2001) reported a significantly higher percentage of chromate tolerant cell counts in Cr polluted soils compared to unpolluted control soils. However, their observation was based on a single soil sample in each group. All this suggests that, unsurprisingly, a complex interplay of factors (e.g., soil pH, Cr speciation, the presence of other toxic elements, organic matter content) rather than the Cr content alone, determine the bacterial abundance.

Another notable finding was that about half of the cultivable population (47.5±21.6 %) was highly chromate sensitive and was inhibited even by the lowest tested concentration of Cr(VI) in cultivation media (0.25 mM). This shows that even the soils with high concentration of Cr can serve as a habitat for strains with low Cr(VI) tolerance. The reduction of hexavalent to less toxic trivalent Cr in soils and the existence of microenvironments with low Cr and/or Cr(VI) levels in the soil provide environmental conditions suitable for sensitive microorganism (Branco et al., 2005).

Certain samples contained >10% of chromate tolerant bacteria which successfully grew even in the medium with the highest tested concentration of 8 mM Cr(VI) (samples 2, 3, 8, 10, 11, 14, 15 and 17). However, it should be noted these particular samples belong to all the three soil groups. Accordingly, soil Cr content nor origin could not be considered as a main contributing factor for their exceptional percentage of chromate tolerant bacteria. Also, no other chemical or physical soil property was found to be responsible.

The study did not find any statistically significant correlation between the soil Cr concentration and the ratio of Gram-positive to total cultivable bacteria. This leads to the conclusion that other factors may contribute to the distribution of Gram-positive and Gram-negative bacteria within cultivable bacteria and that total Cr content may or may not be among those factors. Our results are in contrast with earlier reports showing that the percentage of Gram-positive bacteria increased with increasing Cr concentration in the soil of a polluted leather tannery (Viti and Giovannetti 2001). Others have also suggested that Gram-positive bacteria were a group with greater metal

tolerance capabilities as compared to Gram-negative bacteria (Konopka et al., 1999; Pacheco et al., 1995; Ross et al., 1981; Trajanovska et al., 1997).

To summarize, this study section sought to understand the impact of elevated Cr soil concentrations on the cultivable bacterial soil community abundance and structure. Cultivable bacterial counts were not affected by the corresponding soil's high Cr concentration. However, addition of even a low concentration of chromate in the growth medium inhibited growth of half of the cultivable soil bacteria. Similarly, the percentage of Gram-positive and Gram-negative bacteria as well as the percentage of chromate tolerant population, were not directly affected by the soil Cr level. Results of the present study coincide with earlier findings which did not find a simple and direct relationship between soil Cr concentration and soil cultivable bacteria community abundance, diversity and structure.

6.2. CHROMATE TOLERANCE OF ENVIRONMENTAL BACTERIA

We isolated diverse chromate tolerant bacterial strains from a wide variety of environmental samples. Strains were isolated both from environments with high Cr levels of natural (NCr) and anthropogenic (PCr) origin, as well as from sites with low background Cr concentration (LCr). Based on the source of the isolation strains were designated with different prefixes - PCr for strains from Cr polluted sites, NCr for strains from sites with high Cr of natural geogenic origin and LCr for strains from sites with background Cr level. In total, 33 highly chromate tolerant strains from 26 environmental samples were isolated.

Since bioreduction and bioaccumulation are promising and economically viable methods for the bioremediation of chromium contaminated sites (Dhal et al., 2013), strains with such abilities have a potential for practical application. Numerous studies of chromate reducing bacterial strains isolated from polluted environments have been conducted. In the present study, in addition to chromium polluted environments, we attempted isolation of Cr(VI) tolerant strains from low Cr samples and samples with naturally elevated levels of Cr. We identified chromate tolerant strains in all the sample groups regardless of Cr level or origin. Results indicate that even environments without previous chromium exposure contain chromate tolerant strains, suggesting that tolerance can exist without direct selective pressure from this metal. This finding could be explained by the fact that chromate tolerance is likely related to determinants whose primary function is not chromate resistance (e.g., unspecific chromate reduction, sulfur or iron homeostasis, and reactive oxygen species detoxification) (Ramírez-Díaz et al., 2008), which are present in the bacteria regardless of Cr level in their habitat.

Such ubiquitous presence of chromate tolerant and chromate reducing strains provides the basis for the natural attenuation process in contaminated natural environments. Reduction of highly toxic Cr(VI) to less toxic Cr(III) is favored under most environmental conditions and Cr(VI) concentration is expected to decrease over time (Motzer, 2005). This process of natural attenuation of Cr(VI) pollution is realized through action of indigenous microbial community without any artificial manipulation. In addition to microbial activity, Cr(VI) can be reduced by the organic matter in the soil which can quickly adsorb and reduce Cr(VI) to Cr(III) (Bartlett and James, 1979). However, if the input of Cr(VI) is high, the natural attenuation reduction capacity can be exceeded and high levels of the hexavalent form can persist in the environment (Cervantes et al., 2001).

Our results confirm findings of previous studies in which heavy metal tolerant strains were isolated from soils regardless of their previous metal contamination (Caliz et al., 2012; Roane and Kellogg, 1996; Viti et al., 2006). It should be noted, however, that soils

with high Cr of either anthropogenic or geogenic origin yielded more morphologically diverse chromate-tolerant isolates than low Cr soils. This indicates that a greater diversity of chromate tolerant strains is present in samples with previous Cr exposure, which is likely an effect of the long-term exposure of the microbial community to Cr.

The 33 strains isolated represented 12 species (considering the *B. cereus* group as one species), all of which were previously known for their chromate tolerance. Majority of them belonged to Gram-positive bacteria. Here we will give a brief overview of published findings. The chromate tolerant strain *Cellulosimicrobium cellulans* KUCr3, isolated from a sludge waste canal, exhibited chromate reduction activity and promoted the growth of plants in chromium-contaminated soil (Chatterjee et al., 2009). The tannery wastewater multi-drug and multi-metal resistant isolate *Cellulosimicrobium* sp. KX710177 was also characterized for its Cr(VI) reduction ability (Bharagava and Mishra, 2018). The enhancement of plant growth on Cr contaminated soils and the reduction of chromate have been reported for *Rhodococcus erythropolis* (Banerjee et al., 2017; Trivedi et al., 2007). *Microbacterium* sp. GM-1 was used for the biocementation of chromium slag based on microbially induced calcium carbonate precipitation leading to lower leaching toxicity of Cr(VI) waste (Lun et al., 2016), while polyvinyl alcohol bead-immobilized *M. liquefasciens* MP30 was used for Cr(VI) bioreduction in batch and continuous-flow bioreactors (Pattanapipitpaisal et al., 2001). Chromate tolerance and reduction of *Arthrobacter* sp. has been documented by several studies (Córdoba et al., 2008; Dey and Paul, 2015; Kristene L Henne et al., 2009; Kristene L. Henne et al., 2009), and it was revealed that chromate efflux was the main resistance mechanism. Chromate tolerance of *Staphylococcus haemolyticus* and *S. warneri* strains has been briefly reported (Roychowdhury et al., 2016; Singh et al., 2016), while chromate resistance was characterized in more detail in other species of this genus, namely *S. aureus, S. saprophyticus, S. epidermidis* and *S. arlettae,* (Claude and Adolphe, 2013; Sagar et al., 2012; Vatsouria et al., 2005; Zhang et al., 2016).

Strains of four Gram-negative species were also isolated in the present study: *Serratia fonticola* and three species of *Ochrobactrum* genus. A *Serratia* biofilm formed on activated carbon was utilized for Cr(VI) removal from tannery waste water (De Bruijn and Mondaca, 2000). *Serratia* strain Cr-10 isolated from a chromium contaminated site was tested for Cr(VI) removal from aqueous solutions and it was found that reduction had more significant role compared to bioadsorption (Zhang and Li 2011). The chromate resistance of *Ochrobactrum* species is well characterized. The genes *chrA* and *chrB* located on transposon element Tn*OtChr* in *O. tritici* 5bvl1 were found to be essential for chromate resistance by means of efficient efflux of chromate out of the cell. This primary resistance mechanism was accompanied with a specific or unspecific Cr(VI) reduction, free-radical detoxifying activities, and DNA damage repair (Morais et al., 2011). This strain was also successfully bioengineered for the use as a microbial bioreporter for the measurement of bioavailable chromate in contaminated water (Branco et al., 2013).

Strains characterized in all the above mentioned published studies were isolated from contaminated environments such as waste materials, wastewater and contaminated soil. To remind, we isolated strains both from polluted and unpolluted sites. Thus, in future studies, it will be of interest to compare chromate tolerance characteristics of abovementioned strains to strains *S. warneri* LCr1, *S. haemolyticus* LCr9b and *B. cereus* group strains (LCr2, to LCr8 and LCr9a) isolated in the present study from unpolluted low chromium environments.

It is argued that halotolerance could be a useful trait for survival in polluted environments such as tannery wastewater effluents which, usually, beside high Cr levels, contain high salt concentrations (Branco et al., 2004). Therefore, chromate tolerant strains from halophilic habitats could have an advantage in the bioremediation of waste material with high salt levels. In the present work, six *B. cereus* group strains and one *S. haemolyticus* strain were obtained from soil and water samples collected at the salt marshes Slano Kopovo and Medura. From this point of view they could be interesting candidates for further testing.

The majority (28 out of 33) of the Cr(VI) tolerant strains isolated were Gram-positive. This result should not necessarily be construed as a preponderance of Gram-positive bacteria amongst Cr(VI)-tolerant bacteria. It is likely due to a bias in colony selection, which could also be a reason for the prevalence of the *Bacillus* genus (20 out of the 28 strains) amongst Cr(VI)-tolerant Gram-positive strains isolated in this study (Table 17). Isolation procedure in the present study included confirmation of bacterial growth on solid media with 2 mm Cr(VI). *Bacillus* spp. usually form large spreading colonies, which could have resulted in their easier detection on high Cr(VI) solid media. Contrary, strains which normally form small colonies will form even smaller colonies if grown on high Cr(VI) plates. This could cause that they are not detected during isolation of chromate tolerant strains by culturing on agar plates.

Moreover, for cultivation-based methods, the presence of spores is likely to lead to an overestimation of the importance of sporulating bacteria in soil processes (Mandic-Mulec and Prosser, 2011). Still, it is helpful to have identified so many spore-forming bacteria as they clearly offer an advantage in that they can resist extreme conditions (e.g., heat, desiccation, toxicity). The 13 *B. cereus* group strains were selected for further detailed characterization, which will be discusses in more detail in section 6.3.

Comparison of metal tolerance and reduction data between published studies is a difficult, if not impossible venture, as many experimental parameters are variable (e.g. incubation time and temperature, inoculum preparation, and starting cell density, medium used, preculture conditions, growth measurement method). Therefore, exact comparison of chromate tolerance and reduction ability of isolated strains to those previously described will not be attempted in this work. We will only give an overview of the previous findings.

For instance, strain *Arthrobacter* sp. SUK 1205 (Dey and Paul, 2015) reduced 64 % of the starting 2 mM Cr(VI) after 8 days of incubation at 35°C and 120 rpm in minimal

defined Vogel-Bonner broth, with a starting cell density of 10^6 CFU/ml. *Bacillus cereus* Cr1 isolated from tannery wastewater was used for effluent treatment in laboratory scale experiments. Free and cells immobilized in alginate were effective in reduction of chemical oxygen demand (COD), biological oxygen demand (BOD), total dissolved solids, electrical conductivity and total chromium (Kumari et al., 2016). *S. arlettae* Cr11 reduced 98% of the starting 100 mg/l (1.92 mM) Cr(VI) after 24h incubation in tryptic soy broth at 37°C and 120 rpm. At a starting concentration of 500 and 1000 mg/l (9.6 and 19.2 mM) Cr(VI), reduction capacity and growth were highly impaired due to the inhibitory effect of Cr(VI) (Sagar et al., 2012).

Ochrobactrum anthropi strains reduced 19.6 to 34.2 % of starting 1mM Cr(VI) in nutrient broth at 30°C after 72h (Francisco et al., 2002). *Ochrobactrum tritici* 5bvl1 reduced 57% of 3 mM starting Cr(VI) in minimal medium after 140h, while prolonged incubation did not have impact on further reduction (Branco et al., 2004). Our results indicate similar comparably low level of Cr(VI) reduction by *Ochrobactrum* species which ranged from 0 to 31% at 0.5 mM starting Cr(VI) level. Their high growth in Cr(VI) containing media accompanied with comparably low reduction, leads to a conclusion other resistance mechanism such as reduced chromate intake or effective chromate efflux have a significant role in their chromate resistance.

Cellulosimicrobium genus is known by its high Cr(VI) tolerance, Cr(VI) reduction and plant growth promoting properties (Bharagava and Mishra, 2018; Chatterjee et al., 2011; Karthik et al., 2017). Namely, it reduced 99.33 % in LB with 50 mg/l (0.96 mM) Cr(VI) after 24h incubation at 35°C and 120rpm (Bharagava and Mishra, 2018).

In general, all isolates derived from polluted sites exhibited >80% maximum relative growth in medium with 0.5 mM Cr(VI). No clear correlation between amount of growth in Cr(VI) containing media and chromate reduction was found. Similar conclusions were found in previous research of *Arthrobacter* (Megharaj et al., 2003) and *Pannonibacter phragmitetus* strains (Chai et al., 2009), where no link between amount of growth and reduction was noted.

Out of the 33 examined isolates certain are more interesting for prospective studies:

- High reduction and high growth strains: *Bacillus cereus* group strains NCr1a, NCr2, PCr2a and PCr3a and *Cellulosimicrobium cellulans* PCr3b –of interest in further studies of chromate bioreduction for practical bioremediation application.
- Low reduction and high growth strains: *Serratia fonticola* NCr1d, *Staphylococcus haemolyticus* LCr9b and PCr8, *Ochrobactrum tritici* PCr6 and *Microbacterium maritypicum* PCr11 – of interest in further studies of alternative chromate resistance mechanisms such as chromate efflux, blocking of chromate intake and oxidative stress response.

- *Bacillus cereus* group strains in general as they were predominant among all of the isolated chromate tolerant strains.

Isolated bacteria provide a starting culture collection of highly chromate tolerant strains encompassing wide phylogenetic range and source of origin, which is a valuable tool for further investigation of chromate tolerance mechanisms.

6.3. CHROMATE TOLERANCE OF *BACILLUS CEREUS* GROUP

As mentioned before, among the isolated chromate tolerant strains in this study, the most prevalent were those belonging to *B. cereus* group. Although it is an important member of microbial communities, abundantly and ubiquitously present in various environments, this groups' chromate response is less studied compared to some other species such as Gram-negative *O. tritici* and *P. aeruginosa*. Therefore, we selected 13 isolates of this group for more detailed examination, including molecular, biochemical, morphological, heavy metal resistance, chromate reduction and biofilm formation properties.

The first approach to **identification** of these strains was MALDI TOF analysis. Some of the advantages of this method are high speed, low work load and relatively low price (Kostrzewa and Schubert, 2016). However some disadvantages were also encountered in this study. In particular, MALDI TOF analysis was not able to clearly discriminate between species of *B. cereus* group. Member species of this group are highly similar and very difficult to distinguish by classical biochemical, morphological and molecular methods (Dworkin et al., 2006; Logan and De Vos, 2009). Due to high similarity, some authors even suggest certain species of this group can be regarded as single species based on genetic evidence (Helgason et al., 2000).

Difficulties in standard MALDI TOF methodology for identification of this group have been reported previously (Egli et al., 2015; Pfrunder et al., 2016). Therefore, several studies worked on improvement of the analysis by implementation of new diagnostic peptides (Pfrunder et al., 2016), geometric approach in spectra analysis and development of open-access reference databases (Starostin et al., 2015). Commercially available databases and programs for MALDI TOF analysis are mostly designed for identification of clinically important strains (Starostin et al., 2015). However, applicability of such databases on environmental isolates proved to be limited, stressing the need for continual database refinement and enrichment (Kostrzewa and Schubert, 2016).

Similarly to MALDI TOF classification, 16S rRNA gene sequence analysis, which is a method of choice for bacterial identification, was of limited competence for discriminating between member species of *B. cereus* group in the present work. Similar conclusions were noted in previous studies, in which *pycA*, *ccpA* (Liu et al. 2015) and *rpoB* gene sequence analysis (Blackwood et al., 2004) were suggested for more precise species identification.

Many characteristics crucial for classical identification of this group are located on plasmids, which can be easily lost from the cell or gained through horizontal transfer (Helgason et al., 2000). For instance, *cry* gene coding insecticidal delta-endotoxin crystal

can be easily spread from Cry+ to Cry- strains of *B. thurigiensis* (González et al., 1982). That way, a crucial property for identification of *B. thuringiensis* (production of parasporal crystal body) can be lost and strain could be falsely identified.

For the purpose of this study, precise species identification and phylogenetic analyses were not of paramount importance. More important was to definitely prove isolated strains were not highly pathogenic *B. anthracis* and thus do not pose serious safety risk. This was accomplished through testing of morphological, biochemical and genetic properties (Papaparaskevas et al., 2004). First, none of the isolates contained virulence genes *pagA* and *capA* present in *B. anthracis*. Second, strains were all beta-hemolytic while alpha-hemolysis is present in *B. anthracis*. Third, strains were motile contrary to nonmotile *B. anthracis*. Only exception was strain NCr1a which was nonmotile, but its rhizoidal colony morphology clearly indicated it belonged to *B. mycoides* or *B. pseudomycoides*. Furthermore, BLASTn search of *pycA* gene sequence never gave hits for *B. anthracis.*

If morphological and biochemical properties are taken into consideration, strains could belong to *B. cereus, B. thuringiensis, B. mycoides* or *B. pseudomycoides*, while *B. anthracis* and *B. weichenstephanensis* are eliminated. Contrary, based on MALDI TOF, *pycA* and 16S rRNA gene sequence analysess some strains could be identified as *B. weichenstephanensis*.

These contradictory findings just go to additionally highlight the complexity of the *B. cereus* group taxonomy (Fritze, 2004). Also, our findings stress the need for multiple approaches in characterization of this group, which should include classical biochemical and morphological as well as more modern molecular analyses. Nevertheless, even with such multi-approach, conclusive species identification has proved to be more or less unfeasible.

Ability to grow in the form of a **biofilm** could be a useful trait for practical use of microorganisms in bioremediation (Harrison et al., 2007). Biofilms are microbial communities consisting of microbial cells which are attached to surfaces and encased within EPS (extracellular polymeric substances) matrix. EPS matrix usually consists of proteins, polysaccharides, lipids, DNA and other substances and can contribute to higher metal resistance and metal biosorption (Kantar et al., 2011). Moreover, microorganisms in a biofilm form are more resilient to antibiotics, chemical agents, desiccation, host's immune system and many other factors in comparison to planktonic form. Increased resistance to heavy metals in biofilm form facilitated by different mechanisms has been proven in many studies as reviewed by Harrison et al. (2007). Finally, secondary waste water treatment is often based on the activity of a biofilm formed on various types of carriers (e.g. trickling sand filters, rotating biological contactors, sequencing batch reactors) (Lewandowski and Boltz, 2011). Taken together, strong biofilm formation potential could be considered as an advantage for strain's prospective bioremediation use.

Treatment of Cr contaminated wastewater has been conducted by biofilms on different types of carrier materials. For instance, glass beads, pebbles, coarse sand (Sundar et al., 2011), activated carbon granules (De Bruijn and Mondaca, 2000; Quintelas et al., 2008), alginate (Dey and Paul, 2015) or granular biofilms (Nancharaiah et al., 2010) have been employed. Biofilm-based reduction of Cr(VI) has certain advantages: (i) the cells in biofilm form can tolerate higher Cr(VI) concentrations compared to planktonic cells, and (ii) separation of the treated liquid from the biomass is comparably easier (Nancharaiah et al., 2010). *Bacillus coagulans* supported on granular activated carbon has been employed in Cr biosorption with a metal uptake value of 5.35 mg per gram of biosorbent for an initial Cr(VI) concentration of 100 mg/l (Quintelas et al., 2008). Cr(III) bioremoval by *B. subtilis* and *B. cereus*, and their consortium in continuous flow bioreactors was also investigated. As expected, biofilm was more efficient than planktonic cells, while coarse sand was a better carrier material compared to glass beads and pebbles (Sundar et al., 2011). However, mentioned studies were conducted on laboratory scale and further pilot scale studies on the subject are needed for full understanding of capabilities of such chromium bioremoval methods.

Unfortunately, strains isolated in the present study did not exhibit particularly high biofilm formation potential. However, their high chromate tolerance even in the planktonic form offers the opportunity of their application in waste treatments with free suspended cells, such as activated sludge (especially strain NCr1a which forms flocculent growth in liquid culture).

Waste materials rarely contain only one or few pollutants. In majority of cases, waste contains complex mixture of polluting substances belonging to various chemical types. Consequently, it would be beneficial if microorganisms employed in Cr bioremoval are tolerant to a wider range of pollutants. We tested **heavy metal tolerance** of the selected chromate tolerant isolates by determining their heavy metal MICs. Reference *Bacillus* sp. and *E. coli* strains were also included in the measurements.

Reference strain *B. subtilis* PY79 proved to be the most sensitive to many heavy metals tested (Hg, Cd, Cu, Co, Ni and Zn), while *E. coli* ATCC 25922 was extremely sensitive to Cr(VI). All in all, Ag was the most, while Mo and W were the least toxic.

Trivalent chromium was less toxic to strains than hexavalent form. Although it is uniformly regarded that Cr(VI) is far more toxic than Cr(III) (e.g. Ramírez-Díaz et al. 2008; Cheung & Gu 2007), some studies found significant negative effects of trivalent chromium as well. For instance, among bacteria isolated from treated tannery effluents and agricultural soil irrigated with these effluents, more were resistant to Cr(VI) than to Cr(III) (Alam et al., 2011). Trivalent Cr cannot easily be transported into the cell, but it can be formed inside the cell by intracellular Cr(VI) reduction. It transiently stays in the form of Cr^{3+} or hydroxyl anions before it is quickly complexed with intracellular organic compounds. During brief period before complexation, it can affect DNA transcription and/or replication, by forming DNA adduct (Bencheikh-Latmani et al., 2007b). Toxicity of exogenous exposure to Cr(III) is realized mainly through extracellular interactions

which cause abnormal cell morphology and ultimately lead to cell death (Parker et al., 2011).

In general, chromate tolerant isolates as well as reference *B. cereus* group strains were comparably more metal tolerant than *B. subtilis*. Specifically, *B. cereus* group strains were more resistant to Hg, Cd, Cu, Co, Ni and Zn. This multiple metal tolerance could be advantageous for their application in bioremediation. All tested strains had almost identical MIC values, indicating their metal homeostasis is based on conserved mechanisms innately and constitutively present in all the strains of the group.

Metals exhibit stronger inhibiting effect on bacteria on liquid media compared to solid media (Bajkić et al., 2013; Hassen et al., 1998). This was also found in the present study, as Cr(VI) MIC value was higher on solid M9 medium compared to liquid medium. In particular, MIC on solid medium determined by agar spot method was 10 mM for all the strains, while it was mostly 4 mM if determined by macrodilution method on liquid medium. However, this discrepancy could be caused by the testing of different concentration ranges in the two experiments. In agar spot method we tested 0.1, 2, 10 and 30 mM Cr(VI), while in liquid M9 medium we tested 0.5, 1, 2, 4 and 8 mM. Therefore, agar spot dilution gave only a very crude estimation of MIC values, which could be anywhere between 2 and 10 mM in the case of Cr(VI). To precisely measure influence of agar in medium on heavy metal toxicity testing, more experimental data are needed.

Comparison of metal tolerance of different strains reported in published studies is problematic due to the lack of standard testing conditions. Numerous experimental parameters can strongly influence tolerance measurements, for instance incubation time and temperature, inoculum preparation, and starting cell density. Also, tolerance can be expressed through different criteria such as minimal inhibitory concentrations, maximal OD600 reached, percentage of growth compared to no Cr(VI) control, growth rate, etc. Most importantly, the medium composition greatly affects MIC. For instance, rich broth media yielded 2 to 5 times higher MIC values than TRIS minimal medium (Mergeay, 1995). This was even more pronounced in the present study - MIC on nutrient rich LB medium was at least 15 times higher as compared to that obtained with minimal defined media. The two used minimal media (AMM and M9) gave similar or identical MIC values for all of the strains. A likely explanation for this discrepancy is the role of organic carbon in complexing Cr(III) produced by Cr(VI) reduction in the LB medium (Bencheikh-Latmani et al., 2007a). Nonetheless, use of undefined nutrient rich media for testing of metal tolerance is widely spread in scientific studies. LB broth is often used for such assessment as it is one of the most common media in microbiology research. Chromate anions are complexed or reduced by organic matter in such media and thus lose its toxic effect on bacteria. Amounts of available metals (Cd, Cu) measured by ion selective electrode were lowered to even less than a 0.2 % of initially added metal concentration in undefined rich media such as trypticase soy broth (Rathnayake et al., 2013). Use of nutrient rich media with undefined organic components such as

tryptone and yeast extract, may falsely give higher estimate of bacterial metal resistance (Kumar et al., 2013; Rathnayake et al., 2013), and also make comparison of different strains tested on different media batches inaccurate, if not impossible. Composition and effect of LB medium can vary significantly from batch to batch, brand-to-brand and cause lack of reproducibility (Ezraty et al., 2014).

Determination of Cr(VI) MIC values in this study was repeated many times in slightly different experimental conditions (data not shown). Differences in conditions included inoculum preculture conditions, inoculum concentration, mixing conditions, different type of glassware in which media were poured (tubes, flasks, bottles). More often than not, MIC values varied between measurements (in the range from 2 to 8 mM Cr(VI)). Therefore, differences in determined growth in the presence of Cr(VI) and ability to reduce chromate in different experimental setups through the study can be noticed.

Taking everything into account, metal tolerance measurements should not be taken as an absolute values, but rather as a relative measure for comparing metal tolerance of strains within a specific study.

Chromate tolerance of *B. cereus* group strains has been previously documented. However, strains described in published studies mostly originated from sites highly contaminated with chromium (e.g. Chen et al. 2012; He et al. 2010; Singh et al. 2006; Murugavelh & Mohanty 2013; Kumari et al. 2016). In contrast, isolates in the present study originated from unpolluted low Cr environments and samples with either anthropogenic or naturally elevated levels of Cr. Chromate tolerance level of isolates was very similar despite their distinct origins. Chromate tolerance of reference strains of *B. cereus* group was similar to that ofs environmental *B. cereus* group isolates, while *B. subtilis* PY79 reference strain proved to be far more sensitive. This may indicate that *B. cereus* group species inherently have a high level of chromate tolerance mediated through a conserved mechanism within the species of this group. Similar conclusion can be drawn from their highly similar level of tolerance to other heavy metals, as stated previously.

Prior studies have considered the mechanism of Cr(VI) uptake, as well as Cr(VI) bioreduction by *B. cereus* strains (Chen et al., 2012; He et al., 2010; Murugavelh and Mohanty, 2013), but no specific characteristic of *B. cereus* group species has been uncovered. Their chromate tolerance may be a secondary function of certain constitutive metabolic processes (e.g. unspecific chromate reduction, sulfur or iron homeostasis, and reactive oxygen species detoxification) (Ramírez-Díaz et al., 2008) and is thus present in the bacterium regardless of its previous chromium exposure. Thus, high chromate tolerance of this group could be a species specific characteristic rather than a consequence of a selective adaptation to high Cr environments. Also, natural competence and horizontal gene transfer common in these species could contribute to overall increased chromate tolerance as they could quick obtain resistance determinants and adapt to chromate stress (He et al., 2010). We suspect that the existence of a long chain chromate transporter protein (LCHR) in the *B. cereus* group

instead of short chain chromate transporter protein (SCHR) present in other *Bacillus* species such as *B. subtilis* could contribute to the group's higher chromate resistance, through the more efficient chromate efflux.

Biological **reduction of highly toxic Cr(VI) to less toxic Cr(III)** has been proposed as an effective means of hexavalent chromium bioremediation. Extensive reviews on this subject have been published in recent years (Barrera-Díaz et al., 2012; Cheung and Gu, 2007; Chirwa and Molokwane, 2011; Dhal et al., 2013; Fernández et al., 2018; Jobby et al., 2018; Joutey et al., 2015; Kamaludeen et al., 2003a; Pradhan et al., 2017; Thatoi et al., 2014).

Intracellular Cr(VI) reduction results in high amount of produced ROSs and contributes to cell damage (Thatoi et al., 2014). Effective tolerance mechanisms require mitigation of the oxidative stress generated during the reduction process. Several studies showed an increase in expression of superoxide dismutase (SOD), catalase, glutathione S-transferase, thioredoxin, and glutaredoxin during exposure of bacteria to chromate (Ackerley et al., 2006; Hu et al., 2005; Samuel et al., 2013). Therefore, the ability to reduce toxic heavy metals extracellularly would be a favorable feature for the survival of an organism involved in Cr(VI) reduction and would make it a good candidate for engineered Cr removal (Thatoi et al., 2014). Another viable method of diminishing chromate-induced oxidative stress is a Cr(VI) reduction through pathways which produce far less ROSs. For instance, soluble flavoprotein YieF of *E. coli* is a four-electron chromate reducer, which in one step transfers three electrons to chromate and one to molecular oxygen, resulting in far lesser oxidative stress compared to other reduction pathways (Ackerley et al., 2004b, 2004a).

In the present study, chromate tolerant *B. cereus* group strains exhibited varying level of reduction. Three strains (NCr1b, NCr2, and NCr4) reduced over 90 % of Cr(VI) in minimal medium (or >0.18 mM of a starting 0.2 mM Cr(VI)) in 72 h (Figure 31). However, beside high reduction activity, robust growth in the presence of Cr(VI) is also needed for potential practical application. In this respect, the best combination of reduction and growth was noted in strain NCr1a and PCr12, which thus are promising candidates for the investigation of chromate resistance mechanisms and engineered Cr removal. Strain PCr12 originated from a chromium polluted sample of solid waste material of a leather tannery. Strain NCr1a originated from unpolluted agricultural soil with naturally high level of Cr present in the form of mineral trivalent compounds. Beside moderately high Cr(VI) reduction, it exhibited the highest tolerance to Cr(VI) among the tested strains. It should be noted that in the separate testing of chromate reduction and tolerance on a higher starting Cr(VI) level of 0.5 mM (Figure 24), strain PCr12 had a low reduction potential, indicating strain NCr1a is the best candidate for further studies.

It is interesting to note that the greatest extent of reduction was not necessarily accompanied by the most substantial growth. Similar conclusion was found in previous testing of all isolates including those not belonging to *B. cereus* group, as no statistically

significant correlation was found between OD600/growth percentage and amount/percentage of Cr(VI) reduced.

The most chromate tolerant **strain NCr1a** was chosen for further testing. Detailed account on its morphological, cultivable and molecular characteristics was reported in the present study.

Strain exhibited the typical phenotype characteristics of *B. mycoides* / *B. pseudomycoides*. These two species of *B. cereus* group are the only ones with rhizoidal colony morphology, which is one of the main characteristics to differentiate them from the other species in the group. Also, they are nonmotile, as is NCr1a strain. Based on 16S rRNA BLASTn search, *B. pseudomycoides* Acc. No. KY625609.1 was the highest ranked hit for NCr1a. However, *B. mycoides* Acc. No. CP009651.1 was the highest hit based on *pycA* sequence. To definitively differentiate these two highly similar species, the analysis of fatty acid profiles (level of 12:O iso and 13:0 anteiso fatty acid in particular) content is required (but not done in this study) (Nakamura, 1998; Nakamura and Jackson, 1995). Therefore, NCr1a is presumed to belong to *B. pseudomycoides* or *B. mycoides,* based on its phenotypic characteristics and sequence analysis.

Bacillus pseudomycoides has been described as a new species in 1998 by Nakamura (1998). It was defined based on genetic differences in one group of *B. mycoides* strains, which was phenotypically not different. 16S rRNA gene sequences of *B. mycoides* and *B. pseudomycoides* are highly similar (98%). The two species are not distinguishable by physiological and morphological characteristics, but were clearly separable based on fatty acid composition. The optimum growth temperature of *B. pseudomycoides* is 28°C, the maximum 40°C, and the minimum 15 °C. Vegetative cells are nonmotile, 1.0 µm wide and 3.0-5.0 µm long (Nakamura, 1998).

As mentioned before, NCr1a has striking rhizoidal colony morphology. NCr1a filaments form in counter-clockwise direction (as observed from the bottom of the plate) (DX morphotype). Direction stayed unchanged through the study on all the incubation conditions used. This property has been studied in detail in *B. mycoides* (Di Franco et al., 2002). It was discovered direction of filament rotation is a strain-specific genetically directed characteristic. Colony morphology responds to mechanical properties of the agar surface, such as tension, compressing and bumps on agar surface (Stratford et al., 2013). Filament formation differed on nutrient rich and limited media (LB agar versus R2A agar). Filaments were much thinner and more spreading on oligotrophic medium, presumably as bacterium tends to spread out in the search of limited nutrients. Similar conclusions were made in previous research (Di Franco et al., 2002). Primary habitat of this species group is soil, which is a very competitive environment with highly diverse and abundant microbial community. Nutrients are limited and competition for them is fierce. Therefore, property of spreading growth in nutrient limited conditions could be naturally selected trait as stated in previous research (Di Franco et al., 2002). Both colony and microscopic appearance of NCr1a can give an impression of a mixed culture. This is a common trait of many *Bacillus* species (Dworkin et al., 2006; Logan and De Vos,

2011). Pleomorphism – a variation of cell shape and/or size in response to environmental conditions - is a well-known trait of *B. mycoides,* documented in the 1930s (Lewis, 1932) and was also detected in our study in NCr1a strain. Sporulation efficiency of NCr1a is lower compared to the *B. pseudomycoides* reference strain DSM 12242. We could not find an explanation of this in the literature data.

To conclude, chromate tolerance of *B. cereus* group environmental strains was similar for strains obtained from contaminated and unpolluted sites, regardless of previous exposure to chromium. Molecular and phenotypical identification revealed that all strains belonged to the *B. cereus* group. Reference strains of this group also showed similarly high level of chromate tolerance, thus indicating this group possibly possesses improved intrinsic chromate tolerance mechanisms. Long chain chromate transporter gene *chrA* detected in the most tolerant strain NCr1a could be involved in its high resistance. Reported data on the chromate tolerance of strains from unpolluted habitats is of particular importance as such strains were so far scarcely studied. Isolation of strains with high chromate tolerance even from samples with no previous Cr exposure could enable us to get insights into intrinsic metal homeostasis mechanisms. Certain strains exhibited strong chromate reduction activity coupled with robust growth in Cr(VI) containing medium and tolerance to other heavy metals, making them a potential candidates for engineered Cr removal.

6.4. DIFFERENTIAL RESPONSE OF ENVIRONMENTAL CHROMATE TOLERANT *B. CEREUS* GROUP STRAIN NCr1a AND REFERENCE STRAIN *B. SUBTILIS* PY79

Bacillus cereus group strain NCr1a, tentatively identified as *B. pseudomycoides* or *B. mycoides,* expressed the highest Cr(VI) tolerance level compared to all the tested environmental *B. cereus* group isolates. Contrary, reference laboratory strain *B. subtilis* PY79 (BS) proved to be much more chromate sensitive. In order to compare chromate response of these two Gram-positive sporulating bacteria we conducted a series of STEM-EDS analyses of chromium cellular and endospore content.

Analysis of bacterial response to chromium by electron microscopy coupled with EDS provides valuable data on Cr localization and amount in cells. EDS with both SEM and TEM allows rapid and precise detection of sample's elemental composition qualitatively and quantitatively in the range of 0.1 wt% on flat, uniform samples (Wesenberg et al., 2007). This method of analysis has been employed in a number of studies examining bacterial response to chromate (e.g. Zhu *et al.*, 2008; Chen *et al.*, 2012; Singh *et al.*, 2015).

Singh *et al.* (2015) examined reduction of hexavalent chromium by the thermophilic methanogen *Methanothermobacter thermautotrophicus.* By TEM (transmission electron microscopy) EDS they detected extracellular particles of Cr, mostly in the form of chromium hydroxide precipitates as determined by X-ray absorption near-edge structure (XANES). Cells grown in Cr(VI) medium had rough surface. As mentioned, reduced form of Cr was found mainly outside, but also inside the cells, indicating both extracellular and intracellular Cr(VI) reduction. TEM-EDS was used for detection of intracellular accumulation of reduced chromium in *Pseudochrobactrum asaccharolyticum* LY6 (Long, 2013). A study using SEM-EDS has found *Achromobacter* sp. Ch1 cultivation medium contained extracellular Cr precipitates, tentatively identified as insoluble chromium hydroxide (Zhu et al., 2008). Cells of *Cellulosimicrobium* sp. SCRB10 grown on LB agar amended with 100 mg/L (1.92 mM) of Cr(VI) contained 0.71 weight % (wt%) or 0.19 atomic % (at%) of Cr (Bharagava and Mishra, 2018). In comparative study of *E. coli* and *B. subtilis* response to Cr it was found by EDS that intracellular content of Cr did not vary between these species and was around 0.36-0.37 wt% or 0.12 at% after growth in 100 mg/l Cr(VI) medium (Samuel et al., 2013).

As mentioned, strain NCr1a, belongs either to *B. pseudomycoides* or *B. mycoides,* based on its typical rhizoidal morphology and 16S rRNA and *pycA* gene sequence analysis. Chromate tolerance of *B. mycoides* and *B. pseudomycoides* strains has been characterized in only a few papers (Singh et al. 2006; Wang et al. 2016). First study found Cr(VI)

uptake by a *B. mycoides* wastewater isolate was increased under higher Cr(VI) concentrations and in sulfate limiting conditions (Singh et al. 2006). Second reported *B. mycoides* strain 200AsB1, isolated from the rhizosphere of the arsenic-hyperaccumulator *Pteris vittata,* tolerated 2.4 mM Cr(VI) in LB medium. Strain could remove Cr(VI) from aqueous solution by both bioadsorption and bioaccumulation, while reduction to Cr(III) was moderate (Wang et al. 2016). Studies on *B. pseudomycoides* chromate response have not been published to date, to the best of our knowledge, but its response could probably be comparable to the *B. mycoides* response as these two species are highly similar. None of the studies of *B. mycoides* and *B. pseudomycoides* chromate response conducted EDS analyses. Therefore, in current study we give first report on STEM EDS analysis of chromium content in cells and endospores of *B. mycoides* and *B. pseudomycoides* species.

Chromium content in cells of the two strains was tested under different conditions. Growth of *B. subtilis* PY79 was strongly inhibited by 1000 μM Cr(VI) in minimal M9 medium, while NCr1a grew almost as in the absence of Cr(VI) in the same conditions. Both strains could cope with low 10 μM concentration with similar success. Concentration of 100 μM caused significantly higher toxic effect on *B. subtilis* PY79 compared to NCr1a.

Cells of NCr1a have shown lower and stable Cr content upon growth in high Cr(VI) medium. Contrary, *B. subtilis* PY79 cells show much higher Cr content with pronounced variation between the cells, with some cells taking up large amounts of Cr, while others take up small amounts. Such variability is often reported in studies of bacteria under stress conditions. Cells which took up smaller amount of Cr appeared less dense on micrographs, possibly indicating that they are in fact dead and that their cellular content has been released outside. Consequently, Cr could have got out of the dead lysed cells which resulted in a measurement of a low cellular Cr.

Also, cells of *B. subtilis* PY79 are often lysed in the presence of Cr(VI) while no morphological disturbance in NCr1a cells was noted.

Previous exposure to subinhibitory concentrations of pollutants (induction) can impact bacterium's subsequent tolerance (Ackerley et al., 2006; Stoppel and Schlegel, 1995). However, in the case of strain NCr1a, belonging to the *B. cereus* group, induction had no effect on its chromate resistance and Cr cellular content. Similar was found by He et al. (2010) for isolate *B. cereus* SJ1 whose tolerance to 5 mM Cr(VI) was not improved by induction. On the other hand, on higher Cr(VI) concentration tested (30 mM) induced cells were more tolerant than uninduced (He et al., 2010). Chromate tolerance and reduction of *Lysinibacillus fusiformis* ZC1 (He et al., 2011) and *B. firmus* (Sau et al., 2010) were not affected by induction, indicating their constitutive nature. Stated studies used LB for chromate tolerance testing, hence direct comparison of tolerance and reduction to our strains is not possible.

Contrary to NCr1a, tolerance of *B. subtilis* PY79 was improved by induction, as significantly less Cr was accumulated inside the cell. It appears that *B. subtilis* PY79 cells

are capable of maintaining low cellular Cr content, if they are induced by low Cr(VI) concentration prior to full exposure to high Cr(VI) level. Induction resulted in *B. subtilis* PY79 cellular content which was not significantly different than content in cells of NCr1a. Low Cr content could be the consequence of: *a)* decreased Cr intake and/or *b)* efflux of Cr out of the cell. Subinhibitory Cr(VI) concentration could induce expression of chromate tolerance mechanisms such as chromate transporter proteins coded by the *ywrB* and *ywrA* genes present in *B. subtilis* PY79 (Aguilar-Barajas et al., 2013; Díaz-Magaña et al., 2009). Subsequently, cells will be “prepared” for exposure to chromate toxicity and could consequently cope better with high Cr(VI) levels. Contrary to our findings for *B. subtilis* PY79, other studies found no influence of induction on chromate tolerance of *B. subtilis* laboratory strain 168 (Aguilar-Barajas et al., 2013; Díaz-Magaña et al., 2009). Disagreement in induction effect could be consequence of differences in experimental set up or of some actual difference among the strains.

Chromium content in endospores of the two strains was also tested under different treatments. *B. subtilis* PY79 could not efficiently sporulate in DMS medium containing 1 mM Cr(VI). Thus, its endospores formed in the presence of chromate could not be tested by STEM EDS. Contrary, NCr1a successfully formed spores. NCr1a endospores formed in medium with high Cr(VI) did not appear morphologically different than those formed in medium without Cr(VI), indicating no obstruction of sporulation process occurred. After 6 days of exposure, mature spores of NCr1a take up 3.7 times more Cr than *B. subtilis* PY79 spores, probably because they possess exosporium which binds more Cr, contrary to *B. subtilis* PY79 spores which do not have exosporium. However, after short 1h exposure both strains take up similar amounts of Cr. From EDS elemental mapping it can be putatively concluded Cr is mostly located in the outer layers of NCr1a endospore (coat and exosporium). NCr1a spores formed in the presence of Cr(VI) took up the largest amount of chromium compared to all the spore treatments. They contained 4.5 times more Cr(VI) after than if mature spores were exposed for the same period of time (6 days). This indicates Cr is incorporated into the spore core during the formation as well, although outer layers have primary role in Cr binding.

Although spores are regarded as metabolically dormant, they still can be involved in certain biochemical reactions, due to the presence of functional groups on the outer layer components. Exosporium in *B. cereus* group is mostly composed of proteins, polysaccharides, and lipids (Matz et al., 1970), with many functional groups which could facilitate Cr binding (carboxyl, phosphoric acid sites, amino groups, etc.) (Kantar et al., 2011). Furthermore, exosporium proteins could exhibit enzymatic activity. Several exosporium proteins in *B. cereus* have homologues in other organisms which pertain enzymatic activities, indicating functional activity of spores (Charlton et al., 1999).

Role of endospores in metal redox reactions has been scarcely documented (Chinni et al., 2008; de Vrind et al., 1986; Junier et al., 2009). Spore coats and exosporium of marine *Bacillus* spp. were responsible for oxidation and immobilization of manganese (de Vrind et al., 1986; Francist and Tebo, 2002). Oxidation of Mn to MnO_2 followed by

adsorption and immobilization was proceeding until all active sites were encrusted and masked, after which no further oxidation occurred. Similarly, uranium was associated with the coat of a *Desulfomaculum reducens* spores. These findings go to confirm predominant localization of Cr in outer layers of endospore (coat and exosporium) of NCr1a. However, to precisely map Cr localization, imaging of microtome sections with uniform thickness would be warranted.

To summarize, based on our results of STEM EDS analysis of chromium cellular and endospore content, NCr1a is far more resistant to chromate compared to *B. subtilis*. First, NCr1a grows to a degree similar to no Cr(VI) control in 1 mM Cr(VI) media, while growth of *B. subtilis* PY79 is strongly inhibited. Secondly, cells of NCr1a have low and stable Cr content, while *B. subtilis* PY79 cells show much higher content with more variation between the cells. Besides, no morphological disturbance in NCr1a cells was noted, while cells of *B. subtilis* PY79 are often lysed in the presence of Cr(VI). Finally, sporulation of NCr1a is unhindered, while *B. subtilis* PY79 fails to form endospores in the presence of high Cr(VI)concentration. Taken all in account, NCr1a strain can be considered as highly resistant to chromate, while *B. subtilis* PY79 is much more chromate sensitive.

7. CONCLUSIONS

Elucidation of impact of chromium, an important metallic pollutant, on a microbial community and on specific bacterial isolates, was the main aim of this doctoral dissertation. Main conclusions of the research, divided according to the four main research sections, are given below:

Influence of chromium on the cultivable soil bacterial community

- High soil total Cr concentration did not affect soil cultivable bacteria counts.
- Percentage of chromate tolerant population within total cultivable bacterial population was not affected by the soil Cr level.
- Percentage of Gram-positive and Gram-negative bacteria within total cultivable bacterial population was not affected by the soil Cr level.
- Addition of even a low 0.25 mM concentration of chromate in the growth medium inhibited growth of half of the cultivable soil bacteria.
- Around 10 % of cultivable soil bacteria are resistant to 2 mM Cr(VI) in cultivation media.

Isolation of chromate tolerant bacterial strains

- Both samples with high Cr and low Cr concentration contained chromate tolerant strains. However strains' diversity was seemingly higher in samples polluted with Cr compared to samples with naturally high Cr or with low Cr.
- A total of 33 chromate tolerant bacterial strains with the ability to grow on 2 mM Cr(VI) in minimal media were isolated.
- Isolated strains belong to the 12 different species and 8 genera; 28 were Gram-positive, while 5 were Gram-negative bacteria.
- Strains belonging to the *Bacillus cereus* group were predominant among the isolated chromate tolerant strains (20 out of 33 isolates).
- *Bacillus cereus* group strains NCr1a, NCr2, PCr2a and PCr3a and *Cellulosimicrobium cellulans* PCr3b are the best candidates for prospective bioremediation application, due to their high growth and high chromate reduction in conditions of 0.5 mM starting Cr(VI) concentration.
- Strains *Serratia fonticola* NCr1d, *Staphylococcus haemolyticus* LCr9b and PCr8, *Ochrobactrum tritici* PCr6 and *Microbacterium maritypicum* PCr11, characterized by low reduction and high growth, are of interest in further studies of alternative chromate resistance mechanisms such as chromate efflux, blocking of chromate intake and oxidative stress response.
- Reported data on the chromate tolerance of strains from unpolluted habitats (*S. warneri* LCr1, *S. haemolyticus* LCr9b and *B. cereus* group strains LCr2 to LCr8 and LCr9a) is of particular importance as this type of strains was so far scarcely studied.

Characterization of selected chromate tolerant *Bacillus cereus* group strains

- *Bacillus cereus* group isolates originating both from high and low Cr environments exhibited similar level of chromate tolerance.
- Reference *B. cereus* group strains were Cr(VI) resistant to a similar degree, indicating innate nature of this group's chromate tolerance.
- *B. cereus* group isolates exhibited tolerance to other heavy metals, while reference strain *B. subtilis* PY79 was comparably very sensitive.
- Strain NCr1a had the highest chromate tolerance of all the *B. cereus* group isolates.

STEM EDS analysis of chromium content and localization in cells and endospores of strains NCr1a and *B. subtilis* PY79

- Based on the results of growth measurements and STEM EDS analysis of cell and spore Cr content, NCr1a strain can be considered as highly resistant to chromate, while *B. subtilis* PY79 is much more chromate sensitive. Namely:
    - NCr1a grows to a degree similar to no Cr(VI) control in 1mM Cr(VI) medium, while growth of *B. subtilis* PY79 is strongly inhibited;
    - Cells of NCr1a have low and stable Cr content, while *B. subtilis* PY79 cells show much higher content with more variation between the cells;
    - No morphological disturbance in NCr1a cells was noted, while cells of *B. subtilis* PY79 are often lysed in the presence of Cr(VI);
    - Sporulation of NCr1a is unhindered, while *B. subtilis* PY79 fails to form endospores in the presence of high Cr(VI)concentration.

Taking all in account, results obtained in this doctoral dissertation are of significance both from practical bioremediation perspective as well as from fundamental aspect of bacterial metal homeostasis research.

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9. APPENDIX

9.1. ENVIRONMENTAL SAMPLES DESCRIPTION

Table A - 1 Description of environmental samples with high and low chromium level from which isolation of chromate tolerant strains was performed

Sample group	Sample type	Sample	Location	Site description	Total Cr (mg/kg)[a]
High Cr[b]	NCr[c] soil	NCr1	Zlatibor	agricultural soil	470
		NCr2	Zlatibor	nearby oak forest	1287
		NCr3	Zlatibor	meadow by the road	395
		NCr4	Zlatibor	grazing meadow	835
		NCr5	Maljen	clearing in coniferous forest	663
	PCr[d] soil	PCr1	Subotica	tannery; wastewater lagoon 2 of former leather tannery; soft soil covered with grass, presence of annelid and snails; layer 10-20 cm depth	419
		PCr2	Subotica	tannery; road in front of the tannery	84
		PCr3	Subotica	tannery; wastewater lagoon 2 of former leather tannery; soft soil covered with grass, presence of annelid and snails; layer 0-10 cm depth	496
		PCr4	Subotica	tannery; wastewater lagoon 2 of former leather tannery; soft soil covered with grass, presence of annelid and snails, layer 20-30 cm depth	797
		PCr5	Subotica	tannery; wastewater lagoon 1 of former leather tannery; soft soil covered with grass, layer 0-10 cm depth, black color	112.1
		PCr6	Subotica	tannery; wastewater lagoon 1 of former leather tannery; soft soil covered with grass, layer 10-20 cm depth, clay-like	92.4[e]
		PCr7	Subotica	chemical factory Zorka; near the former chromic acid production facility, by the railway, layer 0-10 cm depth	119
		PCr8	Subotica	chemical factory Zorka; near the former chromic acid production facility, by the railway, layer 10-20 cm depth	534
		PCr9	Subotica	chemical factory Zorka; near pyrite piles, layer 10-20 cm depth, purple color	117
		PCr10	Ruma	tannery; soil irrigated by leather tannery contaminated water	175
	PCr sediment	PCr11	Ruma	sediment from irrigation canal receiving tannery wastewaters	334
	PCr solid waste	PCr12	Zrenjanin	tannery; dry solid waste from container for industrial waste materials of tannery	85[e]

Table A – 1 continued

Sample group	Sample type	Sample	Location	Site description	Total Cr (mg/kg)[a]
Low Cr	soil	LCr1	Subotica	mesophile meadow by the water plant	39.3
	soil	LCr2	Fruška gora	lime and oak forest	25
	soil	LCr3	Subotica	near chemical factory Zorka	13.5
	Salt march, soil	LCr4	Slano Kopovo	flooding soil with salty crust; vegetation *Carex,* layer 0-5cm depth	9.25
	Salt march, soil	LCr5	Slano Kopovo	soil not affected by flooding with salty crust; vegetation *Carex,* layer 0-5cm depth	12.13
	Salt march, soil	LCr6	Medura	surface soil sample	7.7
	Salt march, water	LCr7	Slano Kopovo	water from main water body	1.46
	Salt march, water	LCr8	Medura	water from main water body	1.33
	Salt march, water	LCr9	Slano Kopovo	water from orange puddles, remaining from receding water on the surface of soil	1.52

[a] unit for water samples is µg/l, [b] samples with >100 mg/kg Cr, [c] NCr – samples with natural geogenic Cr, [d] PCr – samples polluted with Cr; [e] sample was collected from Cr polluted site and has elevated Cr compared to background Cr level in the same region, thus it is considered as high Cr soil

9.2. 16S rRNA GENE SEQUENCES OF CHROMATE TOLERANT *BACILLUS* ISOLATES

>NCr1a (MH587689)
TAACTGCAGTCGAGCGGATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGT
AACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGCACCGCATGGTGCG
AAATTCAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTC
ACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTA
CGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCT
TTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAAC
CAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGG
CGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAAC
TGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAAT

>NCr1b (MH587690)
TGCAGTCGAGCGATGGATTGAGAGCTTGCTCTCAAGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCT
GCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACTGCATGGTTCGAAATT
GAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAA
GGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA
GGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGG
GTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAA
AGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAA
AGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGA
GACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGTAAA

>NCr1c (MH587691)
TATACATGCAGTCGAGCGATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGG
GTAACCTACCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATATTTTGAACTGCATAGTT
CGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGC
TCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAG
GCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCT
AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATT
GGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGA
AACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGGCGTAGAAACA

>NCr2 (MH587692)
ACATGCAGTCGAGCGGATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGT
AACCTACCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATATTTTGAACTGCATAGTTCG
AAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTC
ACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTA
CGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCT
TTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAAC
CAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGG
CGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAAC
TGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTAAA

>NCr3 (MH587693)
CTATAACATGCAGTCGAGCGGATGGATTGAGAGCTTGCTCTCAAGAAGTTAGCGGCGGACGGGTGAGTAACACGT
GGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACTGCATGG
TTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACG
GCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACT

CCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAA
GGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACC
TAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTAT
TGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGG
AAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTAAA

>NCr4 (MH587694)

TGCAGTCGAGCGGATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACC
TGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAAT
TGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCA
AGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCG
GGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGA
AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTA
AAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGG
AGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGT

>PCr1 (MH587695)

TATACATGCAGTCGAGCGGATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTG
GGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATATTTTGAACTGCATGGT
TCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGG
CTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAG
GCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCT
AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATT
GGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGA
AACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGGAAATTTGGCT

>PCr2a (MH587696)

TAATACATGCAGTCGAGCGATGGATTGAGAGCTTGCTCTCAAGAAGTTAGCGGCGGACGGGTGAGTAACACGTG
GGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATATTTTGAACTGCATGGT
TCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGG
CTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAG
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AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATT
GGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGA
AACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGTAAAAT

>PCr12 (MH587697)

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TAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATATTTTGAACTGCATGGTTC
GAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCT
CACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGC
TTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAA
CCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGG
GCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAA
CTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGTAAAA

>LCr6 (MH587698)

TAACATGCAGTCGAGCGGATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGG
GTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTT
CGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGC

TCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAG
GCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCT
AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATT
GGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGA
AACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGGAAAATGGGCTT

>LCr7 (MH587699)

CTAAACATGCAGTCGAGCGGATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTG
GGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGT
TCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGG
CTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAG
GCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCT
AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATT
GGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGA
AACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTAAA

>LCr8 (MH587700)

TAACATGCAGTCGAGCGATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGG
TAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTC
GAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCT
CACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGC
TTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAA
CCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGG
GCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAA
CTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGGAAAATGCGCGT

>LCr9a (MH587701)

TGCAGTCGAGCGATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCT
GCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACTGCATGGTTCGAAATT
GAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAA
GGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA
GGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGG
GTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAA
AGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAA
AGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGA
GACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGTAAA

9.3. *pycA* GENE SEQUENCES OF CHROMATE TOLERANT *BACILLUS* SP. ISOLATES

>NCr1a (MH646621)
CAAAAAGTAGTTGAAATTGCGCCAAGCGTGTCACTTTCAGATGATCTTCGTCATCGTATTTGTGAAGCAGCGGTA
CAGTTAACGAAAAATGTAAATTATTTAAATGCTGGTACGGTTGAGTTCCTTGTAAAAGGCGATGACTTTTATTT
CATTGAAGTAAATCCACGTGTTCAAGTAGAACATACAATTACAGAGATGATTACAGGCATTGATATTGTTCAGT
CACAAATTTTAATTGCAGATGGACACGCTTTACATAGCCCAATCGTAAGTATACCAAA

>NCr1b (MH646622)
CAAAAAGTTGTAGAAATTGCACCAAGTGTGTCGCTTTCAGATGATTTACGTCATCGTATTTGTGATGCTGCTGTG
AAATTAACGAAAAATGTAAACTATTTAAATGCAGGAACAGTAGAATTTCTTGTAAAAGGTGATGAGTTTTACTT
TATTGAAGTAAATCCGCGTGTTCAAGTTGAACATACGATTACAGAGATGATTACAGGAGTCGATATCGTTCAAT
CGCAAATTTTAATAGCTGATGGACATGCATTACATAGTAAAATAGTAGGTGTTCCAAA

>NCr1c (MH646623)
CAAAAAGTAGTTGAAATTGCACCGAGCGTGTCGCTTTCAGATGATTTGCGTCAGCGTATTTGTGATGCTGCAGTA
AAGTTAACGAAAAATGTAAATTATTTAAATGCTGGAACGGTAGAATTCCTTGTAAAAGGTGATGAGTTTTACTT
CATTGAAGTAAACCCACGTGTTCAAGTTGAACATACGATTACAGAGATGATTACAGGAGTGGATATCGTTCAAT
CGCAAATTTTAATAGCTGATGGACATGCATTACATAGCAAAATGGTAGGTGTTCCAAA

>NCr2 (MH646624)
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AAGTTAACGAAAAATGTAAATTATTTAAATGCTGGAACGGTAGAATTCCTTGTAAAAGGTGATGAGTTTTACTT
CATTGAAGTAAACCCACGTGTTCAAGTTGAACATACGATTACAGAGATGATTACAGGAGTGGATATCGTTCAAT
CGCAAATTTTAATAGCTGATGGACATGCATTACATAGCAAAATGGTAGGTGTTCCAAA

>NCr3 (MH646625)
CAAAAAGTGGTAGAAATCGCACCTAGTGTGTCGCTTTCAGATGATTTGCGTCAGCGTATTTGTGATGCTGCTGTG
AAATTAACGAAAAATGTAAATTATTTAAACGCAGGAACAGTAGAATTTCTTGTAAAAGGTGATGAGTTTTACTT
TATTGAAGTAAATCCGCGTGTTCAAGTTGAACATACAATTACAGAAATGATCACAGGGGTAGATATCGTTCAAT
CGCAAATTTTAATAGCTGATGGACATGCATTACATAGTAAAATGGTAGGTGTTCCAAA

>NCr4 (MH646626)
GCACCGAGTGTATCACTTTCAGATGATCTGCGTCAGCGTATTTGTGATGCTGCTGTGAAGTTAACGAAAAATGTA
AGTTATTTAAATGCAGGAACAGTAGAATTCCTTGTAAAAGATGATGAGTTTTACTTTATTGAAGTAAATCCACG
TGTTCAAGTAGAACATACGATTACAGAAATGATTACGGGAGTAGATATTGTTCAATCACAAATTTTAATAGCTG
ATGGACATGCATTACATAGCAAAATGGTAGGTGTTCCAAA

>PCr1 (MH646627)
CAAAAAGTTGTAGAAATTGCACCGAGTGTATCACTTTCAGATGATCTGCGTCAGCGTATTTGTGATGCTGCTGTG
AAGTTAACGAAAAATGTAAACTATTTAAATGCAGGAACAGTAGAATTCCTTGTAAAAGATGATGAGTTTTACTT
TATTGAAGTAAATCCACGTGTTCAAGTAGAACATACGATTACAGAAATGATTACGGGAGTAGATATTGTTCAAT
CACAAATTTTAATAGCGGATGGACATTCATTACATAGCAAAATGGTAGGTGTTCCAAA

>PCr2a (MH646628)
CAAAAAGTTGTCGAAATCGCACCAAGCGTTTCGCTTTCAGATGATTTGCGTCGACGTATTTGTGAAGCTGCTGTG
AAGTTAACGAAAAATGTAAACTATTTAAACGCAGGAACGGTAGAATTTCTTGTAAAAGGTGATGAGTTTTACTT
CATAGAAGTAAACCCACGTGTTCAAGTTGAACATACGATTACAGAGATGATTACAGGAGTGGATATCGTTCAAT
CGCAAATTTTAATAGCTGATGGACATGCATTACATAGTAAAATGGTAGGTGTTCCAAA

>PCr12 (MH646629)
CAAAAAGTTGTAGAAATTGCACCGAGTGTATCACTTTCAGATGATCTGCGTCAGCGTATTTGTGATGCTGCTGTG
AAGTTAACGAAAAATGTAAACTATTTAAATGCAGGAACAGTAGAATTCCTTGTAAAAGATGATGAGTTTTACTT
TATTGAAGTAAATCCACGTGTTCAAGTAGAACATACGATTACAGAAATGATTACGGGAGTAGATATTGTTCAAT
CACAAATTTTAATAGCGGATGGACATTCATTACATAGCAAAATGGTAGGTGTTCCAAA

>LCr6 (MH646630)

CAAAAAGTTGTAGAAATTGCACCGAGTGTATCACTTTCAGATGATCTGCGTCAGCGTATTTGTGATGCTGCTGTG
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TATTGAAGTAAATCCACGTGTTCAAGTAGAACATACGATTACAGAAATGATTACGGGAGTAGATATTGTTCAAT
CACAAATTTTAATAGCTGATGGACATGCATTACATAGCAAAATGGTAGGTGTTCCAAA

>LCr7 (MH646631)

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TATTGAAGTAAATCCACGTGTTCAAGTAGAACATACGATTACAGAAATGATTACGGGAGTAGATATTGTTCAAT
CACAAATTTTAATAGCTGATGGACATGCATTACATAGCAAAATGGTAGGTGTTCCAAA

>LCr8 (MH646632)

CAAAAAGTTGTAGAAATTGCACCGAGTGTATCACTTTCAGATGATCTGCGTCAGCGTATTTGTGATGCTGCTGTG
AAGTTAACGAAAAATGTAAGTTATTTAAATGCAGGAACAGTAGAATTCCTTGTAAAAGATGATGAGTTTTACTT
TATTGAAGTAAATCCACGTGTTCAAGTAGAACATACGATTACAGAAATGATTACGGGAGTAGATATTGTTCAAT
CACAAATTTTAATAGCTGATGGACATGCATTACAT

>LCr9 (MH646633)

CAAAAAGTTGTAGAAATTGCACCGAGTGTATCACTTTCAGATGATCTGCGTCAGCGTATTTGTGATGCTGCTGTG
AAGTTAACGAAAAATGTAAACTATTTAAATGCAGGAACAGTAGAATTCCTTGTAAAAGATGATGAGTTTTACTT
TATTGAAGTAAATCCACGTGTTCAAGTAGAACATACGATTACAGAAATGATTACGGGAGTAGATATTGTTCAAT
CACAAATTTTAATAGCTGATGGACATTCATTACATAGCAAAATGGTAGGTGTTCCAAA

9.4. SEQUENCE OF CHROMATE TRANSPORTER GENE OF NCr1a STRAIN

***>chrA* gene of strain NCr1a**

TATCACTCAAGTTAGGCCTTACTTCTTTCGGAGGACCCGTGGCTCACCTTGGGTACTTTCATCATGAATATGTAC
AAAAACGAAAATGGATGGATGAGCGGAGCTATGGGGATTTAGTGGCACTCTGTCAATTCCTACCCGGTCCTGCAA
GCAGCCAAGTTGGAATTGGCACCGGTTTATCACGAGGCGGGCTCCTTGGGGCTGTAGTCGCTTGGATTGGCTTTA
CACTCCCGTCAGTTCTCTTTCTTGTTTTTTCCGCTTCACTACTCCAGCAATTTCATGTGGTGGAAGCTGGCTGGAT
TCATGGATTAAAACTTGTAGCAGTAGCGATTGTCGCACATGCAATATGGGGAATGGCACAGAAATTAACACCGG
ACCGTAACCGAGCAACAATTGCAATTATTACTGCTGCAATTGCGTTATTATTTCCAAGCGCTTGGACACAAGTTA
CGCTTATTCTACTTGCTGGTCTTCTCGGTTGGCTCTTATATCGTAAGCATGAAATTCCATCTGCTAGCAAAACAC
ATATTCCTGTTTCACGTATAACAGCTGCCCTTTGTCTCTTACTATTCTTTAGTTTATTATTCTTGTTACCACTATT
ACGGCCATTTTCGCACTGGATTGCCATGTTTGATAGTTTCTATCGCTCTGGCTCACTCGTGTTTGGCGGAGGACA
TGTCGTTCTTCCCCTTCTTGAAAATGAGTTTGTATTAAATGGCATGATGACAAAAGAACAGTTTCTAGCAGGATA
CGGCTTAACCCAAGCGGTTCCAGGTCCTCTTTTTACATTTGCATCTTACATAGGAACGGTACTAGACGGGGCACT
GGGAGCTACTATTGCGACAATCGCTATCTTTCTCCCGGCATTCTTACTTGTCATTGGTGTATTGCCATTTTGGGA
TCAAGTGAGACGCTTGTCTTATGTACAAGGGGCGCTCCTTGGCATTAATGCCGCCGTCGTCGGTATTTTACTAGC
TGCTTTTTATGACCCTATTTGGACAAGCACAATCATGAGTAAAACTGATTTTGTTATTGCTCCCCCCTAATTTGT
CTATTAGCATTTTGGAAAGCACCACCATGGTATCGTTGTTGTTCTTGGTGCTTTAAGCGGTTAAATTCTTTCTAT
TACCTAGGGATCCACGGGCCCCCCTCAAGGTCAAGGGATCAAAAAACTTGAATACAAATTCGAATTCTAAAGCGG
CCCGCAATTTCAAGCCTCGACGCCGGTCCATCCATTACCAGTTGGTCGGGGGCAAAAAAAAAAAACCGGGAAGGC
CTGGTTGCCCACGTTTCCCTAAAGAATCCTTTTGGACTATATATTCCCGGGAAACCCTCGCCAAAACAGATTTAT
ATTGTGGGTTACTATGTCCTTA

10. EXTENDED ABSTRACT IN SERBIAN LANGUAGE (PROŠIRENI IZVOD NA SRPSKOM JEZIKU)

Uvod.

Hrom (Cr) je teški metal koji ima široku primenu u industriji i kao rezultat toga čest je uzrok zagađenosti zemljišta, sedimenta, površinskih i podzemnih voda. U životnoj sredini hrom se najčešće nalazi u dva stabilna oksidaciona stanja: šestovalentnom [Cr(VI)] i trovalentnom [Cr(III)]. Hrom prirodnog porekla (npr. serpentinska zemljišta potekla od ultramafičnih matičnih stena) se skoro isključivo nalazi u trovalentnoj formi, dok se antropogenim zagađenjem uglavnom ispuštaju jedinjenja sa šestovalentnim hromom. Trovalentni hrom se smatra slabo biodostupnim u životnoj sredini i pokazuje uglavnom slab efekat na žive organizme, jer formira slabo rastvorljive hidrokside i komplekse sa organskim ligandima na vrednostima pH uobičajenim u životnoj sredini (Bencheikh-Latmani *i sar.*, 2007). U poređenju sa trovalentnim, šestovalentni hrom pokazuje značajne toksične, mutagene i teratogene efekte, zbog čega je klasifikovan kao humani kancerogen 1. grupe (International Agency for Research on Cancer, 2012). Šestovalentni hrom je dobro rastvorljiv u vodi, izraženo biodostupan i lako se transportuje kroz ćelijsku membranu u citoplazmu ćelija, gde ispoljava snažne toksične efekte, uglavnom preko oksidativnog stresa (Ramírez-Díaz *i sar.*, 2008).

Pojedini mikroorganizmi imaju sposobnost ne samo da rastu u prisustvu visokih koncentracija hroma, već i da redukuju visoko toksičnu šestovalentnu formu u manje toksičnu trovalentnu formu. Takvi sojevi mogu biti upotrebljeni u bioremedijaciji zagađenih sredina na ekološki prihvatljiv i ekonomski isplativ način. Niža cena bioremedijacije u poređenju sa drugim metodama remedijacije je posebno od značaja u manje razvijenim zemljama, u kojima je problem zagađenja hromom i najveći usled korišćenja zastarelih ili neadekvatnih metoda industrijske proizvodnje, kao i lošeg upravljanja otpadom. Za uspešnu upotrebu mikroorganizama u bioremedijaciji, detaljno poznavanje njihove biologije, fiziologije i mehanizama tolerancije teških metala je od presudnog značaja.

Efekat hroma na mikrobnu zajednicu zemljišta i akvatičnih sredina je manje izučen u poređenju sa efektom hroma na čiste kulture mikroorganizama (npr. Cheung i Gu, 2007; Ramírez-Díaz *i sar.*, 2008; Dhal *i sar.*, 2013; Ahemad, 2014). Postojeća znanja o odgovoru mikrobne zajednice na prisustvo hroma su većinom zasnovana na manje ili više kratkoročnim eksperimentima sprovedenim u mikrokosmos uslovima (Viti *i sar.*, 2006). Iako rezultati mikrokosmos eksperimenata daju kontrolisane i ponovljive rezultate, oni ne mogu u potpunosti da replikuju uslove dugotrajnog izlaganja hromu, koji su normalni u realnim terenskim uslovima. Dakle, postoji jasna potreba za daljim istraživanjima odgovora mikrobne zajednice na prisustvo hroma u životnoj sredini.

Dugotrajno izlaganje hromu navodno dovodi do selekcije i obogaćivanja mikrobne zajednice članovima sa povišenom hromatnom tolerancijom. Zbog toga se zagađeni lokaliteti smatraju obećavajućim izvorom bakterijskih sojeva sa visokom hromatnom tolerancijom. Stoga je većina dosadašnjih studija bila fokusirana na izolaciju Cr(VI) rezistentnih bakterija iz zagađenih sredina (e.g. Alam *i sar.*, 2011; Camargo *i sar.*, 2005; Patra *i sar.*, 2010). Međutim, izolacija hromat tolerantnih sojeva iz nezagađenih sredina je takođe moguća i sprovedena u vrlo malom broju studija (Wani *i sar.*, 2007). Moguće je, dakle da se među bakterijama hromatna tolerancija javlja i bez selektivnog efekta od

strane prisustva hroma, ali takođe i da povišeni nivoi hroma dovode do značajnog povećanja udela hromat tolerantnih članova u mikrobnoj zajednici.
Ispitivanje mehanizama hromatne rezistencije i njihove genetičke regulacije je prvenstveno sprovedeno kod Gram-negativnih bakterija kao što su *Pseudomonas* spp. (Alvarez, Moreno-Sánchez i Cervantes, 1999; Ackerley *i sar.*, 2004), *Shewanella oneidensis* (Middleton *i sar.*, 2003), *Cupriavidus metalidurans* (Nies i Silver 1989) i *Ochrobactrum tritici* (Branco *i sar.*, 2008), ali ne i kod Gram-pozitivnih bakterija. Uprkos pomenutim iskustvima pojedine studije otkrile su da hromat tolerantni sojevi izolovani iz hromom zagađenih sredina dominantno pripadaju grupi Gram-pozitivnih bakterija (Branco *i sar.*, 2005; Caliz *i sar.*, 2012; Viti i Giovannetti, 2001). Štaviše, Gram-pozitivne bakterije su brojna i značajna grupa u zemljišnoj mikrobnoj zajednici, a osobina nekih od njih da formiraju endospore omogućava im preživljavanje i u najnepovoljnijim uslovima životne sredine. Prema tome, ispitivanje mehanizama hromatne rezistencije kod Gram-pozitivnih bakterija uključujući i sporulišuće vrste bi moglo da obezbedi nove vredne uvide u bakterijsku homeostazu hroma.

Ciljevi istraživanja.

Hrom je ozbiljan zagađivač životne sredine koji se koristi u brojnim industrijskim aktivnostima. Određeni mikroorganizmi imaju sposobnost da redukuju visoko toksični šestovalentni u manje toksični trovalentni hrom. Shodno tome, oni se mogu koristiti u ekološki održivoj i ekonomičnoj bioremedijaciji zemljišta, sedimenata i otpadnih voda zagađenih hromom. Zbog toga je izolacija i karakterizacija mikroorganizama sa visokom tolerancijom na hromate i visokom sposobnošću njihove redukcije od velikog ekološkog i naučnog značaja.
Imajući sve ovo u vidu, ***opšti cilj*** ove disertacije bio je ispitivanje uticaja hroma na zemljišne bakterije. Opšti cilj je ostvaren kroz sledeće ***specifične ciljeve***: *(i)* utvrđivanje uticaja hroma na kultivabilnu bakterijsku zajednicu zemljišta (uticaj hroma na brojnost kultivabilne bakterijske zajednice, udeo hromat tolerantnih bakterija, proporciju Gram-pozitivnih i Gram-negativnih bakterija); *(ii)* izolacija i karakterizacija Cr(VI) tolerantnih bakterija iz zemljišta odnosno sredina sa visokim i niskim sadržajem hroma; *(iii)* utvrđivanje nivoa tolerancije Cr(VI) tolerantnih bakterijskih izolata; *(iv)* utvrđivanje nivoa redukcije šestovalentnog u trovalentni oblik hroma od strane izolovanih bakterija; *(v)* utvrđivanje razlika u odgovoru na Cr(VI) između referentnih i izolovanih hromat tolerantnih *Bacillus* sp. sojeva.

Materijal i metode.

Uzorkovanje je izvršeno odgovarajućim propisanim aseptičnim tehnikama.

Fizičko-hemijski parametri uzoraka zemljišta su određeni standardnim metodama: koncentracija hroma (USEPA 7000B), pH vrednost (ISO 10390:2005), granulometrijski sastav (ISO 11277:2009), sadržaj organske materije (ISO 12879:2000) i sadržaj suve materije (BS EN 15934:2012).

Mikrobiološke analize kultivabilne bakterijske zajednice zemljišta su obuhvatale: određivanje otpornosti kultivabilne zajednice na hrom metodom selektivnog obogaćenja na acetatnoj minimalnoj podlozi sa rastućim koncentracijama hroma (od

0,25 do 2 mM Cr(VI)); određivanje brojnosti kultivabilnih bakterija i udela hromat tolerantnih kultivabilnih bakterija u ukupnoj populaciji kultivabilnih bakterija odgajivačkom metodom na R2A agaru sa različitim koncentracijama hroma (od 0,25 do 8 mM Cr(VI)); određivanje udela Gram-pozitivnih i Gram-negativnih bakterija u ukupnoj populaciji kultivabilnih bakterija bojenjem preparata tehnikom po Gramu i svetlosnom mikroskopijom svetlog polja.

Izolacija hromat tolerantnih sojeva izvršena je tehnikom selektivnog obogaćenja na acetatnoj minimalnoj podlozi sa rastućim koncentracijama hroma, iščišćavanjem kultura, proverom rasta na podlogama sa hromom. Sojevi su identifikovani MALDI TOF analizom prema uputstvu proizvođača i sačuvani na -70°C. Testiranjem intenziteta rasta na minimalnoj podlozi M9 sa različitim koncentracijama hroma određen je nivo hromatne tolerancije. Na istoj podlozi utvrđena je sposobnost sojeva da redukuju šestovalentni u trovalentni hrom. U ispitivanju tolerancije korišćene su minimalne podloge sa definisanim hemijskim sastavom, jer one daju preciznije rezultate merenja u odnosu na nedefinisane nutrijentima bogate podloge.

Karakterizacija odabranih hromat tolerantih sojeva B. cereus grupe obuhvatala je: određivanje morfologije ćelija i kolonija, određivanje biohemijskih osobina klasičnim biohemijskih testovima, analizu sekvenci 16S rRNK i *pycA* gena, određivanje potencijala formiranja biofilma standarnom metodom u mikrotitar pločama, određivanje tolerancije na hrom i druge teške metale testiranjem intenziteta rasta na podlogama sa različitim koncentracijama metala.

STEM EDS (skenirajuća transmisiona elektronska mikroskopija sa energodisperzionom spektroskopijom) analiza sadržaja i lokalizacije hroma u ćelijama i endosporama sojeva NCr1a i referentnog soja *B. subtilis PY79* izvršena je pri različitim uslovima laboratorijske kultivacije, uključujući različite periode izlaganja i uslove sa ili bez indukcije. Nakon inkubacije vršeno je fiksiranje preparata i mapiranje elementarnog sastava pomoću STEM EDS.

Rezultati i diskusija.

Rezultati istraživanja su podeljeni u 4 dela u okviru kojih je data kratko tumačenje rezulata:

I. Ispitivanje uticaja hroma na kultivabilnu bakterijsku zajednicu zemljišta;
II. Izolacija hromat tolerantnih sojeva;
III. Karakterizacija odabranih hromat tolerantnih sojeva *B. cereus* grupe;
IV. STEM EDS analiza sadržaja i lokalizacije hroma u ćelijama i endosporama sojeva NCr1a i *B. subtilis* PY79.

I. Ispitivanje uticaja hroma na kultivabilnu bakterijsku zajednicu zemljišta

Da bi se utvrdio uticaj hroma na kultivabilne bakterije zemljišta sakupljeno je ukupno 17 uzoraka zemljišta , koji su na osnovu koncentracije i porekla hroma razvrstani u 3 grupe:

– uzorci sa niskom koncentracijom hroma – LCr – oznake uzoraka: 1-4 – opseg koncentracije hroma 25-47 mg/kg;

- uzorci sa povišenom koncentracijom hroma antropogenog porekla – PCr – oznake uzoraka: 5-8 – opseg koncentracije hroma 112-818 mg/kg;
- uzorci sa povišenom koncentracijom hroma prirodnog porekla – NCr – oznake uzoraka: 9-17 – opseg koncentracije hroma 342-1287 mg/kg.

Na uzorcima zemljišta izvršena su sledeća ispitivanja:

- Fizičko-hemijske analize uzoraka zemljišta (koncentracija hroma, pH vrednost, granulometrijski sastav, sadržaj organske materije, sadržaj suve materije);
- Određivanje otpornosti kultivabilne zajednice na hrom metodom selektivnog obogaćenja;
- Određivanje brojnosti kultivabilnih bakterija i određivanje udela hromat tolerantnih kultivabilnih bakterija u ukupnoj populaciji kultivabilnih bakterija;
- Određivanje udela Gram-pozitivnih i Gram- negativnih bakterija u ukupnoj populaciji kultivabilnih bakterija.

Određivanje otpornosti kultivabilne zajednice na hrom metodom selektivnog obogaćenja. Da bi se utvrdila opšta Cr(VI) tolerancija kultivabilne mikrobne zajednice, uzorci zemljišta su podvrgnuti selektivnom obogaćenju (testiranju rasta u medijumu sa rastućim koncentracijama Cr(VI) od 0,5 do 2,5 mM). Najviša hromatna tolerancija na nivou zajednice određena selektivnim obogaćenjem je zabeležena u uzorcima sa antropogeno povišenom koncentracijom hroma.

Brojnost kultivabilnih bakterija i udeo hromat tolerantnih kultivabilnih bakterija u ukupnoj populaciji kultivabilnih bakterija. Brojnost kultivabilnih zemljišnih bakterija je određena odgajivačkom metodom na R2A agaru. Brojnost na R2A agaru sa dodatkom različitih koncentracija Cr(VI) (0,25 do 8 mM) je poređena sa brojnošću na agaru bez hroma i na osnovu toga je računat procenat hromat tolerantnih kultivabilnih bakterija unutar ukupne populacije kultivabilnih bakterija. Tako je određeno koji udeo ukupne populacije kultivabilnih bakterija pokazuje povišen nivo hromatne tolerancije. Takođe, utvrđeno je da li povišena koncentracija hroma u zemljištu dovodi do povećanog prisustva hromat tolerantnih bakterija. Brojnost kultivabilnih bakterija u zemljištima sa niskom pozadinskom koncentracijom hroma je bila u rasponu od 10^6 do 10^7 CFU/g, dok je brojnost u uzorcima sa antropogeno povišenim hromom bila u rasponu od 10^6 do 10^8 CFU/g. Dodavanjem Cr(VI) u R2A agar, zabeleženo je da je čak i relativno niska koncentracija Cr(VI) od 0,25 mM dovela do velikog pada brojnosti bakterija od čak 47,5±21,6% u poređenju sa istim medijumom bez hroma . Kod svih grupa uzoraka, nezavisno od njihovog sadržaja hroma, utvrđeno je da je brojnost bakterija eksponencijalno opadala sa porastom koncentracije Cr(VI) u podlozi. Veličina kolonija je bila uočljivo manja na agaru sa koncentracijom Cr(VI) od ≥1 mM ukazujući na toksični efekat. Sličan procenat kultivabilnih bakterija sposobnih za rast na povišenim koncentracijama Cr(VI) u podlozi je zabeležen u sve tri grupe uzoraka zemljišta. Nije bilo statistički značajne razlike između procenata hromat tolerantnih bakterija u ove tri grupe (one-way ANOVA, $p>0,05$). Zabeležena je umerena korelacija procentualnog udela hromat tolerantnih bakterija i ukupne brojnosti kultivabilnih bakterija ($r=-0,547$, $p<0,05$). Međutim, nije bilo korelacije sa koncentracijom Cr, pH, granulometrijskim

sastavom i sadržajem organske materije u zemljištu. U proseku, 86,6±9,6% kultivabilne bakterijske populacije inhibirano je koncentracijom od 2 mM Cr(VI) u R2A agaru, i dalje povećanje koncentracije na 4 i 8 mM nije dovelo do dalje inhibicije. Moguće je da se na 2 mM i višim koncentracijama beleže slične bakterijske brojnosti, jer se već na 2 mM dostigne kritični nivo toksičnosti, koji eliminiše većinu osetljivih sojeva, te dalje povećanje nivoa Cr(VI) nema značajnijeg efekta. Uzimajući sve u obzir, dobijeni rezultati ukazuju da brojnost kultivabilnih bakterija i proporcija hromat tolerantnih kultivabilnih bakterija nisu pod direktnim uticajem koncentracije hroma u zemljištu niti njegovog porekla.

Udeo Gram-pozitivnih i Gram-negativnih bakterija u ukupnoj populaciji kultivabilnih bakterija. U ranijim studijama je ukazano na to da Gram-pozitivne bakterije pokazuju višu hromatnu toleranciju i da su one dominantno prisutne u zemljištima zagađenim sa Cr(VI). Zbog toga smo testirali udeo Gram-pozitivnih bakterija u ukupnoj populaciji kultivabilnih bakterija u uzorcima zemljišta iz tri različite grupe. Udeo Gram-pozitivnih bakterija je značajno varirao unutar svih grupa. Nije bilo statistički značajne linearne korelacije između udela Gram-pozitivnih bakterija i koncentracije Cr u zemljištu (Pearson-ov koeficijent korelacije r=-0,200, koeficijent determinacije R^2=0,0399). Takođe, nije bilo statistički značajne razlike u udelu Gram-pozitivnih bakterija u različitim grupama zemljišta (one-way ANOVA, p=0,195). Ustanovljena je umerena korelacija sa pH vrednošću zemljišta (r=0,686, p<0.01), ali nije bilo korelacije sa sadržajem suve materije i granulometrijskim sastavom zemljišta. Dakle, u ovom istraživanju koncentracija i poreklo hroma u zemljištu nisu mogli biti povezani sa proporcijom Gram-pozitivnih bakterija.

II. Izolacija hromat tolerantnih bakterijskih sojeva

Uzorci različitih sredina sa niskom i visokom koncentracijom hroma su iskorišćeni za izolaciju hromat tolerantnih sojeva. Sem uzoraka zemljišta koji su korišćeni za ispitivanje uticaja hroma na kultivabilnu bakterijsku zajednicu, dodatno su sakupljeni i drugi tipovi uzoraka kao što su sediment, voda i otpadni materijal. Svrha izolacije iz različitih tipova uzoraka je bila da se izoluju raznovrsni sojevi i da se utvrdi da li se hromat tolerantni sojevi javljaju podjednako u sredinama sa visokom i niskom koncentracijom hroma.

Ukupno 26 sredinskih uzoraka su sakupljeni i iskorišćeni za izolaciju hromat tolerantnih sojeva. Uzorci su na osnovu koncentracije i porekla hroma razvrstani u 3 grupe: (I) 9 uzoraka sa niskom koncentracijom hroma <50 mg/kg i oznakom LCr; (II) 12 uzoraka sa povišenom koncentracijom hroma antropogenog porekla (konc. hroma 84 do 496 mg/kg i oznakom PCr); (III) 12 uzoraka sa povišenom koncentracijom hroma prirodnog porekla (konc. hroma od 395 do 1287 mg/kg i oznakom NCr).

Izolacija Cr(VI) tolerantnih sojeva iz uzoraka je vršena metodom selektivnog obogaćenja na acetatnoj minimalnoj podlozi sa rastućim koncentracijama hroma, nakon čega je vršeno iščišćavanje kulture i čuvanje sojeva na -70°C. Izolovani sojevi su identifikovani pomoću MALDI TOF analize. Nivo hromatne tolerancije je određen merenjem sposobnosti rasta na podlogama sa različitim koncentracijama šestovalentnog hroma.

Na kraju, određen je i bioremedijacioni potencijal sojeva u vidu sposobnosti redukcije toksičnog Cr(VI) u netoksični Cr(III).

Izolovano je ukupno 33 hromat tolerantna bakterijska soja sa sposobnošću rasta na 2 mM Cr(VI) u minimalnoj M9 hranljivoj podlozi. Prema rezultatima MALDI TOF analize, ispitivani sojevi su identifikovani kao predstavnici 12 bakterijskih vrsta iz 8 različitih rodova. Većina izolata su bili Gram-pozitivni (28 od 33), dok je samo 5 bilo Gram-negativno. Rod *Bacillus* je bio dominantno prisutan (21 od 33 izolata). Među njima, jedan soj (obeležen sa PCr7) je klasifikovan kao *B. subtilis,* dok su ostali pripadali *B. cereus* grupi. U uzorcima sa povišenim koncentracijama hroma (NCr i PCr grupe uzoraka) konstatovana je veća raznovrsnost hromat tolerantnih sojeva – 11 različitih vrsta iz 17 uzoraka. Posebno veliki broj različitih vrsta zabeležen je među PCr uzorcima (9 vrsta iz 12 uzoraka), dok su tri različite vrste izolovane iz 5 uzoraka iz NCr grupe. Nasuprot tome, uzorci sa niskom koncentracijom hroma (LCr grupa uzoraka) pokazali su niži nivo diverziteta hromat tolerantnih sojeva, jer su izolovane samo 3 vrste iz 9 uzoraka. Prema tome, grupa uzoraka PCr sa antropogeno povišenim nivoom hroma je pokazala najviši diverzitet hromat tolerantnih sojeva. Iz većine ispitivanih uzoraka (21 od 26) izolovan je samo jedan soj po uzorku. Ovo je verovatno posledica sposobnosti datog soja da preraste ostale prisutne hromat tolerantne sojeve tokom procedure selektivnog obogaćenja, jer je malo verovatno da je stvarno samo jedan soj u bakterijskoj zajednici tolerantan na Cr(VI). Treba naglasiti da je moguće da je metodologija izolacije limitirala diverzitet izolata. Moguće objašnjenje dominacije *Bacillus* sp. među izolatima je morfologija njihovih kolonija na čvrstim podlogama. Naime, kada su tokom procedure izolacije sojevi proveravani na rast na agaru sa 2 mM Cr(VI), moguće je da su sojevi roda *Bacillus* bili lakše primećeni i selektovani za dalje presejavanje zbog veličine njihovih kolonija.

Hromatna tolerancija i hromatna redukcija je testirana kod svih izolata. Hromatna tolerancija je iskazana kao procenat rasta na podlozi sa Cr(VI) u poređenju sa podlogom bez Cr(VI). Koncentracije koje su testirane su bile 0,5 mM, 2 mM i 4 mM Cr(VI). Hromatna redukcija je merena nakon 24 h inkubacije na podlozi sa startnom koncentracijom od 0,5 mM Cr(VI). Za sva testiranja je korišćena minimalna podloga M9, jer nedefinisane nutrijentima bogate podloge mogu dati precenjenu procenu metalne tolerancije i redukcije i neponovljive rezultate.

Najveću sposobnost redukcije (preko 50%) imali su sojevi *Bacillus cereus* grupe NCr2, PCr2a i PCr3a i *Cellulosimicrobium cellulans* soj PCr3b. Niža redukcija je zabeležena kod LCr sojeva (sojeva iz sredina sa niskim sadržajem hroma). Međutim, i neki sojevi iz PCr i NCr grupa su takođe imali nisku redukciju, tako da nije bilo moguće utvrditi direktnu vezu između porekla soja i njegove sposobnosti da redukuje Cr(VI). Slično tome, nije bilo direktne veze između pripadnosti rodu i vrsti i stepena redukcije hromata. Tako na primer neki sojevi *Bacillus cereus* grupe su pokazali visoku, dok su drugi imali nisku sposobnost redukcije. Dva soja vrste *Staphylococcus haemolyticus* su imali podjednako nisku sposobnost redukcije, dok je soj *S. warneri* pokazao umeren stepen redukcije. Sojevi roda *Ochrobactrum* su imali umerenu ili nisku redukciju.

Nije bilo jasne korelacije između procenta rasta i hromatne redukcije (Pearson-ov koeficijent korelacije r <0,40; koeficijent determinacije R^2=0,1222). Pojedini sojevi su imali visok procenat rasta i redukcije, a drugi visok procenat rasta, a nisku redukciju.
Na osnovu dobijenih podataka, sojevi koji su najinteresantniji sa aspekta njihovog bioremedijacionog potencijala su sojevi *Bacillus cereus* grupe NCr1a, NCr2, PCr2a i PCr3a i *Cellulosimicrobium cellulans* PCr3b. Ovi sojevi pokazuju visoku sposobnost redukcije Cr(VI) u Cr(III) praćenu izraženom hromatnom tolerancijom, što bi im omogućilo da uspešno rastu i redukuju Cr(VI) uslovima sredina zagađenih sa Cr(VI). Sa aspekta ispitivanja alternativnih mehanizama hromatne rezistencije kao što je efluks hromata (transport hromata van ćelije), interesantni su sojevi sa niskom sposobnošću redukcije Cr(VI) u Cr(III), a visokom hromatnom tolerancijom, kao što su *Serratia fonticola* NCr1d, *Staphylococcus haemolyticus* LCr9b i PCr8, *Ochrobactrum tritici PCr6* i *Microbacterium maritypicum* PCr11.

III. Karakterizacija odabranih hromat tolerantnih sojeva *B. cereus* grupe

Većina izolovanih hromat tolerantnih sojeva pripadala je *Bacillus cereus* grupi, koja je vrlo značajna u mikrobnim zajednicama, ali je manje izučena sa aspekta hromatne homeostaze. Zbog toga smo dalje ispitivanje fokusirali na 13 odabranih sojeva *B. cereus* grupe (od 20 ukupno izolovanih). Sojevi su odabrani tako da su različite grupe uzoraka predstavljene proporcionalnim brojem uzoraka.
Na odabranim sojevima su izvršene sledeće analize: MALDI TOF identifikacija, ispitivanje morfoloških i biohemijskih osobina, analiza sekvenci 16S rRNK i *pycA* gena, određivanje potencijala formiranja biofilma, određivanje tolerancije na druge teške metale, poređenje tolerancije na hrom izolata sa referentnim sojevima roda *Bacillus*, određivanje sposobnosti redukcije Cr(VI) u Cr(III), određivanje morfoloških i molekularnih osobina soja NCr1a.

MALDI TOF identifikacija je klasifikovala sve sojeve kao članove *B. cereus* grupe, ali nije mogla da obezbedi pouzdanu identifikaciju do nivoa vrste, zbog visokog nivoa sličnosti između vrsta ove grupe.

Biohemijske i morfološke osobine predložene za identifikaciju vrsta *B. cereus* grupe su: hemoliza, rezistencija na penicilin (ampicilin), pokretljivost, morfologija kolonije i prisustvo parasporalnih kristala (Turnbull *i sar.*, 2004; Dworkin *i sar.*, 2006; Luna *i sar.*, 2007; Logan i De Vos, 2009). Nijedan od sojeva nije rastao na 5°C, što eliminiše mogućnost da sojevi pripadaju vrsti *B. weihenstephanensis*. Vrsta *B. anthracis* sadrži gene virulencije, ne produkuje hemolizu, nepokretna je, i većina sojeva su osetljivi na ampicilin. Od 13 sojeva, svi su bili negativni na prisustvo *B. anthracis* gena virulencije, ispoljili su beta-hemolizu, bili su pokretni (izuzev soja NCr1a, koji je bio nepokretan) i rezistentni na ampicilin. Na osnovu toga je potvrđeno da nijedan od sojeva nije pripadnik vrste *B. anthracis*. Izolat NCr1a je imao rizoidalnu morfologiju kolonije i bio nepokretan, što je tipično za vrste *B. mycoides* odnosno *B. pseudomycoides*. Drugi sojevi su imali tipičnu *B. cereus* morfologiju kolonija (velike, nepravilnog oblika, hrapave, blago ispupčene, neprovidne, beličaste do krem boje) i bili su pokretni. Dakle, kada se morfološke i biohemijske osobine uzmu u obzir, sojevi mogu da pripadaju vrstama *B. cereus, B. thuringiensis, B. mycoides* ili *B. pseudomycoides*, dok su vrste *B.*

anthracis i *B. weichenstephanensis* eliminisane. Treba napomenuti da je ova klasifikacija zasnovana na osobinama koje ispoljava većina sojeva date vrste (>85%) i da sojevi sa atipičnim karakteristikama postoje u svakoj vrsti (Dworkin *i sar.*, 2006; Logan i De Vos, 2009).

Analiza sekvenci 16S rRNK i pycA gena je potvrdila da su svi sojevi blisko povezani sa *B. cereus* grupom vrsta. Univerzalni bakterijski prajmeri 27F i 685R korišćeni u ovom ispitivanju dozvoljavaju dobijanje V1-V3 varijabilnog regiona 16S rRNK gena, koji je predložen kao najadekvatniji za identifikaciju vrsta roda *Bacillus* (Blackwood *i sar.*, 2004). Međutim, BLASTn pretraga rezultovala je brojnim pogocima iz *B. cereus* grupe i *Bacillus* sp. sa identičnim merilima pouzdanosti, što je onemogućilo preciznu identifikaciju vrste. Dakle, sekvenca 16S rRNK gena je otkrila samo blisku povezanost sa *B. cereus* grupom, ali nije dovela do identifikacije do nivoa vrste. Sekvenciranje *pycA* gena se pokazalo kao primenljivije i ograničilo je BLASTn pogotke samo na članove *B. cereus* grupe, ali i dalje nije dovelo do nedvosmislene identifikacije do nivoa vrste. Filogenetska stabla zasnovana na 16S rRNK i *pycA* sekvenci su konstruisana, pri čemu je *Clostridium acetobutylicum* korišćen kao out-grupa, a takođe su uključeni i referentni sojevi različitih vrsta roda *Bacillus*. Vrste roda *Bacillus* koje ne pripadaju *B. cereus* grupi (*B. subtilis, B. pumilus, B. licheniformis, B. firmus*) su bile jasno odvojene od naših izolata na oba stabla, dok su se referentni sojevi *B. cereus* grupe grupisali sa našim izolatima. Nekoliko jasnih zaključaka se može izvesti na osnovu dobijenih filogenetskih stabala. Prvo, sojevi NCr1c i NCr2 su blisko povezani sa *B. mycoides* i *B. weihenstephanensis* (zasnovano na oba stabla). Drugo, soj NCr1a je blisko povezan sa *B. pseudomycoides*. Treće, sojevi PCr1, PCr12 i LCr9a su blisko povezani. I na kraju, sojevi LCr6, LCr7, LCr8 i NCr4 su blisko povezani. Međutim, na osnovu rezultata dobijenih analiza može se reći da u većini analiziranih slučajeva ni na osnovu analize sekvenci nije bilo moguće precizno definisati pripadnost vrsti.

Potencijal formiranja biofilma. Život u formi biofilma pruža mikroorganizmima povećanu zaštitu od mnogih biotičkih i abiotičkih faktora životne sredine, uključujući i toksičnost teških metala. Stoga bi formiranje biofilma mogla biti korisna osobina za praktičnu upotrebu mikroorganizama u bioremedijaciji. Iz tog razloga ispitali smo sposobnost produkcije biofilma odabranih izolata u dve hranljive podloge (nutrijentima bogatoj LB i minimalnoj M9 podlozi) nakon 1 i 7 dana inkubacije. Ispitan je i uticaj različitih koncentracija Cr(VI) na potencijal formiranja biofilma. Potencijal formiranja biofilma je bio niži na nutrijentima bogatoj LB podlozi u poređenju sa minimalnom M9 podlogom. Duži period inkubacije nije pospešio formiranje biofilma. U LB podlozi sojevi su uglavnom bili neadherentni ili slabo adherentni. Formiranje biofilma je bilo najviše u M9 podlozi nakon prvog dana inkubacije. U takvoj postavci eksperimenta sojevi NCr4, PCr12, LCr7, LCr8 i LCr9a su bili umereno adherentni, dok je soj LCr6 bio vrlo adherentan. Koncentracija Cr(VI) od 0,1 mM nije uticala na formiranje biofilma, dok je koncentracija od 1 mM izazvala smanjenje bakterijskog rasta, što je propraćeno i smanjenim formiranjem biofilma. Referentni sojevi *B. cereus* grupe (*B. cereus* ATCC 14579 i *B. pseudomycoides* DSM 12442) pokazali su sličan potencijal formiranja biofilma kao i naši izolati. Bili su neadherentni ili umereno adherentni u svim eksperimentalnim postavkama. Referentni soj *B. subtilis* PY79 je pokazao veći potencijal formiranja

biofilma, posebno u LB podlozi nakon 1 dana, kada je bio vrlo adherentan. Referentni soj *P. aeruginosa* ATCC 15692 je bio adherentniji u LB u poređenju sa M9 podlogom.

Tolerancija na druge teške metale. Pošto se zagađenje retko javlja u formi jednog izolovanog polutanta, multipla tolerancija na više teških metala se smatra poželjnom osobinom soja za primenu u bioremedijaciji. Stoga smo testirali toleranciju na teške metale odabranih sojeva *B. cereus* grupe. Većina sojeva je ispoljila sličan obrazac tolerancije. Među testiranim metalima srebro (Ag) je bio najtoksičniji, dok su volfram (W) i molibden (Mo) bili najmanje toksični.

Tolerancija na hrom. Nivo hromatne tolerancije je kod odabranih sojeva *B. cereus* grupe dodatno opisan preko minimalne inhibitorne koncentracije (MIK) šestovalentnog hroma određene na različitim podlogama: minimalnim definisanim (M9 i acetatnoj minimalnoj podlozi (AMM)) i visoko-nutritivnoj LB podlozi. MIK na LB podlozi je bio najmanje 15 puta viši u odnosu na minimalne podloge. Minimalne podloge su dale međusobno slične ili identične vrednosti MIK kod svih sojeva. Jedino se soj NCr1a isticao po vrednosti MIK od 8 mM Cr(VI) na M9 podlozi. Soj NCr1a formira pahuljičast rast u tečnim podlogama, što može dovesti do zaštite ćelija unutar pahuljica od toksičnih efekata šestovalentnog hroma. Takođe, formiranje pahuljica može da rezultuje lakše uočljivim rastom, što bi dovelo i do povećanja određene MIK vrednosti. Ostalih 12 sojeva su imali isti nivo hromatne tolerancije (MIK od 4 mM), uprkos njihovom različitom poreklu.

Hromatna tolerancija ispitivanih izolata i referentnih laboratorijskih sojeva roda *Bacillus* je upoređena merenjem rasta na OD600 u modifikovanoj podlozi M9 sa 0,5, 1 i 2 mM Cr(VI). Izolat iz serpentinskog zemljišta NCr1a se pokazao kao najtolerantniji, pošto nije bilo uticaja na rast čak ni na 2 mM Cr(VI). Izolat PCr12 i referentni sojevi *B. cereus* grupe (*B. pseudomycoides* DSM 12442 i *B. cereus* ATCC 14579) nisu inhibirani koncentracijom od 0,5 mM Cr(VI), dok je rast na 1 i 2 mM bio sličan i iznosio oko 60%. Suprotno tome, referentni soj *B. subtilis* PY79 je bio vrlo osetljiv na Cr(VI), pa je čak i najniža koncentracija Cr(VI) od 0,5 mM snažno inhibirala njegov rast. Ovo ukazuje da su svi ispitivani sojevi *B. cereus* grupe (i sredinski i referentni) generalno mnogo tolerantniji na delovanje hromata u odnosu na *B. subtilis*.

Sposobnost redukcije Cr(VI) u Cr(III). Dodatna detaljnija merenja intenziteta rasta i redukcije šestovalentnog hroma, uključujući ispitivanje vremenske dinamike, su sprovedena za odabrane sojeve *Bacillus cereus* grupe. Intenzitet rasta i sposobnost redukcije šestovalentnog hroma su praćeni svakih 6 sati, do ukupnog vremena inkubacije od 72 sata, pri čemu je korišćena M9 podloga sa startnom koncentracijom od 0,2 mM Cr(VI), uz kontrole bez Cr(VI) i abiotičke kontrole. Rast meren preko OD600 je izražen kao maksimalni relativni rast u procentima u odnosu na rast u kontroli bez Cr(VI). Koncentracija Cr(VI) je praćena pomoću DPC testa, a sposobnost redukcije izražena kao procenat redukcije Cr(VI) u odnosu na početnu koncentraciju. Sojevi su ispoljili različit procenat redukcije Cr(VI), koji je varirao od 22,9% (soj PCr2a) do 98,5% (soj NCr4). Maksimalni relativni rast varirao je od 29,5% do 65,3% u zavisnosti od soja. Tri soja (NCr1b, NCr2 i NCr4) pokazala su najvišu redukciju od preko 90% nakon 72h. Interesantno je da najveća sposobnost redukcije nije bila praćena i najizraženijim rastom, jer maksimalni relativni rast ova tri soja nije bio među najvišim (42,5%, 35,8% i

41,8%). Takođe, ovi sojevi su ispoljili nisku OD600, čak i u odsustvu Cr(VI). Nepostojanje korelacije između rasta i redukcije je evidentno iz veoma niskog koeficijenta korelacije R^2 od 0,07. Cr(VI) je imao najmanji negativni uticaj na rast sojeva NCr1a i PCr12 (maksimalni relativni rast od 65,3%, odnosno 64,9%) što je praćeno i visokom redukcijom Cr(VI) od 73,7%, odnosno 74,4%. Ostali sojevi (NCr3, PCr1, PCr2a, LCr6, LCr7, LCr8 i LCr9a) su ispoljili niži procenat redukcije šestovalentnog hroma (<50%). Verovatno neki drugi mehanizam hromatne rezistencije kao što je smanjeni unos hroma ili poboljšan efluks hroma (transport hroma van ćelije), igraju značajniju ulogu u hromatnoj rezistenciji ovih sojeva. Na višoj startnoj koncentraciji od 1 mM, sposobnost redukcije i maksimalni relativni rast sojeva su bili značajno smanjeni. Redukcija je bila ispod 10% kod svih sojeva. U većini slučajeva, razlika u redukciji među sojevima je bila u rasponu standardne devijacije. Rast je takođe umanjen do nivoa ispod 18,3% za sve sojeve izuzev soja NCr1a, koji je zabeležio 34,3% rasta.

Određivanje morfoloških i molekularnih osobina soja NCr1a. Soj NCr1a imao je najvišu hromatnu toleranciju od svih testiranih sredinskih izolata iz *B. cereus* grupe. Pokazao je snažan rast u podlozi sa Cr(VI) i visoku do umereno visoku hromatnu redukciju, u zavisnosti od eksperimentalne postavke. Stoga je on odabran za detaljnije ispitivanje kulturelnih karakteristika, ćelijske morfologije i molekularnih osobina.

Soj NCr1a ispoljava karakterističnu rizoidalnu morfologiju kolonija tipičnu za vrste *B. pseudomycoides* i *B. mycoides*. Smer formacije filamenata posmatrano sa donje strane ploče je suprotan smeru kazaljke na satu (DX morfotip prema Di Franco *i sar.* (2002)). U početku kolonija je uniformnog izgleda na čitavoj površini (prvi dan inkubacije). Međutim, nakon duže inkubacije dolazi do pojave heterogene boje, teksture i generalnog izgleda kolonije. Soj spada u Gram-pozitivne štapiće u kratkim ili dužim lancima. Endospore su subterminalne i eliptične. Citoplazma često izgleda zrnasto, usled akumulacije granula polihidroksibutirata, posebno ukoliko se rast vrši na podlogama sa visokim C:N odnosom (npr. podloge sa visokim sadržajem ugljenih hidrata). Ćelijska morfologija varira tokom starenja.

Soj NCr1a je podvrgnut detekciji i sekvenciranju *chrA* gena za hromatni transporter, koji je poznat po svojoj ulozi u hromatnoj toleranciji (Cervantes *i sar.* 1990; Díaz-Pérez *i sar.* 2007; Nies *i sar.* 1990). Dobijeni produkt je bio dužine od 1373 bazna para. Sekvenca je imala 97% identičnosti sa sekvencom genoma dva soja *B. pseudomycoides*, konkretno sojem BTZ (Acc. No. CP009651.1) i sojem 219298 (Acc. N. CP007626.1). Sa drugim članovima *B. cereus* grupe je nađeno niže poklapanje (≤75% identičnosti). Hromatni transporter soja NCr1a pripada superfamiliji transportera hromatnog jona, a familiji hromatnih transportera dugog lanca (Nies *i sar.*, 1998; Díaz-Pérez *i sar.*, 2007).

IV. STEM EDS analiza sadržaja i lokalizacije hroma u ćelijama i endosporama sojeva NCr1a i *B. subtilis* PY79

U prethodnim testovima je utvrđeno da soj NCr1a, uslovno identifikovan kao *B. pseudomycoides,* ima najviši nivo hromatne tolerancije među svim testiranim izolatima *B. cereus* grupe. Referentni soj *B. subtilis* PY79 se pokazao kao mnogo osetljiviji na dejstvo Cr(VI). Stoga, ova dva soja su izabrana za dalje testiranje sadržaja i lokalizacije hroma pomoću elementarnog mapiranja upotrebom STEM-EDS. Količina unetog hroma

i njegova lokalizacija je određena u ćelijama koje su rasle u prisustvu ili bez prisustva Cr(VI), sa ili bez prethodne indukcije i nakon različitih perioda izlaganja. Takođe, ispitane su i endospore formirane u prisustvu Cr(VI), kao i endospore koje su nakon formiranja izložene delovanju Cr(VI).

Sadržaj hroma u ćelijama dva soja je analiziran nakon kultivacije u M9 medijumu sa 1 mM Cr(VI). Ćelije su sakupljene u dve vremenske tačke: eksponencijalnoj fazi rasta (4h rasta) i stacionarnoj fazi (18h rasta). Akumulacija hroma unutar ćelija NCr1a sakupljenih u eksponencijalnoj fazi je bila vrlo niska i sa malom standarnom devijacijom i vrednostima bliskim nivou detekcije instrumenta – 0,03±0,01 atomskih procenata (at%). Nakon duže inkubacije od 18h, sadržaj hroma u ćelijama NCr1a je porastao na 0,09±0,03 at%. Ćelije soja *B. subtilis* PY79 pokazale su visoku varijaciju u sadržaju hroma (od 0,04 do 0,70 at%), pošto su neke akumulirale veliku količinu, dok su druge akumulirale količinu sličnu ćelijama soja NCr1a. Nije bilo statistički značajne razlike između sadržaja Cr u ćelijama *B. subtilis* PY79 nakon 4 i 18h inkubacije (0,27±0,20 at%, odnosno 0,30±0,23 at%). Ćelije soja NCr1a su i nakon 4h i nakon 18h imale statistički značajno manju količinu akumuliranog hroma u poređenju sa ćelijama soja *B. subtilis* PY79.

Mnoge ćelije *B. subtilis* PY79 su bile oštećene/lizirane u prisustvu Cr(VI). Suprotno tome, ćelije soja NCr1a nisu pokazale nikakve znakove oštećenja, lize ili atipičnog izgleda u prisustvu Cr(VI). Ovo dodatno ukazuje na to da *B. subtilis* PY79 trpi teže posledice toksičnosti Cr(VI) u poređenju sa NCr1a.

Ispitali smo takođe i uticaj indukcije niskim koncentracijama Cr(VI) pre pune ekspozicije ćelija visokim koncentracijama Cr(VI). Sojevi su kultivisani u M9 medijumu bez Cr(VI) i sa prisustvom niske subinhibitorne koncentracije Cr(VI) (10 µM). Ćelije su sakupljene u eksponencijalnoj fazi rasta i isprane. Nakon toga, izložene su delovanju 1 mM Cr(VI) u fosfatnom puferu u trajanju od 1h, fiksirane i analizirane pomoću STEM-EDS. Koncentracija za indukciju od 10 µM je odabrana, jer je prethodno utvrđeno da nije značajno uticala na rast dva soja u poređenju sa kontrolom bez Cr(VI).

Indukcija nije imala efekta na sadržaj hroma u ćelijama NCr1a - i sa i bez indukcije sadržaj je bio nizak, stabilan i iznosio je 0,02±0,01 at%. Suprotno tome, kod soja *B. subtilis* PY79 uočen je statistički značajan efekat indukcije na smanjenje sadržaja hroma u ćelijama. Sadržaj hroma u indukovanim ćelijama *B. subtilis* PY79 (0,03±0,02 at%) je bio 5,7 puta niži nego u neindukovanim ćelijama (0,17±0,05 at%). Indukcija je rezultovala time da sadržaj hroma u ćelijama *B. subtilis* PY79 nije bio značajno drugačiji nego u ćelijama NCr1a. Na osnovu ovih rezultata izgleda da su ćelije *B. subtilis* PY79 sposobne da održavaju nizak ćelijski nivo hroma, ali samo ukoliko su indukovane niskim koncentracijama Cr(VI) pre izlaganja visokim koncentracijama Cr(VI).

Sadržaj hroma u endosporama. Ispitali smo sadržaj i lokalizaciju hroma u endosporama sojeva NCr1a i *B. subtilis* PY79. Prvo, ispitane su spore formirane u podlozi sa Cr(VI) od samog starta rasta. Drugo, testirane su spore koje su formirane bez prisustva Cr(VI) i koje su nakon formiranja izložene delovanju Cr(VI) u različitom trajanju (izlaganje zrelih spora).

- *Endospore formirane u prisustvu hroma.* Testiran je sadržaj hroma u sporama koje su formirane u nakon 6 dana rasta u Difco sporulacionoj podlozi sa 1 mM Cr(VI). *Soj B.*

subtilis PY79 nije rastao i efikasno formirao spore u prisustvu Cr(VI), dok je neometano rastao i sporulisao u istoj podlozi bez Cr(VI). Dakle, nije bilo moguće ispitati sadržaj hroma u sporama ovog soja koje su formirane u prisustvu hroma. Endospore soja NCr1a formirane u podlozi sa 1 mM Cr(VI) sadržale su 0,50±0,28 at% Cr i nisu bile morfološki različite u odnosu na spore formirane u podlozi bez Cr(VI).

- *Izlaganje zrelih spora.* Zrele spore su sakupljene nakon inkubacije u podlozi bez hroma i nakon toga izložene delovanju Cr(VI) u trajanju od 1 sat ili 6 dana. Nakon kratkog izlaganja od jednog sata zrele spore oba soja vezuju slične količine hroma (0,01 do 0,02 at%). Nakon dužeg izlaganja od 6 dana, spore soja NCr1a sadrže 0,11±0,04 at%, dok spore *B. subtilis* PY79 sadrže 0,03±0,01 at%. Dakle, spore NCr1a vezuju 3,7 puta više hroma od spora soja *B. subtilis* PY79. Veći sadržaj hroma kod spora NCr1a je verovatno posledica toga što one, za razliku od spora *B. subtilis* PY79, poseduju egzosporijum, koji značajno učestvuje u vezivanju hroma.

Iz elementarnog mapiranja može se uslovno zaključiti da je hrom većinski lociran u spoljašnjim slojevima endospore NCr1a (omotaču i egzosporijumu). Ovo zapažanje zasnovano je na odvojenim merenjima u oblasti jezgra i spoljašnjih slojeva spore, pri čemu je razlika u sadržaju hroma bila statistički značajna na $p>0.01$ (t-test). Međutim, da bi se definitivno mapirala lokalizacija hroma, bilo bi neophodno posmatranje tankih preseka endospore uniformne debljine.

Sumirano, sporulacija NCr1a u prisustvu Cr(VI) je neometana, spore zadržavaju normalnu morfologiju i akumuliraju značajno veće količine hroma u odnosu na druge preparate spora. Hrom je većinski lociran u spoljašnjim slojevima spore (omotač i egzosporijum). Suprotno tome, *B. subtilis* PY79 ne uspeva da formira spore u prisustvu Cr(VI). Ukoliko se zrele spore *B. subtilis* PY79 formirane bez prisustva Cr(VI) njemu naknadno izlože, onda one akumuliraju značajno manje hroma u odnosu na spore NCr1a pod istim uslovima. Ova pojava je verovatno posledica toga što endospore *B. subtilis* PY79 ne formiraju egzosporijum, koji dominantno učestvuje u vezivanju hroma.

Zaključci.

Rasvetljavanje uticaja hroma, značajnog polutanta iz grupe teških metala, na bakterijsku zajednicu i na pojedinačne bakterijske izolate, je bilo tema ove doktorske disertacije. Glavni zaključci istraživanja, podeljeni prema glavne 4 sekcije istraživanja, su sledeći:

Ispitivanje uticaja hroma na kultivabilnu bakterijsku zajednicu zemljišta

- Visoka koncentracija ukupnog Cr u zemljištu nije imala efekat na brojnost kultivabilnih zemljišnih bakterija.
- Udeo hromat tolerantne populacije u ukupnoj populaciji kultivabilnih zemljišnih bakterija nije zavisio od koncentracije Cr u zemljištu.
- Udeo Gram-pozitivnih i Gram-negativnih bakterija u ukupnoj populaciji kultivabilnih zemljišnih bakterija nije bio pod uticajem koncentracije Cr u zemljištu.
- Dodatak čak i niskih koncentracija Cr(VI) od samo 0,25 mM hranljivoj podlozi inhibirao je rast polovine kultivabilnih zemljišnih bakterija.
- Oko 10% kultivabilnih zemljišnih bakterija su rezistentne na 2 mM Cr(VI) u kultivacionoj podlozi.

Izolacija hromat tolerantnih bakterijskih sojeva

- I uzorci sa visokom i uzorci sa niskom koncetracijom hroma sadrže Cr(VI) tolerantne sojeve. Međutim, diverzitet izolovanih sojeva je viši u uzorcima zagađenim hromom u poređenju sa uzorcima sa prirodno povišenom koncentracijom hroma i uzorcima sa niskom koncentracijom hroma.
- Izolovano je ukupno 33 Cr(VI) tolerantna bakterijska soja sa sposobnošću rasta na minimalnom medijumu sa 2 mM Cr(VI).
- Izolati su predstavnici 12 vrsta iz 8 različitih rodova, pri čemu je 28 izolata Gram-pozitivno, a 5 Gram-negativno.
- Sojevi *Bacillus cereus* grupe su bili dominantno prisutni među Cr(VI) tolerantnim izolatima (20 od 33 izolata).
- Sojevi *Bacillus cereus* grupe NCr1a, NCr2, PCr2a i PCr3a i *Cellulosimicrobium cellulans* PCr3b su najbolji kandidati za dalje ispitivanje primene u bioremedijaciji, zbog njihove visoke sposobnosti redukcije šestovalentnog hroma pri startnoj koncentraciji od 0,5 mM i visokog intenziteta rasta u prisustvu 2 mM hroma.
- Sojevi *Serratia fonticola* NCr1d, *Staphylococcus haemolyticus* LCr9b i PCr8, *Ochrobactrum tritici* PCr6 i *Microbacterium maritypicum* PCr11, sa niskom sposobnošću redukcije šestovalentnog hroma i visokim intenzitetom rasta u prisustvu 2 mM hroma, su od interesa u daljem ispitivanju drugih mehanizama hromatne rezistencije, kao što su efluks, blokiranje unosa hromata i odgovor na oksidativni stres.
- Podaci o sojevima iz nezagađenih sredina (*S. warneri* LCr1, *S. haemolyticus* LCr9b i sojevi *B. cereus* grupe LCr2 do LCr8 i LCr9a) sa niskim sadržajem hroma su od posebnog značaja, zbog toga što su do sada sojevi poreklom iz takvih sredina vrlo retko ispitivani.

Karakterizacija odabranih hromat tolerantnih sojeva *B. cereus* grupe

- Sojevi *Bacillus cereus* grupe i iz sredina sa visokom i sa niskom koncentracijom hroma su pokazali sličan nivo hromatne tolerancije.
- Referentni sojevi *B. cereus* grupe su bili podjednako tolerantni na Cr(VI), što ukazuje na urođenu prirodu hromatne tolerancije ove grupe vrsta.
- Izolati *B. cereus* grupe su pokazali toleranciju i na druge teške metale, dok je u poređenju sa njima referentni soj *B. subtilis* PY79 bio mnogo osetljiviji.
- Soj NCr1a se pokazao kao najotporniji na Cr(VI) među svim izolatima *B. cereus* grupe.

STEM EDS analiza sadržaja i lokalizacije hroma u ćelijama i endosporama sojeva NCr1a i *B. subtilis* PY79

- Na osnovu dobijenih rezultata merenja rasta u prisustvu Cr(VI) i STEM EDS analize sadržaja hroma u ćelijama i endosporama, soj NCr1a se može smatrati visoko otpornim na Cr(VI), dok je soj *B. subtilis* PY79 vrlo osetljiv. Konkretno:
    - Soj NCr1a raste neometano u prisustvu 1 mM Cr(VI), dok je rast soja *B. subtilis* PY79 je snažno inhibiran;
    - Ćelije soja NCr1a imaju nizak i stabilan sadržaj hroma, dok ćelije soja *B. subtilis* PY79 imaju mnogo viši i varijabilniji sadržaj hroma;

- Kod ćelija soja NCr1a ne dolazi ni do kakvih morfoloških promena, dok ćelije *B. subtilis* PY79 često liziraju u prisustvu Cr(VI);
- Soj NCr1a neometano sporuliše, dok *B. subtilis* PY79 ne uspeva da izvrši efektivnu sporulaciju u prisustvu Cr(VI).

Uzimajući sve u obzir, rezultati dobijeni u okviru ove doktorske disertacije su od velikog značaja ne samo sa praktičnog aspekta primene u bioremedijaciji, već i sa fundamentalnog naučnog aspekta izučavanja mehanizama hromatne tolerancije kod bakterija.

BIOGRAPHY

Dragana Tamindžija (née Čučak) was born on May 19th 1988 in Bihać, Bosnia and Hercegovina. She finished secondary medical school in Novi Sad and gained degree of Pharmacy technician. In 2007 she started higher education at the Faculty of Sciences of the University of Novi Sad and received undergraduate degree of Bachelor of Science in Biology with a 8.88/10 average. At the same institution in 2012 she graduated as a Master in Biology - module microbiology with 9.5/10 average after defending master thesis on the subject "Microbiological quality of pig farm waste waters". The same year she starts PhD studies of ecological sciences at the same institution.

She gained the title of a Research trainee in 2012 and title of a Teaching assistant at the Department of Chemistry, Biochemistry and Environmental Protection in 2015. During her PhD studies she participated in two national scientific projects of the Ministry of education, science and technological development of the Republic of Serbia (TR37004 lead by professor Srđan Rončević, PhD and III43005 lead by professor Božo Dalmacija, PhD), during which she worked on biodegradation of polycyclic aromatic hydrocarbons in polluted sediment. She participated in an international project "The role of metal homeostasis, reduction and sporulation in the metal resistance of Gram-positive bacteria" funded by the Swiss National Science Foundation. In the scope of this project she worked on chromium homeostasis in environmental bacterial isolates and went on two two-month-long study visits to Imrich Barák's laboratory at the Institute of Molecular biology in Bratislava Slovakia.

She participated in teaching activities on several courses of undergraduate and graduate studies, including: Practicum in Microbiology, General Microbiology, Microbial Ecology, Environmental Microbiology, Microbiological Monitoring at the Department of Biology and Ecology, and Ecotoxicology, Basics of environmental protection, Microbiology in Environmental Protection and Fundamentals of Biological Principles in Environmental Protection on the Department of Chemistry, Biochemistry and Environmental Protection.

She coauthored 1 paper in leading academic journal of M21a category, 1 paper in prominent academic journal of M22 category, 3 papers in category M23. Also, she is author of 6 international conference papers (category M33), 10 international conference abstracts (category M34), 3 national conference papers (category M63), and 1 technical solution (category M83).

She actively participated in organization, modernization, procurement and financial planning of the Laboratory of microbiology. She is a member of Federation of European Microbiological Societies, Serbian Microbiological Society, Serbian Chemical Society and Serbian Biological Society.

In Novi Sad, 30.01.2019.

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U ovom radu ispitana je tolerantnost zemljišnih bakterija na šestovalentni hrom (Cr(VI)). Prvo, ispitan je uticaj visokog nivoa hroma antropogenog i geogenog porekla na kultivabilnu bakterijsku zajednicu zemljišta. Dalje, izolovani su bakterijski sojevi sa visokom tolerancijom na Cr(VI) iz različitih sredinskih uzoraka kao što su zemljište, sediment, voda i otpadni materijal. Sojevi su identifikovani i određen je nivo njihove Cr(VI) tolerancije i sposobnost redukcije toksičnog Cr(VI) u manje toksični Cr(III). Odabrani sojevi *Bacillus cereus* grupe su dalje karakterisani – određene su njihove morfološke i biohemijske karakteristike, 16S rDNK i *pycA* sekvence, potencijal formiranja biofilma i otpornost na druge teške metale. Takođe, sprovedeno je detaljnije ispitivanje njihove tolerancije i redukcije Cr(VI). Soj sa najvišom otpornošću je uporedo sa kontrolnim osetljivim sojem analiziran pomoću STEM EDS na sadržaj hroma u ćelijama i endosporama u različitim uslovima. Rezultati ukazuju da su bakterije tolerantne na Cr(VI) prisutne i u sredinama sa niskim i sa visokim koncentracijama hroma. Većina izolata pripadala je *B. cereus* grupi što ukazuje na njenu uopšteno visoku otpornost na Cr(VI). Pojedini sojevi su pokazali visoku otpornost i sposobnost redukcije Cr(VI), što ukazuje na mogućnost njihove praktične primene u bioremedijaciji. STEM EDS analiza osetljivog *B. subtilis* PY79 soja i Cr(VI)-rezistentnog soja *B. cereus* grupe NCr1a otkrila je značajne razlike u njihovom odgovoru na Cr(VI) i sadržaju Cr u njihovim ćelijama i endosporama.

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In this study, tolerance of soil bacteria to hexavalent chromium (Cr(VI)) was investigated. First, influence of high chromium levels of anthropogenic and geogenic origin on the soil cultivable bacterial community was examined. Next, a number of bacterial strains with high Cr(VI) tolerance were isolated from diverse environmental samples such as soil, sediment, water and waste material. Strains were identified and tested for the level of Cr(VI) tolerance and the ability to reduce toxic Cr(VI) to more innocuous Cr(III). Selected *Bacillus cereus* group strains were further characterized - their morphological and biochemical characteristics, 16S rRNA and *pycA* gene sequences, biofilm formation potential and resistance to other heavy metals were determined. Also, more detailed study of their tolerance level and Cr(VI) reduction was conducted. Strain with the highest resistance together with the control chromate sensitive strain were analyzed by STEM EDS for their cellular and endospore Cr content under different conditions. Results indicate Cr(VI) tolerant bacteria are present both in low and high Cr environments. Majority of isolates belonged to the *B. cereus* group indicating its overall high tolerance to Cr(VI). Certain strains exhibited high tolerance and reduction ability, indicating their possible usefulness in practical bioremediation application. STEM EDS analysis of Cr(VI)-sensitive *B. subtilis* PY79 strain and Cr(VI)-resistant *B. cereus* group strain NCr1a revealed significant differences in their response to Cr(VI) and in their Cr cellular and endospore content.

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