Science of the Total Environment 563–564 (2016) 822–834

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Combined nano-biotechnology for in-situ remediation of mixed contamination of groundwater by hexavalent chromium and chlorinated solvents

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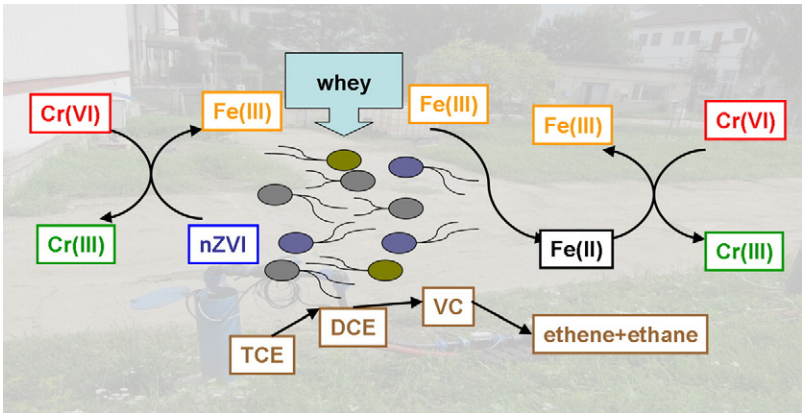
HIGHLIGHTS

- A co-mingled plume was successfully remediated by *in-situ* nZVI and whey application.
- Cr(VI) was removed completely after nZVI and remained stable after whey injection.
- Subsequent whey application also resulted in a high removal of chlorinated ethenes.
- Application of whey assisted microbial partial regeneration of the spent nZVI.
- Detected chlororespiration activity documents utility of the combined technology.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 2 September 2015
Received in revised form 3 January 2016
Accepted 4 January 2016
Available online 2 February 2016

Keywords:
Chlorinated solvents
Hexavalent chromium
NZVI
Bioreduction
Dechlorination
Indigenous microorganisms

ABSTRACT

The present report describes a 13 month pilot remediation study that consists of a combination of Cr(VI) (4.4 to 57 mg/l) geofixation and dechlorination of chlorinated ethenes (400 to 6526 μg/l), achieved by the sequential use of nanoscale zerovalent iron (nZVI) particles and *in situ* biotic reduction supported by whey injection. The remediation process was monitored using numerous techniques, including physical–chemical analyses and molecular biology approaches which enabled both the characterization of the mechanisms involved in pollutant transformation and the description of the overall background processes of the treatment. The results revealed that nZVI was efficient toward Cr(VI) by itself and completely removed it from the groundwater (LOQ 0.05 mg/l) and the subsequent application of whey resulted in a high removal of chlorinated ethenes (97 to 99%). The persistence of the reducing conditions, even after the depletion of the organic substrates, indicated a complementarity between nZVI and the whey phases in the combined technology as the subsequent application of whey phase partially assisted the microbial regeneration of the spent nZVI by promoting its reduction into Fe(II), which further supported remediation conditions at the site. Illumina sequencing and the detection of functional

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http://dx.doi.org/10.1016/j.scitotenv.2016.01.019

Co-mingled plume
PLFA

vcrA and *bvcA* genes documented a development in the reducing microbes (iron-reducing, sulfate-reducing and chlororespiring bacteria) that benefited under the conditions of the site and that was probably responsible for the high dechlorination and/or Cr(VI) reduction. The results of this study demonstrate the feasibility and high efficiency of the combined nano-biotechnological approach of nZVI and whey application *in-situ* for the removal of Cr(VI) and chlorinated ethenes from the groundwater of the contaminated site.

1. Introduction

Chromium is one of the most abundant and toxic metals that cause the pollution of groundwater and soil due to its frequent industrial application. Chromium generally exists in water with two stable oxidation states, the trivalent Cr(III) and hexavalent Cr(VI) forms. The hexavalent form Cr(VI) is well known to be mutagenic, carcinogenic and toxic (Nriagu and Nieboer, 1988). In contrast to the less reactive and toxic Cr(III), which is almost insoluble and can be readily precipitated out of solution, the adverse environmental effects of Cr(VI) are closely linked to its solubility and mobility, that leads to health problems such as liver damage, pulmonary congestion, vomiting and severe diarrhea. Therefore, the geo-fixation of Cr(VI) by its reduction into Cr(III) and subsequent formation of insoluble Cr(III) compounds represent the majority of *in-situ* remediation methods employed to-date, as represented by numerous publications that describe various biological or chemical approaches to achieve this transformation (Jardine et al., 1999; Barrera-Diaz et al., 2012; Dhal et al., 2013; Němeček et al., 2014; Xu and Zhao, 2007; Flury et al., 2009). In this regard, the application of reduced forms of iron materials, mainly zerovalent iron and its nanoscale forms (nZVI), represents one of the promising chemical remediation methods (see Gheju, 2011 and references therein).

Another option is to utilize microbial processes to reduce and transform Cr(VI) to Cr(III). A number of aerobic as well as anaerobic microorganisms are capable of reducing Cr(VI) (Barrera-Diaz et al., 2012) by employing different mechanisms. For instance mechanisms related to chromium resistance are utilized by the aerobic bacteria in order to detoxify hexavalent chromium (Dhal et al., 2013; Cheung and Gu, 2007) whereas the anaerobic bacteria utilize Cr(VI) as an electron acceptor in the electron transport chain related to their respiratory reactions (Barrera-Diaz et al., 2012) or reduce chromates in the periplasmatic space by hydrogenase or cytochrome c3 (Dhal et al., 2013).

Besides chromium, chlorinated organic compounds also pose a serious problem for the environment. For example, tetrachloroethene and other related organochlorine aliphatic compounds have been massively used in the past in various industry-related technological processes, resulting in a large number of contaminated sites (Tiehm and Schmidt, 2011; Koenig et al., 2015). Interestingly, nanoscale zerovalent iron (nZVI) can act on chlorinated ethenes through a sequential mechanism that involves reduction and dechlorination of the ethene structure, (Katsenovich and Miralles-Wilheirn, 2009) and the validity of this process has already been tested *in-situ* (Lacinová et al., 2012; Wei et al., 2012). As well as, the subject of bioremediation of the contaminated sites by chlorinated ethenes has been studied for more than two decades and bacterial reductive dechlorination has already been introduced into practice. Chlorinated ethenes can be dechlorinated under favorable redox conditions by abiotic and biotic reductive processes—β-elimination and/or hydrogenolysis (Cwiertny and Schrerer, 2010). During this process of reductive dechlorination, indigenous microflora chlororespirates upon biostimulation with organic substrates and the hydrogen atoms replace the chlorine substituents one-at-a-time, in a sequential manner, resulting in the production of less chlorinated analogues, starting from tetrachlorethene (PCE), via trichloroethene (TCE), dichloroethene (DCE) and vinylchloride (VC) to ethene and ethane. Unfortunately, the slowest step in the sequence of hydrogenolysis is the reduction of VC to ethane, a serious drawback because VC represents a carcinogenic metabolite on the pathway (Futagami et al., 2008). It is noteworthy that the transformation relying on a β-elimination pathway does not cause accumulation of VC (Roberts et al., 1996; Wei et al., 2010; Liu et al., 2005).

In the recent past we reported both the successful *in-situ* application of nZVI suspension in removing Cr(VI) from a contaminated aquifer (Němeček et al., 2014) and a multistep remedial approach with long-term effectivity that included geofixation with nZVI followed by whey injection supported microbial reduction (Němeček et al., 2015). This leads us to the challenging question whether nZVI could be successfully applied in case of co-mingled pollution and whether a promising way could be targeting on pollutants that require reductive conditions.

Regarding the new applications of nanomaterials such as nZVI, attention was primarily focused on the concern of its possible negative effect on natural microbiota and the phenomenon of nZVI interaction with chlororespiring bacteria is excellently reviewed by Bruton et al. (2015). However, it is important to bear in mind that contaminated sites that require a call for remediation are generally represented by sufficiently damaged environments where the most toxic component is often represented by a particular pollutant. For example a rapid decrease of the pollutant concentration together with hydrogen evolution and redox potential shifts caused by nZVI can finally lead to favorable conditions for consequent biological processes (Němeček et al., 2015; Fajardo et al., 2012; Liu and Lowry, 2006).

The present study was performed to assess the feasibility of remediating a site co-contaminated by Cr(VI) and chlorinated ethenes by injecting nZVI suspensions and subsequently cheese whey as an organic substrate. In light of our previous successful studies and the promising results obtained in the presented study, we suggest that this remedial design may be employed effectively to enhance the removal of pollutants from a co-mingled plume. The remediation process was monitored using a combination of techniques, ranging from physical–chemical analyses to molecular biology approaches, in order to elucidate the mechanisms involved in pollutant transformations and to describe the background of the treatment process in general.

2. Materials and methods

2.1. Test site

The pilot test was performed at a site in the Czech Republic. The site is polluted with chlorinated ethenes and Cr(VI) originating from historical degreasing and chromium coating activities. The aquifer is developed in Quaternary sandy gravels with silty admixture and is overlain by clay and clayey loam with a thickness of 5 m. The aquifer has a saturated thickness of approximately 4 m. The hydraulic conductivity of the aquifer is 7.6×10^{-4} m/s based on a pumping test. The average seepage velocity of the groundwater flow is 1.5 m/day. The groundwater discharges into a local river located at a distance of 430 m from the site. The groundwater is of the Ca-HCO_3^- type and is characterized by elevated mineralization (total dissolved solids from 0.9 to 1.2 g/l), neutral pH (6.9–7.0), oxidation–reduction potential ranging from 130 to 490 mV and TOC from <1.0 mg/l to 5.4 mg/l. The initial Cr(VI) concentration in the groundwater ranged from 4.4 to 57 mg/l. The total concentration of chlorinated ethenes ranged from 400 to 6526 μg/l. Trichloroethene (TCE) and cis-1,2-dichloroethene (cis-DCE) were the dominant chlorinated contaminants (TCE and cis-DCE represent 45% to 93% and 5% to 53% of the total chlorinated ethenes on a molar basis, respectively).

PCE represented less than 5% and VC was bellow its respective limit of quantification (LOQ; 4.0 μg/l).

2.2. Pilot application of combined technology

Within the first step of the pilot test nZVI was injected twice with a 4-month interval. 20 kg of nZVI (produced by NANO IRON, Ltd., Czech Republic) in a form a suspension was used for each injection using the direct push technology. For the first injection, surface-passivated nZVI NANOFER STAR was used (Zbořil et al., 2012) as the nZVI suspension at a concentration of 1 g/l. For the second injection, 2 g/l of nZVI NANOFER 25S was applied. The suspensions were injected into 3 boreholes located perpendicular to the groundwater flow, see Fig. 1.

Approximately 2.5 months after the second nZVI injection, application of whey was begun using a circulation system. The whey was dosed into the groundwater being pumped from downgradient wells HS-1 and HS-2 (Fig. 1B) and injected into wells IN-1 and IN-2 located up-gradient in a total rate of 0.5–0.7 l/s. The dosing ratio of whey with pumped-injected groundwater was 1:50 and resulted in 60 mg/l of TOC in the groundwater. In total, 8.2 m^3 of whey was injected over approximately 5 weeks. After additional 9 days of groundwater circulation (without dosing of whey), the natural groundwater flow regime was re-established and monitoring continued for the next 5 months. The individual pumping and injection wells were periodically switched on and off in order to change groundwater flow pattern and to achieve homogenous distribution of whey in the aquifer. The whole pilot test lasted approximately 13 months.

2.3. Monitoring

Groundwater samples from different monitoring wells (Fig. 1) were collected during the pilot test: three monitoring wells of the first downgradient monitoring line (MV-2, MV-3, MV-4) and four monitoring wells of the second downgradient monitoring line (MV-5, HV-2, HS-1 and HS-2). Two wells were used for the whey injection (IN-1 A and IN-2 A), in addition to one up-gradient well, which was intended to provide background values (MV-1). In addition, wells HV-1, HV-3, HV-5Z, HV-7Z were sampled less frequently for a reduced range of laboratory analyses. The data from these wells were used mainly for 2D modeling of Cr(VI) and the concentrations of chlorinated ethenes. Moreover, well HV-8, which contained only chlorinated ethenes but no chromium, was used. This well was situated cross-gradient from the source of the chromium pollution (northwest, at a distance of 25 m). The groundwater samples were collected after well purging, using a Gigant (Ekotechnika, Czech Republic) submersible sampling pump. Field parameters including the pH, oxidation–reduction potential, electrical conductivity and temperature were recorded during the sampling. Prior to sampling, approximately 3 borehole casing volumes of groundwater were removed from the groundwater monitoring well to follow the standard procedure (ČSN ISO 5667-11, 1993).

The groundwater samples were analyzed and monitored for various parameters (Section 2.9): Cr(VI), Cr tot., chlorinated ethenes, ethene, ethane, methane, sulfate, hydrogen sulfide, nitrate, ammonium, manganese, total organic carbon, pH, redox potential. At the end of the pilot test, sediment samples were collected from wells HV-8, MV-1, MV-3, MV-4 and MV-5 for microbial community analysis (Section 2.6.). In addition, two types of passive samplers were emplaced in wells for solid phase and molecular biology analyses (Section 2.4).

2.4. Passive samplers in wells

Two types of passive samplers were used, placed in the monitoring wells. One of them contained the aquifer soil samples collected during the drilling of up-gradient well MV-1 and monitoring wells MV-3, MV-4 and MV-5 in late November 2013. The soil samples were mixed from the whole profile submerged in the saturated zone and placed into the cartridges made of screened 1 1/2″ OD PVC tubes used for direct push soil sampling coated with a mesh with hole size of 0.1 mm. The 1.2 m long cartridges were then placed into up-gradient well MV-1 and monitoring wells MV-3, MV-4 and MV-5 on the same day when the drilling was performed into the depth of 6 to 7 m below ground level. At the end of the pilot test, the soil from the cartridges was collected, transferred into 2 ml vials, frozen and delivered to the laboratory for soil phase analyses on the same day. The oxidation state of Cr in the soil

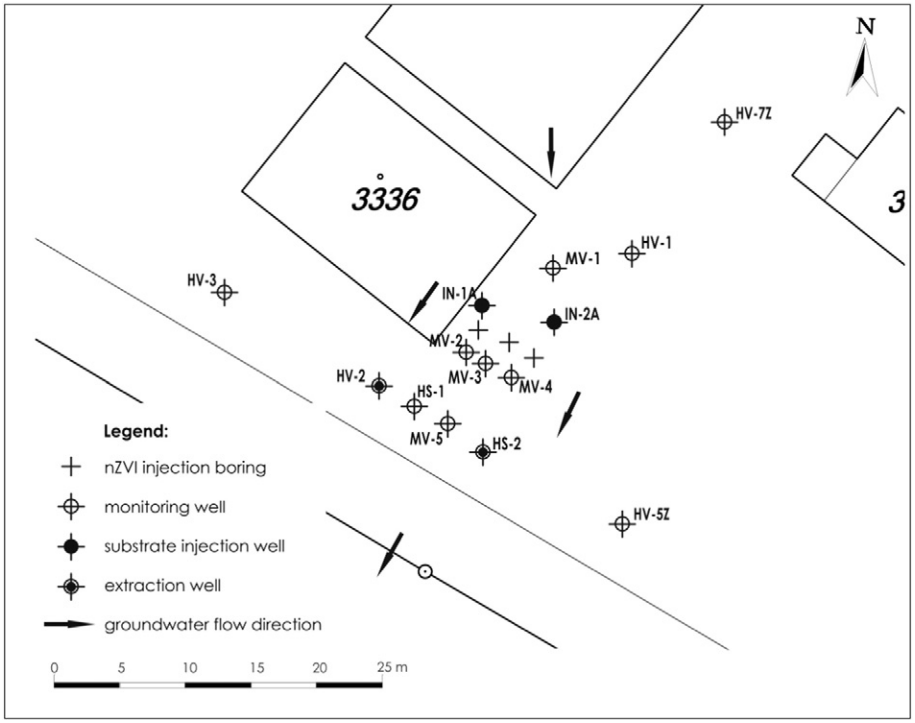

Fig. 1. Layout of the pilot test wells.

phase was determined using X-ray photoelectron spectroscopy (XPS), the oxidation state of Fe was determined using ^{57}Fe Mössbauer spectroscopy (only in the sample from well MV-4).

The second type of the passive sampler contained nanofiber carriers, which were placed in wells MV-1 and MV-2 prior to the first step of the remediation. The carrier consisted of polyurethane nanofibers spread on polyester threads in a high voltage electrostatic field, produced at Technical University Liberec. This sampler was used to detect the presence of dechlorinating bacteria using the polymerase chain reaction (PCR) with specific primers (Section 2.5). The samples were also used for quantification of functional dechlorinating genes *vcrA* and *bvcA* using quantitative PCR (qPCR). In addition, the total bacterial biomass from the nanofibers was quantified by qPCR using 16S rRNA gene.

2.5. qPCR

Total DNA was extracted from the nanofiber carriers (prior, during and after both steps of the applied combined technology) using the FastDNA Spin Kit for Soil (MP Biomedicals, CA, USA) and following the manufacturer's protocol. Extracted DNA was quantified with a Qubit 2.0 fluorometer (Life Technologies, MA, USA).

Reactions for qPCR were prepared as follows: 5 μl LightCycler® 480 SYBR Green I Master (Roche, Switzerland), 0.4 μl of 20 μM forward and reverse primer mixture and 3.6 μl of ultra-pure water were used for 10 μl reaction volume. Specific primers of the functional genes *vcrA* and *bvcA* (vcrA880F, vcrA1018R and bvcA277F, bvcA523R) are listed in STab. 1 (Behrens et al., 2008). 1 μl of DNA was added to the PCR reaction. The samples were measured in duplicate. qPCR was performed in a LightCycler® 480 instrument (Roche, Switzerland) with reaction conditions as follows: initial denaturation for 5 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 15 s at 60 °C and 20 s at 72 °C. Cp values were obtained using the Second Derivative Maximum method available in LightCycler® 480 Software. The amplification efficiency of each primer set was determined by measuring standard curves using serial dilution of template DNA from 5 different environmental samples. Relative quantification of each parameter was expressed as a fold change between two states (given sampling time and a sampling time for zero) using the delta Cp method. Total bacteria were quantified using U16SRT-F and U16SRT-R (STab. 1) according to Clifford et al. (2012).

The presence of *Dehalococcoides spp.* was detected by amplification of 16S rDNA using specific primers DHC587F and DHC1212R (Hendrickson et al., 2002; listed in STab. 1). PCRs were carried out with Ready-to-Go-PCR Beads (GE Healthcare Life Sciences, UK). Amplifications were prepared for a 25 μl final volume. The PCR consisted of an initial denaturation step at 95 °C (4 min), followed by 35 cycles of 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C; the PCR schedule ended with final extension for 7 min at 72 °C. The PCR products were visualized under UV light on 1% agarose gels stained with SYBR Safe DNA gel stain (Molecular Probes, Eugene, OR, USA).

2.6. Illumina sequencing

Total DNA for microbial community analysis was extracted in triplicate from all the well sediment samples using a PowerSoil DNA Isolation Kit (MoBio Laboratories Inc., CA, USA), cleaned with a Geneclean Turbo Kit (MP Biomedicals, CA, USA) and the concentration was measured using a Qubit 2.0 Fluorometer (Life Technologies, CA, USA). For the microbial community analysis, three consecutive PCR reactions per sample were performed to amplify DNA from the V4 region of bacterial 16S rRNA was amplified using the barcoded primers 515F and 806R described elsewhere (Caporaso et al., 2012). The V4 variable region of the bacterial 16S rRNA gene was amplified using the barcode 515F (5′-GTG CCA GCM GCC GCG GTA A-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′) primers (Caporaso et al., 2011). Each sample was tagged with a unique barcode attached to the reverse primer 806R. The PCR cycle conditions were as follows: 94 °C for 4 min; 35 cycles at 94 °C for 45 s, 50 °C for 60 s and 72 °C for 75 s, with a final extension at 72 °C for 10 min. Each sample was amplified in triplicate, pooled and purified using the MinElute Kit (Dynex, Czech Republic). The concentration of purified PCR products was measured with a Qubit 2.0 Fluorometer (Life Technologies, CA, USA). Then the barcode-tagged amplicons from different samples for either bacterial community were mixed in equimolar concentrations. The library was constructed using the TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, USA) and sequencing of bacterial amplicons was performed on the Illumina MiSeq platform as an external service (GeneTiCA, Czech Republic).

The amplicon sequencing data were processed using the SEED 1.2.1 pipeline (Větrovský and Baldrian, 2013). Briefly, pair-end reads were merged using fastq-join (Aronesty, 2013). Chimeric sequences were detected using Usearch 7.0.1090 (Edgar, 2010) and deleted and the sequences were clustered using UPARSE implemented within Usearch (Edgar, 2013) at a 97% similarity level. Representative sequences for each operational taxonomic units (OTUs) were randomly selected and the closest hits at a genus or species level were identified using BLASTn against the RDP (Cole et al., 2014) and GenBank databases. From 16S rRNA in DNA, bacterial genome count estimates were calculated based on the 16S copy numbers in the closest available sequenced genome as described previously (Větrovský and Baldrian, 2013).

2.7. Mössbauer spectroscopy analyses of soil samples

^{57}Fe Mössbauer spectra were collected in transmission geometry (constant acceleration mode) with a ^{57}Co(Rh) radioactive source (1.85 GBq). A magnetically pre-concentrated sample was fast-frozen and measured at a temperature of 100 K in order to avoid iron oxidation during measurement (data collection time about 4 days). The values of hyperfine parameters (i.e., isomer shift values) were calibrated against a metallic iron (α-Fe) foil at room temperature. The spectra were fitted by Lorentz functions using the CONFIT2000 software (Žák and Jirásková, 2006). The experimental error is ± 0.02 mm s^{-1} for the hyperfine parameters and $\pm 2\%$ for the relative spectral areas.

2.8. X-ray photoelectron spectroscopy (XPS) analyses of soil samples

The speciation of selected metals in the soil from passive samplers (*i.e.* metals associated with the surface of the nZVI particles) was determined by PHI VersaProbe II X-ray photoelectron spectrometer (XPS) with a monochromatic AlK_α X-ray source (1486.6 eV). Binding energies were conventionally corrected by the adventitious C 1 s peak (set at 284.8 eV). Charge-neutralization was employed during all the measurements. Samples were prepared by placing air-dried soil on adhesive double-sided tape. The data were processed using software MultiPack (Ulvac-PHI, Inc., Japan).

2.9. Physical, chemical and inorganic parameters of the groundwater

Cr_{total}, Ca, Mg, Na, K, Fe and Mn dissolved in groundwater were analyzed using inductively coupled plasma optical emission spectrometry—ICP-OES (Optima 2100, Perkin Elmer, USA) according to ČSN EN ISO 11885 (2007). Cr(VI) in the groundwater was determined photometrically according to ISO 11083 (1994). The groundwater samples were filtered through a 0.45 μm membrane filter prior to the metal analyses.

Ammonium (NH_4^+) and sulfane were determined spectrophotometrically according to ČSN ISO 7150-1 (1994) and ČSN 83 0530–31 (1980), respectively.

Total organic carbon (TOC) was determined according to ČSN EN 1484 (1998) using a TOC analyzer (MULTI N/C 2100S, Analytik Jena, Germany).

The oxidation–reduction potential and pH of the groundwater were measured electrochemically in the field using a Multi 350i Multimeter (WTW, Germany). The pH and oxidation–reduction probes were placed in a flow-through cell.

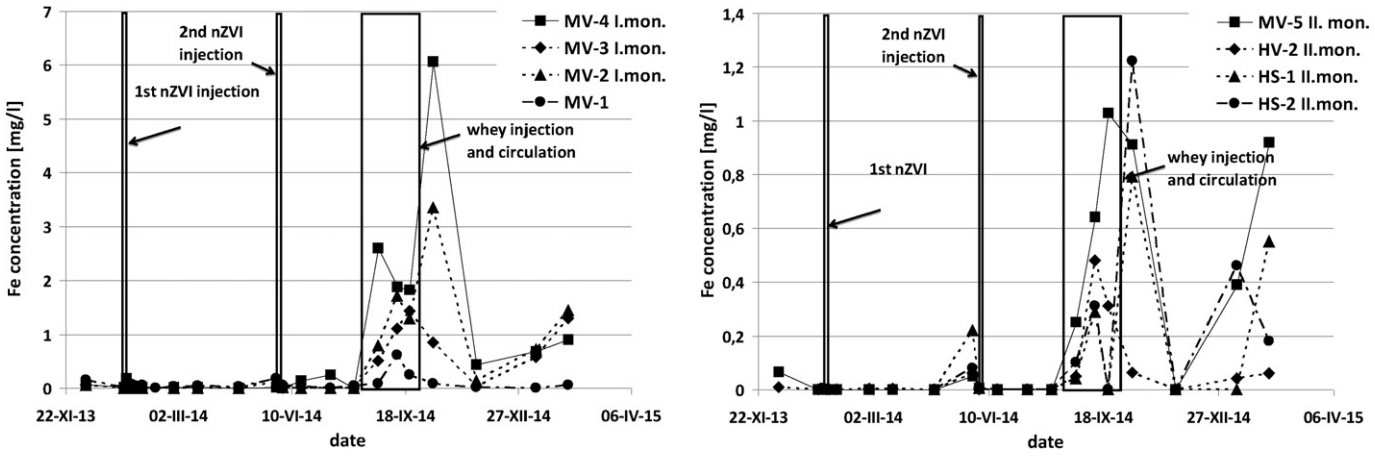

Fig. 2. Concentration of dissolved iron in the groundwater during the pilot test.

Chloride, nitrate and sulfate were analyzed by ion chromatography according to EN ISO 10304-1 (2007) and bicarbonates and carbonates were determined by titration according to EN ISO 9963–1 (1994).

Volatile organic carbons including chlorinated ethenes, ethene, ethane, and methane were analyzed by gas chromatography – mass spectrometry (GC–MS; CP 3800, Saturn 2200, Varian, USA) using a VF-624 ms column (Varian, USA) injected with CTC Combipal (CTC Analytics, USA) and equipped with a headspace agitator.

2.10. Phospholipid fatty acid analysis

Groundwater samples for PLFA analyses were prepared by filtration (1 l) through microbial filters (0.2 μm). These filters were then extracted with a mixed chloroform–methanol–phosphate buffer (1:2:0.8). Phospholipids were separated using solid-phase extraction cartridges (LiChrolut Si 60, Merck) and the samples were subjected to mild alkaline methanolysis. The free methyl esters of phospholipid fatty acids were analyzed by GC–MS (456-GC, SCION SQ mass detector, Bruker, USA) (Němeček et al., 2015). Methylated fatty acids were identified according to their mass spectra using a mixture of chemical standards obtained from Sigma. Biomass G + bacteria were quantified as the sum of i14:0, i15:0, a15:0, i16:0, i17:0 and a17:0. G- bacteria were determined on the basis of 16:1 Ω7, 18:1 Ω7, cy17:0, cy19:0, and 16:1 Ω5. Anaerobic bacteria were quantified using cy17:0, cy19:0, and 18:1 Ω9 (Šnajdr et al., 2011).

2.11. Toxicological test of Cr(VI) using indigenous bacteria

This test was performed to assess the viable amount of indigenous cultivable psychrophilic bacteria under the site conditions. The method was based on international standard ISO 8199 (2005) and accredited by the Czech Accreditation Institute. Psychrophilic bacteria were determined according to the Czech National Standard ČSN 75 7842.

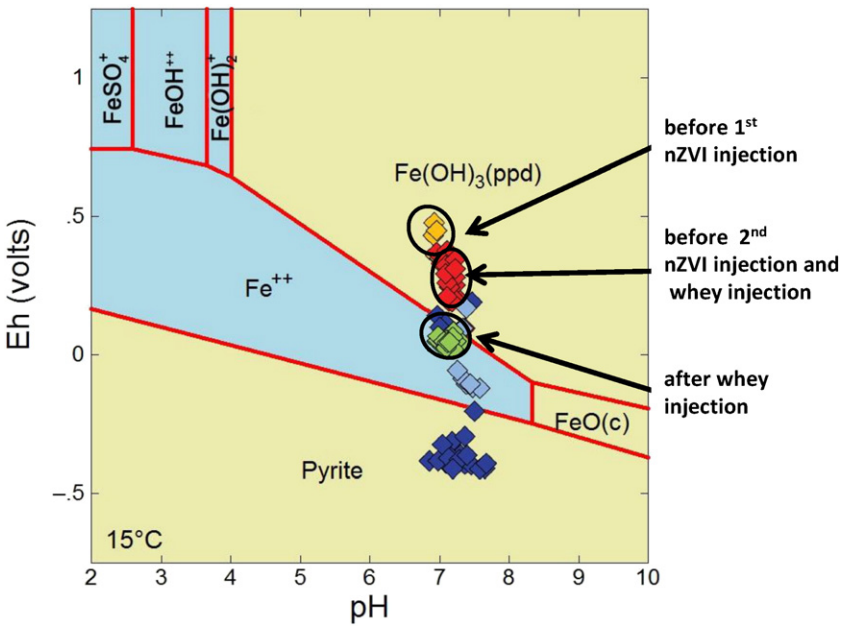

Fig. 3. Eh–pH data plotted on an iron–sulfur–water system stability diagram calculated using the Geochemist's Workbench software (Aqueous Solutions LLC, USA) and its default thermodynamic database thermo.dat (suppressed hematite, goethite and magnetite): Colour of symbols: yellow—before 1st nZVI injection, dark blue—after 1st nZVI injection, red—before 2nd nZVI injection and before whey injection, light blue—after 2nd nZVI injection and green—after whey injection.

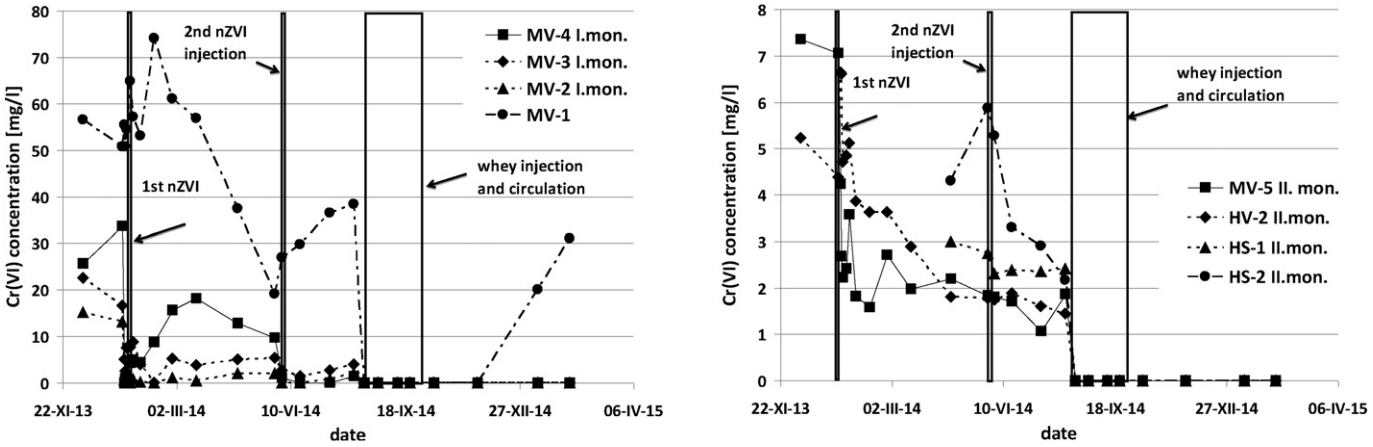

Fig. 4. Cr(VI) concentrations in groundwater during the pilot test in the monitoring wells (left: wells of the first monitoring line, right: wells of the second monitoring line).

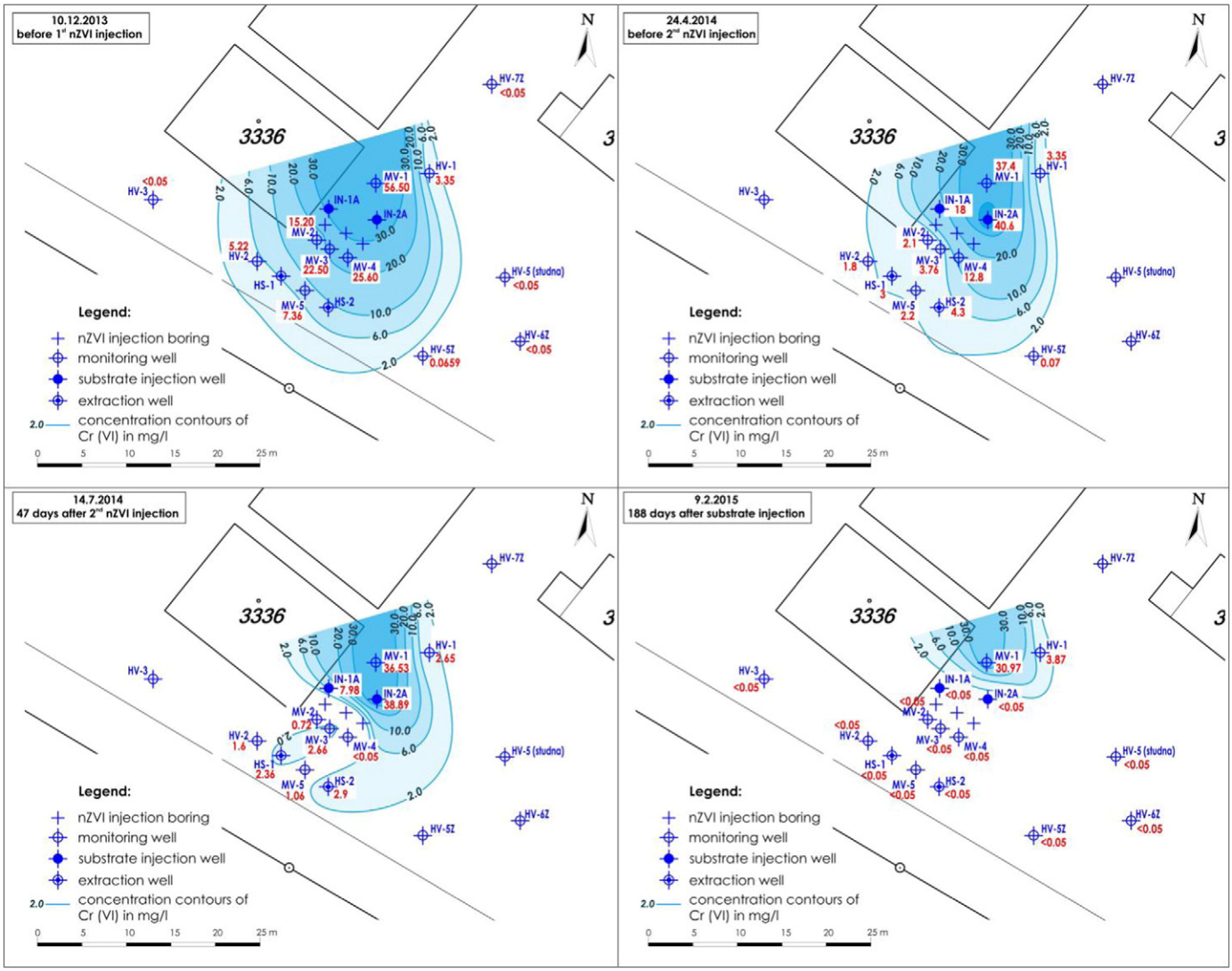

Fig. 5. 2D model of Cr(VI) concentrations in groundwater: A—before the first nZVI injection; B—before the second nZVI injection; C—47 days after the second nZVI; D—188 days after whey application (prepared using Toolbox SANACE software, Czech Republic).

3. Results and discussion

3.1. Physical–chemical and inorganic parameters of groundwater

After the first nZVI injection, the Eh of the groundwater decreased to −400 mV in all the monitoring wells taken from both monitoring lines (SFig. 1). The second injection of nZVI resulted in a less significant drop in Eh, especially in the groundwater of wells of the second monitoring line, possibly due to the lower total volume of the injected nZVI suspension. The subsequent application of the organic substrate again caused a decrease in Eh and after approximately 1 month, Eh stabilized at the level of approximately 50 mV and 100 mV in the wells of the first and second monitoring lines respectively. Both remained stable for the rest of the test. *In-situ* measurements of the pH revealed that the value increased slightly from 7.0 to 7.5 during the nZVI injections but returned back to almost the original values after a short period of time. These data and the results of other inorganic/organic parameters are shown in Supplementary Figs.2–7. The course of reducing conditions at the site after the application of nZVI is well documented by the gradual decrease and further disappearance of nitrates after the whey injection, that probably acted as an electron acceptor during the microbial processes (SFig. 3; Němeček et al., 2015). The reductive conditions further caused an increase in the amount of Mn in the groundwater (from 1 mg/l to 4 mg/l in the first line of monitoring wells; SFig. 4), probably due to partial reduction of Mn(VI) oxides into soluble Mn(II). This was even more pronounced after the whey injection, resulting in a dissolved Mn concentration of 11 mg/l in both monitoring lines. These data document that hydrogeochemical conditions of the aquifer were influenced by the injection to the similar extent within the whole area that was monitored. The course of production of methane (SFig. 6) after the whey application was also measured. Taken together, the increase in the ammonia concentration (SFig. 3), the persisting reducing conditions, the dramatic drop in the active microbial biomass estimated by PLFA analysis and 16S rRNA (see below) indicate that the decaying microbial biomass acted as another reducing agent.

The original amount of dissolved Fe was close to its respective ICP-OES LOQ (0.1 mg/l) at the site (Fig. 2). The application of nZVI temporarily led to an increase which was negligible; however, after the application of whey, the concentration increased substantially to 2 to 6 mg/l in the first line of monitoring wells. The presence of soluble Fe(II) is probably a consequence of microbial processes and the reducing conditions of the site. This explanation is supported by the Eh-pH data plotted in Fig. 3 using the Pourbaix stability diagrams of an iron–sulfur-water system calculated using The Geochemist's Workbench software. The stability diagram shows an evident shift in the Eh–pH data caused by the first nZVI injection; however, this effect became apparent mainly

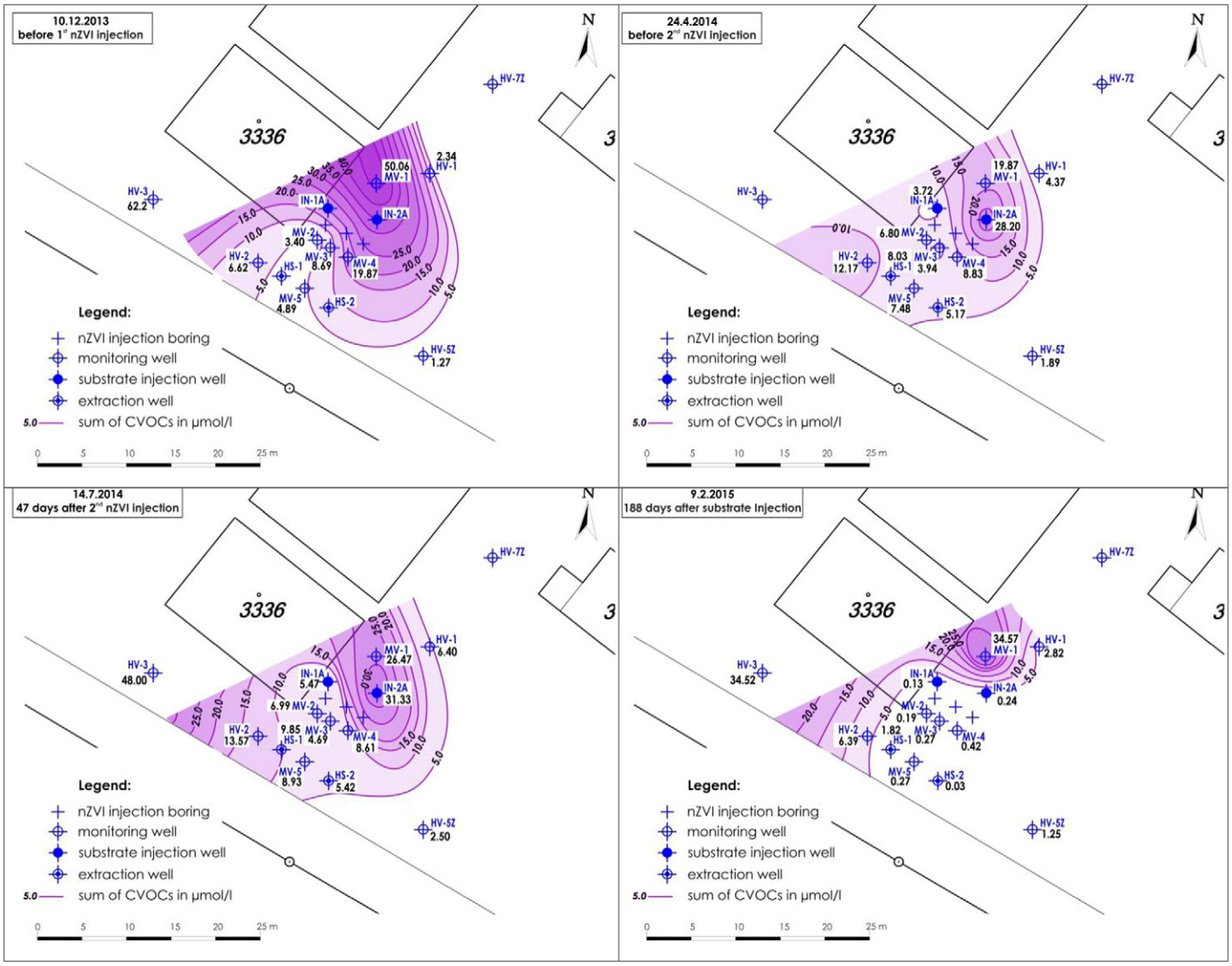

Fig. 6. 2D model of cVOCs concentrations in groundwater: A—before the first nZVI injection; B—before the second nZVI injection; C—47 days after the second nZVI; D—188 days after whey application (prepared using Toolbox SANACE software, Czech Republic).

after the whey injection that caused biological reduction of the oxidized Fe(III) to Fe(II) in the stability region of Fe^{2+}(aq). These data are also in accordance with our previous study (Němeček et al., 2015) where we suggested that this Fe(II) is derived not only from the naturally present iron but is also recycled from the used nZVI that becomes oxidized to Fe(III). This statement is supported by the fact that despite the similar redox potentials in both the lines of the monitoring wells, significantly higher concentrations of dissolved Fe(II) were detected in the monitoring wells which were closer to the nZVI injection boreholes. Considering the fact that Mn(II) originating from the minerals present in the site was detected at the same concentrations regardless of the distance from the injection wells; we can exclude the notion that Fe(II) originated dominantly from the iron bearing minerals. In order to monitor the oxidation state of Fe in solid phase collected from the passive samplers, we employed ^{57}Fe Mössbauer spectroscopy that indicated the presence of Fe in the form of Fe(0)—6% (residual nZVI), Fe(II)—21% and Fe(III)—73% (wt%) in a frozen sample from well MV-4 at the end of the pilot test (the spectrum is shown in SFig. 8). Moreover, Fe(II) and also part of Fe(III) were present as green rust, originating from Fe(0) corrosion (Lee and Batchelor, 2002). The rest of the Fe(III) was identified as nearly-amorphous ferric oxides.

3.2. Concentrations of Cr(VI) and Cr_{total} in the groundwater

The concentrations of Cr(VI) in the groundwater of the monitoring wells are depicted in Fig. 4. In the groundwater from the wells of the first monitoring line (2 m from the nZVI injection wells), the first and the second nZVI injection resulted in a decrease in the concentrations of Cr(VI) below the respective ICP-OES LOQ (0.05 mg/l). After a certain time, rebounds in the concentrations were observed; however, even

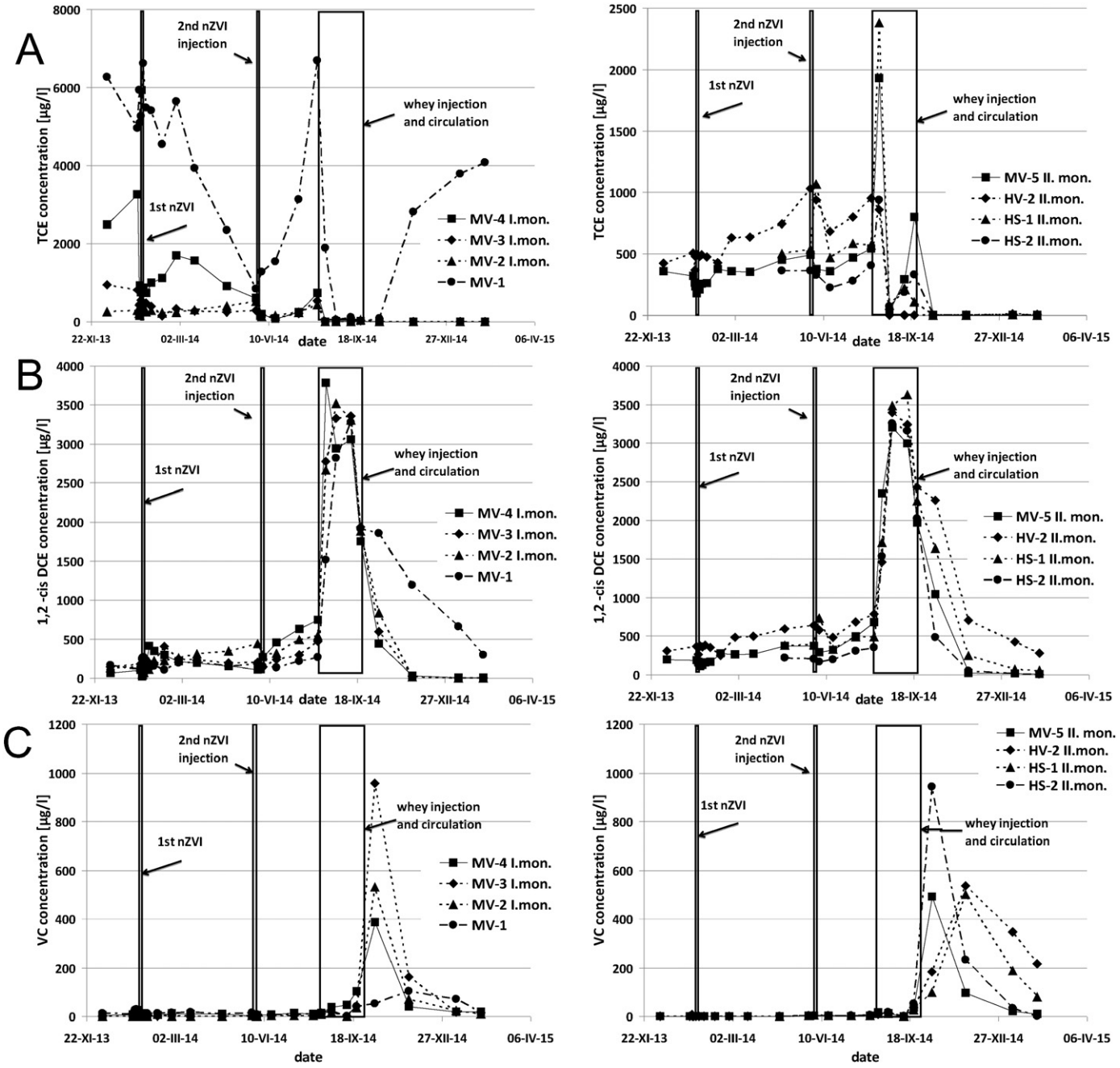

Fig. 7. Concentration of TCE in groundwater (A), cis-1,2-DCE concentration (B) and VC concentration (C) in the monitoring wells groundwater during the pilot test. (left: wells of the first monitoring line, right: wells of the second monitoring line).

after the second nZVI injection this reached only 10%–15% of the initial concentrations. Addition of the organic substrate (whey) resulted in a rapid decrease in the Cr(VI) concentrations in all of the monitoring wells and the concentrations remained below LOQ until the end of the test. The Cr_{total} concentrations were almost identical to the concentrations of Cr(VI) indicating that the reduced Cr(III) was well fixed in the soil matrix (SFig. 7).

The initial concentrations of Cr(VI) were significantly lower in the wells of the second monitoring line (situated 8 m from nZVI injection boreholes) compared to the wells of the first monitoring line (by an order of magnitude). The effect of the nZVI injections were not as pronounced as compared to the wells of the first monitoring line, although the Cr(VI) concentrations also rapidly decreased below the LOQ after the application of substrate.

The overall distribution of Cr(VI) concentration during the individual phases of the pilot test is presented in Fig. 5. The figures document the efficiency of the whey application where, even 188 days after the application, the contamination of the surrounding environment by Cr(VI) was completely suppressed. In order to examine the process of Cr geofixation, the XPS method was used to analyze the Cr forms in the soil samples from the up-gradient and monitoring wells (MV-1, MV-3, MV-5), where Cr(III) were dominantly found in the oxide and/or hydroxide forms (data not shown).

It is worth noting that reduction and immobilization of Cr(VI) were probably facilitated by soluble Fe(II) produced by the respiration of Fe(III) during the whey phase. This phenomenon was described using iron-bearing minerals in a laboratory-scale test by several authors (e.g. Wielinga et al., 2001) and in the field by Faybishenko et al. (2008).

The decrease in the sulfate concentrations after whey injection (SFig. 5) together with the production of hydrogen sulfide—an important agent for Cr(VI) reduction (Kim et al., 2007; Kirschling et al., 2010; Barrera-Diaz et al., 2012)—indicates the presence of another microbial process reducing Cr(VI). The detection of green rust in the soil sample from the cartridge placed in monitoring well MV-4 suggests that Cr(VI) was also reduced by Fe(II) present in precipitates formed by processes related to nZVI corrosion (Wilkin et al., 2005). Both the green rust forms, i.e. the sulphate ($Fe^{II}_4Fe^{III}_2(OH)_{12}SO_4.yH_2O$) and the carbonate ($Fe^{II}_4Fe^{III}_2(OH)_{12}CO_3.yH_2O$), are able to reduce Cr(VI) at a similar or even higher rate than direct reduction by microorganisms (Williams and Scherer, 2001).

3.3. Concentrations of chlorinated ethenes in groundwater

As in the case of Cr(VI), chlorinated ethenes can also be removed from groundwater by combined nano-bio reductive treatment. However, the injection of nZVI did not cause the expected long-term decrease in the concentration of chlorinated ethenes in the groundwater of the monitoring wells of the first monitoring line (see Figs. 6 and 7). The effect of the nZVI injection was almost undetectable in the wells of the second monitoring line. The results indicate that the efficiency of nZVI to reduce chlorinated ethenes is limited in the presence of Cr(VI), which is a thermodynamically more favored reducible compound for nZVI. The substrate injection stimulated sequential reductive dechlorination of the parent TCE and DCE to less chlorinated or dechlorinated ethenes (Fig. 7). The reasoning of the low efficiency of the nZVI treatment toward chlorinated aliphatics under the conditions at the site is also supported by the results shown in Fig. 8 where ethane and ethene were close or below their LOQ (1.0 μg/l) before the whey injection.

The course of the TCE concentrations during the pilot test is depicted in Fig. 7A. TCE concentration decreased below its respective LOQ (1.0 μg/l) in the wells of the first monitoring line after the whey injection and remained at this level over the following 6 months. This trend was followed by a temporal increase in the concentrations of the transformation products: cis-1,2-DCE and VC (Figs. 7B,C and 8). Subsequently, the cis-1,2-DCE and VC concentrations dropped and the concentrations of ethene and ethane increased (Fig. 8) depicting the situation in well MV-2. As shown in Figs. 6 and 7, 97 to 99% of the total chlorinated ethenes was removed from the groundwater in the wells of the first monitoring line at the end of the pilot test and the values were in the range of 36 to 97% in the second monitoring line (including PCE and VC). The pattern of the dechlorination of chlorinated ethenes, as expressed by chlorine number in well MV-2, is depicted in Fig. 8 (Bewley et al., 2015). Data for other wells are shown in SFig. 11. The chlorine number (weighted average number of Cl atoms per molecule of ethene) in the groundwater ranged from 2.6 to 3 prior to the pilot test. After the injections of nZVI, the chlorine number temporarily decreased slightly, indicating that some reductive dechlorination occurred; however, the chlorine numbers returned to their original values within two months (SFig. 11). During, and especially after the

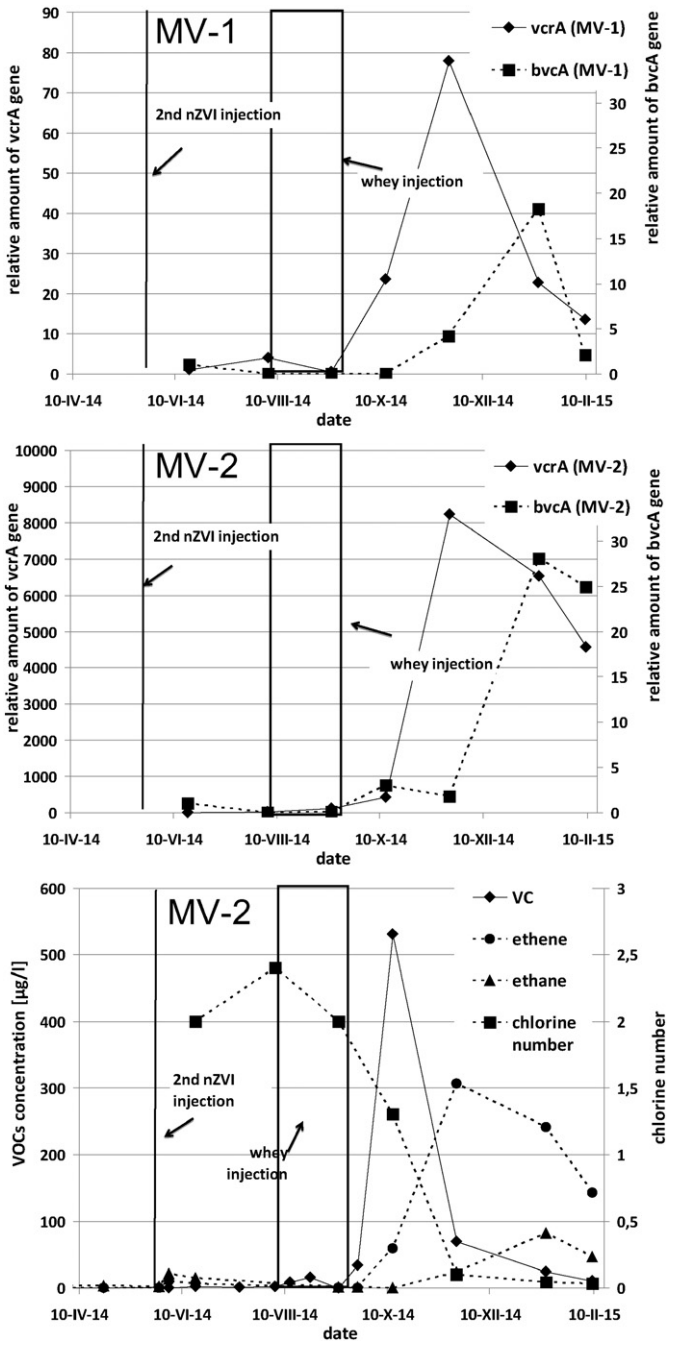

Fig. 8. Relative amount of dechlorination genes in the MV-1 upgradient well (upper part), MV-2 monitoring well (middle part) and MV-2 concentrations of the transformation products with the respective chlorine number (lower part).

application of whey, the chlorine number decreased rapidly to 0.1–0.2 in well MV-2 (Fig. 7) and also decreased in well MV-5 (SFig. 11) within approximately 3 months after addition of whey. In the up-gradient well MV-1, the decrease was only temporary and terminated after the circulation period of the groundwater; when the flow of natural groundwater was re-established, the chlorine number gradually returned back to the initial level (SFig. 11). In addition, we detected the presence of chloroethene-reductive dehalogenase bacterial genes (*vcrA* and *bvcA*) involved mainly in the transformation of VC (Cupples, 2008). The results in Fig. 8 also document the significant increase in the number of *vcrA* copies in MV-2, which was shown to correlate with the presence of ethene (van der Zaan et al., 2010). The number of *vcrA* gene copies (compared to the first sampling) increased 80 times in well MV-1 and more than 8000 times in well MV-2 (Fig. 8).The relative increase in *vcrA* resulted in a decrease in the concentration of vinylchloride and increase in concentrations of ethene and ethane.

3.4. Microbial biomass, ecotoxicity and community analysis

The injection of whey temporarily increased the TOC value to 60 and 50 mg/l in the monitoring wells of the first and second line, respectively. However, it dropped below 10 mg/l in the first line wells in a relatively rapid manner and in the second line wells it reached the initial values within one month after the circulation period.

These results are in agreement with the PLFA analysis, which estimates microbial biomass in the groundwater. The results of PLFA from the wells of the first monitoring line are summarized in Fig. 9 The PLFA analysis was employed during the first nZVI and whey injections. As expected, a massive development in the microbial biomass was observed after the whey injection and was approximately 10 times, as detected in the first line monitoring wells. However, as the substrate was depleted, the total amount of bacteria rapidly decreased within two months after the circulation period. These results were verified using 16S rRNA qPCR method with samples from the nanofiber passive samplers that exhibited similar increase with a certain delay (SFig. 9). On the other hand, the PLFA results after the first nZVI injection are not in agreement with our previous finding when we recorded stimulation of G+ bacteria with no influence on G- after a nZVI injection (Němeček et al., 2014). In the present study, we observed a significant stimulation of G- bacteria (t-test, $P < 0.05$). However, it should be noted that in this case we used the groundwater from the site instead of the soil in our previous study.

In addition, we used a cultivation based test in an attempt to monitor the changes in the microbial population of psychrophilic bacteria in order to determine the toxicity of the groundwater. The results showed a positive influence of all the nZVI and whey injections on the numbers of psychrophilic bacteria and the bacterial density increased to 10^4 CFU/ml after the injections in all the cases. However, within a short period of time, the value decreased again to the original level of about 10^2 CFU/ml (SFig. 10). This is in agreement with what Xiu et al. (2010b) suggested in the case of chlorinated ethenes, we documented in our previous work (Němeček et al., 2014) for Cr(VI) and by Qiu et al. (2013) at laboratory scale as well, that nZVI application can result in a decrease in the pollutant concentrations below the threshold toxicity levels.

Barcoded amplicon sequencing from the sediment samples collected at the end of the test revealed that the bacterial community structure was different from well to well, although some phylotypes, namely *Geobacter*, *Rhodoferax*, *Hydrogenophaga* and *Clostridium*, showed a high relative abundance in all samples (Fig. 10). Whereas *Geobacter* and *Rhodoferax* were already described as dehalorespiring and metal-reducing bacteria (Paes et al., 2015; Ise et al., 2011; Zhuang et al., 2011) *Clostridium* and other members of the phylogenetic group *Clostridiales* are fermentative prokaryotes that benefit from fermentable electron donors (whey) to reduce metals (Kostka and Green, 2011). Contrary to what has been reported by Merlino et al. (2015), who described the suppression of *Hydrogenophaga* upon lactate supplementation, this bacterium was not significantly affected by the injection of whey. Given the known toxic effects of nZVI towards bacteria, it seems that either the prokaryotes mentioned above were not negatively affected by nZVI suspensions or that their communities were re-established after the nZVI oxidation and they benefited from the whey injection better than other members of the eubacterial community.

In the control well (HV-8), located cross-gradient with respect to the Cr(VI)-contaminated plume that is contaminated only by chlorinated ethenes, the predominant bacterial phylotype was represented by *Imtechium assamiensis* (27% out of all detected OTUs). The high abundance of this bacterium could be due to its capability to adapt to an environment heavily polluted by chlorinated organics, such as chlorinated biphenyls (PCBs), as already pointed out by Macedo et al. (2006). Consequently, the ability of this bacterium to dechlorinate chloroethenes in addition to PCBs should not to be excluded, since the existence of analogous metabolic capabilities have been reported in other prokaryotes (Fennell et al., 2004). In spite of the scanty knowledge about the metabolic capabilities of *Imtechium*, we also postulate that its reduced abundance in the downstream wells (from MV-1 to MV-5) could be due to the toxic effects of either Cr (VI) or nZVI. Similar assumptions could be made for *Simplicispira*, a facultative anaerobic bacterium that was abundant (9%) only in the HV-8 well. In contrast, the *Rhodoferax* ribotype mentioned above (Fig. 10) is a well-known chlororespiring bacterium (Paes et al., 2015) and they were detected in all the analyzed wells (HV-8 to MV-5) at a concentration ranging between 2 and 5%. It is

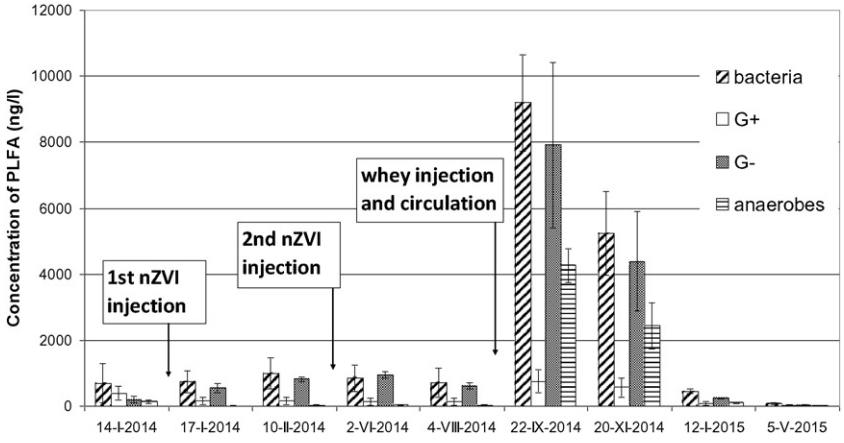

Fig. 9. Concentration of PLFA representing bacterial biomass during the pilot test. The data represent means of MV-2, MV-3 and MV4 analyses. The error bars are standard deviations.

therefore likely that *Rhodoferax* was primarily involved in the chloroethene respiration (in control well HV-8) and due to its metabolic versatility towards metals (Zhuang et al., 2011) also participated in the reduction of Cr(VI) and Fe(III) in the presence of whey in wells MV-3, 4 and 5; as well as in the MV-1 well which was partially influenced be the whey injection.

In the up-gradient well (MV-1), the most abundantly detected phylotypes were *Altererythrobacter* (6%), *Bacteroides* (5.4%) and

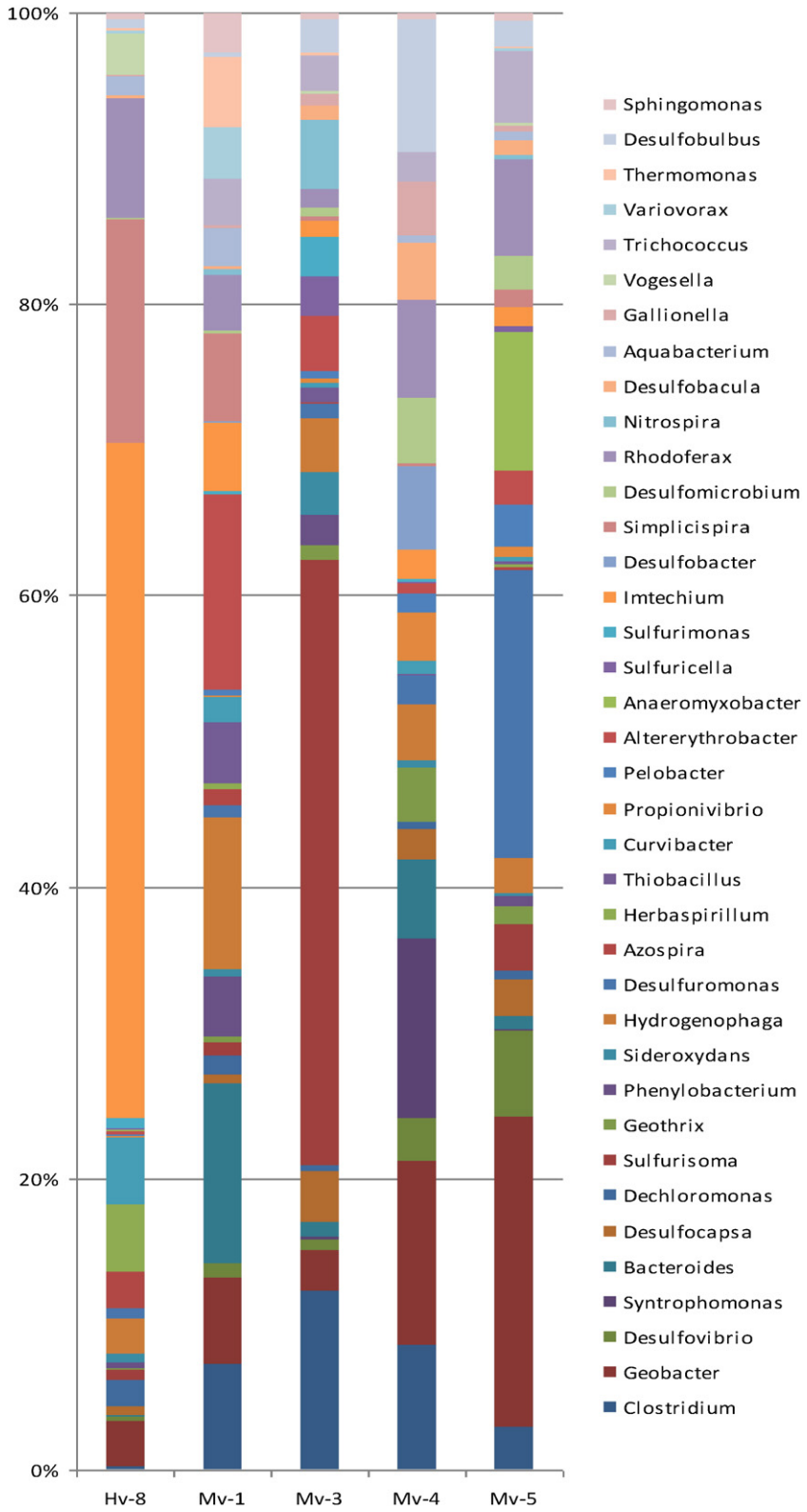

Fig. 10. Results of bacterial community Illumina sequencing of the sediment samples at the end of the pilot test. The most abundant bacterial genera (>1%) above the 95% similarity threshold are displayed.

Hydrogenophaga (4.6%). While the former and the latter are known NO_3^- reducers, similar properties have been reported only for a few *Bacteroides* spp. However, all three genera were detected in consortia actively involved in the reduction of chlorinated ethenes in contaminated aquifer/sediments/lab reactors (Merlino et al., 2015).

In the samples from MV-3, MV-4 and MV-5 wells where both Cr(VI) along with tetra-, tri- and di-chlorinated ethenes were completely removed but VC were present in the range of units of μg per liter (Figs. 7 and 8), important members of the eubacterial community that could be detected were *Desulfovibrio*, *Desulfuromonas*, *Syntrophomonas*, *Sulfurisoma*, *Desulfobulbus* and *Anaeromyxobacter*. The latter is a well-known facultative anaerobic halorespiring bacterium (HRB) which has been shown to reduce nitrate, metals and radionuclides, e.g. Fe(III), U(VI) and As(V) (Sanford et al., 2002; Smidt and de Vos, 2004; Kudo et al., 2013) while the others belong to the sulfate-reducing bacteria (SRB) (Muyzer and Stams, 2008). Numerous present SRB representatives in the samples are in agreement with our previous study, where we employed 454-sequencing to characterize the microbial community after *in-situ* whey application (Němeček et al., 2015). As evidenced from the current paper, the detected genera and families indicate that the Cr(VI) reducing process could be mediated by sulfate-reducing bacteria that are known to react up to 100 times faster than chromium-reducing bacteria from other laboratory studies (Somasundaram et al., 2011).

Other specific chlororespiring bacteria such as *Dehalococcoides*, *Dehalobacter* and *Sulfurospirillum* (Smidt and de Vos, 2004) were detected at relatively low concentrations, probably due to the fact that their abundance declined as a consequence of chloroethene removal. On the other hand, the detection of the functional genes *vcrA* and *bvcA*, which are assigned to the activity of *Dehalococcoides* (Behrens et al., 2008), suggests their elevated activity in the previous phases (Fig. 8). In addition, we performed semiquantitive PCR detection of this group in MV-1 and MV-2 samples and the results exhibited an increased activity in MV-2 as compared to the up-gradient well MV-1 (SFig. 12), documenting the presence of the bacterial genera (Hendrickson et al., 2002). Xiu et al. (2010a, 2010b) observed inhibition of trichloroethylene dechlorinating anaerobes after nZVI application; however, the results of our study document that the community can be re-established after biostimulation with the addition of whey. In addition, the chlororespiring anaerobes thrived even after the development of the methanogenic activity as documented in Figs. 7 and 8 (and SFig. 6), which is contradictory to the observations made by Xiu et al. (2010a, 2010b).

4. Conclusions

The results of this study document the feasibility and high efficiency of the combined nano-biotechnological approach of nZVI and *in-situ* whey application in removing Cr(VI) and chlorinated ethenes from the groundwater of the contaminated site.

The repeated injection of nZVI was shown to be efficient toward Cr(VI) removal. A low effect was observed in the removal of chlorinated ethenes by nZVI in the presence of Cr(VI). On the other hand, the subsequent application of whey resulted in complete reduction of Cr(VI) to Cr(III), (s) as supported by XPS and high removal of chlorinated ethenes.

Persistence of reducing conditions even after the depletion of substrate and a corresponding decrease in the microbial biomass indicate a reciprocal effect of nZVI and whey phases used in the combined technology, in agreement to what we have suggested in our previous work (Němeček et al., 2015).

The oxidized iron resulting probably from the initial application of nZVI (in addition to the iron contained in the mineral phase) was partly regenerated, i.e. reduced to Fe(II) by microbes, during the subsequent application of whey, Fe(II), both in the dissolved and solid forms (indicated in green rust) acted as a reducing agent even when the substrate was already depleted and the microbial population decreased back to the initial level.

In addition, the reduction of Cr(VI) and/or chlorinated ethenes was probably also assisted by other reductants, such as reduced forms of sulfur produced or recycled by microbial processes. Moreover, based on the elevated concentrations of NH_4^+ and the decreased PLFA concentrations observed in the final phase of the pilot test, we also hypothesize that the decaying biomass could serve as an electron donor for continuing the bioreduction processes after the depletion of substrate. Molecular biological analyses revealed the presence of iron- and sulfate-reducing bacteria in the groundwater, which may produce these reducing compounds during their catabolic processes.

Despite previous reports indicating possible negative effects of nZVI on the chlororespiring community and its effectiveness (see Bruton et al., 2015 and references therein), the Illumina sequencing data and qPCR analyses of the functional genes from our study indicate re-establishment of the community capable of chlororespiration after the whey application and its depletion.

The successive combination of chemical reduction using nZVI and direct or indirect bioreduction stimulated by the addition of whey seems to be an efficient and sustainable approach for remediation of groundwater with a complex type of contamination, exemplified here by Cr(VI) and chlorinated ethenes.

Acknowledgments

This work was supported by Grant No. TA01021792, by Competence Center TE01020218 of the Technology Agency of the Czech Republic, and in part by Project OP VaVpI Centre for Nanomaterials, Advanced Technologies and Innovation CZ.1.05/2.1.00/01.0005. The research leading to these results has received funding from the Norwegian Financial Mechanism 2009–2014 and the Ministry of Education, Youth and Sports under project contract no. MSMT-23681/2015-1. The authors also thank to Martin Petr for the XPS measurements.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.01.019.

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