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Report

Feasibility study for Life NARMENA (BN200108)

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Report ID	LIFENARVGJ | BN200108

Report comments: Second, final report

Report Date: 14/01/2021

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Feasibility study for Life NARMENA (BN200108)

The object of this study was to perform extended feasibility tests according to the code of good practice, to evaluate which plant species are most suitable for phytostabilisation of chromium (Cr) at the Grote Calie, Turnhout, Belgium. Plant species were collected from the field and evaluated for Cr content in roots and shoots, compared to the rhizosphere soil, Cr-tolerant bacteria were isolated, tested for plant-growth promotion potential and Cr-tolerance, pot test was setup to compare the Cr-uptake of different plant species, and the effect of inoculation or amendments on Cr uptake, and leaching. Finally, chromate reductase genes were searched for in the total microbial communities from the field via next generation sequencing, and the fate of the inoculum was tracked via DNA-sequencing. This feasibility study was performed by UHasselt for OVAM, as part of the NARMENA (Nature-based Remediation of Metal pollutants in Nature Areas) project with number BN200108, where bio2clean is responsible for the phytoremediation projects.

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1 Research questions and overview of the tasks

The following research questions were formulated in the research proposal:

1) Which plant species in the field take up Cr, and in which form (Cr(III), Cr(VI))? What is the chromium translocation per compartment: rhizosphere soil, root, shoots?
2) Which plant species are known in literature to take up Cr and suitable for phytostabilization?
3) Are there Cr-tolerant bacteria in the soil, do we find Cr(VI) reducing genes/strains?
4) Which plant species are most suitable for Cr-phytostabilization, and what is the contribution of inoculation with Cr-reducing and plant growth promoting (PGP)-bacteria, and soil amendments, on the plant Cr concentrations?
5) Is there a seasonal effect, which other plant species (spring, summer, autumn) take up Cr?

Status	Nr	Tasks	Delivery term
✓	1	First screening phytoremediation feasibility: Terrain visit in function of phytoremediation: plant sampling for Cr analyses, and soil collection for pot experiment	19/06/2020
✓	2	Literature study: state-of-the-art Cr phytoremediation	01/07, corrections send 20/07
✓	3	Cr(III) and Cr(VI) analyses in the rhizosphere soil, plant roots, and shoots collected from the field	Cr-results 24/07/20
✓	5	Isolation and characterisation of PGP and Cr tolerant bacteria, and 16S sequencing	09/09/20
✓	6	Basic pot experiment agricultural area: *Achillea millefolium*, *Epilobium hirsutum*	Harvested 24/11/20
✓	7	Basic pot experiment nature reserve area: *Phragmites australis*, and *Dryopteris filix-mas*	Harvested 24/11/20
✓	8	Extended pot experiment agricultural area: *Achillea m.* and *Epilobium h.* x inoculated/non inoculated x basalt/portland cement	Harvested 24/11/20
✓	9	Extended pot experiment nature reserve: *Phragmites australis*, and *Dryopteris felix-mas* x inoculated/non-inoculated	Harvested 24/11/20
✓	10	NGS for Cr-reducing genes (bacteria + fungi)	Analysed 24/12/2020 – 12/01/2021
✓	11	Repetition of plant sampling in spring, P analyses, seasonal effect	Spring 2021

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2. Material and methods

1.1 Field sampling and Cr analyses

The study location is the Grote Calie in Turnhout (51.283199, 4.946097), where the sediment and riverbank, up to 10 meters distant from the river is polluted with chromium (Cr), due to historical release by a leather processing and tannery company. During the field visit on June 19th 2020, eighteen plant samples and rhizosphere soil were collected from both the agricultural area (51.283199, 4.946097) and the nature reserve area (51.2781006, 4.9433023). In total we collected 15 different plant species mainly herbaceous plants, and branches from white willow.

In the lab, roots were separated from the shoots. The roots were washed rigorously with plenty of distilled water to remove all adherend soil particles, and then dried in the oven at 60°C. No washing with lead nitrate was performed because the aim was to assess both the surface-bound Cr and internalised Cr. Shoots with the leaves were washed with distilled water and then dried in the oven prior to chemical analyses.

The soil and plant samples were analysed for Cr(III) and Cr(VI) by SGS, Belgium. For Cr(III) and total Cr, an acid microwave-assisted digestion was performed followed by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) analyses (method ECO/AV/IMA/007). For Cr(VI), an alkaline extraction was performed based on EN71-3 and analyses by Ion Chromatography (IC) coupled with Inductively Coupled Plasma (ICP), High resolution mass spectrometry (HRMS).

An aliquot of the rhizosphere soil samples was flash-frozen in liquid nitrogen and stored at -80°C prior to DNA extraction and Phospholipid Fatty Acid Analysis.

1.2 Isolation of PGP bacteria and Cr-tolerance test

To isolate Cr-tolerant and plant growth promoting bacterial strains, soil was mixed from three sampling buckets and then one gram was suspended in 100 ml of sterile phosphate buffer and used for the extraction of the microbes. Serial dilutions of the soil suspension were plated on agar plates with different nutrient content (Bushnell Haas, 869 rich and artificial mineral salt (AMS) media plates). After one week of incubation, single colonies were picked and purified on 1/10 and 1/100 diluted Nutrient Broth agar plates. Twenty pure colonies were picked and tested for the ability to produce the hormone indole acetic acid (IAA) via the Salkowski test. The bacteria were also screened for their survival on rich medium plates amended with 100 and 150 mg/L Cr(VI) (K_2CrO_4) as previously described (Ramírez et al., 2019).

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1.3 Genomic DNA extraction and taxonomic identification of the chromium tolerant bacterial strains

Total DNA was extracted using the Qiagen Blood and Tissue kit and quantified with Qubit and checked for purity on nanodrop. Near full-length sequence of the 16S rRNA gene was amplified with the primers 27f and 1492r as described previously (Thijs et al., 2018). Products were checked on agarose gel and then shipped to Macrogen for 16S rRNA Sanger sequencing. Sequencing results were quality filtered using Geneious v4.8, were analysed over the ribosomal database, to obtain the taxonomic information of the bacterial strains.

1.4 Total DNA extraction and shotgun metagenome sequencing

Total DNA was extracted from the rhizospheric soil samples collected in the field using a custom phenol-chloroform extraction method. The HMW DNA was subjected to library preparation with the Nextera DNA flex (Illumina) kit prior to sequencing 20 million reads deep on the Illumina Hiseq X (2 x 150 bp) (Macrogen, Seoul, South-Korea).

1.5 Analyses of shotgun metagenome data

The raw reads were quality filtered, trimmed, and assembled using the metagenomics pipeline atlas (Kieser et al., 2020), on the Flemish Supercomputer infrastructure (VSC). Taxonomic identification was performed using Kaiju (Menzel et al., 2016), and Kraken2 (Wood et al., 2019). Custom functional analyses for Cr-reductase genes were performed using MetAnnotate (Petrenko et al., 2015)

1.6 Pot experiment in the green house

To be able to compare different plant species for Cr-uptake, they must be grown on a soil with the same Cr-concentration, therefore we sieved and mixed the two trays of collected top-soil from the agricultural area (see **Figure S1**), and 3 buckets from the nature reserve area, to have one homogenised batch for each location. Four plant species were selected for the test in three replicates, with/without inoculation and with or without soil amendments. In total there were 36 pots for the agricultural area, and 15 pots for the nature reserve area.

Portland cement and basalt, finely ground to powder < 100 µm, were kindly provided by JT minerals. Amendments were added in a 3 % ratio (3 g per 100 gram), the soil was mixed, then filled in the pots (+-400 gr soil/pot) and then stabilised for 3 months. Wild-type plants were bought from Ecoflora Halle, Belgium. The plants were carefully taken out of their pot, the soil was rubbed and shaken off, and roots washed in water, prior to planting in the Cr-polluted soils. Willow cuttings (*Salix alba* Liempde and *Salix alba* Chermesina) were collected at the tree nursery (Ben Verlinden, Aarschot, Belgium) in September 2020, but they did not root, so they were not considered further for the experiments.

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The plants were grown in the greenhouse for 2 months. Then the pots were randomly assigned to treatment groups, and half was inoculated with *Bacillus* sp. Narmena19 (an isolate from the field), and the other pots served as non-inoculated controls. The strain was grown overnight in 1L of rich medium, centrifugated to obtain a pellet, and brought to optical density of 1 (10^9 cells/ml) at 600 nm in sterile $MgSO_4$ solution. The plants were inoculated by pouring 80 ml of 10^9 cells/ml in each plant pot. After inoculation, plants were grown for an additional month prior to harvest at a total of 3 months growth in the Cr-polluted soil.

Plants were harvested and fresh and dry shoot and root weight determined gravimetrically. Dried plant shoots were analysed for Cr(III) (LUFA, Agrolab). Soil samples were subjected to a 24h EUR2 shaking test (L/S: 10) prior to Cr analyses, and to a total bioavailability test. For this last test, Cr was measured in the third eluate fraction, after leaching with the soil with a solution at pH 7 and pH 4 (methods: CMA/2/I/B.1; CMA/2/II/A.1; CMA/2/II/A.19; CMA/2/II/A9.1/A9.3/A9.4/A9.5/A12/A19; CMA/5/B.3 ; CMA/5/B.4 en NEN 7371 for total bioavailability and neutralising capacity at pH=4 and pH=7, AL-West NV, Agrolab, Netherlands). A leaching test was also conducted to simulate heavy rain, for this 1 L of water was poured on top of the soil, and the leachate collected for anion analyses (chloride, sulfaat, nitraat, nitriet, carbonaat, bicarbonaat, fosfaat, method 946 at AL-West, Netherlands).

To estimate bacterial abundance, 20-gr rhizosphere soil samples were flash-frozen in liquid nitrogen and stored at -80°C for PLFA analyses. In addition, rhizosphere soil samples were collected for bacterial amplicon sequencing to track the fate of the inoculum.

1.7 Statistical analyses

Graphs were made in RStudio, R-version 3.6.2, and in Microsoft excel.

3. Results

3.1 Cr concentrations in plants from the field

The Cr concentration in the plant samples (**see Figures S2 and S3**) differed between the agricultural area and in the nature reserve area (**Table S1**), that is because the soil Cr concentrations also significantly differed between the two locations (**Figure 1**). A fourfold higher Cr concentration was measured in the topsoil (0-20 cm) in the nature reserve area (up to 2900 mg Cr(III)/kg dw soil) compared to the agricultural area (up to 680 mg Cr(III)/kg dw soil) (**Figure 1, Table S1**). Between plant species within the same location, also differences were observed in the root and shoot Cr-concentrations, which can be related to a plant species effect, but also to the local variations in soil Cr-concentrations in their root zone (**Figure 2**), and distance to the stream.

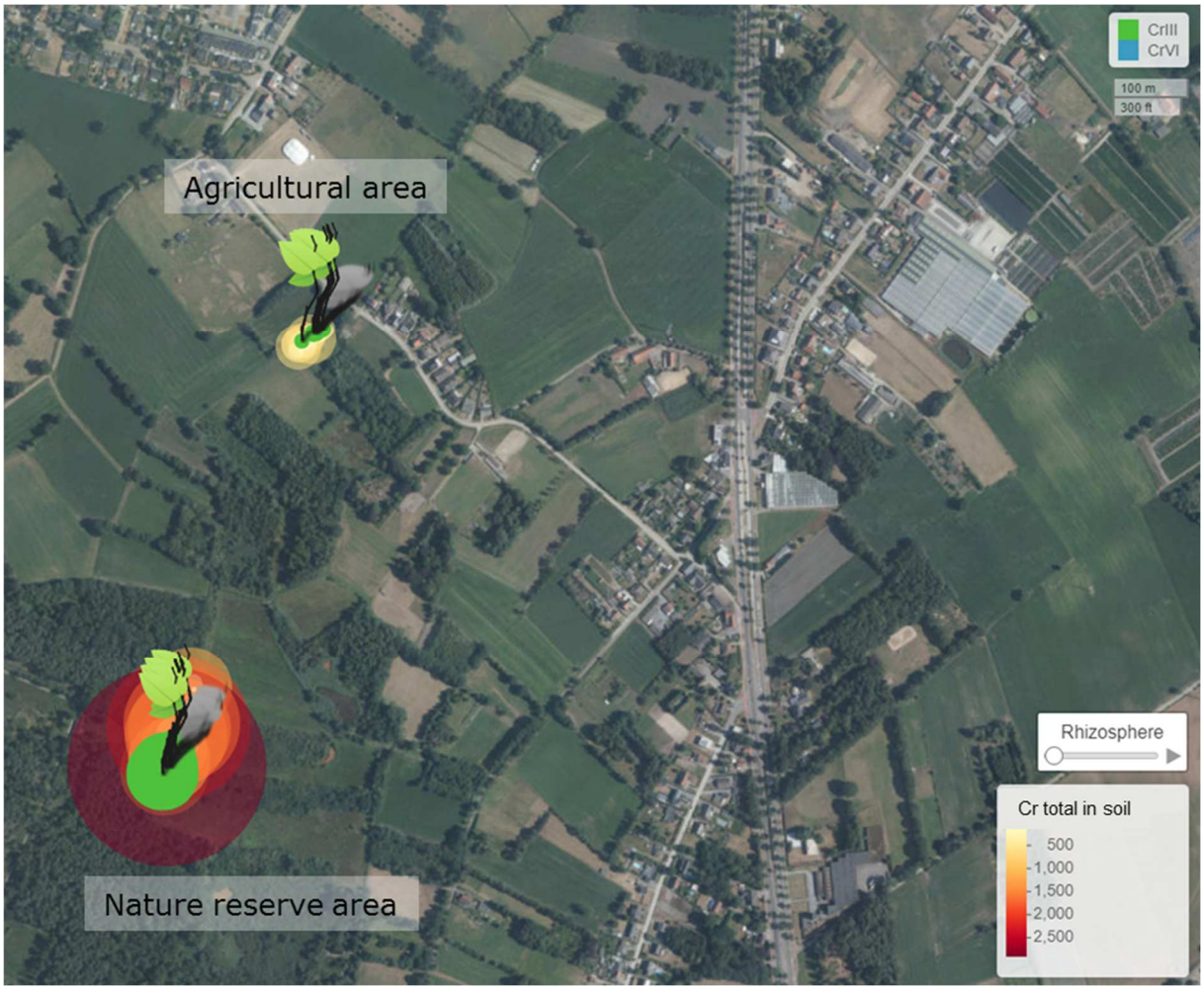

Figure 1: Topographic map overlaid with indications of the sampled plants at the two locations, and the total Cr-concentrations in the rhizosphere soil (Interactive map, click here). Higher Cr(III) concentrations are shown in darker red, and the size of the circle is in proportion to the Cr(III) concentration in the bulk soil. The green circle size is in proportion to the Cr(III) concentrations in the plant rhizosphere.

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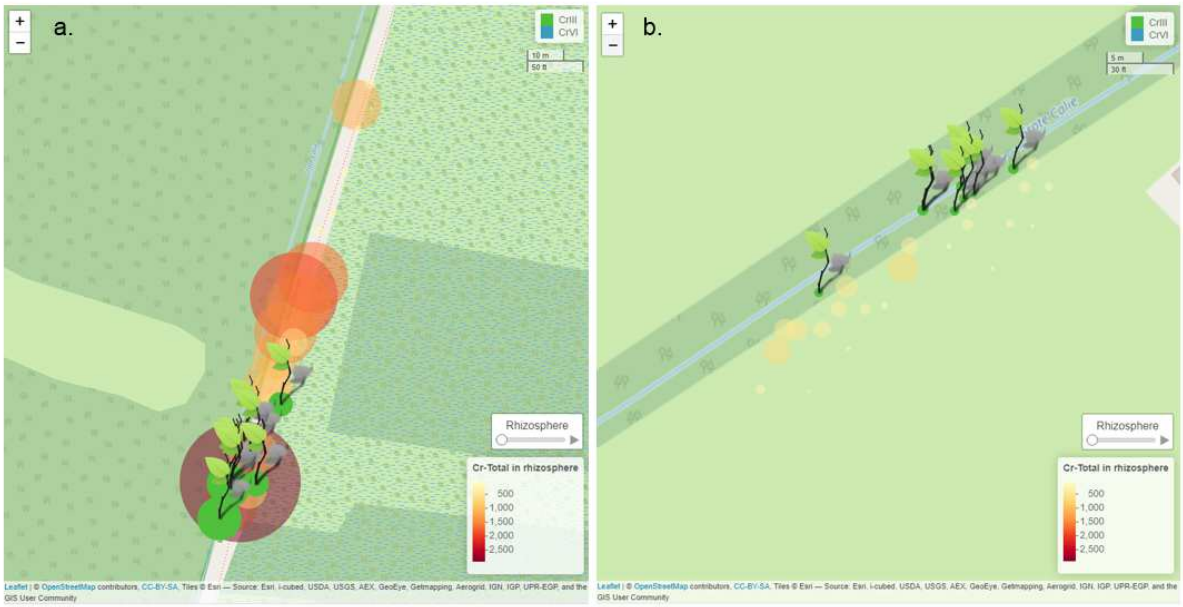


Figure 2: Zoomed in maps of (a) the nature reserve area and (b) the agricultural area. It shows the local differences in Cr-concentrations and the implantation of the plants to the riverbank. See also the interactive charts in attachment to toggle between rhizosphere, root, shoot, and upon hovering over, the plant name is shown and its concentration.

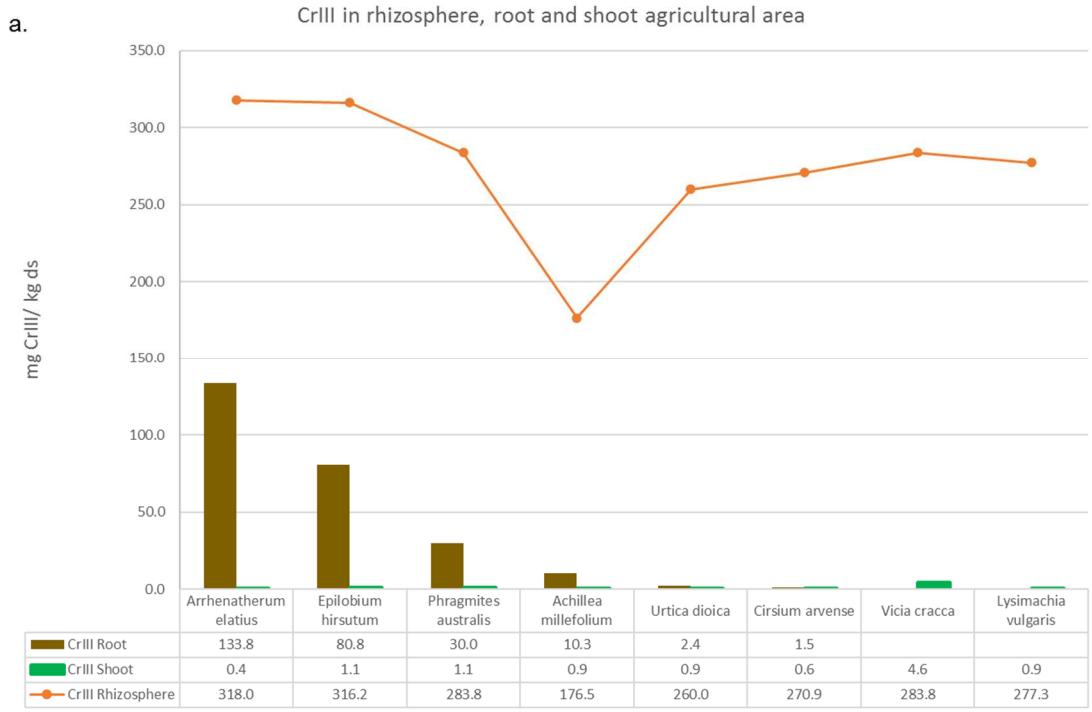
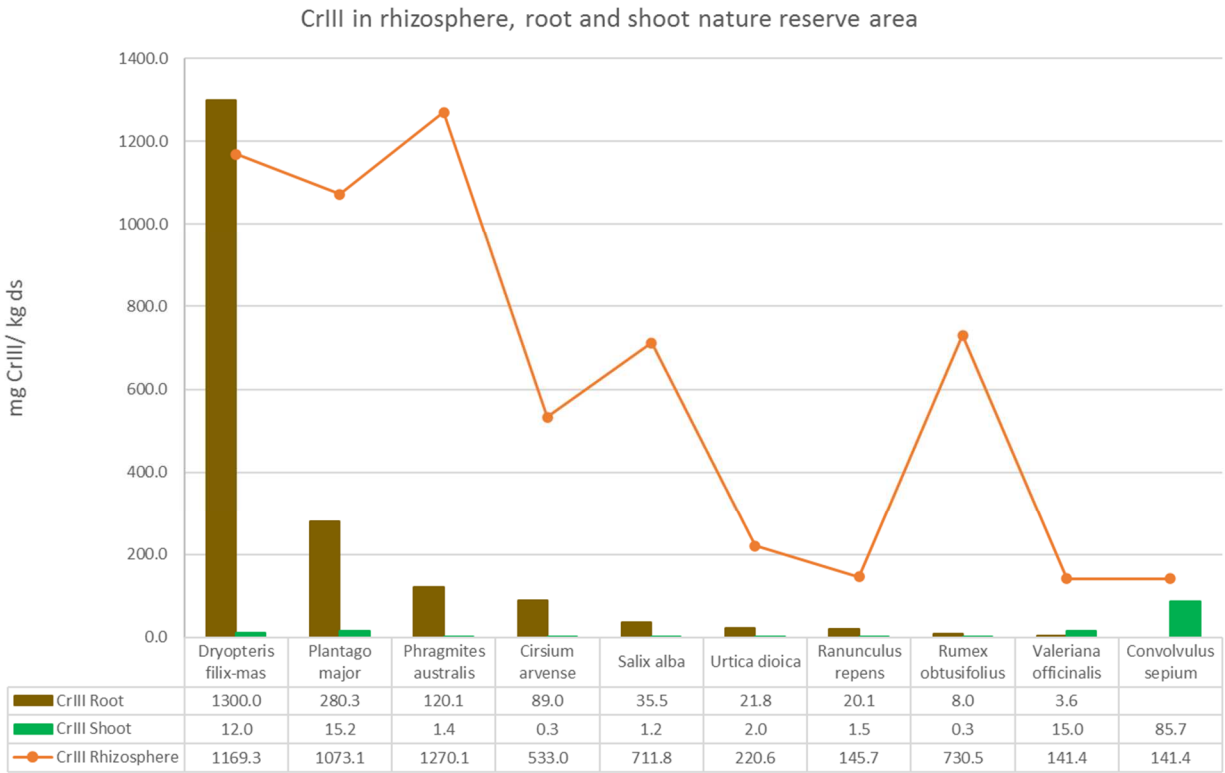


Figure 3: Cr(III) concentrations in rhizosphere soil, root and shoots of plants in the agricultural area. Dutch plant names are in order of appearance: glanshaver, harig wilgenroosje, riet, duizendblad, grote brandnetel, akkerdistel, vogelwikke en grote wederik. (n = 1 replicate plant species. Biological replicate plant material is still available).

False oat-grass (glanshaver) shows the highest Cr(III) concentrations in the root, followed by hairy fireweed (harig wilgenroosje), reed (riet) and yarrow (duizendblad). The root concentrations follow also the rhizosphere soil Cr(III)-concentrations. Though despite high Cr(III) in the soil, common nettle, thistle, bird vetch do not take up or bind Cr(III) strongly to the roots. So, for phytostabilization the first plants sequestering Cr(III) in the roots, with only little or no translocation to the shoots (see also **Table S2**) are most interesting.

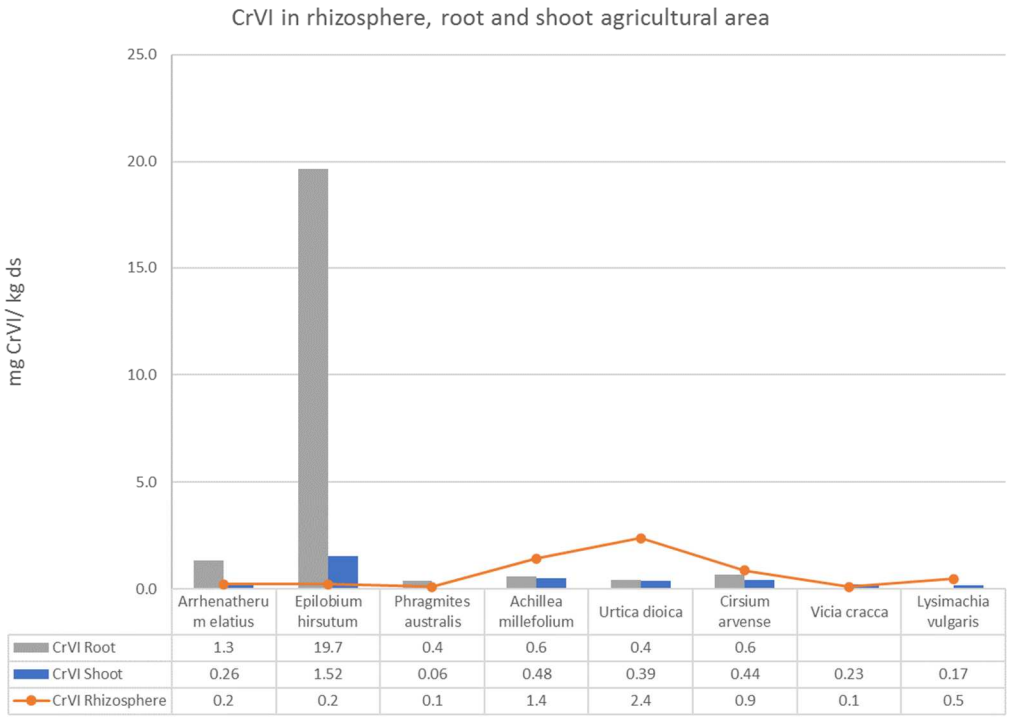
The plant with the highest Cr concentration bound to the roots in the nature reserve area is *Dryopteris filix-mas* or male fern (mannetjesvaren) (**Table S2 and Figure 4**), followed by broadleaf plantain (grote weegbree), reed (riet), thistle (akkerdistel), and white willow (schietwilg). The root Cr-concentrations are in relation to the soil Cr-concentrations around the roots (very high for the fern because it was growing on the river border). Despite having high Cr concentrations in the rhizosphere, the *Rumex* sp. (zuring) does not seem to take up much Cr. Every plant species was measured only once because this was a first open screening. In spring this can be repeated to confirm the findings with more replicate plant species sampled over a larger area (five replicates).



	Dryopteris filix-mas	Plantago major	Phragmites australis	Cirsium arvense	Salix alba	Urtica dioica	Ranunculus repens	Rumex obtusifolius	Valeriana officinalis	Convolvulus sepium
CrIII Root	1300.0	280.3	120.1	89.0	35.5	21.8	20.1	8.0	3.6	
CrIII Shoot	12.0	15.2	1.4	0.3	1.2	2.0	1.5	0.3	15.0	85.7
CrIII Rhizosphere	1169.3	1073.1	1270.1	533.0	711.8	220.6	145.7	730.5	141.4	141.4

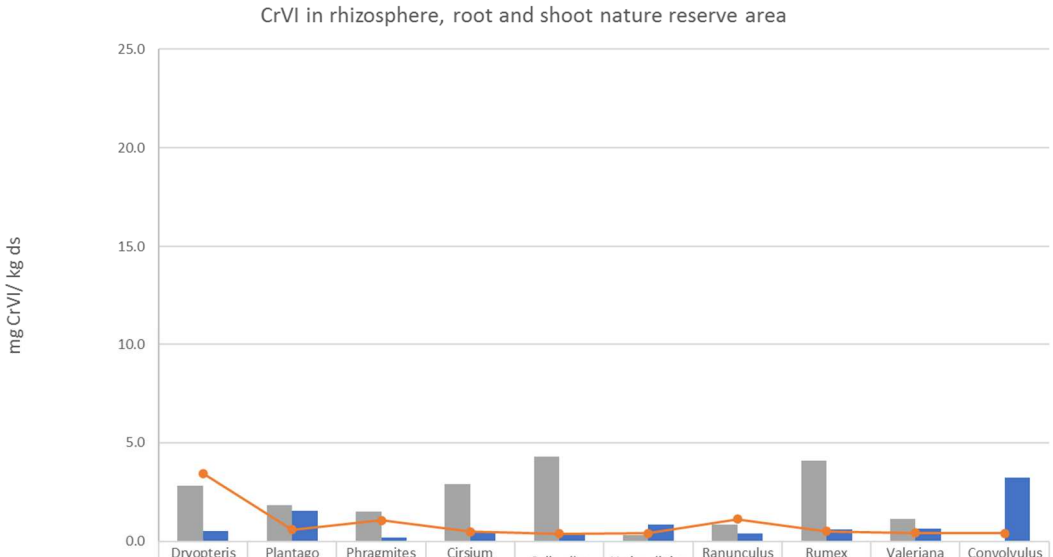
Figure 4: Cr(III) concentrations in rhizosphere soil, root and shoots of plants in the nature reserve area. Dutch plant names are, respectively from left to right: mannetjesvaren, grote weegbree, riet, akkerdistel, schietwilg, grote brandnetel, kruipende boterbloem, ridderzuring, echte valeriaan, haagwinde. (n = 1 replicate plant species, more biological replicates will be analysed).

a.



	Arrhenatherum elatius	Epilobium hirsutum	Phragmites australis	Achillea millefolium	Urtica dioica	Cirsium arvense	Vicia cracca	Lysimachia vulgaris
CrVI Root	1.3	19.7	0.4	0.6	0.4	0.6		
CrVI Shoot	0.26	1.52	0.06	0.48	0.39	0.44	0.23	0.17
CrVI Rhizosphere	0.2	0.2	0.1	1.4	2.4	0.9	0.1	0.5

b.



	Dryopteris filix-mas	Plantago major	Phragmites australis	Cirsium arvense	Salix alba	Urtica dioica	Ranunculus repens	Rumex obtusifolius	Valeriana officinalis	Convolvulus sepium
CrVI Root	2.8	1.8	1.5	2.9	4.3	0.3	0.8	4.1	1.1	
CrVI Shoot	0.54	1.54	0.19	0.51	0.36	0.86	0.41	0.62	0.64	3.23
CrVI Rhizosphere	3.5	0.6	1.1	0.5	0.4	0.4	1.1	0.5	0.4	0.4

Figure 5: Cr(VI) concentrations in the rhizosphere, root and shoot of plants in the agricultural area (a) and nature reserve (b). (n = 1 replicate plant species).

The results of hexavalent chromium (Cr(VI)) concentrations in plant root and shoot tissues (**Figure 5**) shows only one 'outlier' that is hairy fireweed (*Epilobium hirsitum*) for the agricultural area with 20 mg Cr(VI) per kg dw. Analysing Cr(VI) in plant material is challenging, because Cr(VI) reacts very

fast with organic material. So, we do not know the 'true' Cr(VI) concentrations (SGS), and thus Cr(VI) measurements in plants is not a recommended analysis. Compared to the phytotoxicity threshold in plants, Kabata-Pendias et al., 2000 speak about 5-30 mg Cr/kg DW as being phytotoxic, and 0.45-2.7 mg Cr/ kg FW. Hence all shoot Cr concentrations are still under this previously set norm (Kabata-Pendias, 2000).

Comparing and integrating ABOs soil Cr analyses data with plant, rhizosphere soil Cr analyses, we can make the following interpretations. First, based on the soil analyses and distance to the riverbank (**Figure 6**), we see that the highest concentrations, or risk is at 1, or 2 m from the riverbank, which is expected with where the sludge is laid down after cleaning of the stream.

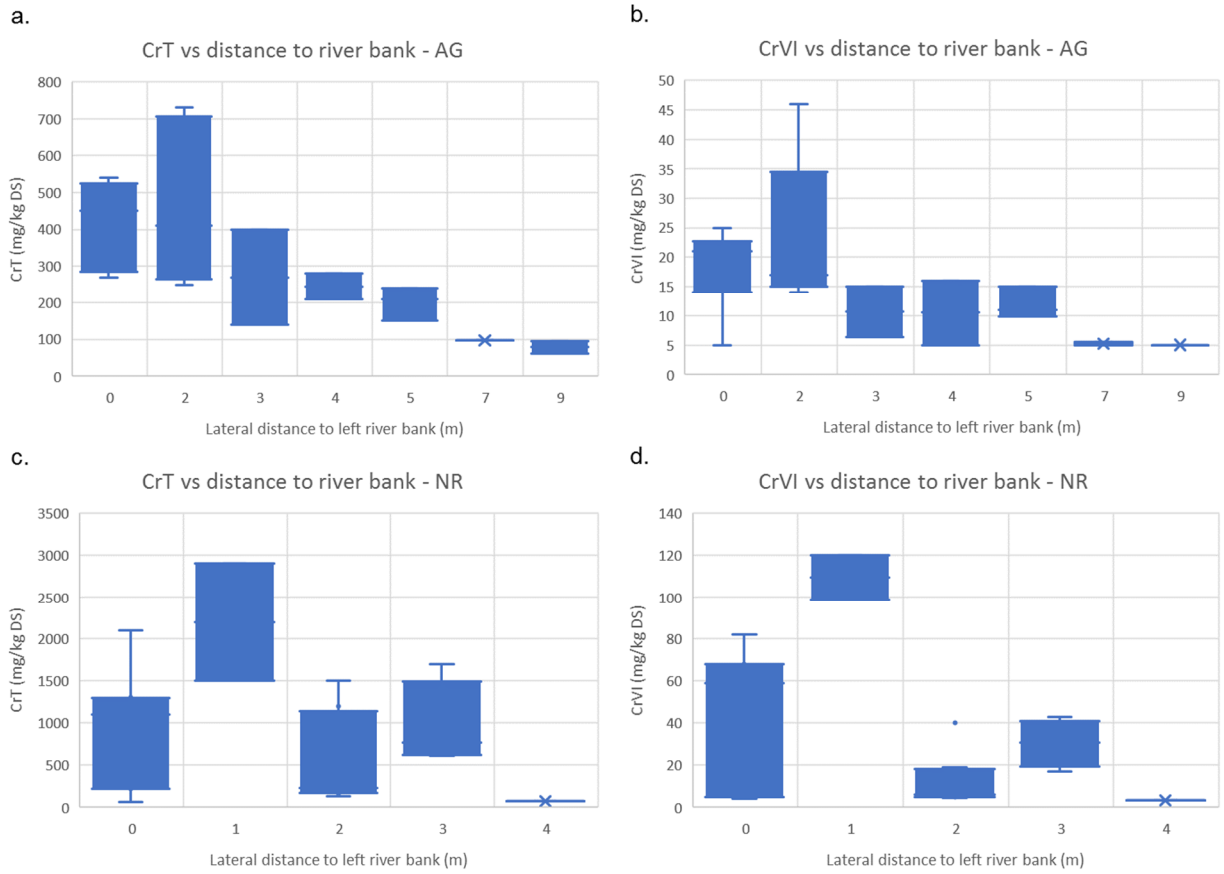


Figure 6: Total Cr (CrT) and Cr(VI) concentrations in the soil in function of the lateral distance to the river.

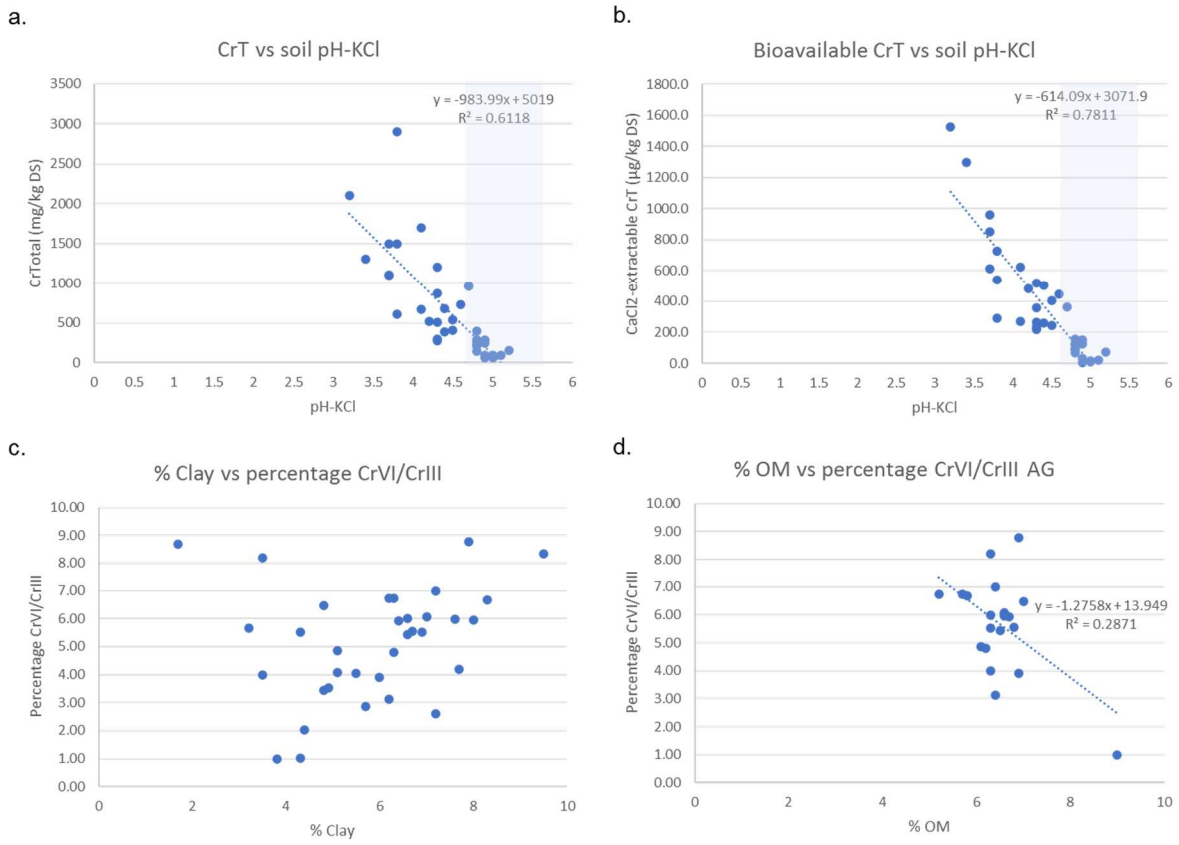


Figure 7: Soil CrTotal (CrT) versus pH-KCl (a), bioavailable CrT vs soil pH-KCl, and the percentage Cr(VI)/Cr(III) to %organic matter (OM) and clay.

There is a negative correlation between pH and total or bioavailable chromium concentration in soil, whether this is a true effect of pH on the form of Cr, or a co-occurrence that high Cr-pollution goes together with lower pH is also possible, because the tannery industry used acids in the production process of leather. The nature reserve area with highest Cr-concentrations had also the lowest pH, on average 3.9 ± 0.27; while the pH of the agricultural soil is on average 4.7 ± 0.46 (**Figure 8 and table S1**). The effect of increasing pH-KCl from 3.9 to 5.5 on Cr-bioavailability can be empirically tested in a pot experiment to better understand the effects of a higher pH on the Cr-form. Cr(III) is only limited mobile or bioavailable and is naturally present in insoluble complexes at pH < 4. At higher pH, Cr(III) is more present in the hydrolysed forms, but also not very mobile and complexed to organic matter (Ertani et al., 2017). There are more forms of Cr than Cr(III) and Cr(VI), but the previous mentioned ones are the most occurring and stable in the environment. Cr(VI) is in contrast to Cr(III) very mobile, it forms soluble organic compounds. It is usually present in association with oxygen to form chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$), these are the compounds that are extremely toxic and carcinogenic to all living organisms.

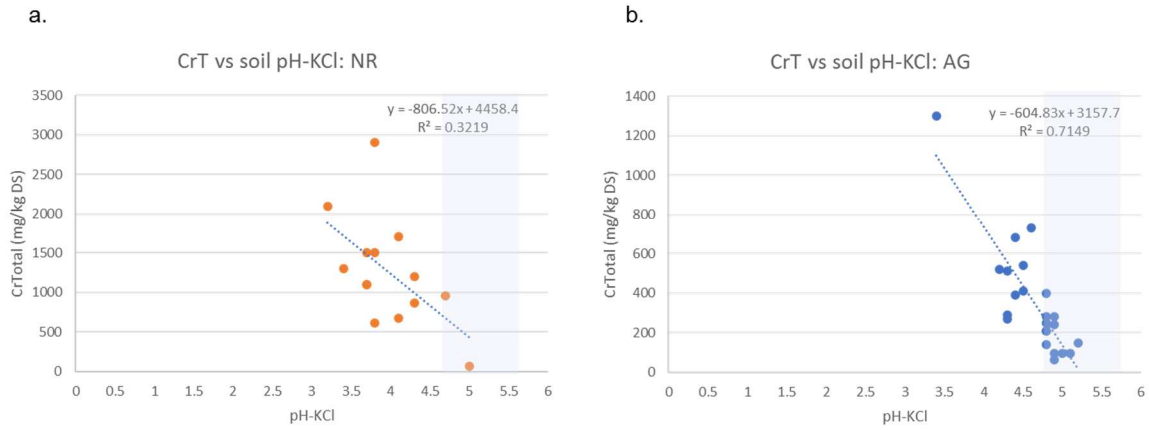


Figure 8: CrT versus pH-KCl for the nature reserve area (NR) and agricultural soil (AG). In light blue are the optimal pH-KCl values indicated for nature reserve soil.

Besides pH, there is also a negative correlation between the % of organic matter and CrT. This is interesting as it has been described before that the reduction of Cr(VI) to Cr(III) is enhanced in soils with high organic matter content and under acidic conditions (Deng and Stone, 1996). The opposite reaction can also happen but is less likely, the oxidation of Cr(III) to Cr(VI) in the presence of oxygen and manganese oxides. Cr(VI) is much more toxic and water soluble than Cr(III), so the oxidation of Cr(III) to VI should be avoided. Mn mediates the transfer of electrons between Cr(III) and the oxygen in the air, and other studies have shown that the amount of Cr(III) that is oxidised to Cr(VI) is proportional to the reduction rate of Mn.

To interpret the results with the current soil remediation norms for Cr, we see that both sites significantly exceed the soil remediation norm (BSN) for total Cr (**Table 1, 2 and 3**), and that also the norm for Cr(VI) is exceeded. The agricultural area exceeds 2.4 times the BSN for total Cr concentration (311 mg/kg vs 130 mg/kg norm), and the nature reserve area has on average 909.36 mg/kg total Cr, with a maximum of 2900 mg CrT/kg, about 20 times more than the norm. With an average of 14.8 mg Cr(VI)/kg dw (maximum: 68 mg/kg), the norm for Cr(VI) of 9.3 mg/kg is exceeded 1.5 times for the agricultural area, and almost four times for the nature reserve area, with an average of 34.24 mg Cr(VI)/kg dw (maximum: 120 mg Cr(VI) /kg dw).

Table 1: Soil remediation norm of Cr

BSN	BSN (mg/kg ds) type II (agriculture area)	BSN (mg/kg ds) type III (living area)	BSN (mg/kg ds) type IV (recreational area)	BSN (mg/kg ds) type V (Industrial area)
Cr(III)	70.3	240	746	11944
Cr(VI)	6.6	4.3	6.6	3.7

Ecotoxicological BSN

Cr(III)	560	560	560	880

From the document: "Voorstel voor herziening bodemsaneringsnormen voor chroom, OVAM, 2010".

Table 2 : Target value for the soil remediation norm for CrTotal (destination type I, nature reserve, in mg/kg ds)

	Abbrev.	**Target value (RW)**	**Soil remediation norm (BSN)**
[Cr] determined by chemical analyses (in mg/kg)	[Cr]	91	130

Table 3: Target value and soil remediation norm for Cr(VI) (agricultural are, in mg/kg ds) (more stringent than the recreational area).

	Abbrev.	**Target value (RW)**	**Soil remediation norm (BSN)**
[Cr(VI)] determined by chemical analyses (in mg/kg)	[Cr(VI)]	6.5	9.3

*source: from ABO

An interesting calculated metric is the ratio of Cr(VI) to Cr(III) (**Figure 9**). Graphs in **Figure 9** show the percentage of Cr(VI)/Cr(III) and % of OM in **the bulk soil** for the agricultural area (a) and the nature reserve (c), while in b and d, the percentage of CrVI/CrIII **in the rhizosphere** is shown. From this we can see that while the Cr(VI)/Cr(III) percentage is around 6 for the bulk soil samples, the rhizosphere soil samples have ratio's close to 1. Thus, proportionally less Cr(VI) is found in the rhizosphere of plants than in the bulk soil. This can be related to the presence of more organic substances in the plant rhizosphere *i.e.*, release of root exudates, which can easily reduce Cr(VI) to Cr(III). Thus, for the investigated plant species, a vegetation border at the Grote Calie is beneficial to phytostabilise Cr. The lower the Cr(VI)/Cr(III) ratio is the more interesting the plant species is for phytostabilization (see **Figure 9, b and d**). To confirm the reducing power, the reduction potential (Eh) in the rhizosphere can be measured, though this is not evident. There is high spatial and temporal variability, *e.g.,* rice roots could increase Eh of over 300 mV and exert effect to 4 mm distant from the root. Eh in a soil aggregate core can also be 200 mV higher than on the surface. So, you must adapt the technique to deal with the high variability.

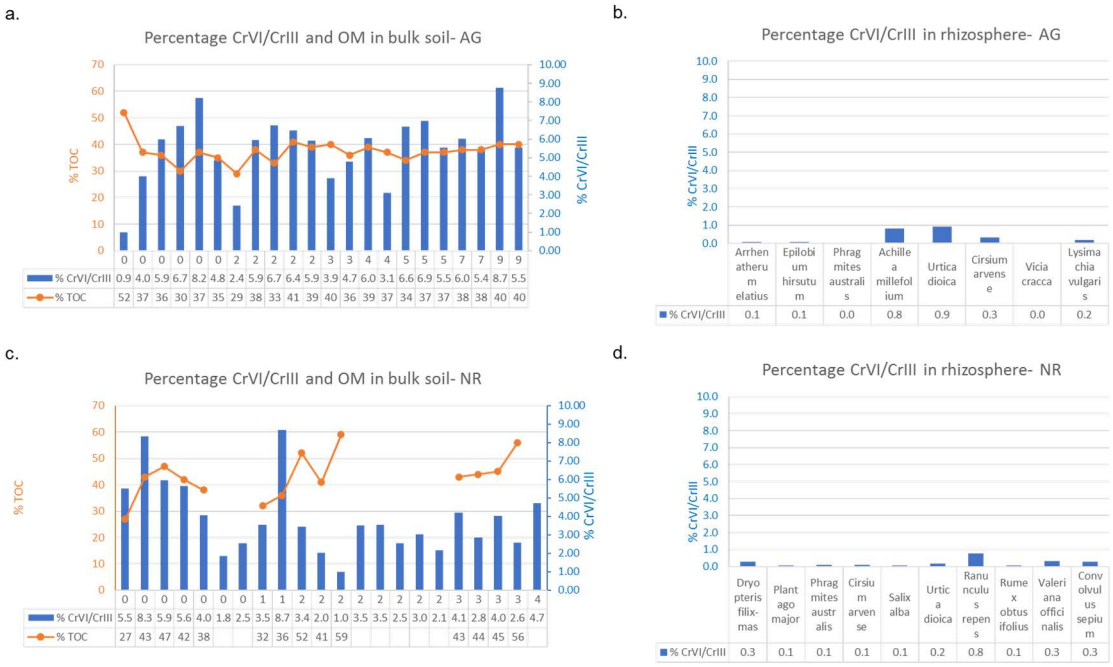


Figure 9: Percentage Cr(VI)/Cr(III) in relation to soil organic matter content. (a) Showing this relationship for the agricultural area, and (c) the nature reserve. On the right, the rhizosphere Cr(VI)/Cr(III) concentrations are shown for (b) agricultural area and (d) nature reserve. AG: agricultural area, NR: nature reserve.

Isolation and characterisation of Cr-reducing PGP-bacteria

Twenty bacterial strains grew in liquid media tolerating 100 mg/L Cr(VI). The concentration of Cr(VI) was chosen based on the Cr(VI) concentrations in the field (highest of 120 mg Cr(VI)/kg in the nature reserve area). The positive Cr-tolerant bacteria were subsequently subjected to indole acetic acid production test.

Six strains (tested in triplicate) scored positive for indole production. Indole biosynthesis is a characteristic that is relatively widespread among plant-associated bacteria (Spaepen et al., 2007). Indole belongs to the auxin phytohormones and has shown before to have phytostimulatory effects. In particular, the production of IAA stimulates root growth and the proliferation and elongation of the root hairs. It is believed that bacteria produce this hormone to interact with plants as part of their colonisation strategy, and/or to confer phytostimulation, or to escape the basal plant defence mechanism (Spaepen et al., 2007). Using 16S rRNA gene Sanger sequencing we found that all isolates belonged to the genus *Bacillus*, which is interesting because *Bacillus* spp. have been isolated before that showed hyper-tolerance to Cr(VI) of up to 15 000 mg/l, and when inoculated to *Prosopis laevigata* (mesquite trees), the bacteria showed to enhance Cr phytoremediation (Ramírez et al., 2019).

Based on the positive test results of both Cr-tolerance and IAA-production, we chose for *Bacillus* sp. Narmena_19 for the inoculation of the pot experiment. This was done by pouring the bacterial

inoculum on top of the soil near the roots, as described in the material and methods. Non-inoculated controls were taken along, as well as no plant controls.

Greenhouse pot experiment

The schematic of the pot experiment is shown in **Figure 10**. All plants for the pot experiment showed good growth, see **Figure 11**. After 2 months, the bacteria inoculum treated pots received an inoculum with the *Bacillus* sp. N19 and grown for additional month prior to soil and plant analyses.

Location	Agricultural area			
Plant sp.	*Achillea millefolium*		*Epilobium hirsutum*	
Treatment	Inoc	nonInoc	Inoc	nonInoc
No amendment	3	3	3	3
Portland cement	3	3	3	3
Basalt	3	3	3	3

Location	Nature reserve area				
Plant sp.	*Phragmites australis*		*Dryopteris filix-mas*		No plant control
Treatment	Inoc	nonInoc	Inoc	nonInoc	nonInoc
No amendment	3	3	3	3	3

Figure 10: Schematic of the greenhouse pot experiment. Numbers are the replicate pots.

Figure 11: Pictures of the plants used in the pot experiment growing for 2 months in Cr-polluted soil.

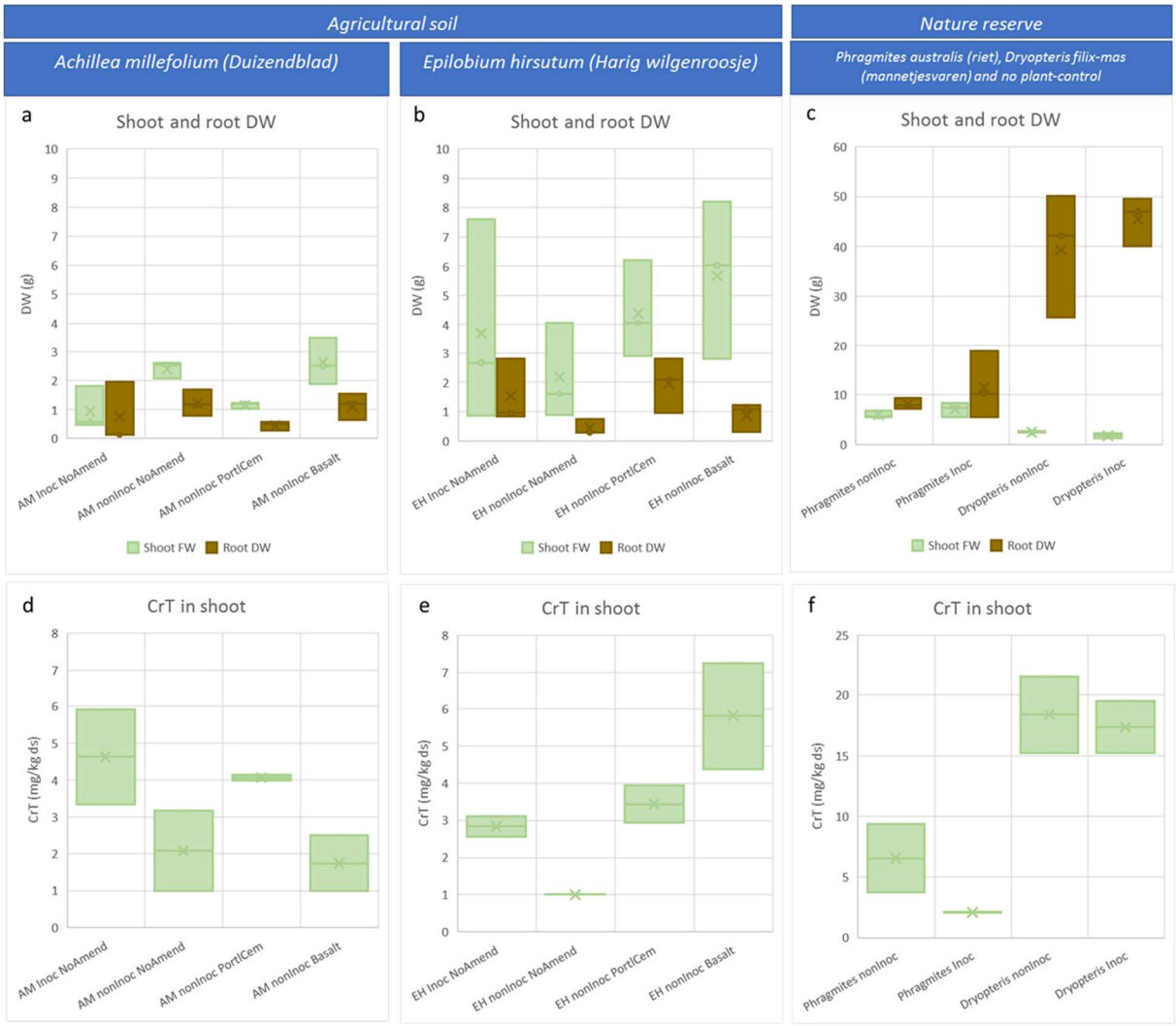


Figure 12: Above- and belowground plant biomass and Cr(T) concentrations in the shoot. Abbreviations: AM: *Achillea millefolium*; EH: *Epilobium hirsutum*. (n = 3 plant pot replicates, for shoot analyses 2/3 replicates were send for analyses).

As can be seen from **Figure 12**, the plant with the highest root biomass is fern (*Dryopteris filix-mas*), followed by *Phragmites australis*. The most variable shoot biomass is observed for *Epilobium hirsutum*. We have equally divided the plants over the treatments but because it were pre-grown wild plants there was variation in above-and belowground biomass between plants from the beginning of the experiment. So, the plant biomass is merely informative to get an idea of the size of the plant we worked with (see also **Figure 11**).

The total chromium concentration in the shoots (**Figure 12 d, e, f**) is better interpretable than the biomass. First the total Cr concentration is presented because this is the chemical-extracted Cr (Cr(III) + Cr(VI)) by the LUFA-lab using acid extraction. No separate Cr(VI) extraction was possible on the plant tissue (AL-West), or not recommended (SGS) because Cr(VI) reacts away very fast to Cr(III) in an organic matrix as plant tissue. First the highest Cr concentration was observed in the shoots of *Dryopteris* (15-20 mg CrT per kg soil), and this is followed by the three other plant species,

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which have on average 4.3; 4.0; and 3.8 mg Cr per kg dw shoot biomass for *Phragmites*, *Epilobium* and *Achillea* respectively (see also **Table S3**). There is more plant-to-plant variation in total Cr then a significant treatment effect, so within one plant species, there is no statistical effect of either the inoculation or the mineral amendment on the shoot CrT concentrations. The strongest effect visible from this pot experiment is the difference between plants (*e.g.* the large difference between *Dryopteris* and the other plants). For observing inoculation and amendment effects either many more plant replicates need to be considered for the pot experiment because of the natural variability of the plant biomass/phenotype at the start of the pot experiment, or repeating the experiment with *e.g.* similar sized willow cuttings to reduce the inter-plant variability, and so the inoculation and amendment effect will be the single varying factors. This range-finding pot experiment was also useful to understand that after 3 months, it is possible to measure Cr in the aboveground plant tissues (given we had to remove and wash the soil from the roots, and that the plants needed to establish new roots in the Cr-polluted soil and this takes some time). Further, the CrT concentrations in the shoots measured are in the range of the concentrations we measured for the plants in the field. We focussed initially on the aboveground CrT concentrations, but dried root samples are still left from the pot experiment which can be analysed.

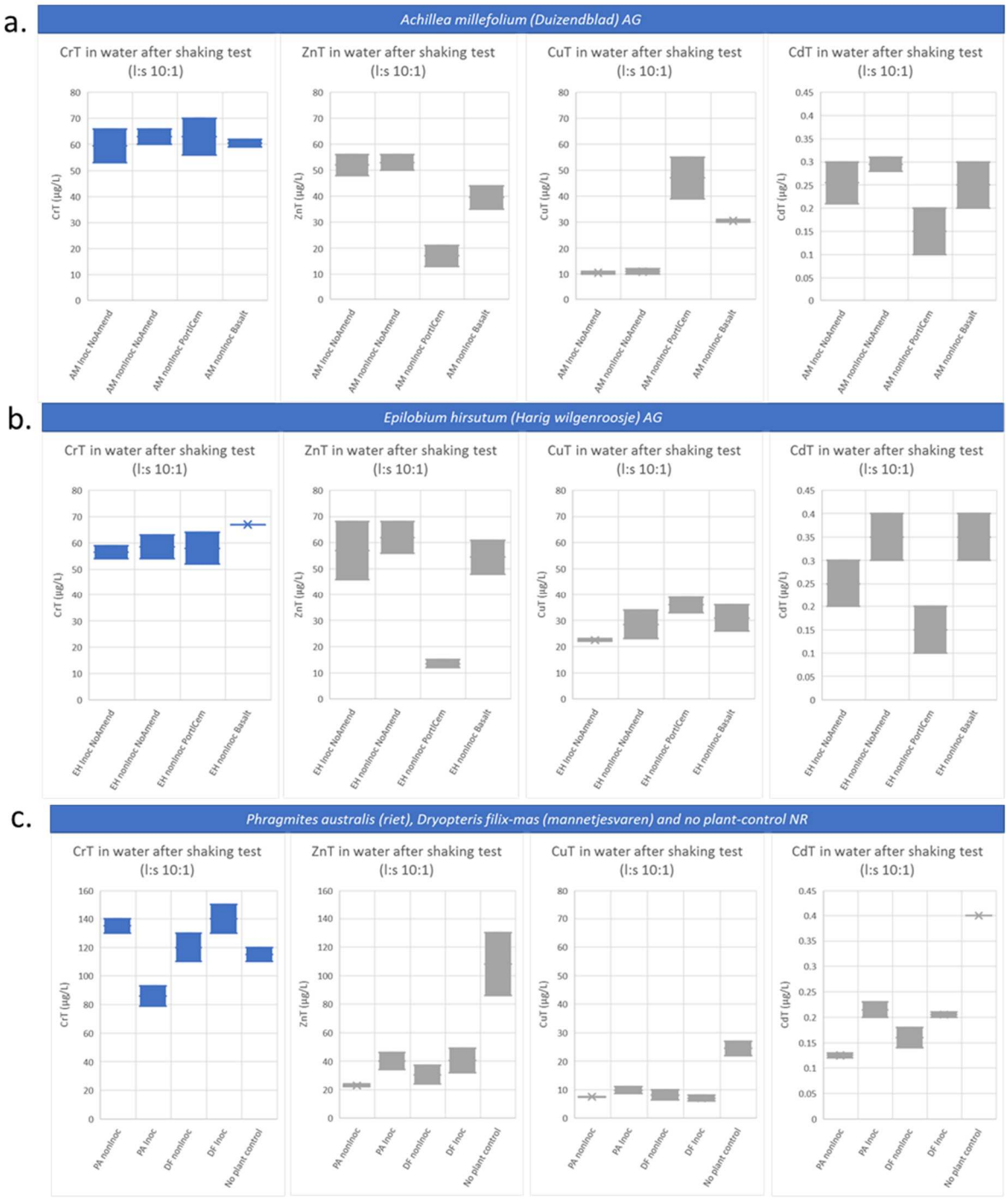

Figure 13: Concentrations of total Cr, Zn, Cu and Cd in the leachate of the soil after the EUR2 (LS 10:1) 24h shaking test. (n = 3 plant pot replicates). The total soil Cr concentrations was for the agricultural soil, for Cr(III) and Cr(VI) respectively (in mg/kg): 420 mg, and 11; whereas for the nature reserve soil: 880 and 26.

At the end of the experiment, we also performed a shaking test to measure the 'availability' of Cr in the soil, and the effect of the inoculant and/or minerals on this (**Figure 13**). Except for *Phragmites*, there is no significant effect of inoculation on the Cr in the leaching water. For *Phragmites*, inoculation reduced the total Cr concentration in the leachate. When bacteria can enhance Cr complexation, and/or making it less bioavailable by enhancing complexation in plant tissues, the risk for leaching

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and off-site migration is reduced. Previously, a study proposed that the Cr that remained in an oxidized form in the soil and cannot be extracted from a 10 mM solution of $(KH)_2PO_4$ at pH 7.2, is considered immobilized, because of precipitation or strong absorbance. So here the shake test does something similar and estimates how much Cr can be released from the soil after 24h shaking at neutral pH, the less leached, the more it was in an immobilised form.

To be complete, also the results of the leaching of other trace elements (Zn, Cu, Cd) from soil is shown (**Figure 13**). Interesting to note is that much more Zn, Cu, Cd is leached from a non-planted soil compared to planted soils, as expected, but for Cr this effect between planted and non-planted soil is not so clear, perhaps because of the excess Cr in the soil (440 and 880 mg/kg) that gets released from the soil fast, even when plants take up Cr form the soil matrix, while Zn, Cu, Cd sequestrated in the plant biomass, is not 'refilled' from the element pool. The different oxidation states that Cr can take, may also mask the effects or complicate interpretation of the results. Portland cement interestingly reduces Zn and Cd leaching, so for Cd/Zn polluted soils this amendment is promising. The reduced leaching can be explained by the increase in soil pH, from pH 6 to 8, **Figure 14**. The addition of 3 % basalt did not change soil pH compared to the non-amended controls. Portland cement also increased the soil electrical conductivity, that is explainable because Portland cement is rich in CaO (67 %) that is released to the soil upon weathering. Basalt has a larger proportion of SiO_2, besides CaO (15 %) and MgO (10 %), the chemical composition is given in **Table S5**. To note is that basalt contains also relatively high Cr itself, 93 mg/kg, so this is not the most suitable mineral amendment for a Cr-polluted soil. Portland cement has much lower Cr-content (37 mg/kg).

While Zn, Cd seem to 'respond' to the increased soil pH installed by Portland cement, Cr not. So, increasing soil pH from 6 to 8 at least, does not reduce Cr-leaching. That is also explainable, because Cr-immobilisation and/or Cr(VI) reduction to Cr(III) is higher in acidic soils (pH 4) (Deng et al., 2003; Ertani et al., 2017). In the pot test, we also see that the pH is 6 of the agricultural and nature reserve soil, while in the field the pH-KCl was 4.5 and 3.9, respectively. It is commonly observed that soil pH in pots is very different from the field, because of carbonation/oxidation/reduction reactions that initiate upon mixing, homogenising, and filling the pots (trait-off between homogenising the soil and deviation from the field condition). An alternative is to work with soil cores in pot experiments to minimally disturb soil processes and pH, this works for starting plants from seeds, but is hard if we transplant pre-grown plants.

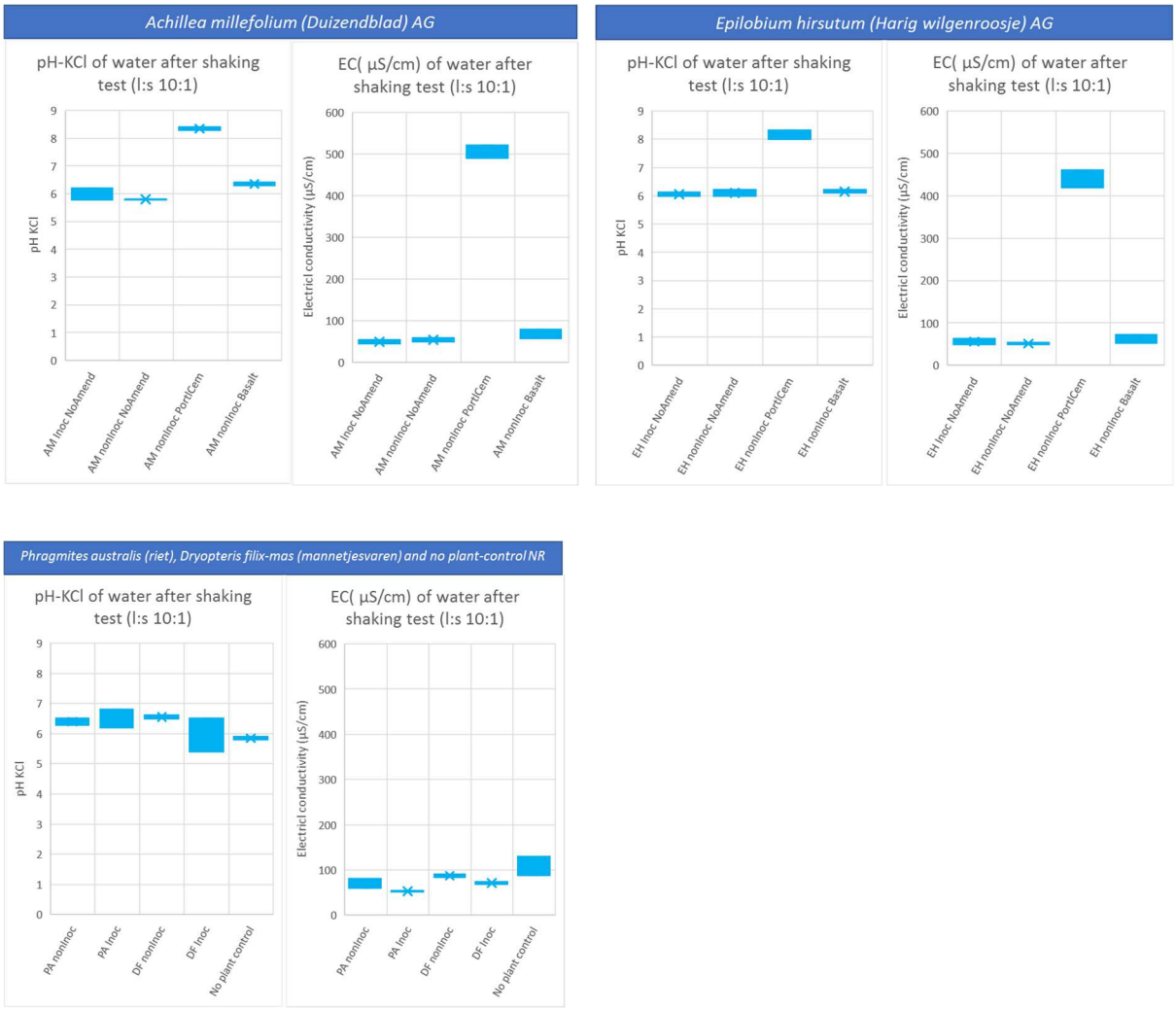

Figure 14: pH-KCl of the leaching water and electrical conductivity.

Figure 15: Bacterial community structure composition at the order level in the rhizosphere of *Epilobium hirsutum* (EH). (n = 3 replicate pots). Abbreviations: inoc: inoculated. NoAmend: no amended. PortCem: Portland Cement.

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Rhizosphere soil samples were also collected at the end of the experiment to track the inoculated bacterium and observe if the mineral amendments changed the overall rhizospheric bacterial community composition (**Figure 15**). Soil microbes are key to nutrient and metal cycling, plant growth, vigour, and health, so changes in the ratio of bacteria:fungi, or on a smaller scale in individual members (relative abundance or presence/absence of certain taxa) can influence plant Cr-uptake. Inoculation did not lead to pronounced differences in bacterial community composition at the order level (**Figure 15**), 1 month after inoculation, so either the inoculated *Bacillus* sp. (order of Bacillales) did not establish itself well in the existing microbial community, is < 2 % abundant in the soil, or we did not see the Bacillus effect anymore 1 month after inoculation. *Epilobium* itself can exert a strong selective pressure and/or its native rhizosphere community is maybe not easily affected or disturbed by exogenous application of *Bacillus*. The fact that the rhizospheric communities are similar, can explain also why we do not see an inoculation effect on the shoot Cr concentrations or Cr-leaching. We plan to also sequence the other plant rhizospheres to see if the positive effect on Cr-leaching by *Phragmites* inoculation can be explained by inoculation. We do see a pronounced effect of Portland cement on the microbial community composition, with a higher abundance of the Xanthomonadales, Chtoniobacterales, Frankiales, and Burkholderiales in the disadvantage of other taxa which made up the smaller groups. This reduced and skewed diversity is due to the increase in pH, which has a significant effect on which bacteria flourish/survive in the soil. We do not know yet the full consequences of this bacteria-shift in the Portland cement condition for plant growth and Cr-uptake, as longer term, time-series studies are needed.

Metagenomics insights in the total bacterial communities of the field

The total DNA was extracted from the rhizosphere soil of the plants growing in the field and subjected to an open screening with shotgun DNA metagenomics to find Cr-cycling genes. Ten representative plant species were selected, and in total 143 Gbp DNA was obtained or 482,292,694 reads (analysed on the Tier2 Genius cluster, 36 threads, 195 Gb RAM, 360 hours of analyses, VSC).

First protein queries were constructed from proteins and genes found in papers describing bacteria with Cr tolerance and reductase genes (Ramírez et al., 2019) and a KEGG orthologs table was constructed (**Table 4**). From these KEGG orthologs, a Hidden-Markov-Model (HMM) model was obtained from the KOFAM database, and this was used to query the metagenome, using MetAnnotate.

Table 4: KEGG Ortholog table with bacterial genes related to chromium

Proteins related to chromium metabolism in bacteria		
KEGG-ortholog (KOFAM)	**Function**	**Gene name**
K07240	Chromate transporter	chrA
K07240	Chromate transporter	chrA
K02045	Chromate resistance protein, sulfate/thiosulfate transport system ATP-binding protein	cysA, chrB

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K19784	NADPH-dependent FMN reductase family protein	chrR, chrT
K11753, K00861	Riboflavin kinase/FMN adenylyltransferase	ribF
K01118	FMN-dependent-NADH-azoreductase	acpD
K01118	FMN-dependent-NADH-azoreductase	acpD
K10679	Nitroreductase	nfsB
K10678	Nitroreductase	nfsA
K09019	NAD(P)H nitroreductase	rutE

The results (**Figure 16**) show in the first place that there are bacteria in the rhizosphere of all plant species that contain one or more copies of chromate transporters and resistance proteins. It seems that *Achillea millefolium*, followed by *Phragmites* (only ChrT), *Dryopteris*, and *Ranunculus repens* contain the highest abundance of ChrA, ChrB, ChrT bacterial genes in their root zone, mostly affiliated to Rhizobiales, Burkholderiales, and Streptomycetales, and unclassified (*i.e.* Cr genes belonging to bacterial taxa not described before). This result is positive as it shows that the plant species in Turnhout have already an association with bacteria harbouring chromate transporters and resistance genes. This can maybe explain why both *Dryopteris* and *Phragmites* can tolerate or take-up higher amounts of Cr in roots, and shoots, than the other plants. *Dryopteris* was sampled from the nature reserve area, and the *Achillea* and *Phragmites* from the agricultural area. Further customised bioinformatics software is needed to investigate the DNA-sequences for Cr-tolerance genes. This is the first study ever applying deep sequencing to a Cr polluted soil, and in which we discovered novel chromate reducing bacteria. Only, the effect of Cr nanoparticles on bacteria has been described this year (Zheng et al., 2020) but insights into the effects of high levels of Cr-pollution on the diversity of microbes from the field has not been described before.

Genome studies have shown that the proteins involved in the reduction of Cr(VI) to Cr(III) in bacteria belong to the classes of the NAD(P)H-dependent flavoprotein reductase, dehydrogenase, nitroreductase and azoreductase (Mugerfeld et al., 2009; Sarangi and Krishnan, 2016; Dong et al., 2018). For example, the chromate transporter A (ChrA), is a transmembrane protein, which pumps Cr(VI) outside of the cells, thereby reducing the toxicity for the strain. The function of ChrB is still a bit unclear, but studies show that when ChrB is present, the bacterial resistance to Cr(VI) is enhanced in bacteria as *Shewanella* (Mugerfeld et al., 2009). It is hypothesised that the protein plays a role in reducing Cr(VI) to III before it is pumped out of the cell. Since an substantial set of the Cr related genes are found in the bacterial metagenome of the plants, it suggests that the plants must rely in way or another on these bacteria to grow and survive in the high Cr-polluted soil.

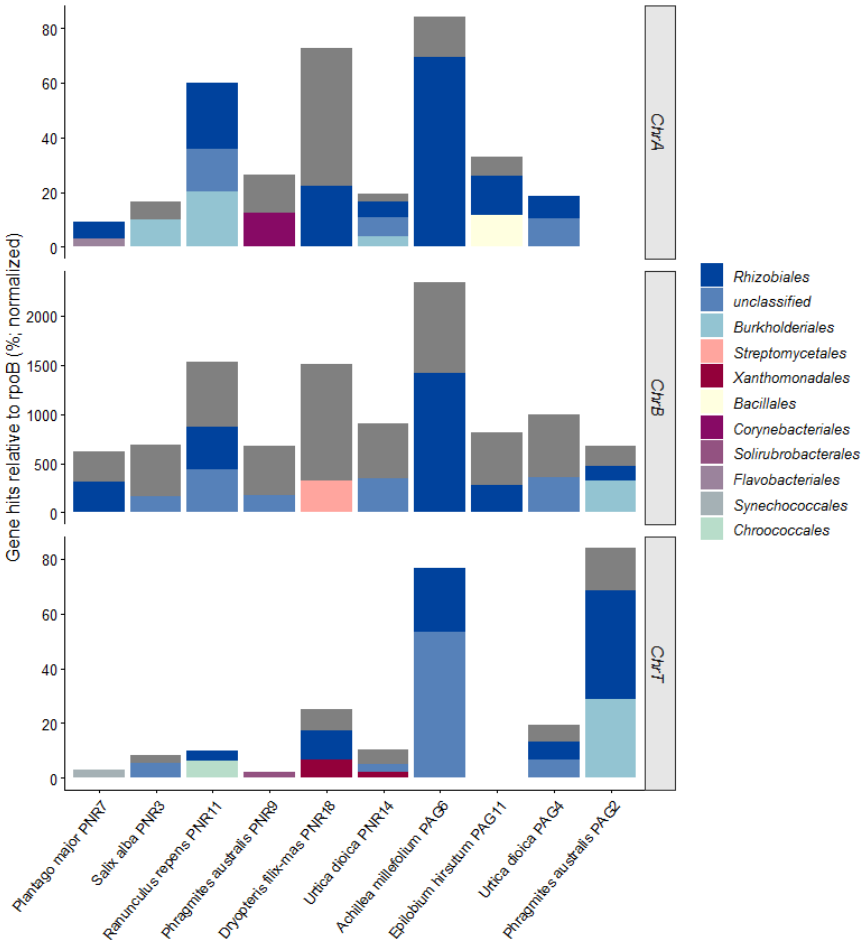

Figure 16: Hits with the chromate transporter A, B and NADPH-dependent FMN reductase family protein (ChrT) in the rhizosphere of plants sampled in Turnhout.

Lastly, a taxonomic overview is given of the distribution of DNA-reads that we picked up in all 4 domains of life, majority of bacteria, and archaea, followed by Eukaryota (plants), and then also some viruses. A further breakdown of the phyla and orders of the bacteria is shown (**Figure 17**), which shows that the dominant bacteria belong to the Proteobacteria phylum and Actinobacteria, and on the order level, the Rhizobiales and Burkholderiales. Rhizobia are known for their nitrogen fixation and high pollutant tolerance. *Salix* seems to have more Rhodobacterales present in the rhizosphere, what this means needs to be further investigated.

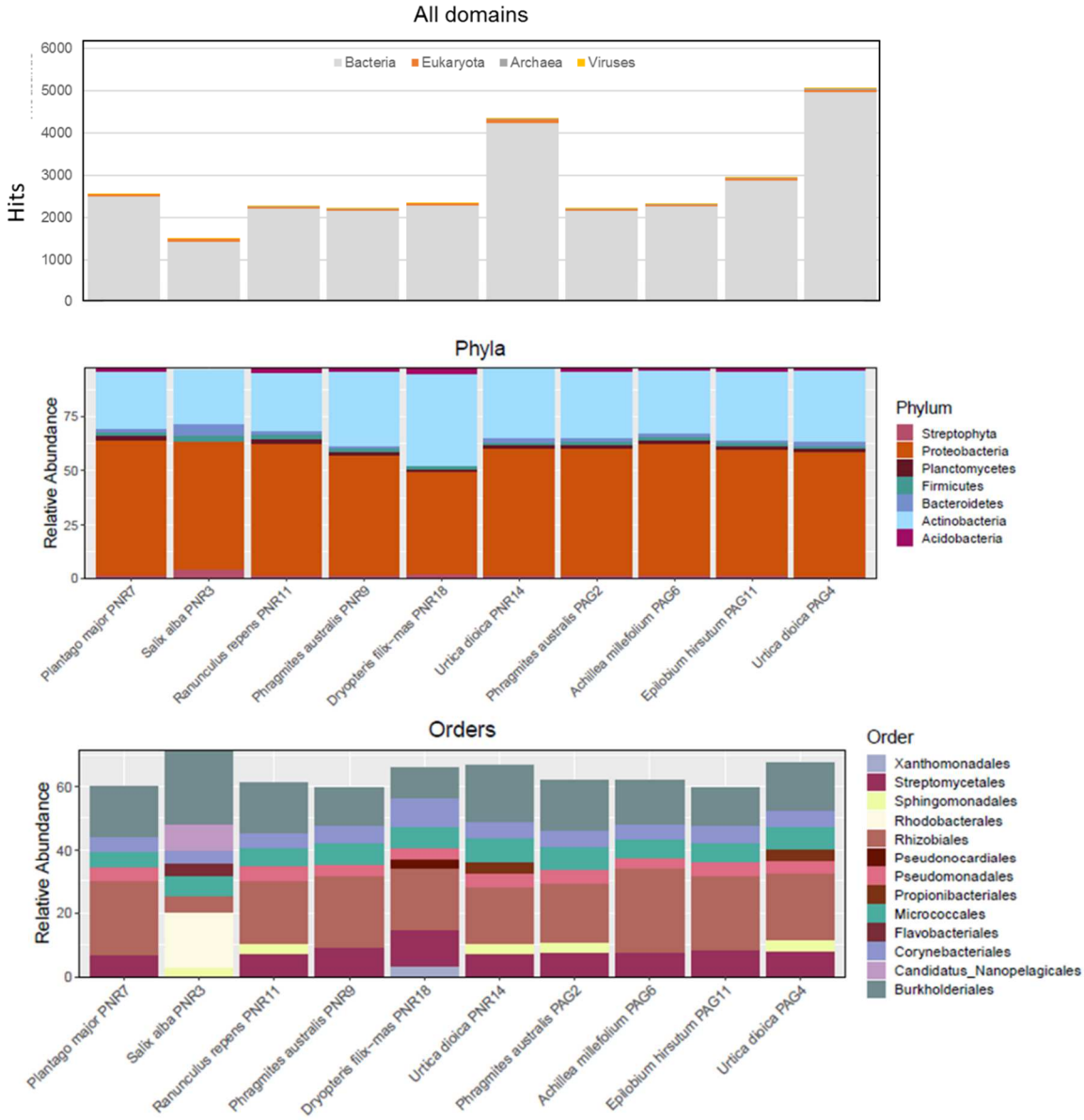

Figure 17: Taxonomic classification of the metagenome reads in the 4 domains of life, bacterial phyla and orders (using Kraken2), see here for interactive charts.

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General conclusions

To sum up the most important findings of this feasibility study and assist the Cr-phytostabilization:

1) The plant species to consider for phytostabilization are, in order of highest root Cr concentration, for the agricultural area: *Arrhenatherum elatius, Epilobium hirsutum, Phragmites australis, Achillea millefolium*, and for the nature reserve area: *Dryopteris filix-mas, Plantago major, Phragmites, Circium arvense, Salix, Urtica dioica* and *Ranunculus repens*

2) pH-KCl of both locations is quite low (3.9-4.5). Ideally the soil for the region of Turnhout has a pH-KCl of 5.5. Slight acidic pH is good because it enhances the Cr(VI) to III reduction.

3) The pot experiment showed the highest shoot Cr concentration in *Dryopteris-filix-mas*, providing additional evidence that this plant takes up Cr, hence in a way making it less bioavailable. In autumn when the leaves fall, and decay, Cr can be released again.

4) The minerals portland cement and basalt (applied at 3 % ratio), were not effective to bind Cr and reduce leaching. Portland cement was effective to reduce Zn and Cd leaching though, due to a pH increasing effect.

5) Inoculation with *Bacillus* N19 reduces Cr-leaching in combination with *Phragmites*.

6) The inoculated strain shows *in vitro* plant growth promotion traits and is Cr-tolerant, does not establish itself in high numbers in the rhizosphere of *Epilobium*, or disappears fast (< 2 % relative abundance). Therefore, the success for inoculation in the field is questionable. The highest chance of success is inoculating the bacterium at the time of planting, in the planting hole, to stimulate root growth.

7) The native rhizosphere bacterial communities harbour already Cr(VI) reducing strains. To further increase their population, more *Dryopteris* can be planted, *Achillea*, *Phragmites* or *Ranunculus repens*.

8) Considering the Cr(VI)/Cr(III) ratio, it is better to vegetate the site than to leave it bare, as the rhizosphere contains much lower Cr(VI) to Cr(III) ratio's then non-vegetated soil.

Future

We will repeat the pot test with *Salix alba* cuttings from the field. *Bacillus N19* is currently being genome sequenced, and in its genome, we can search for ChrA, B, genes. More in-depth analyses of the metagenomes to understand the effect of Cr pollution on the composition of the soil microbial communities and functioning is needed. Sending additional replicate root, shoot samples for Cr-analyses.

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Supplementary tables and figures

Figure S1: Photo of the surface collected soil for the pot experiments.

Figure S2: Photos of the plants from the agricultural area. In order of appearance (in Dutch): a. Glanshaver, b. Harig wilgenroosje, c. riet, d. duizendblad, e. grote brandnetel, f. akkerdistel, g. vogelwikke, h. grote wederik.

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Figure S3: Photos of the plants from the nature reserve. In order of appearance: a. mannetjesvaren, b. grote weegbree, c. riet, d. akkerdistel, e. schietwilg, f. grote brandnetel, g. kruipende boterbloem, h. ridderzuring, i. echte valeriaan, j. haagwinde.

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Table S1: Soil physical and chemical data

						Acid extr. total Cr			$CaCl_2$ exrtr Cr	pH KCl	TOC	OM	Clay (< 2µm)	Kjeldahl N	NO3-N	P-Olsen	Ktotal
Sample name	Depth	Latitude	Longitude	Loc.	Lateral distance to the riverbank (in m)	CrIII	CrVI	% CrVI / CrIII	CrT	-	g/ kg ds	% (m/m) ds	%	g/kg ds	mg/ kg ds	mg/ kg ds	mg/ kg ds
						mg/kg ds			mg/ kg ds								
L2	0-20	4.94582984	51.28305444	AG	0	505	5	1.0	517	4.3	52	9	3.8	4.2	6.1	49	4.4
L4	0-20	4.94594961	51.28310618	AG	0	500	20	4.0	481	4.2	37	6.3	3.5	2.3	2.6	56	4.6
L6	0-20	4.94607121	51.28315654	AG	0	368	22	6.0	258	4.4	36	6.3	7.6	3.4	2	42	4.7
L8	0-20	4.94619051	51.28320637	AG	0	253	17	6.7	217	4.3	30	5.2	6.3	2.4	2	34	4.1
L10	0-20	4.94631022	51.28325527	AG	0	268	22	8.2	231	4.3	37	6.3	3.5	3.7	2	25	4.6
L14	0-20	4.94585236	51.2830543	AG	0	515	25	4.9	407	4.5	35	6.1	5.1	0.6	3.1	52	5
L24	0-20	4.94581565	51.28302764	AG	2	664	16		503	4.4	29			3.2	2	41	4.9
L26	0-20	4.94593474	51.28307915	AG	2	387	23	5.9	246	4.5	38	6.6	8	3.7	5.2	43	5.1
L28	0-20	4.94606031	51.28312945	AG	2	684	46	6.7	449	4.6	33	5.7	6.2	3.6	2	28	5
L30	0-20	4.94618378	51.28318175	AG	2	263	17	6.5	151	4.9	41	7	4.8	3.7	2.2	41	4.8
L32	0-20	4.94629345	51.28322796	AG	2	236	14	5.9	119	4.8	39	6.7	6.4	3.5	3.7	32	5.3
L36	0-20	4.94590231	51.28305451	AG	3	385	15	3.9	156	4.8	40	6.9	6	3.5	2	18	5.2
L40	0-20	4.94614804	51.28315561	AG	3	133.6	6.4	4.8	66	4.8	36	6.2	6.3	3.4	4.2	38	5
L47	0-20	4.94598484	51.28307929	AG	4	264	16	6.1	123	4.8	39	6.6	7	3.6	4.6	29	5
L50	0-20	4.94634123	51.28322727	AG	4	160	5	3.1	90	4.8	37	6.4	6.2	3.8	4.7	38	5.4
L51	0-20	4.94578295	51.28298154	AG	5	225	15	6.7	122	4.9	34	5.8	8.3	3.7	3.6	18	5.2
L53	0-20	4.94602291	51.28308338	AG	5	140.2	9.8	7.0	71	5.2	37	6.4	7.2	3.6	4	24	4.7
L55	0-20	4.94626623	51.28318315	AG	5	199	11	5.5	93	4.8	37	6.3	6.9	3.5	6.4	42	5.4
L58	0-20	4.9459528	51.28303134	AG	7	91.5	5.5	6.0	21	5.1	38	6.6	6.6	3.4	2.6	19	5.2
L61	0-20	4.94630804	51.28318011	AG	7	92	5	5.4	32	4.9	38	6.5	6.6	3.4	11	56	5.2
L62	0-20	4.94587481	51.28297909	AG	9	57	5	8.8	7	4.9	40	6.9	7.9	3.7	5.1	18	5.2
L65	0-20	4.94623178	51.28312834	AG	9	90	5	5.6	17	5	40	6.8	6.7	3.1	6.2	46	5.5
N2	0-20	4.94331719	51.27804867	NR	0	1232	68	5.5	1298	3.4	27	4.7	4.3	3.3	8.9	59	5.6
N6	0-20	4.94341141	51.27824479	NR	0	60	5	8.3	12	5	43	7.4	9.5	4.4	2	35	5.6
N8	0-20	4.94345917	51.27834648	NR	0	1038	62	6.0	854	3.7	47	8.1		3.9	2	34	5
N9	0-20	4.94349214	51.27840719	NR	0	1041	59	5.7	961	3.7	42	7.2	3.2	3.6	6.4	42	4.7
N12	0-20	4.94355944	51.27855249	NR	0	2018	82	4.1	1525	3.2	38	6.6	5.1	4.7	5.8	84	6.1

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N14	0-20	4.94365967	51.27871021	NR	0	216	4	1.9	11					7.1	9.8	78	4.9
N17	0-20	4.94380099	51.27901324	NR	0	1170	30	2.6	373					4.1	3.1	60	5.2
N22	0-20	4.94335364	51.27810624	NR	1	2801	99	3.5	726	3.8	32	5.5	4.9	5.2	2	27	5.6
N29	0-20	4.94352763	51.27846481	NR	1	1380	120	8.7	613	3.7	36	6.2	1.7	3.6	2	31	5.3
N35	0-20	4.94340459	51.27817205	NR	2	1160	40	3.4	360	4.3	52	9	4.8	4.2	2	33	5.2
N38	0-20	4.94347749	51.27832618	NR	2	941	19	2.0	362	4.7	41	7.1	4.4	4.5	2.8	47	5.4
N42	0-20	4.94357987	51.27853232	NR	2	1485	15	1.0	539	3.8	59	10.2	4.3	5.6	18	62	4.9
N44	0-20	4.94364812	51.27864951	NR	2	125.6	4.4	3.5	17					6.1	4.4	60	5
N45	0-20	4.94370807	51.27874888	NR	2	183.5	6.5	3.5	19					6.9	4	59	4.8
N46	0-20	4.94376033	51.27885399	NR	2	195	5	2.6	21					4.2	3.4	33	5.8
N47	0-20	4.94380827	51.27895491	NR	2	155.3	4.7	3.0	18					9.9	5.1	28	5
N48	0-20	4.94385539	51.27906562	NR	2	254.5	5.5	2.2	7					6.8	8.9	29	5
N51	0-20	4.9433855	51.2780831	NR	3	835	35	4.2	266	4.3	43	7.5	7.7	4.6	2.5	57	5.2
N55	0-20	4.94348122	51.27828896	NR	3	593	17	2.9	289	3.8	44	7.6	5.7	4	4.9	52	4.8
N58	0-20	4.94356166	51.27844018	NR	3	644	26	4.0	268	4.1	45	7.8	5.5	4.7	7.7	74	4.9
N61	0-20	4.94363299	51.2785967	NR	3	1657	43	2.6	622	4.1	56	9.6	7.2	5.8	7.7	50	5.3
N65	0-20	4.94385902	51.27900536	NR	4	67.8	3.2	4.7	11	1.5				3.3	3.3	29	5.4

*Data from ABO

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Table S2: Cr concentrations in rhizosphere, root, and shoot.

						Rhizosphere			Root			Shoot		
Sample name	**Latin name**	**Latitude**	**Longitude**	**Loc.**	**Lateral distance to the bank (in m)**	**CrIII**	**CrVI**	**% CrVI/ CrIII**	**CrIII**	**CrVI**	**% CrVI/ CrIII**	**CrIII**	**CrVI**	**% CrVI/ CrIII**
						mg/kg ds			**mg/kg ds**			**mg/kg ds**		
Glanshaver PAG8	*Arrhenatherum elatius*	51.283199	4.946097	AG	0.5	318	0.2	0.1	133.8	1.3	1	0.4	0.26	65
Harig wilgenroosje PAG11	*Epilobium hirsutum*	51.283249	4.946272	AG	1.5	316.2	0.2	0.1	80.8	19.7	24.32	1.1	1.52	135.1
Riet PAG2	*Phragmites australis*	51.2832288	4.9461702	AG	0.5	283.8	0.1	0	30	0.4	1.28	1.1	0.06	5.6
Duizendblad PAG6	*Achillea millefolium*	51.2832182	4.946196	AG	2	176.5	1.4	0.8	10.3	0.6	5.5	0.9	0.48	54.6
Grote brandnetel PAG4	*Urtica dioica*	51.2832136	4.9461759	AG	2	260	2.4	0.9	2.4	0.4	17.99	0.9	0.39	45
Akkerdistel PAG13	*Cirsium arvense*	51.2830992	4.9458953	AG	2	270.9	0.9	0.3	1.5	0.6	44.36	0.6	0.44	76.7
Vogelwikke PAG1	*Vicia cracca*	51.2831981	4.9461584	AG	3	283.8	0.1	0				4.6	0.23	4.9
Grote wederik PAG10	*Lysimachia vulgaris*	51.2831995	4.9460997	AG	0.2	277.3	0.5	0.2				0.9	0.17	19.5
Mannetjesvaren PNR18	*Dryopteris filix-mas*	51.2781006	4.9433023	NR	0.5	1169.3	3.5	0.3	1300	2.8	0.22	12	0.54	4.5
Grote weegbree PNR7	*Plantago major*	51.2780883	4.9433169	NR	1	1073.1	0.6	0.1	280.3	1.8	0.66	15.2	1.54	10.2
Riet PNR9	*Phragmites australis*	51.2780165	4.9432726	NR	0.2	1270.1	1.1	0.1	120.1	1.5	1.26	1.4	0.19	13.6
Akkerdistel PNR5	*Cirsium arvense*	51.2780986	4.943412	NR	5	533	0.5	0.1	89	2.9	3.27	0.3	0.51	182
Schietwilg PNR3	*Salix alba*	51.2782911	4.9435084	NR	6	711.8	0.4	0.1	35.5	4.3	12.05	1.2	0.36	29.6
Grote brandnetel PNR14	*Urtica dioica*	51.2781118	4.9433242	NR	0.8	220.6	0.4	0.2	21.8	0.3	1.43	2	0.86	43.5
Kruipende boterbloem PNR11	*Ranunculus repens*	51.2781786	4.943392	NR	3	145.7	1.1	0.8	20.1	0.8	4.16	1.5	0.41	27.5
Ridderzuring PNR1	*Rumex obtusifolius*	51.2780996	4.9434161	NR	5	730.5	0.5	0.1	8	4.1	51.26	0.3	0.62	184.3
Echte valeriaan PNR16	*Valeriana officinalis*	51.2781007	4.943317	NR	0.5	141.4	0.4	0.3	3.6	1.1	32.04	15	0.64	4.3
Haagwinde PNR13	*Convolvulus sepium*	51.2782043	4.9433687	NR	2	141.4	0.4	0.3				85.7	3.23	3.8

*n = 1 plant species/analyses

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Table S3: Cr concentration in the leachate of the pot experiment, anions in the leachate, and Cr in the shoot.

					24h shaking test EUR2 L/S=10						Leaching test and nutrient analyses in flow-through water							Cr Shoot
					Cond.	pH-KCl	Cd	Cr	Cu	Zn	CO_2^-	HCO_3	Cl-	NO_3	NO_2	PO_4^{3-}	SO_4^{2}	Cr total
Sample name	Latin name	Loc.	Inoculation	Amendment	µS/cm		µg/L	µg/L	µg/L	µg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/kg DW
Duizendblad	*Achillea millefolium*	AG	Inoculated	No Amendment	53	6.2	0.21	53	10	48	<6	7	1.4	0.06	<0.01	0.02	2.4	5.91
Duizendblad	*Achillea millefolium*	AG	Inoculated	No Amendment	46	5.8	0.3	66	11	56								3.34
Duizendblad	*Achillea millefolium*	AG	non inoculated	No Amendment	58	5.8	0.28	66	12	50	<6	6	1.4	0.06	<0.01	<0.01	1.1	3.16
Duizendblad	*Achillea millefolium*	AG	non inoculated	No Amendment	50	5.8	0.31	60	10	56								<2
Duizendblad	*Achillea millefolium*	AG	non inoculated	portland cement	490	8.3	0.1	56	39	21	<6	15	3.4	0.85	0.01	0.02	6.4	3.99
Duizendblad	Achillea millefolium	AG	non inoculated	portland cement	520	8.4	0.2	70	55	13								4.13
Duizendblad	*Achillea millefolium*	AG	non inoculated	basalt	77.5	6.4	0.3	59	31	44	<6	7	3.3	1.1	0.01	0.04	3.6	<2
Duizendblad	*Achillea millefolium*	AG	non inoculated	basalt	57.5	6.3	0.2	62	30	35								2.49
Harig wilgenroosje	*Epilobium hirsutum*	AG	Inoculated	No Amendment	62.8	6.1	0.2	59	22	46	<6	6	<1	0.42	<0.01	0.03	3.5	2.56
Harig wilgenroosje	*Epilobium hirsutum*	AG	Inoculated	No Amendment	49.5	6	0.3	54	23	68								3.11
Harig wilgenroosje	*Epilobium hirsutum*	AG	non inoculated	No Amendment	49.5	6	0.3	54	23	68	<6	6	<1	<0.05	<0.01	<0.01	16	<2
Harig wilgenroosje	*Epilobium hirsutum*	AG	non inoculated	No Amendment	52.7	6.2	0.4	63	34	56								<2
Harig wilgenroosje	*Epilobium hirsutum*	AG	non inoculated	portland cement	460	8.3	0.1	64	39	15	<6	9	<1	0.12	<0.01	<0.01	2.6	2.94
Harig wilgenroosje	*Epilobium hirsutum*	AG	non inoculated	portland cement	420	8	0.2	52	33	12								3.93
Harig wilgenroosje	*Epilobium hirsutum*	AG	non inoculated	basalt	71	6.2	0.3	67	26	48	<6	6	1.9	0.07	<0.01	0.02	1.5	4.38
Harig wilgenroosje	*Epilobium hirsutum*	AG	non inoculated	basalt	53.6	6.1	0.4	67	36	61								7.25
Riet	*Phragm. australis*	NR	non inoculated	No Amendment	60	6.3	0.13	140	7.6	22	<6	6	1.3	<0.05	<0.01	0.06	17	3.74
Riet	*Phragm. australis*	NR	non inoculated	No Amendment	81	6.5	0.12	130	7.4	24								9.34
Riet	*Phragm.australis*	NR	Inoculated	No Amendment	54	6.2	0.2	79	11	34	<6	7	1.2	<0.05	<0.01	<0.01	1.2	2.09
Riet	*Phragm. australis*	NR	Inoculated	No Amendment	52	6.8	0.23	93	8.7	46								2.12
Mannetjesvaren	*Dryopteris filix-mas*	NR	non inoculated	No Amendment	90	6.5	0.14	130	9.9	24	<6	7	1.5	0.05	<0.01	<0.01	22	21.6
Mannetjesvaren	*Dryopteris filix-mas*	NR	non inoculated	No Amendment	85	6.6	0.18	110	6.4	37								15.2
Mannetjesvaren	*Dryopteris filix-mas*	NR	Inoculated	No Amendment	73	5.4	0.21	130	6	49	<6	46	13	0.61	<0.01	0.06	83	19.5
Mannetjesvaren	*Dryopteris filix-mas*	NR	Inoculated	No Amendment	69	6.5	0.2	150	8	32								15.2
no plant control	no plant control	NR	non inoculated	No Amendment	130	5.8	0.4	120	27	86	<6	5	1.1	0.1	<0.01	<0.01	1.2	
no plant control	no plant control	NR	non inoculated	No Amendment	89.3	5.9	0.4	110	22	130								

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Table S4: Total bioavailable Cr in the leachate after 1 week shaking test, at pH 7 and 4.

					1 week shaking test total bioavailability (pH 7 and pH 4)					
					Conductivity	pH-KCl	Cd	Cr	Cu	Zn
Sample name	Latin name	Location	Inoculation	Amendment	µS/cm		µg/L	µg/L	µg/L	µg/L
Duizendblad	*Achillea millefolium*	Agricultural area	non inoculated	portland cement	430	6.9	1.9	48	14	230
Duizendblad	*Achillea millefolium*	Agricultural area	non inoculated	basalt	120	4.9	2.1	19	9.4	230
Harig wilgenroosje	*Epilobium hirsutum*	Agricultural area	Inoculated	No Amendment	110	5	1.1	10	7.8	220
Harig wilgenroosje	*Epilobium hirsutum*	Agricultural area	non inoculated	No Amendment	93	5.1	1.2	14	5.2	180
Harig wilgenroosje	*Epilobium hirsutum*	Agricultural area	non inoculated	portland cement	720	6.9	1.4	48	11	180
Harig wilgenroosje	*Epilobium hirsutum*	Agricultural area	non inoculated	basalt	110	4.7	1.8	15	5	230

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Table S5: Chemical composition of the minerals used.

			Portland Cement CEM I 52.5 R	Basalt
Chemical XRF	**Oxide**	**Unit**	**Results**	**Results**
	Fe2O3	%	1.44	10.83
	Al2O3	%	3.63	11.60
	TiO2	%	0.18	2.45
	K2O	%	0.69	3.07
	CaO	%	67.53	15.69
	MgO	%	0.59	10.50
	Na2O	%	n.d.	2.91
	SiO2	%	23.42	41.92
	Mn2O3	%	0.05	0.21
	Cr2O3	%		0.0204
	BaO	%		0.09
	ZrO2	%		0.102
	SrO	%		0.056
	P2O5	%	0.14003	0.788655
	SO3	%	3.33	0.04
ICP heavy metals (mg/kg DS)	As	ppm	2.6	1.9
	Cd	ppm	< 0.1	<0.1
	Cr	ppm	37	93
	Cu	ppm	91	110
	Pb	ppm	9.8	4.5
	Ni	ppm	18	95
	Zn	ppm	270	59
XRF minors	Zn	ppm	414.3	67.8
	Cu	ppm	108.3	121.3
	Ni	ppm	40.8	146.7
	Cr	ppm	61.3	408
	V	ppm	50.1	396
	Ba	ppm	595.6	1319.8
	Sc	ppm	23.7	29
	La	ppm	12	90.9
	Ce	ppm	21.6	155.5
	Nd	ppm	11.9	64.2
	U	ppm	1.2	3.4
	Th	ppm	2.1	11.6
	Pb	ppm	14.7	7.2
	Nb	ppm	4.7	118.6
	Zr	ppm	47.8	239.5
	Y	ppm	12	24.2
	Sr	ppm	1227.1	1036.8
	Rb	ppm	36	100.5
Loss on ignition(1100°C)	LOI	%	2.5	0.2

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