Taking Nanotechnological Remediation Processes from Lab Scale to End User Applications for the Restoration of a Clean Environment

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WP 5: Environmental Impact of Reactive Nanoparticles

DL 5.2: Influence of Transformation and Transport on Ecotox

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Executive summary

Samples from the large scale experiments and from four field sites (Spolchemie sites I and II in Ustí nad Labem, CZ; Solvay site, CH; Balassagyarmat, HU) were analysed for their potential toxicity (Task 5.3 *Time-course of ecotoxicity alleviation after interactions with soil constituents*) and their microbial community composition (Task 5.4 *NP-microbial interactions during and after remediation*). Although transient, toxicity alleviation was demonstrated at Spolchemie site II (Nano-Goethite injection) and Balassagyarmat (Carbo-Iron® injection). At the Solvay site, a transient increase in toxicity was observed right after injection of milled Fe particles (FerMEG12). Microbial analyses carried out on samples from the Solvay site and Balassagyarmat indicated a positive effect of NP injection on indigenous microbial communities and more specifically, the apparition of organohalide-respiring bacteria after NP injection.

1 Introduction

A major regulatory obstacle for widespread use of nanoparticles (NP) *in situ* is uncertainty about unintended effects on the environment. Many stakeholders, including NGOs, regulatory authorities, and the general public, have expressed serious concern regarding the effects of unintended release of engineered nanoparticles into the environment. This scepticism is less pronounced when it comes to nano Zero-Valent Iron (nZVI), as the transformation products are iron oxides, which are natural soil constituents. Yet, some uncertainties remain and new mobilising agents and particle types (e.g. bi-metallic nanoparticles) need testing to verify or assess the potential for possible adverse environmental effects. Three important characteristics determine environmental risks: mobility (transport of nanoparticles to deep layers of surface soil or to surface waters via erosion or recharge from groundwater), toxicity (the capacity of reactive nanoparticles to cause harm) and persistence.

During the first half of the NanoRem project, WP5 partners tested the potential ecotoxicity of a range of NPs developed by other NanoRem partners, using a suite of standard and non-standard ecotoxicity tests. The results are compiled in the deliverable 5.1 (Coutris et al. 2015). During the second half of the NanoRem project, WP5 partners worked on the time-course of ecotoxicity alleviation after interactions of nanoparticles with soil constitutents. In this task, water samples from large scale experiments at VEGAS (WP8) and field sites (WP10) were tested for their potential ecotoxicity. Tests included time-course sampling to assess effects of ageing, and account for the (assumed) reduction in toxicity caused by nanoparticles transformation and adsorption to solid matrices. Data from ecotoxicity tests with nanoparticle-treated groundwater and soil were non-existent prior to NanoRem, and therefore represent a strong innovative aspect of the present project. The information provided in this deliverable is essential to furthering a more robust and empirically based understanding of the ecotox aspects of nZVI and other NPs in the environment and how this changes over time.

As bacteria are likely to be among the few organisms that will ever come into contact with reactive nanoparticles used for remediation, the second task was devoted to the study of nanoparticle-microbial interactions during and after remediation. In this task, microbial communities in soils and aquifers were characterized, prior to, during and after nanoparticle application. Metabolic capacities and rates were monitored to assess to what extent and with what delay these functions are affected and restored, if negatively impacted.

2 Time-course of ecotoxicity alleviation after interactions with soil constituents

Despite differences amongst field sites, the sampling strategies were harmonized, both with regards to sampling locations and sampling frequency. Sampling wells were all chosen within the contaminated area, with one well located upstream from the NP injection point, and three wells downstream from the NP injection point. Regarding sampling frequency, several time points were chosen to cover the situation prior to NP injection (as a reference point with maximum toxicity expected), then a few hours after NP injection, two weeks, one month, three months and nine months after NP injection. As the intrinsic physical and chemical properties of the NPs and the aquifer, including groundwater flow velocity, soil composition and porosity are site-specific, adaptations in sampling frequency were made wherever relevant. A few deviations from the original sampling schedule also occured due to

practical constraints.

The testing protocols used for testing water samples from NanoRem field sites have previously been applied to document remediation efficiencies at field sites in complex matrices (Baun et al. 1999). As no specific concerns regarding the samples from NanoRem field sites were identified, the testing protocols were applied as previously described (Baun et al. 2003 and 2004). The whole sample toxicity was measured and no fractionation was carried out. This means that the impact of groundwater quality as well as contaminant mixtures were assessed directly. This whole sample toxicity testing approach enabled identification of the most problematic samples as well as the relative development in toxicity (or reduction) over time, as a function of the remediation action initiated.

Water samples from large scale experiments at VEGAS (WP8) and field sites (WP10) were tested on the root elongation of radish *Raphanus sativus*, growth of green algae *Pseudokirchneriella subcapitata*, growth of anaerobic bacteria *Clostridium perfringens*, and colony formation of cultivable indigenous bacteria. The test protocols used were generic ones to ensure regulatory adequacy of the data generated, and further details can be found in the deliverable 5.1 (Coutris et al. 2015). The sampled groundwater was stored at 4 °C in the dark immediately after sampling (glass bottles filled up to the rim to maintain red-ox conditions) until ecotoxicity tests were performed.

2.1 Ecotoxicity testing on samples from the large scale experiments (WP8)

2.1.1 Large Scale Container (LSC) (Nano-Goethite)

Water samples from several sampling points in LSC (Fig. 1) were tested for their potential ecotoxicity, prior to injection of Nano-Goethite (14 Oct 2014), 8 h (16 Oct 2014) and 4 weeks (12 Nov 2014) after injection of Nano-Goethite.

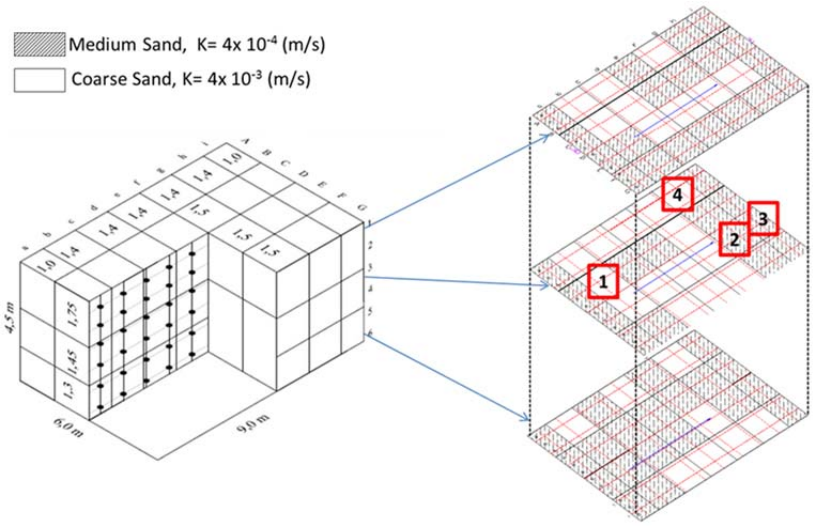


Figure 1 Sampling points in LSC. (1) b3C, positive control (unaffected by NP, but containing residual toluene); (2) h3E, containing toluene and prone to be affected by NPs; (3) i3E, further downstream; (4) h3B, similar to h3E/h3C but in coarse sand. The negative (unpolluted) control was the water entering the container.

Prior to Nano-Goethite injection, the only sample that reduced the root elongation of radish was b3C. None of the samples taken further away in the container reduced root elongation (Fig. 2). These

results seem to indicate that either toluene did not travel far in the container, or that toluene was degraded downstream by bioremediation. No toxicity towards *C. perfringens* was found at any of the sampling times (Fig. 3).

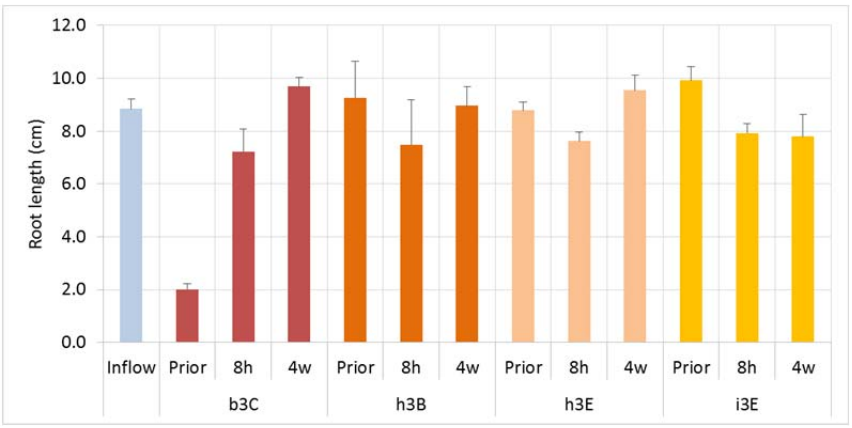


Figure 2: Root length of *Raphanus sativus* after 6-day exposure to water samples from four different sampling ports in LSC, taken before, 8 h and 4 weeks after injection of Nano-Goethite.

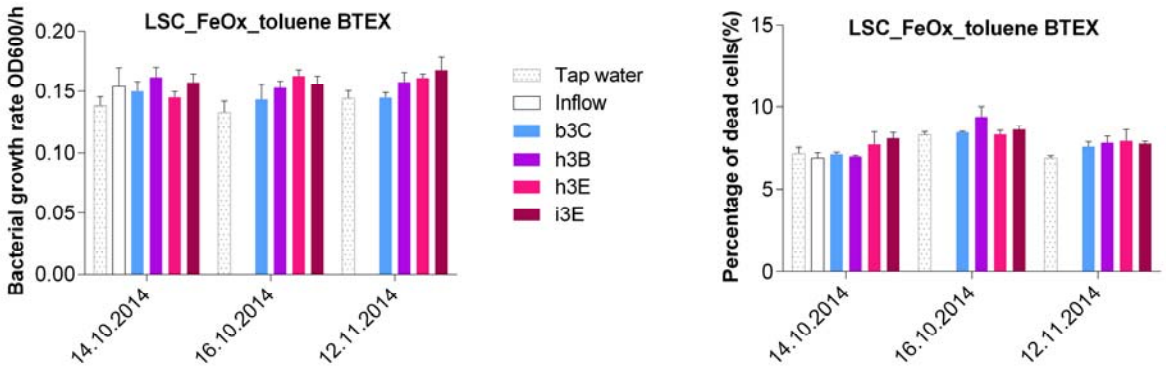


Figure 3: Effects of LSC water samples on *Clostridium perfringens* growth rate (left) and percentage of dead cells (right).

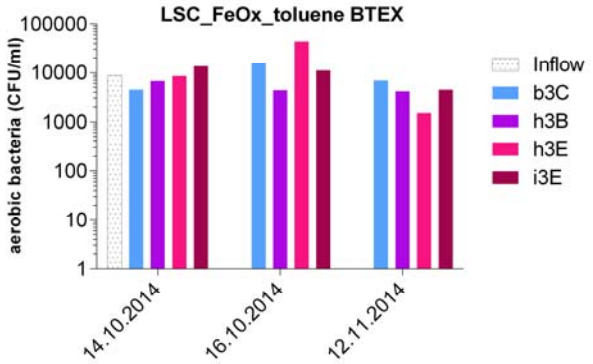
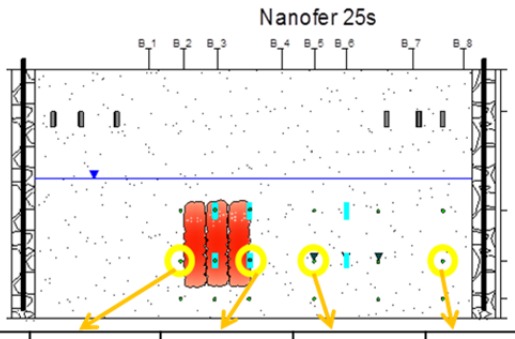


Figure 4: Colony Forming Units (CFU/mL) of aerobic bacteria in LSC water samples. Note the logarithmic scale. Anaerobic bacteria were cultivated as well, but no colony was found.

2.1.2 Large Scale Flume (LSF) (NANOFER 25S)

Water samples from several sampling points (Fig. 5) were tested for their potential ecotoxicity, prior to injection of NANOFER 25S (18 Aug 2014), 8 hours after (21 Aug 2014), 2 weeks after (4 Sep 2014), and 3 months after injection (12 Nov 2014). The average concentration of injected NANOFER 25S was 8.6 g/L.



18 Aug 2014 Prior to NP injection	A1/B1 (a3A)	A1/B3 (c3A)	A1/B4 (d3A)	A1/B7 (f3A)	Inflow
21 Aug 2014 8h after NP injection	A1/B1 (a3A)	A1/B3 (c3A)	A1/B4 (d3A)	A1/B7 (f3A)	

Figure 5: Sampling points in LSF.

None of the water samples reduced the root elongation of the plant species, neither before nor after NANOFER 25S injection (Fig. 6). No toxicity towards *C. perfringens* was found at any of the sampling times (Fig. 7). Indigenous anaerobic bacteria were found in higher abundance at the downstream border of the PCE source (sampling point A3/B3 (c3A), Fig. 8). None of the samples were toxic to the green algae *P. subcapitata* (results not shown).

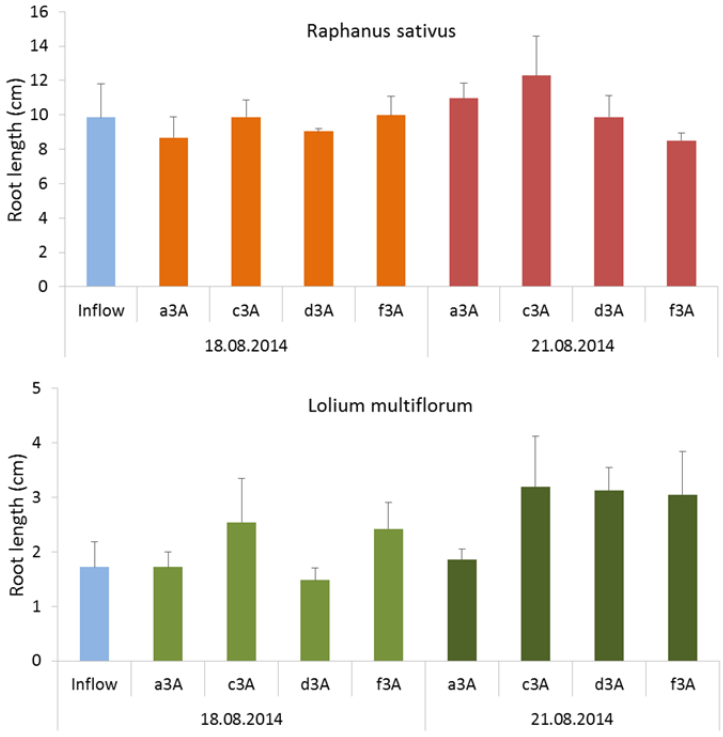


Figure 6: Root length of *Raphanus sativus* and *Lolium multiflorum* after 6-day exposure to water samples from LSF, taken before and 8h after injection of NANOFER 25S.

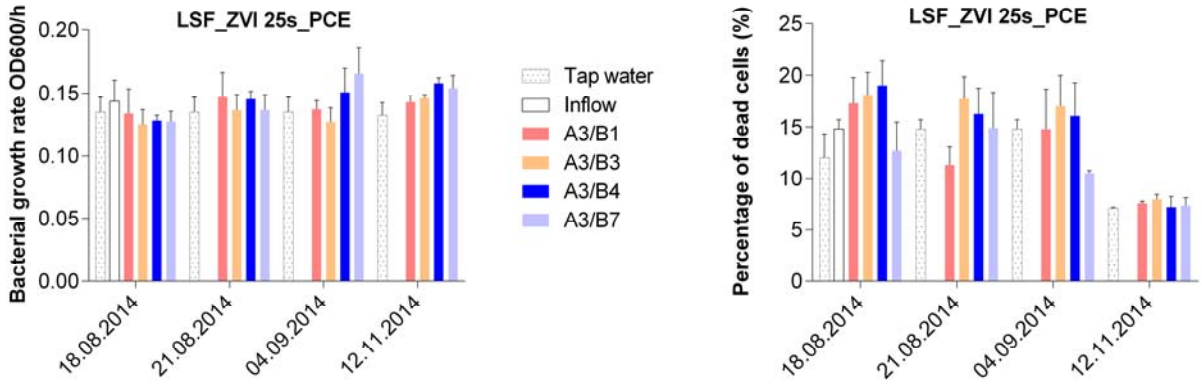


Figure 7: Effects of LSF water samples on *Clostridium perfringens* growth rate (left) and percentage of dead cells (right).

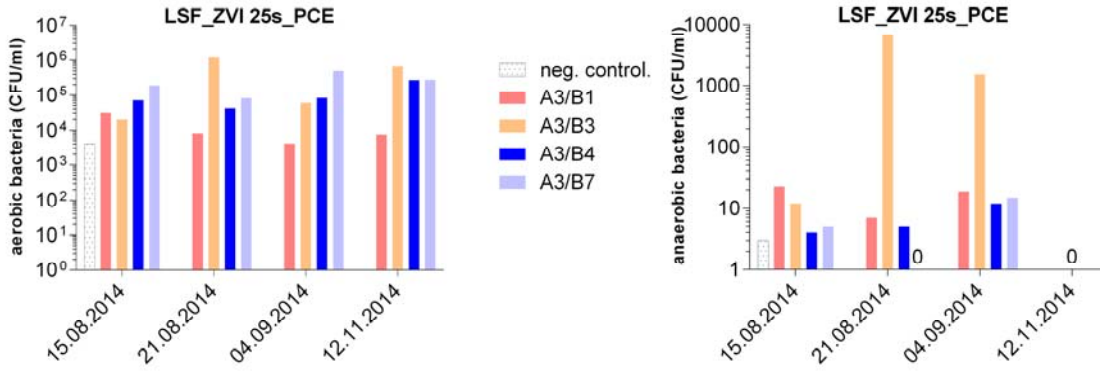


Figure 8: Colony Forming Units (CFU/mL) of indigenous aerobic (left) and anaerobic (right) bacteria in LSF water samples.

2.2 Ecotoxicity testing on samples from the field sites (WP10)

2.2.1 Spolchemie I, CZ (NANOFER 25S)

Water samples from several sampling wells (Fig. 9) were tested for their potential ecotoxicity. Several sampling times were tested: before NP injection (11 Nov 2014), right after (24 Nov 2014), 3 weeks after (15 Dec 2014), 3 months after (10 Feb 2015) and 9 months (25 Aug 2015) after injection of NANOFER 25S.

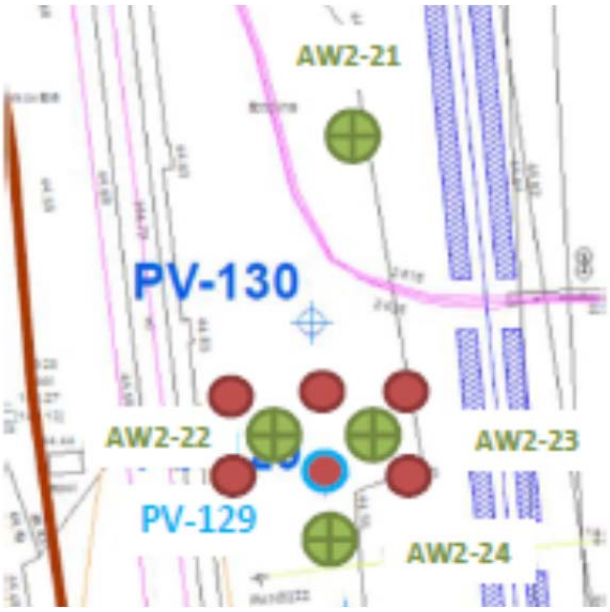


Figure 9: Sampling points at Spolchemie I: AW2-21 (8 m BGL); PV-129 (12 m BGL); AW2-22 (6.5 m BGL) and AW2-24 (6.5 m BGL). Red circles indicate the injection wells for NANOFER 25S.

None of the water samples had a significant toxic effect to the root elongation of *R. sativus* before injection and within 3 weeks after injection (Fig. 10). No toxicity either was observed towards the growth of the anaerobic bacterium *C. perfringens* before injection and within 9 months after injection (Fig. 11, left). The higher percentage of dead cells observed after incubation in water samples from AW2-22 and AW2-21 could be caused by rebounding of DNAPL nine months after nZVI application (Fig. 11, right). However this is not certain, because the sample from AW2-24, which had no negative effect on the number of dead cells, contained a high concentration of chlorinated hydrocarbons (28.2 mg/L) comparable to AW2-22 (31.1 mg/L), and the contamination in AW2-21 was even lower (3.5 mg/L). The highest toxicity towards green algae *P. subcapitata* was observed three months after injection, in wells PV-129 and AW2-24 (Fig. 12).

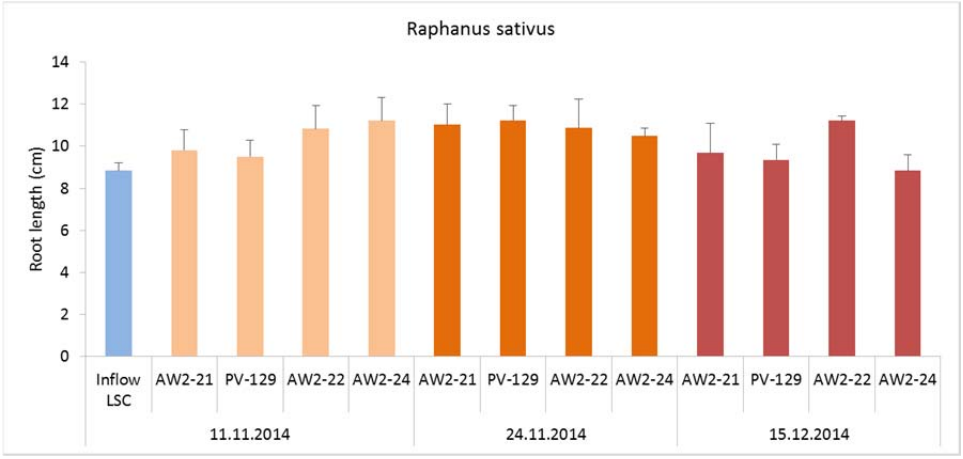


Figure 10: Root length of *Raphanus sativus* after 6-day exposure to water samples Spolchemie I, taken prior to, right after, and 3 weeks after injection of NANOFER 25S.

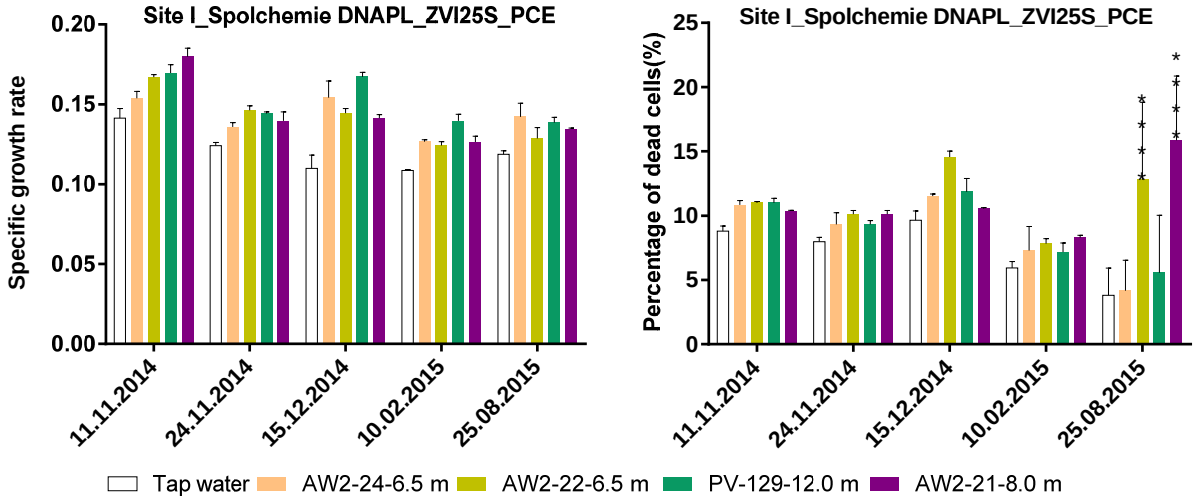


Figure 11: Effects of Spolchemie I water samples on *Clostridium perfringens* growth rate (left, expressed in OD_{600}/h) and percentage of dead cells (right).

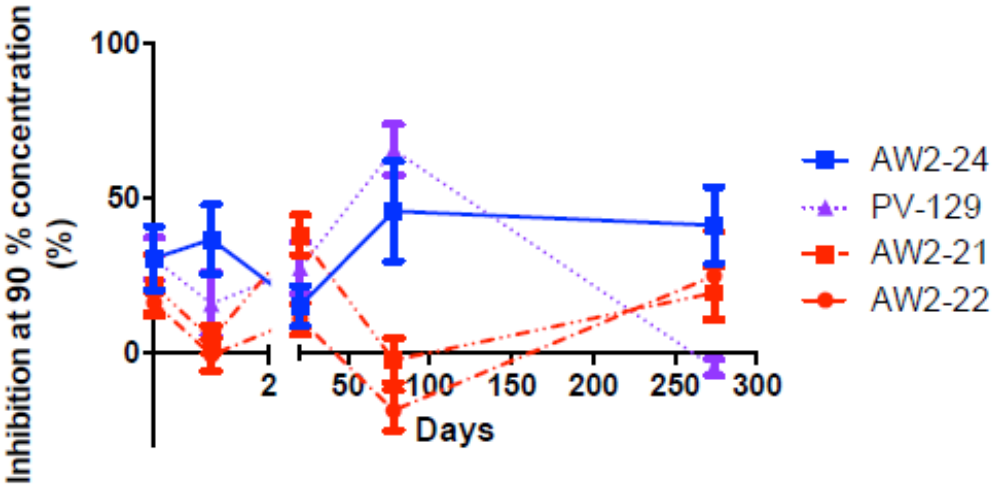


Figure 12: Growth inhibition of green algae *Pseudokirchneriella subcapitata* after 72h exposure to water samples from Spolchemie I, taken prior to, right after, 3 weeks, 3 months and 9 months after injection of NANOFER 25S.

2.2.2 Spolchemie II, CZ (Nano-Goethite)

Water samples from several sampling wells (Fig. 13) were tested for their potential ecotoxicity. Several sampling times were tested: prior NP injection (11 Nov 2014), 8 h (18 Nov 2014), 4 weeks (15 Dec 2014), 3 months (10 Feb 2015) and 9 months (25 Aug 2015) after injection of Nano-Goethite.

In addition, samples taken before injection of nFeOx were diluted with distilled water to produce the following dilution series: 0 % (distilled water, control), 6.25 %, 12.5 %, 25 %, 50 %, and 100 % (original sample). The aim was to determine the dilution resulting in 50% reduction of the root elongation.

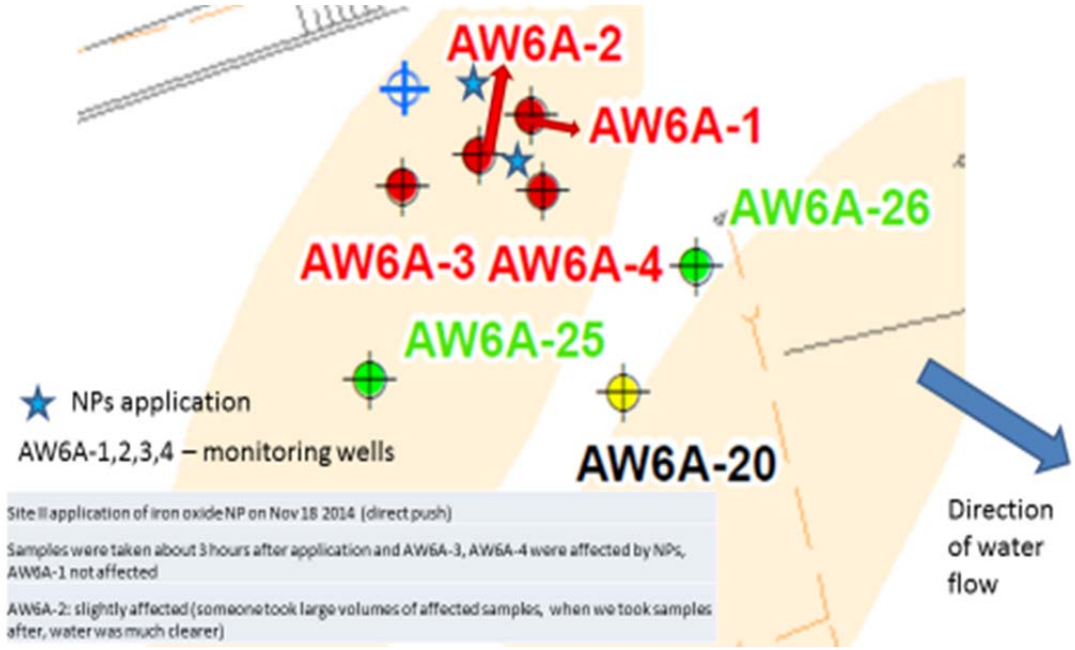


Figure 13: Sampling points at Spolchemie II: AW6A-1, AW6A-2, AW6A-3, AW6A-4.

Prior to injection of Nano-Goethite, the highest toxicity to radish root elongation was observed in AW6A-3, then in AW6A-2 (Fig. 14). The calculated EC_{50} (with 95 % confidence interval) was 25 % (17-34 %) for AW6A-3, and 42 % (23-75 %) for AW6A-2 (Fig. 15). Toxicity alleviation was very clearly observed in these two wells right after injection of Nano-Goethite (after 8h). In well AW6A-2, toxicity alleviation was maintained for at least one month. After 3 months, root lengths were similar to those before injection. In well AW6A-3, toxicity alleviation was very impressive a few hours after injection, but did not last as long as in well AW6A-2: the high toxicity seen before injection of Nano-Goethite was already back one month after injection, and stayed this way 3 months after injection. The difference in toxicity alleviation between AW6A-2 and 3 is probably explained by the fact that AW6A-3 is further away from the Nano-Goethite injection points, and maybe also closer to the core of the polluted plume (higher contamination levels).

Water samples from AW6A-1 were toxic to anaerobic bacteria *C. perfringens* before and 3h after Nano-Goethite injection, but no longer toxic 4 weeks and 3 months after NP injection, an indication of successful treatment (Fig. 16, left). Toxicity alleviation was however transient, since toxicity levels were almost back to pre-treatment levels 9 months after injection. The percentage of dead cells was between 10-15% in all samples (Fig. 16, right).

Transient toxicity alleviation was also demonstrated by toxicity tests on green algae *P. subcapitata* (Fig. 17). All samples inhibited algal growth before injection of Nano-Goethite. Right after injection (8h after), all signs of toxicity had disappeared in samples AW6A-2 and AW6A-3. Nevertheless, toxicity gradually reappeared within the next 3 months. In well AW6A-4, partial toxicity alleviation was only seen at one time point: one month after injection of Nano-Goethite. No toxicity alleviation towards algae was observed in well AW6A-1, contrary to what was found with the anaerobic bacteria *C. perfringens*.

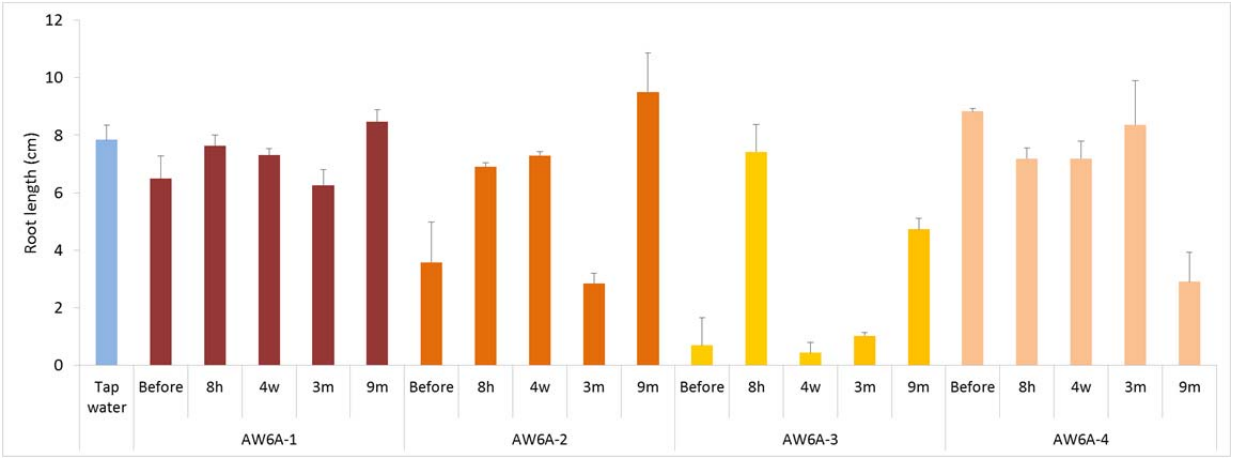


Figure 14: Root length of *Raphanus sativus* after 6-day exposure to water samples from Spolchemie II, taken before and 8h, 4 weeks, 3 months and 9 months after injection of Nano-Goethite.

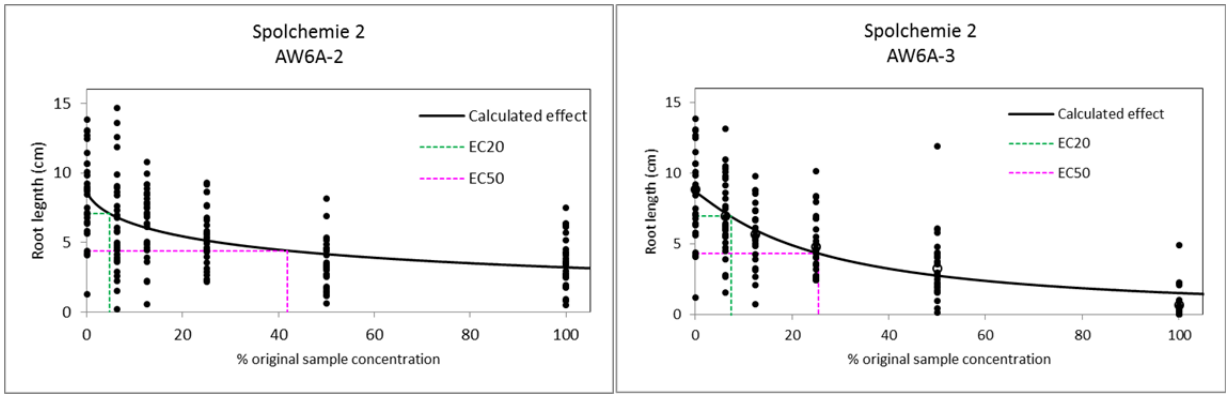


Figure 15: Dose-response relationships for AW6A-2 and AW6A-3 dilution series prior to NP injection. The Hill model was used to fit the data and calculate Effect Concentration (EC) values.

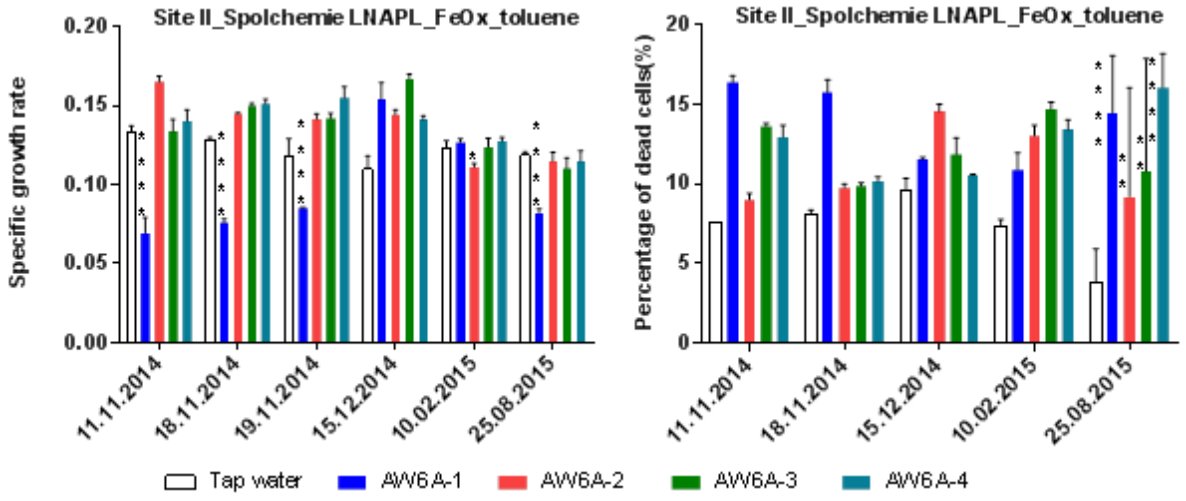


Figure 16: Effects of Spolchemie II water samples on *Clostridium perfringens* growth rate (left, expressed in OD_{600}/h) and percentage of dead cells (right).

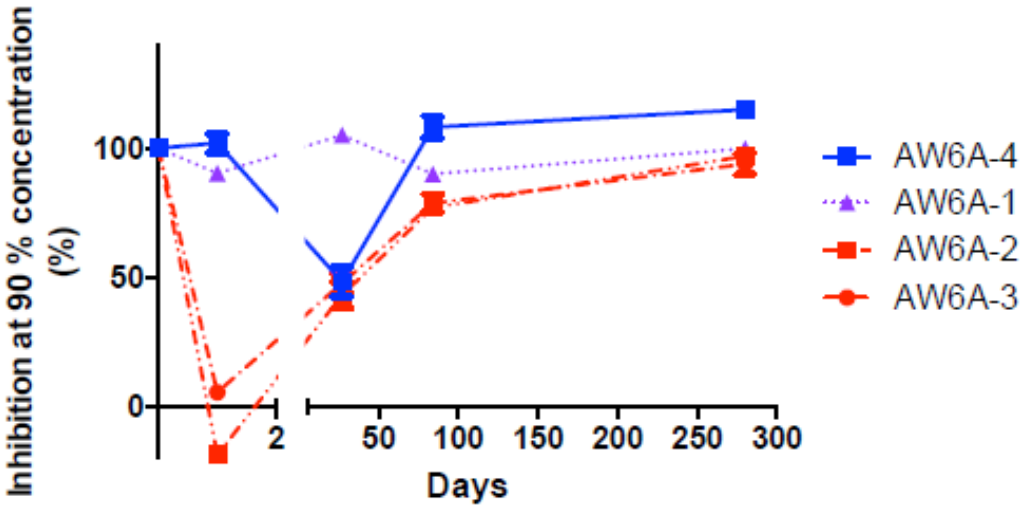
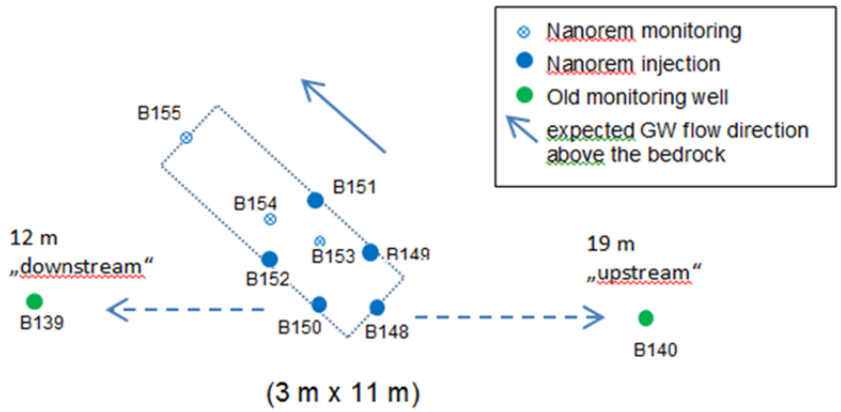


Figure 17: Growth inhibition of green algae *Pseudokirchneriella subcapitata* after 72h exposure to water samples from Spolchemie II, taken prior to, right after, 3 weeks, 3 months and 9 months after injection of Nano-Goethite.

2.2.3 Solvay, CH (FerMEG12)

Groundwater samples from 3 different wells at Solvay (Fig. 18) were tested for their potential ecotoxicity before injection (24 March 2015) of FerMEG12 particles, as well as 2 days (27 March 2015) 2 weeks (7 April 2015), 3 months (23 June 2015) and 8 months (24 Nov 2015) after injection.



Soil	B153	B154	B155
Modified marlstone Σ contam. mg/kg	14 000	12 000	165
Black marlstone Σ contam. mg/kg	20 000	3 000	380

Figure 18: Sampling points at Solvay, with contaminant concentrations for the three wells of interest.

Virtually no toxicity was observed to plant root elongation and bacterial growth, neither before nor after injection of FerMEG12 particles (Fig. 19 and 20). The significantly higher cell death induced by

water samples from well 155/D before NP injection was no longer observed after NP injection. Although a slight decrease in toxicity towards green algae was observed 8 months after injection, all samples were more toxic 2 days after injection than before injection (Fig. 21). Samples 153D and 153M, coming from the deepest layers of the well surrounded by the 5 injection points, total growth inhibition was even observed 2 weeks after injection.

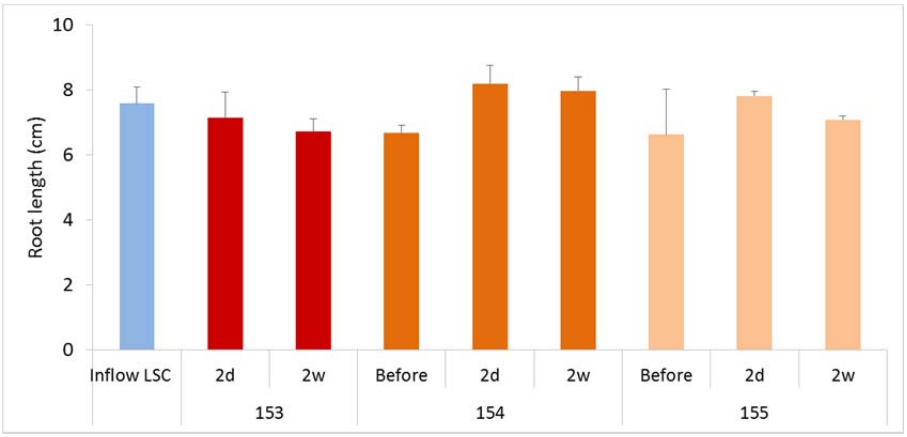


Figure 19: Root length of *Raphanus sativus* after 5-day exposure to water samples from Solvay wells 153, 154 and 155, taken before injection of FerMEG12 particles, as well as 2 days and 2 weeks after injection. Result in inflow water from LSC is shown for comparison.

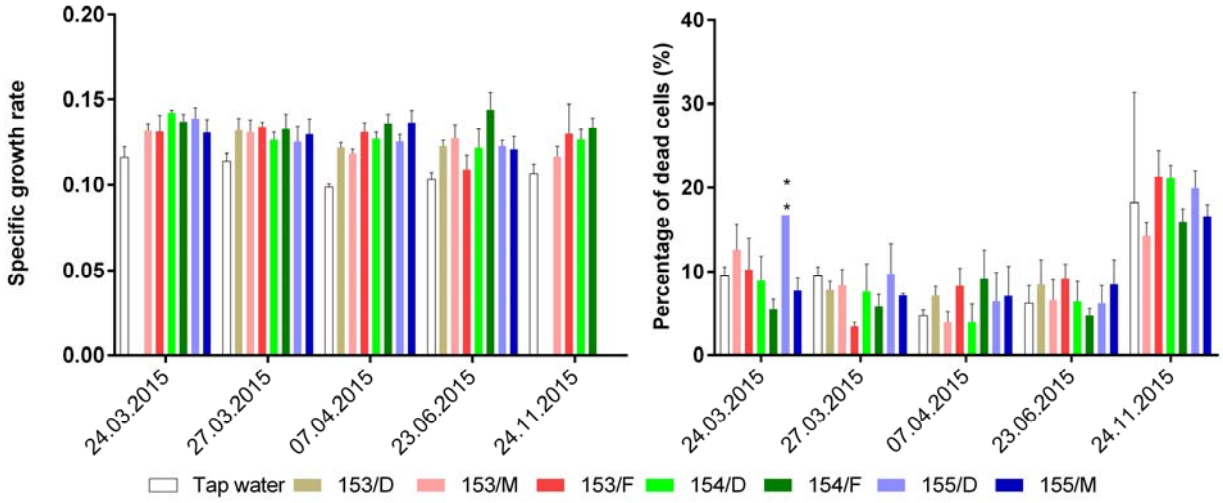


Figure 20: Effects of Solvay water samples from the Solvay site on *Clostridium perfringens* growth rate (left, expressed in OD_{600}/h) and percentage of dead cells (right). Letters F, M and D indicate the depth at which water was sampled, F being the closest to the surface and D, the deepest.

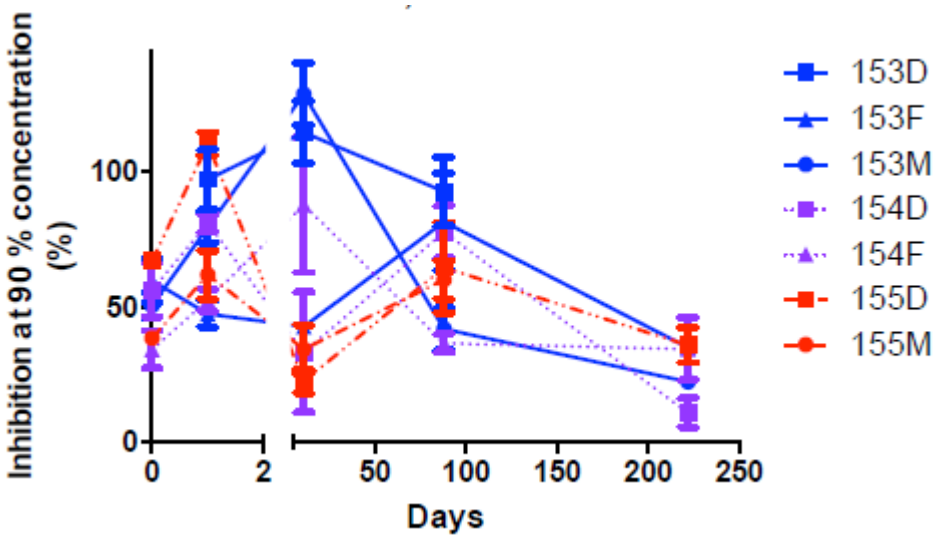


Figure 21: Growth inhibition of green algae *Pseudokirchneriella subcapitata* after 72h exposure to water samples from the Solvay site, taken before injection, 2 days, 2 weeks, 3 months and 8 months after injection of FerMEG12 particles.

2.2.4 Balassagyarmat, HU (Carbo-Iron®)

Groundwater samples from 3 different wells (and two different depths for wells CMT-2 and CMT-3) at Balassagyarmat (Fig. 22) were tested for their potential ecotoxicity before injection (10 Sep 2015), 2 days (17 Sep 2015), 1 week (22 Sep 2015), 1 month (21 Oct 2015) and 3 months (9 Dec 2015) after injection of Carbo-Iron®.

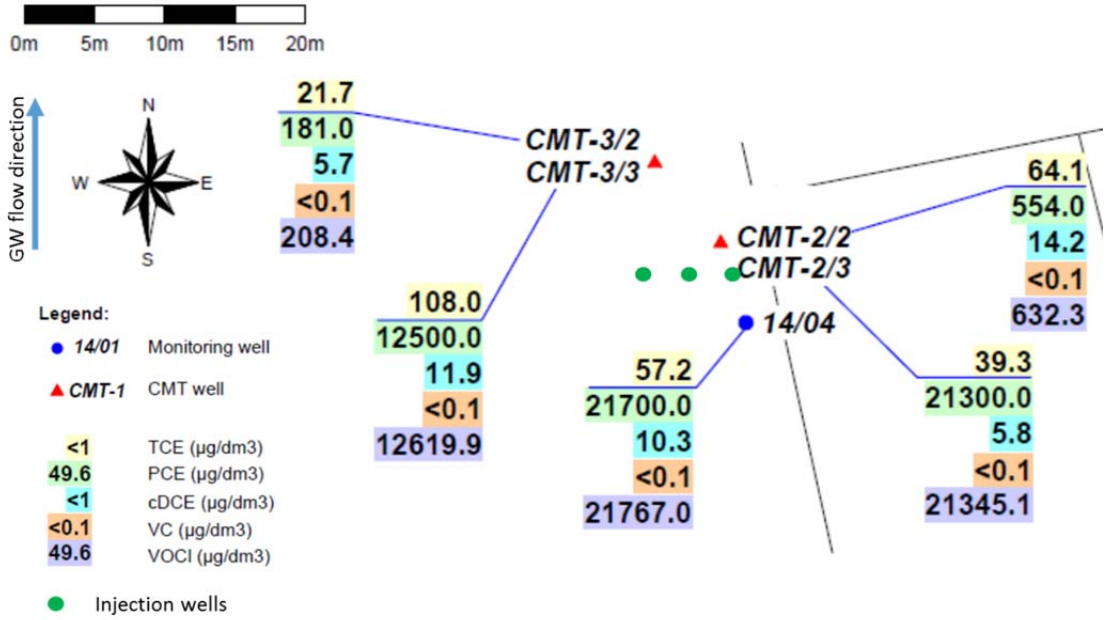


Figure 22: Sampling points at Balassagyarmat, with contaminant concentrations for the wells of interest. For wells CMT-2 and CMT-3, two depth ranges were sampled: 14.5-15.5 m (CMT-2/2 and CMT-3/2) and 18-19 m (CMT-2/3 and CMT-3/3).

Water samples from Balassagyarmat had little effect on the root elongation of radish, although some differences were observed before and after injection in CMT2/2 and CMT3/3 (Fig. 23).

None of the tested samples affected the growth rate of anaerobic soil bacteria *C. perfringens* (Fig. 24, left). However, the percentage of dead cells was significantly higher in wells CMT2/3 and CMT3/3 before injection of Carbo-Iron® (P = 0.002, Fig. 24, right). This result is consistent with the fact that these two sampling points contained high concentrations of PCE and volatile chlorinated ethylene (VOCl). Two days after injection, the percentage of dead cells was back to normal in the sampling well closest to the injection point, while the other one (CMT3/3) still displayed a significantly higher fraction of dead cells (P < 0.0001). One week after injection, only well CMT2/2 had a higher fraction of dead cells (P = 0.0001), for reasons unknown. No further effects were observed 1 month and 3 months after injection.

All water samples affected the growth of green algae before injection of Carbo-Iron® (Fig. 25). In wells 14/04 and CMT3/3, total toxicity alleviation was observed already 2 days after injection and lasted for at least 1 month (toxicity levels were back to pre-injection levels after 3 months). Partial toxicity alleviation was seen in well CMT2/2 two days after injection and lasted for at least one week (the toxicity level was back to its pre-injection level after one month). No toxicity alleviation was observed in wells CMT2/3 and CMT3/2.

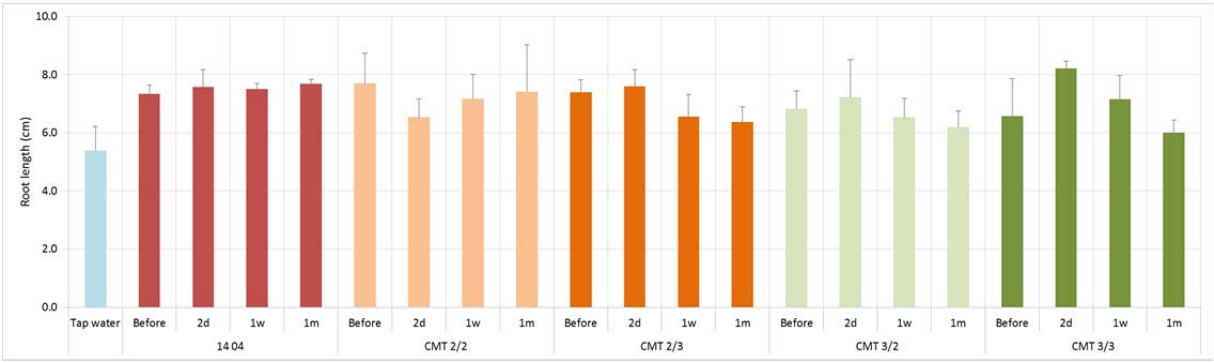


Figure 23: Root length of *Raphanus sativus* after 5-day exposure to water samples from Balassagyarmat, taken before injection of Carbo-Iron®, as well as 2 days, 1 week and 1 month after injection.

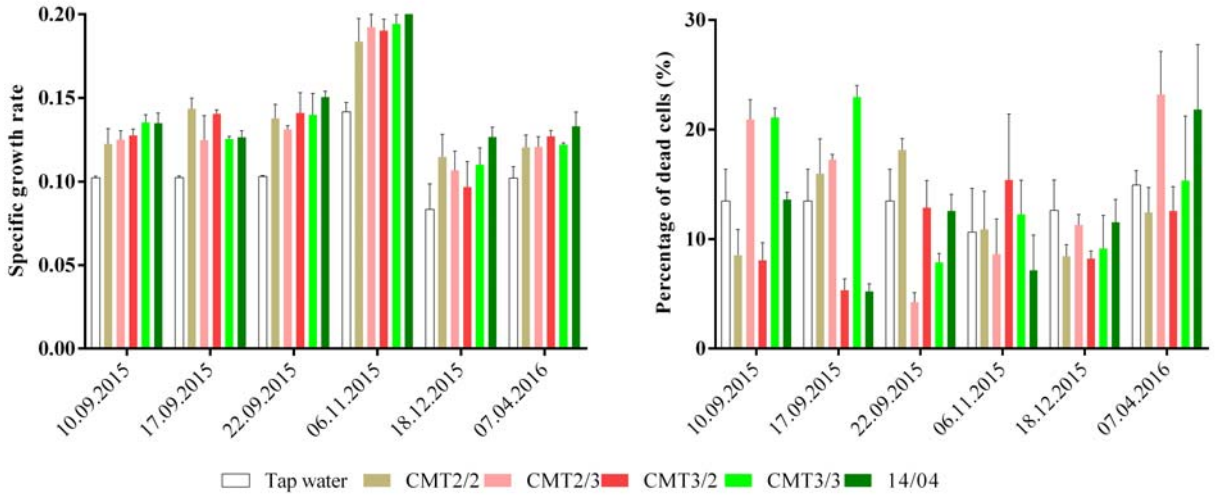


Figure 24: Effects of Balassagyarmat water samples on *Clostridium perfringens* growth rate (left, expressed in OD_{600}/h) and percentage of dead cells (right).

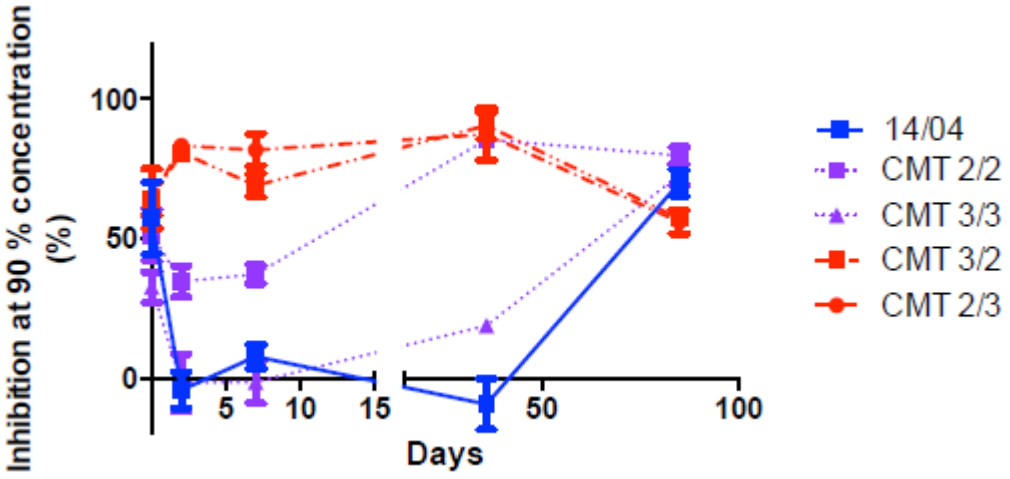


Figure 25: Growth inhibition of green algae *Pseudokirchneriella subcapitata* after 72h exposure to water samples from Balassagyarmat, taken before injection, 2 days, 1 week, 1 month and 3 months after injection of Carbo-Iron®.

3 NP-microbial interactions during and after remediation

The composition of the microbial community (by 16s rRNA gene profiling) was analysed before NP injection and several time points after NP injection, in wells located at different distances from the injection points. The total bacterial biomass (16s rDNA), the presence of dehalogenation genes (vinyl chloride reductase genes vcrA and bvcA) and of organohalide-respiring bacteria, and the expression of enzymes involved in the degradation of organic contaminants (organochlorides TCE, PCE and cDCE) were also monitored. At Spolchemie II, which is contaminated with BTEX, the presence of enzymes involved in BTEX degradation pathways was also analysed: benzylsuccinate synthase (anaerobic pathway) and catechol-2, 3-dioxygenase (aerobic pathway).

Groundwater and sand sampled for microbial community analysis, were stored frozen right after sampling to avoid biological alterations.

3.1 Microbial analyses on samples from the large scale experiments (WP8)

UMAN carried out DNA extraction from water samples taken before, during and after NP injection. Details about the extracted samples are compiled in the tables below.

3.1.1 Large Scale Container (Nano-Goethite)

Table 1: Samples from LSC processed for microbial community composition analysis.

Sampling time	Sampling date	Label	Volume	DNA Extracted	Sample No.
Prior to NP injection	05/10/2014	05/10/2014 Prior b3C	100 mL	Yes - worked	V2-1
		05/10/2014 Prior h3B	100 mL	Yes - worked	V2-2
		05/10/2014 Prior h3E	100 mL	Yes - worked	V2-3
		05/10/2014 Prior i3E	100 mL	Yes - worked	V2-4
2 h after NP injection	16/10/2014	16/10/2014 2h b3C	100 mL	Yes - worked	V2-5
		16/10/2014 2h h3B	100 mL	Yes - worked	V2-6
		16/10/2014 2h h3E	100 mL	Yes - worked	V2-7
		16/10/2014 2h i3E	100 mL	Yes - worked	V2-8
2 weeks after NP injection	01/12/2014	01/12/2014 LSC-2W b3C	100 mL	Yes - worked	V2-9
		01/12/2014 LSC-2W h3B	100 mL	Yes - worked	V2-10
		01/12/2014 LSC-2W h3E	100 mL	Yes - worked	V2-11
		01/12/2014 LSC-2W i3E	100 mL	Yes - worked	V2-12

As microbial profiling has been also been carried out by Helmholtz Zentrum München, DNA was extracted from the samples for future work if needed.

3.1.2 Large Scale Flume (NANOFER 25S)

Table 2: Samples from LSF processed for microbial community composition analysis.

Sampling time	Sampling date	Label	Colour	DNA Extracted	Sample No.
Prior to NP injection	15/08/2014	A3/B1_2 (a3B)	Clear	Yes - worked	V1
		Inflow	Clear	Yes - worked	V2
		A3/B4_2 (d3B)	Clear	Yes - worked	V3
		A3/B7_2 (f3B)	Clear	Yes - worked	V4
2 h after NP injection	21/08/2014	A3/B1 (a3A)	Clear	Yes - worked	V5
		A3/B3 (c3A)	Cloudy	Yes - worked	V6
		A3/B4 (d3A)	Clear	Yes - worked	V7
		A3/B7 (f3A)	Clear	Yes - worked	V8
2 weeks after NP injection	04/09/2014	A3/B1 (a3A)	Clear	Yes - worked	V9
		A3/B3 (c3A)	Clear	Yes - worked	V10
		A3/B4 (d3A)	Clear	Yes - worked	V11
		A3/B7 (f3A)	Clear	Yes - worked	V12
3 months after NP injection		A3/B1		Yes - worked	V13
		A3/B3		Yes - worked	V14
		A3/B4		Yes - worked	V15
		A3/B7		Yes - worked	V16

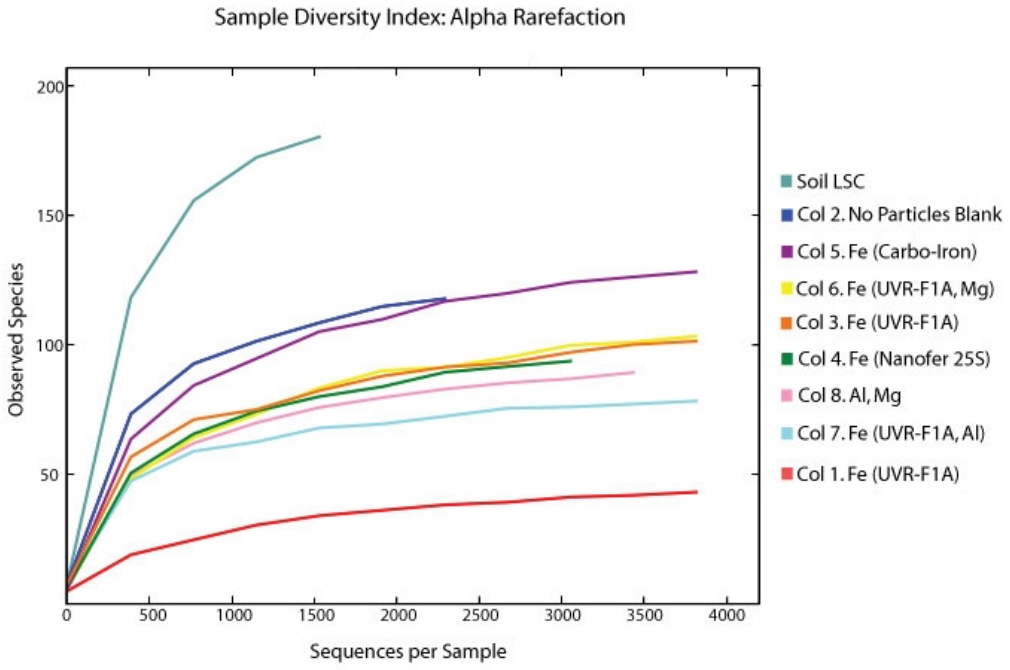
Upstream border of source: a3A, a3B, A3/B1; Downstream border of source: c3A, A3/B3; 50-75 cm downstream: d3A, d3B, A3/B4; 225 cm downstream: f3A, f3B, A3/B7. Extracted volume was 100 mL. DNA amplifications have been saved back and can be further processed if required.

3.1.3 VEGAS columns (all NP types)

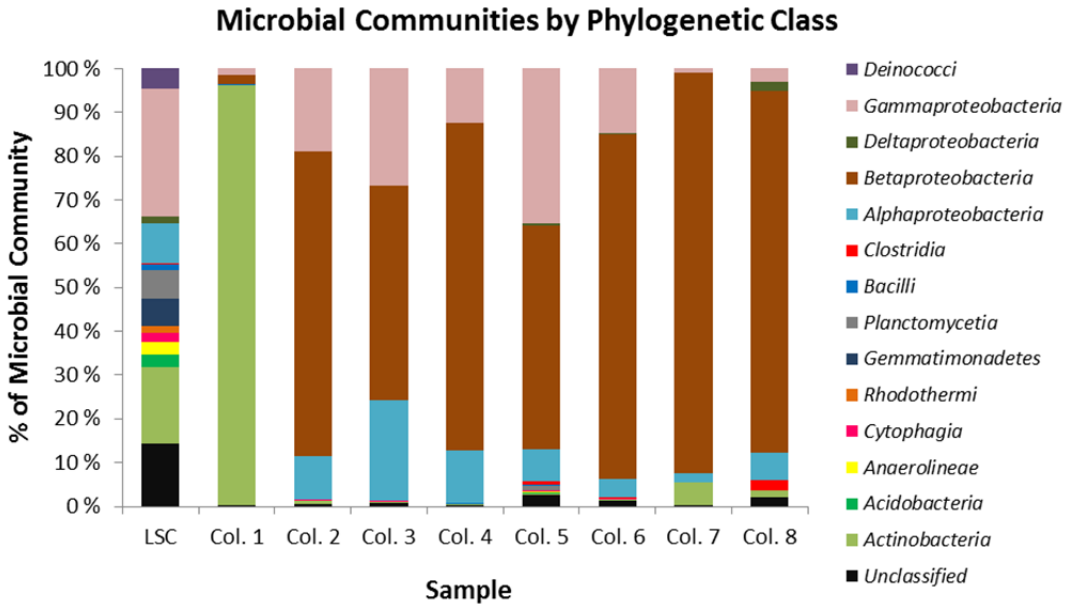
Table 3: Samples from VEGAS processed for microbial community composition analysis.

Sample label	Arrival date	Volume	Type	DNA Extracted
Soil LSC	08/09/2014	100 mL	Sand	Yes - worked
Column 1 - Fe (FerMEG12)	10/11/2014	50 mL	Sand	Yes - worked
Column 2 - No Particles - blank	10/11/2014	50 mL	Sand	Yes - worked
Column 3 - Fe (FerMEG12)	10/11/2014	50 mL	Sand	Yes - worked
Column 4 - Fe (NANOFER 25S)	10/11/2014	50 mL	Sand	Yes - worked
Column 5 - Fe (Carbo-Iron)	10/11/2014	50 mL	Sand	Yes - worked
Column 6 - Fe (FerMEG12), Mg	10/11/2014	50 mL	Sand	Yes - worked
Column 7 - Fe (FerMEG12), Al	10/11/2014	50 mL	Sand	Yes - worked
Column 8 - Al, Mg	10/11/2014	50 mL	Sand	Yes - worked
Soil LSC	10/11/2014	50 mL	Sand	Yes - worked
Column 1 - Fe (FerMEG12)	10/11/2014	50 mL	Sand	Yes - worked

More background information about these columns can be found in Menadier Stavelot, 2014. For these samples, a detailed microbial community analysis has also been carried out (Fig.26).



(A)



(B)

Figure 26: Microbial analyses of samples from VEGAS (A) Sample diversity index, (B) 16S rRNA gene microbial community composition.

The LSC background soil contained a large diversity of mainly novel uncharacterised bacterial species. Column 1 was found to be dominated (~96% of the 16S rRNA microbial community) by a species closely related to *Rhodococcus erythropolis Strain CCM2595*. This species has been shown to utilize phenol, catechol, resorcinol, hydroxybenzoate, hydroquinone, *p*-chlorophenol, *p*-nitrophenol, pyrimidines, and sterols as carbon sources. The other columns all had broadly similar microbial communities comprised mainly of members of the Betaproteobacteria and Gammaproteobacteria.

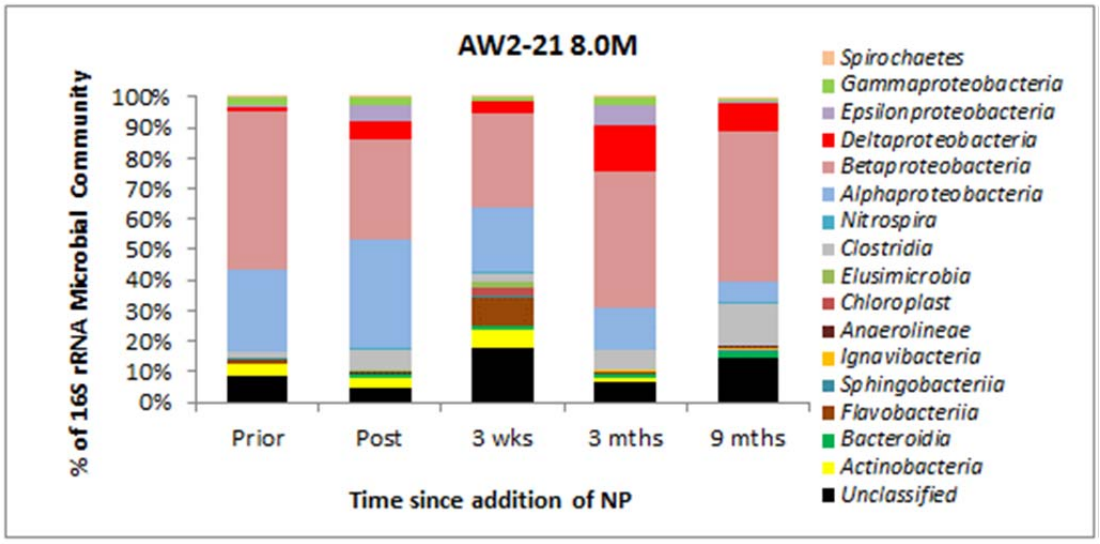
3.2 Microbial analyses on samples from the field sites (WP10)

3.2.1 Spolchemie I, CZ (NANOFER 25S, then NANOFER STAR)

Table 4: Samples from Spolchemie I processed for microbial community composition analysis.

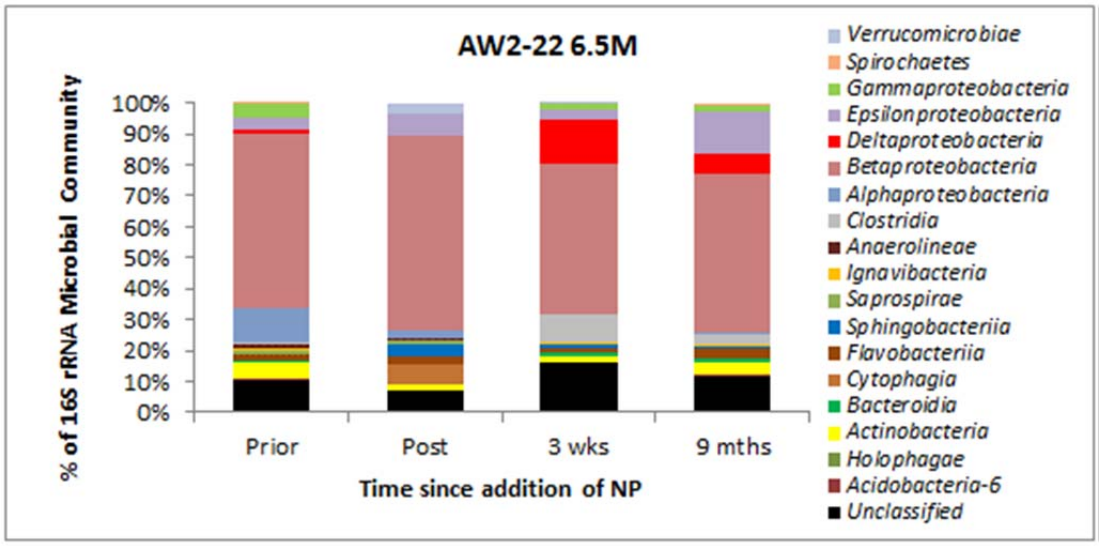
Sampling time	Sample label	Well	Horizon	Pump	DNA Extracted
Prior to NP injection 11.11.2014	1A	AW2-24	6.5 m	Anaerobic Pump	Yes - worked
	2A - BROKEN	AW2-24	8 m	Anaerobic Pump	
	3A - BROKEN	AW2-24	9.5 m	Aerobic Pump	
	1B	AW2-22	6.5 m	Anaerobic Pump	Yes - worked
	2B	AW2-22	8.0 m	Anaerobic Pump	Yes - worked
	3B	AW2-22	9.5 m	Aerobic pump	Yes - worked
	C	PV-129	12.0 m	Aerobic pump	Yes - worked
	D	AW2-21	8.0 m	Anaerobic pump	Yes - worked
24.11.2014	1A	AW2-24	6.5 m	Anaerobic Pump	Yes - worked
	1B	AW2-22	6.5 m	Aerobic pump	Yes - worked
	C	PV-129	12.0 m	Aerobic pump	Yes - worked
	D	AW2-21	8.0 m	Anaerobic pump	Yes - worked
15.12.2014	1A (S1-11)	AW2-24	6.5 m	Anaerobic Pump	Yes - worked
	1B (S1-12)	AW2-22	6.5 m	Aerobic pump	Yes - worked
	C (S1-13)	PV-129	12.0 m	Aerobic pump	Yes - worked
	D (S1-14)	AW2-21	8.0 m	Anaerobic pump	Yes - worked

Additional time points (3 months and 9 months post injection) were analyzed for most wells. An Illumina Miseq next generation sequencer was used to characterize (by 16S rRNA gene profiling) the microbial communities present in groundwater samples taken from test wells at Spolchemie site I.

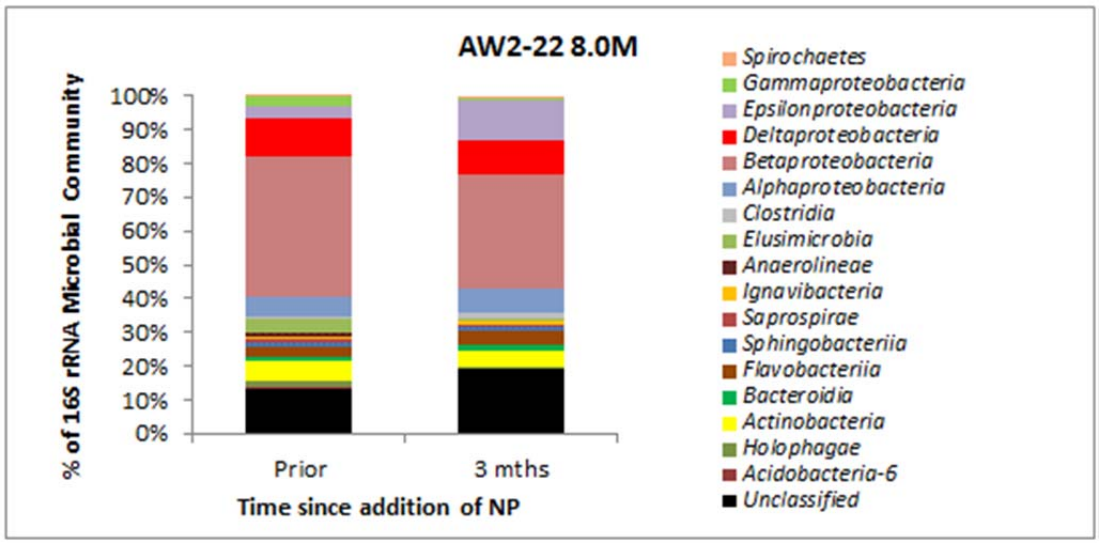


(A)

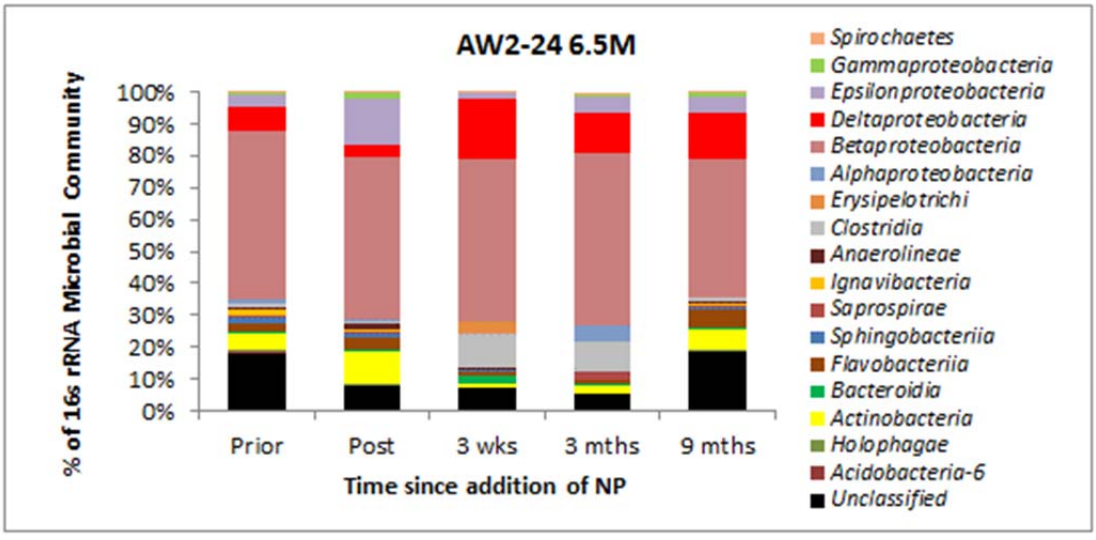
(B)

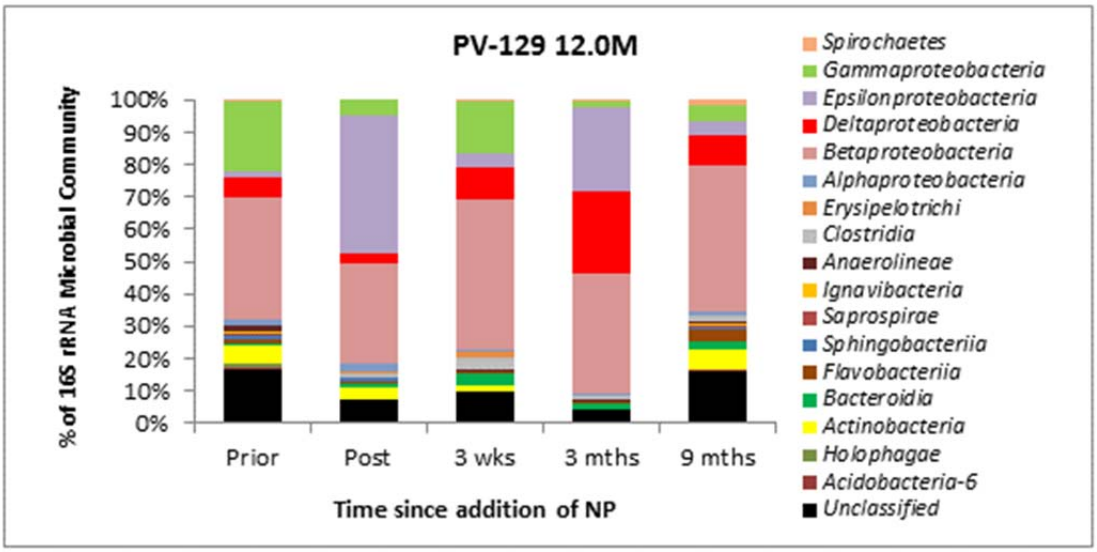

AW2-22 6.5M
% of 16S rRNA Microbial Community
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Prior
Post
3 wks
9 mths
Time since addition of NP
Verrucomicrobiae
Spirochaetes
Gammaproteobacteria
Epsilonproteobacteria
Deltaproteobacteria
Betaproteobacteria
Alphaproteobacteria
Clostridia
Anaerolineae
Ignavibacteria
Saprospirae
Sphingobacteriia
Flavobacteriia
Cytophagia
Bacteroidia
Actinobacteria
Holophagae
Acidobacteria-6
Unclassified

(C)


AW2-22 8.0M
% of 16S rRNA Microbial Community
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Prior
3 mths
Time since addition of NP
Spirochaetes
Gammaproteobacteria
Epsilonproteobacteria
Deltaproteobacteria
Betaproteobacteria
Alphaproteobacteria
Clostridia
Elusimicrobia
Anaerolineae
Ignavibacteria
Saprospirae
Sphingobacteriia
Flavobacteriia
Bacteroidia
Actinobacteria
Holophagae
Acidobacteria-6
Unclassified

(D)


AW2-24 6.5M
% of 16s rRNA Microbial Community
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Prior
Post
3 wks
3 mths
9 mths
Time since addition of NP
Spirochaetes
Gammaproteobacteria
Epsilonproteobacteria
Deltaproteobacteria
Betaproteobacteria
Alphaproteobacteria
Erysipelotrichi
Clostridia
Anaerolineae
Ignavibacteria
Saprospirae
Sphingobacteriia
Flavobacteriia
Bacteroidia
Actinobacteria
Holophagae
Acidobacteria-6
Unclassified



(E)

Figure 27:16S rRNA microbial community analysis of Spolchemie Site I groundwater samples from wells AW2-21 (A), AW2-22 6.5 m (B), AW2-22 8 m (C), AW2-24 (D) and PV-129 (E).

Wells AW2-22, AW2-24, and PV-129 experienced relatively minor shifts in microbial community composition, and after 9 months the communities appeared to be of a very similar composition to those prior to nanoparticle addition (Fig. 27). Well AW2-21 experienced a significant increase in the presence of *Clostridia* species over the 9 months (2% → 14%) but otherwise remained similar. Species important in the degradation of chlorinated hydrocarbons were discovered in all wells; there was a significant presence of *Dechloromonas* species in PV-129 (6.35% of community after 3 weeks), and AW2-21 (11.52% after addition of nanoparticles). *Geobacter* species were present in all wells at site I, comprising up to 12.5% of the 16S rRNA microbial community.

Water samples taken before and after the second NP application (NANOFER STAR injected between 15 and 18 Oct 2015) were sent to TUL for analysis of indigenous microbial communities. Samples were obtained from wells PV-112, PV-129, PV-130, AW2-24 (6.5 m, 8 m, 9.5 m and 11 m below ground level) and AW2-23 (5 m, 7 m and 8 m below ground level) (Fig. 28). Water was sampled before NP injection, and 3 days, 2 weeks, 1 month, 2 months, 3 months and 8.5 months after NP injection.

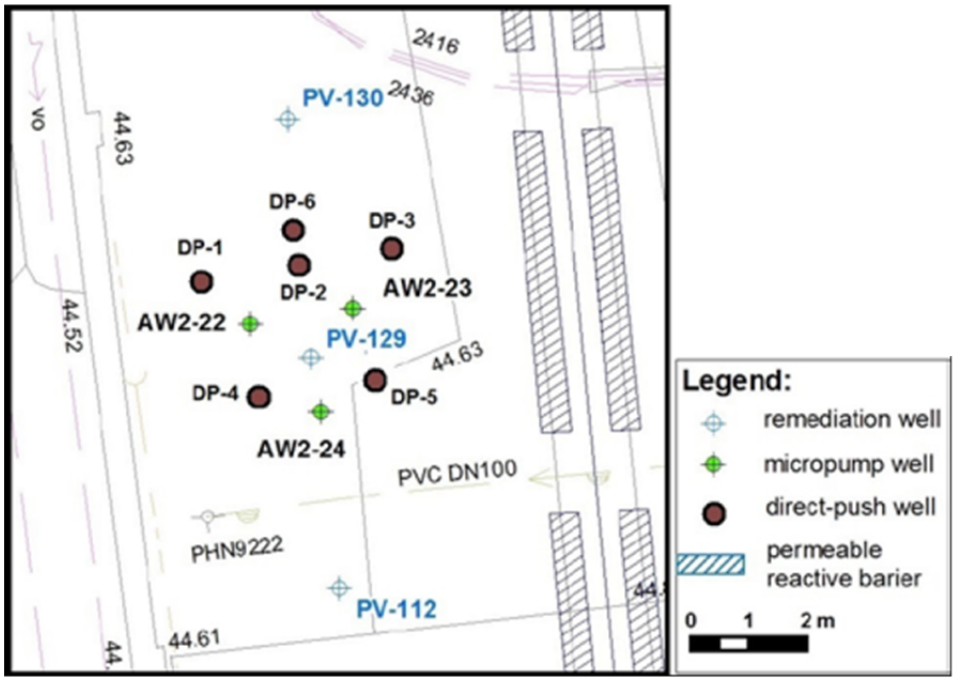


Figure 28: Sampling points for microbial analysis at Spolchemie I: PV-112, PV-129, PV-130, AW2-24 and AW2-23. Situation at the time of NANOFER STAR injection (red circles).

Quantitative polymerase chain reaction (qPCR) was performed to assess the relative abundance of different organohalide-respiring bacteria and vinyl chloride reductase genes level (vcrA and bvcA) (Table 5). The results of qPCR were evaluated as relative quantification, with the condition of the organohalide-respiring bacteria prior to NANOFER STAR injection taken as the starting point.

Table 5: Specific primers used for quantitative PCR.

Name	Sequence (5' → 3')	Product size (bp)	Target organism; genes	Reference
U16SRT-F	ACTCCTACGGGAGGCAGCAGT	180	Bacteria; 16S rRNA genes	Clifford et al. 2012
U16SRT-R	TATTACCGCGGCTGCTGGC			
vcrA880F	CCCTCCAGATGCTCCCTTTA	139	*Dehalococcoides sp.* strain VS; *vcrA*	Behrens et al. 2008
vcrA1018R	ATCCCCTCTCCCGTGTAACC			
bvcA277F	TGGGGACCTGTACCTGAAAA	247	*Dehalococcoides sp.* strain BAV-1; *bvcA*	Behrens et al. 2008
bvcA523F	CAAGACGCATTGTGGACATC			
Dre441F	GTTAGGGAAGAACGGCATCTGT	205	*Dehalobacter sp.*; 16S rRNA genes	Smits et al. 2004
Dre645R	CCTCTCCTGTCCTCAAGCCATA			
DHC793F	GGGAGTATCGACCCTCTCTG	191	*Dehalococcoides sp.*; 16S rRNA genes	Yoshida et al. 2005
DHC946R	CGTTYCCCTTTCRGTTCACT			
Dsb406F	GTACGACGAAGGCCTTCGGGT	213	*Desulfitobacterium sp.*; 16S rRNA genes	Smits et al. 2004
Dsb619R	CCCAGGGTTGAGCCCTAGGT			

Results of qPCR analysis from zonal micropumps from wells AW2-24 and AW2-23 are not shown, because limited volume of water samples caused too low DNA yields that gave very inconsistent results.

Total bacterial biomass decreased right after NANOFER STAR injection in most of the wells, except for PV-112, where it increased 5 times and later decreased again. Well PV-112 was situated on the outflow and was not directly affected by NANOFER STAR nanoparticles contrary to the other wells PV-129 and PV-130, where increase in Fe(II) and lithium chloride tracer were clearly detected. On the other hand, based on a decrease in redox conditions, the well PV-112 was most probably affected indirectly by drilling and by pressure during direct-push injection of NANOFER STAR. Extra nutrients, originally non-available, could be mobilised and utilised by bacteria. This effect disappeared within a few days (November 2nd) due to relatively fast underground water flow (1m/day). Finally, bacterial biomass in all wells was higher than before injection (Fig. 29).

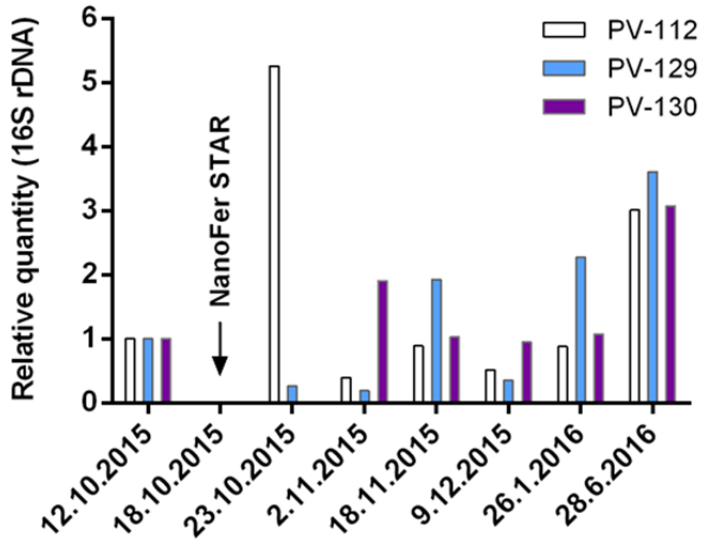
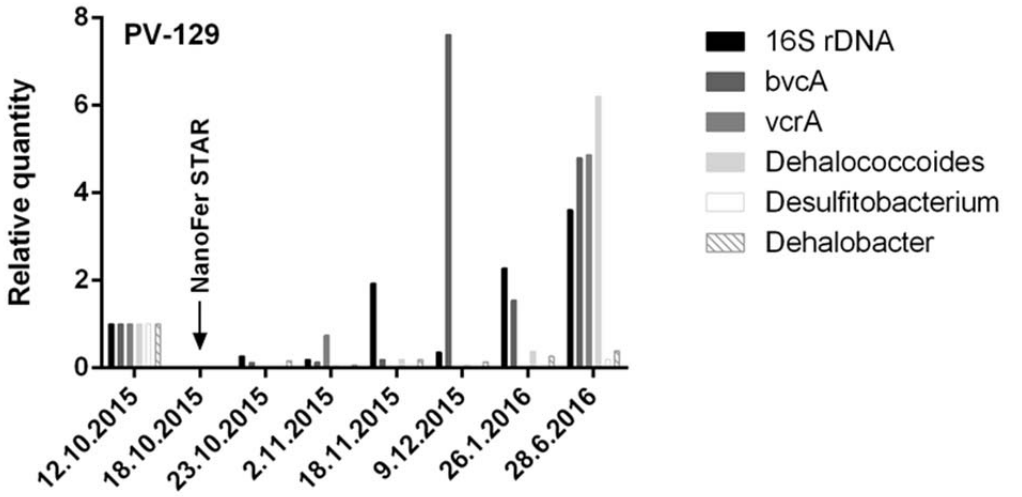
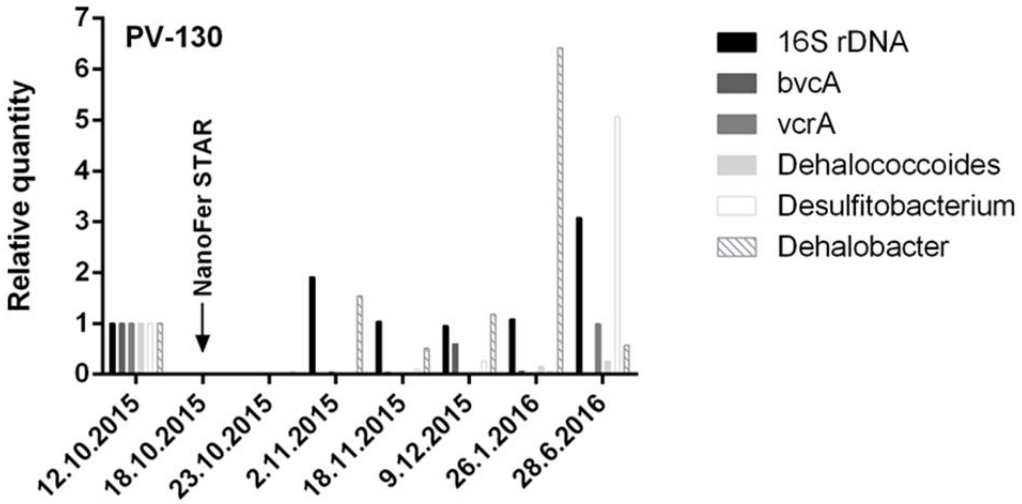


Figure 29: Relative quantification of 16S rDNA in Spolchemie I groundwater samples.

A similar pattern was observed for the relative abundance of organohalide-respiring bacteria and vinylchloride reductase genes vcrA and bvcA (Fig. 30). Right after the NANOFER STAR injection, their signals decreased and most of them returned to the state prior to the application already after one month. The exception was well PV-129 where the signals remained low even after 3 months, nevertheless all monitored bacteria and genes were detected after 8.5 months in higher abundance than prior injection. Interestingly, the two nanoiron-affected wells differed in terms of presence of specific organohalide-respiring bacteria: *Dehalococcoides* were more abundant in PV-129, and *Dehalobacter* and *Desulfitobacterium* in PV-130.



(A)



(B)

Figure 30: Relative quantification of total bacterial biomass, organohalide-respiring bacteria, and vinylchloride reductase genes in NANOFER STAR-affected wells PV-129 (A) and PV-130 (B).

To conclude, the injection of NANOFER STAR caused first a negative effect on selected organohalide-respiring bacteria and bvcA and vcrA genes. However, this effect was transient, and groundwater was colonized again with monitored bacteria within approximately 1 month.

3.2.2 Spolchemie II, CZ (Nano-Goethite)

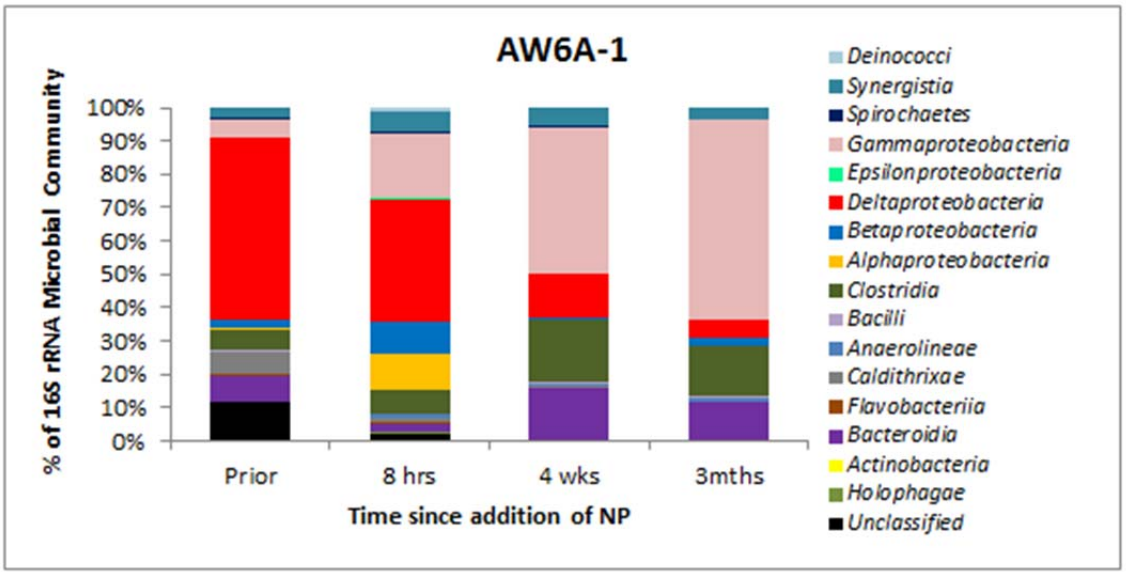
Table 6: Samples from Spolchemie II processed for microbial community composition analysis.

Sampling time	Sample label	Well	Horizon	Pump	DNA Extracted
11.11.2014 Before NP injection	1	AW6A-1	5.5 m	Aerobic pump	Yes - worked
	2	AW6A-2	5.5 m	Aerobic pump	Yes - worked
	3	AW6A-3	5.5 m	Aerobic pump	Yes - worked
	4 - BROKEN	AW6A-4	5.5 m	Aerobic pump	
18.11.2014	1	AW6A-1	5.5 m	Aerobic pump	Yes - worked
	2	AW6A-2	5.5 m	Aerobic pump	Yes - worked
	3 - BROKEN	AW6A-3	5.5 m	Aerobic pump	
	4	AW6A-4	5.5 m	Aerobic pump	Yes - worked
15.12.14	1 (S2-7)	AW6A-1	5.5 m	Aerobic pump	Yes - worked
	2 (S2-8)	AW6A-2	5.5 m	Aerobic pump	Yes - worked
	3 (S2-9)	AW6A-3	5.5 m	Aerobic pump	Yes - worked
	4 (S2-10)	AW6A-4	5.5 m	Aerobic pump	Yes - worked

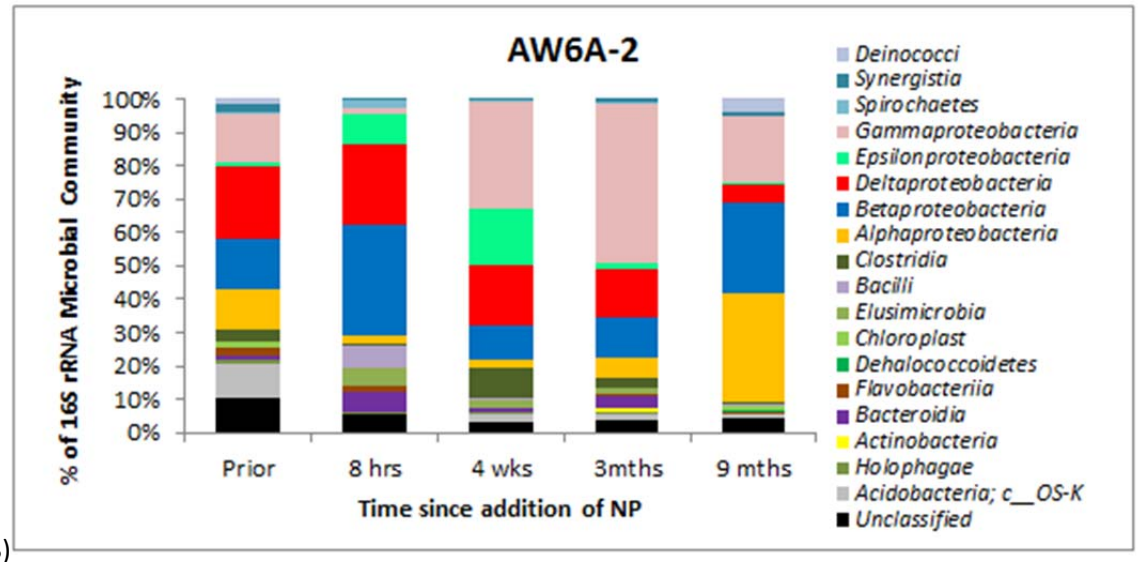
Additional time points (3 momths and 9 months post injection) were also analysed.

An Illumina Miseq next generation sequencer was used to characterize (by 16S rRNA gene profiling) the microbial communities present in groundwater samples taken from test wells at Spolchemie site II.

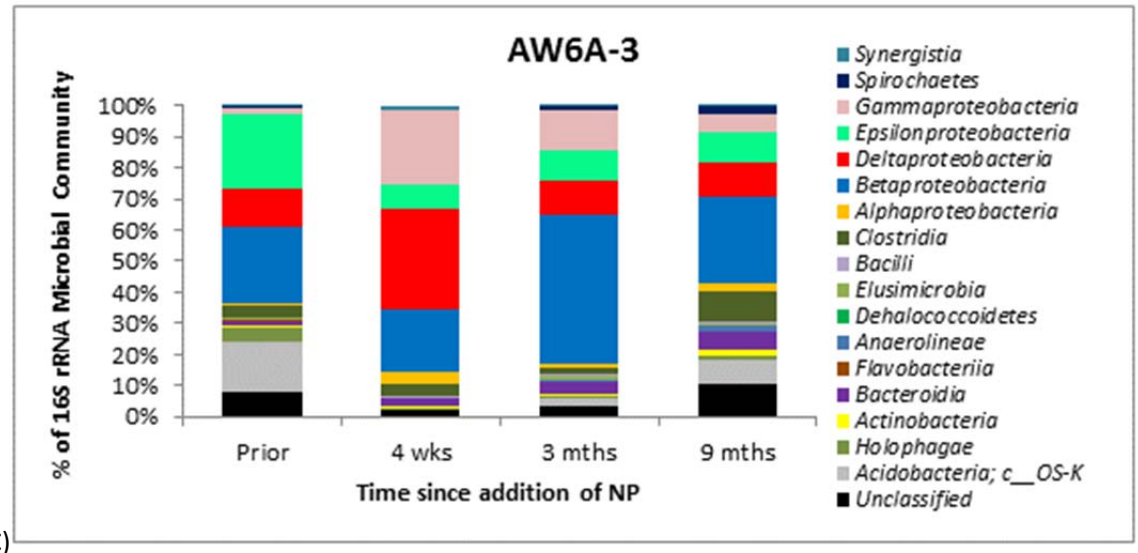
(A)



(B)



(C)



(D)

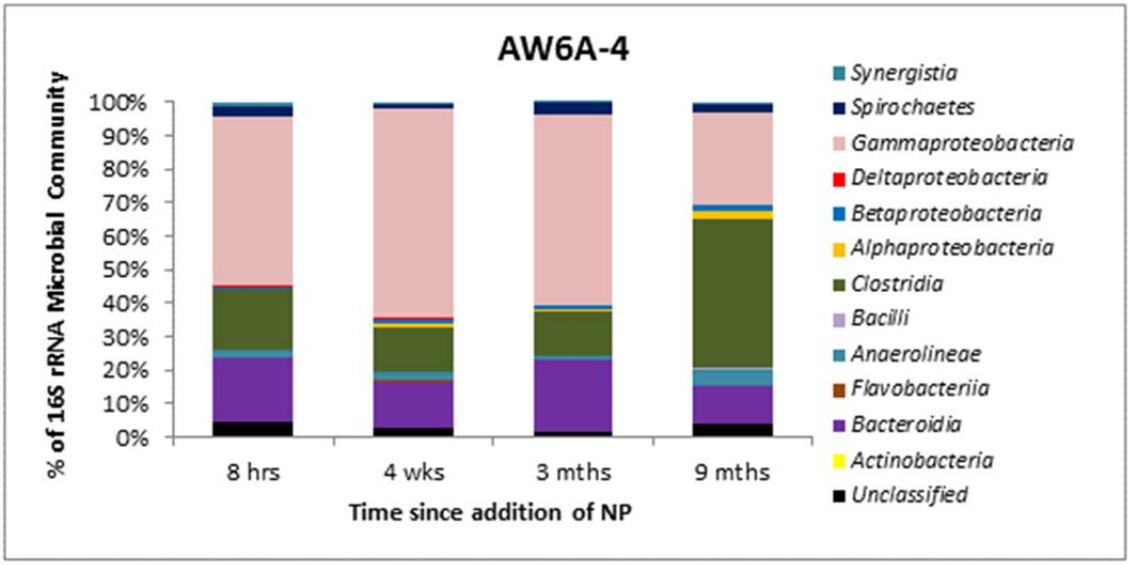


Figure 31: 16S rRNA microbial community analysis of Spolchemie Site II groundwater samples from wells AW6A-1 (A), AW6A-2 (B), AW6A-3 (C) and AW6A-4 (D).

At Spolchemie II most wells experienced more significant shifts in microbial community composition following nanoparticle addition. Well AW6A-1 experienced an increase in Gammaproteobacteria (5% → 60%) and Clostridia (6% → 15%). In well AW6A-2 there was an increase in Alphaproteobacteria (12% → 33%) and Betaproteobacteria (15% → 27%). Well AW6A-3 remained broadly similar across the time course but there was stimulation of Clostridia (4% → 10%) and Bacteroidia (1.5% → 5%). Well AW6A-4 showed an increase in Clostridia (18% → 45%). A species closely related (98% match) to *Georgfuchsia toluolica strain G5G6* was found to be present in all samples at site II, although it was particularly prevalent in well AW6A-3 (46.75% of microbial community after 3 months). This is an anaerobic bacterium which uses nitrate, Fe (III) or Mn (IV) as an electron acceptor in order to carry out the oxidation of toluene and ethylbenzene.

Second Nano-Goethite injection (October 2015)

Water samples taken before and after the second NP application (300 kg of Nano-Goethite suspended in 60 m^3 of river water directly pushed into the 5-8 m BGL between 20 and 23 Oct 2015) were sent to TUL for analysis of indigenous microbial communities. Particularly, samples were obtained from AW6A-30 and AW6A-34 (Fig. 32) before the Nano-Goethite injection, 3 days after the injection, 2 weeks, 1 month, 2 months, 3 months and 8.5 months after the injection.

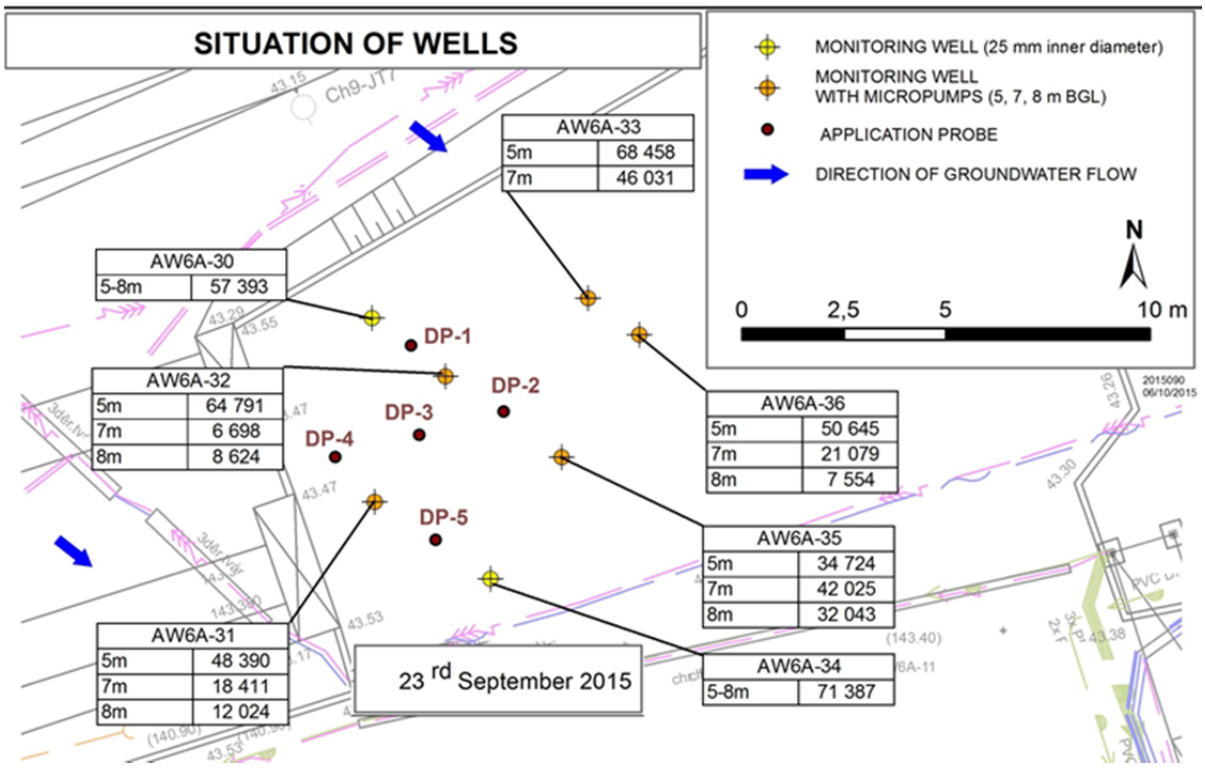


Figure 32: Sampling points for microbial analysis at Spolchemie II, CZ – AW6A-30 and AW6A-34. Situation one month before second Nano-Goethite injection in October 2015.

This site is mainly contaminated by BTEX (roughly tens of mg/l) and partly by chlorinated hydrocarbons (units of µg/l). Samples were processed and DNA was isolated as described above. qPCR with specific primer sets was performed to monitor changes in the quantity of total bacterial biomass (16S rDNA), different organohalide-respiring bacteria, vinyl chloride reductase genes level (vcrA and

bvcA), and were also analysed for the presence of enzymes involved in BTEX degradation - benzylsuccinate synthase (anaerobic pathway) and catechol-2, 3-dioxygenase (aerobic pathway).

Nano-Goethite application had an immediate inhibitory effect on organohalide-respiring bacteria in well AW6A-30, and then all the monitored parameters increased again after 1 month, and after that slowly decreased until the end of the sampling period (Fig. 34). The initial inhibition might be partly caused by O_2-saturated Labe river water in which Nano-Goethite was dispersed, the slow increase was caused by release of the substrate from the soil after the injection of the nanoparticles. The benzylsuccinate synthase gene level was almost unaffected by the application and remained rather stable throughout the experiment but the level of the enzyme for the aerobic BTEX degradation pathway catechol-2,3-dioxygenase remained low even after 8.5 months after Nano-Goethite injection.

BTEX levels did not decline after Nano-Goethite injection over 210 days of monitoring. Concentrations of BTEX increased significantly after the injection of Nano-Goethite particles to the subsurface. This increase is very likely caused by unsaturated and capillary fringe zone washing after a groundwater level increase during the Nano-Goethite injection and the subsequent development of groundwater level on the site. The groundwater level increased during winter 2015/2016 and spring 2016 after a very dry summer 2015, from 4.7 m below surface to more then 3.7 m. Thus, the concentration ratio between the inflow (AW6A-30) and outflow area (AW6A-34) has changed (Figure 33). This fact indicates the ongoing slow process of microbial degradation of BTEX in the area of application.

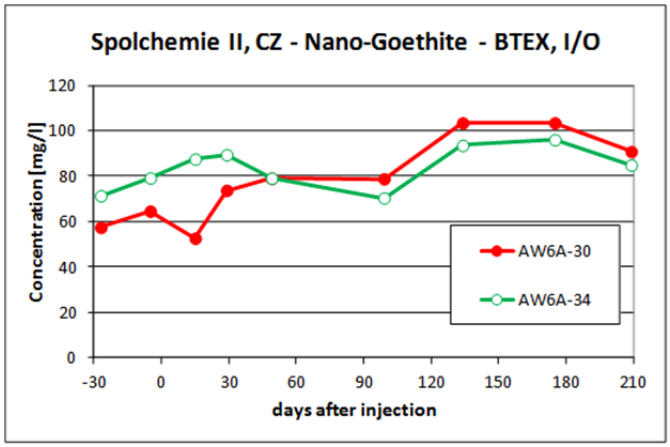


Figure 33: Spolchemie Site II, CZ groundwater monitoring: BTEX concentration development.

Colonization of well AW6A-34 developed differently (Fig. 35). After Nano-Goethite application, growth of organohalide-respiring bacteria and bacteria with anaerobic BTEX degradation enzyme benzylsuccinate synthase were inhibited, while the level of aerobic catechol-2,3-dioxygenase increased. The main reason is that the concentration of total iron was only 60 mg/l in comparison to more than 1 g/l in AW6A-30. All monitored bacterial groups and enzymes were detected 8.5 months after the application. This is particularly important for biodegradation processes and their biostimulation, i.e. when nutrients are added, we can be sure that the important bacterial degraders are present and will be supported.

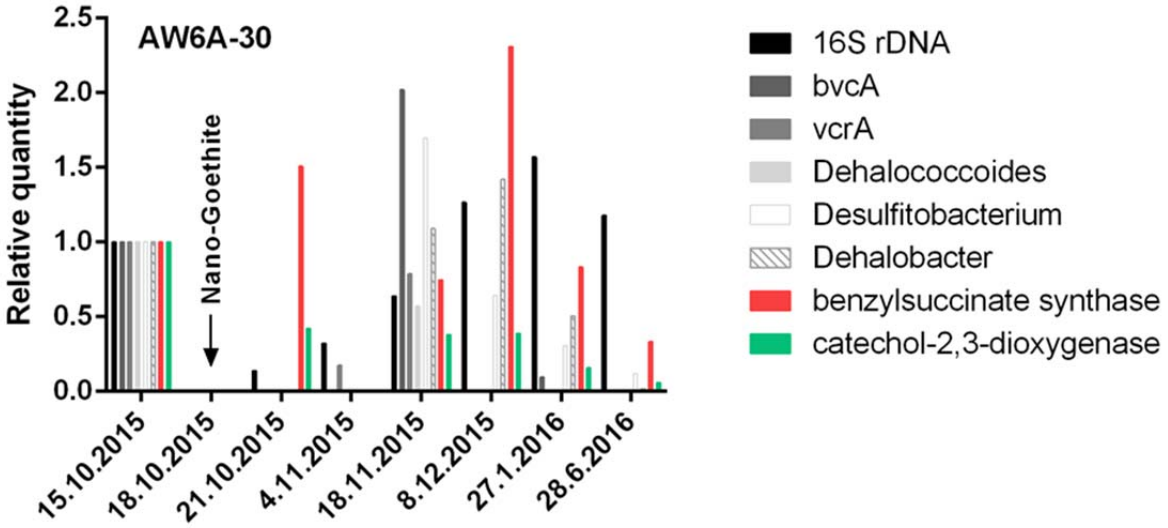


Figure 34: Relative quantification of total bacterial biomass, organohalide-respiring bacteria, vinylchloride reductase genes and BTEX degrading enzymes in well AW6A-30 before and after Nano-Goethite injection.

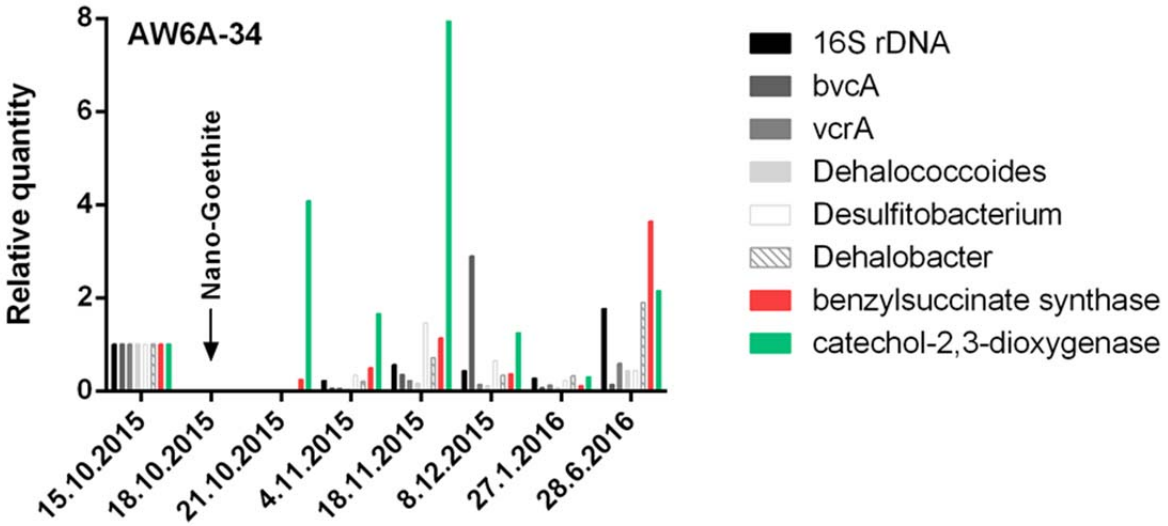


Figure 35: Relative quantification of total bacterial biomass, organohalide-respiring bacteria, vinylchloride reductase genes and BTEX degrading enzymes in well AW6A-34 before and after Nano-Goethite injection.

3.2.3 Solvay, CH (FerMEG12)

Table 7: Samples from the Solvay site processed for microbial community composition analysis.

Sample number	Sample type	Sampling date	Label	DNA Extracted
1	Groundwater	27/03/2015	153/D 27/03/2015 09:00	Yes
2	Groundwater	07/04/2015	153/D 07/04/2015	Yes
3	Groundwater	24/03/2015	153/F 24-3-15	Yes
4	Groundwater	27/03/2015	153/F 27-3-15 09:00	Yes
5	Groundwater	07/04/2015	B 153/F 07-4-15	Yes
6	Groundwater	24/03/2015	153/M 24-3-15	Yes
7	Groundwater	27/03/2015	153/M 27-3-15 09:00	Yes
8	Groundwater	07/04/2015	B 153/M 07-4-15	Yes
9	Groundwater	24/03/2015	154/D 24-3-15	Yes
10	Groundwater	27/03/2015	154/D 27-3-15 09:00	Yes
11	Groundwater	07/04/2015	B 154/D 07-4-15	Yes
12	Groundwater	24/03/2015	154/F 24-3-15	Yes
13	Groundwater	27/03/2015	154/F 27-3-15 09:00	Yes
14	Groundwater	07/04/2015	B154/F 07-4-15	Yes
15	Groundwater	24/03/2015	155/D 24-3-15	Yes
16	Groundwater	27/03/2015	155/D 27-3-15 09:00	Yes
17	Groundwater	08/04/2015	B 155/D 08-4-15	Yes
18	Groundwater	24/03/2015	155/M 24-3-15	Yes
19	Groundwater	27/03/2015	155/M 27-3-15 09:00	Yes
20	Groundwater	08/04/2015	B 155/M 08-4-15	Yes
1	Groundwater	23/06/2015	153/D 23-6-15	Yes
2	Groundwater	23/06/2015	153/F 23-6-15	Yes
3	Groundwater	23/06/2015	153/M 23-6-15	Yes
4	Groundwater	23/06/2015	154/D 23-6-15	Yes
5	Groundwater	23/06/2015	154/F 23-6-15	Yes
6	Groundwater	22/06/2015	155/D 22-6-15	Yes
7	Groundwater	22/06/2015	155/M 22-6-15	Yes

In addition, the expression of specific enzymes connected with degradation pathways of organic contaminants (organochlorides TCE, PCE and cDCE) was determined.

The data evaluation was influenced by very low DNA yield (Table 8). The initial concentration of DNA isolated from all sampled wells, except for the well 155/M, was found to be below the detection limit. Even after FerMEG12 particles application, virtually no DNA could be extracted. An increase in DNA yield was visible 14 days after application. 16S rDNA results were influenced by the extremely

low signal in samples obtained before injection of FerMEG12 particles. Nevertheless, considerable microbial community growth was detected two weeks after NP injection (Table 9). Dehalogenation genes were reported in terms of presence/absence. *VcrA* gene was not detected in any of the samples. The presence of the *bvcA* gene and organohalide-respiring bacteria *Dehalococcoides, Desulfitobacterium* and *Dehalobacter* is reported for each well and sampling time in Table 10.

The data fail to convey significant deleterious impacts associated with the injection of FerMEG12 particles. Prior to the application, some of the monitored organohalide-respiring bacteria and genes for vinyl chloride reductases were detected only in the wells 153F and 155M. Right after the application, *Dehalobacter* and *bvcA* gene were detected also in well 155D and 14 days after application some of the bacterial degraders were detected in all sampled wells, except well 153F, where the degraders appeared after 3 months. It can be concluded that the FerMEG12 treatment had a positive effect on microbial communities and particularly on organohalide-respiring bacteria.

Table 8: DNA yield from samples of the Solvay site.

Wells	**24 Mar 2015**		**27 Mar 2015**		**7 Apr 2015**		**23 Jun 2015**		**4 Nov 2015**	
	Filtered vol (L)	cDNA (ng/µL)	Filtered vol (L)	cDNA (ng/µL)	Filtered vol (L)	cDNA (ng/µL)	Filtered vol (L)	cDNA (ng/µL)	Filtered vol (L)	cDNA (ng/µL)
153/F	0.4	too low	0.55	too low	0.25	too low	0.465	5	0.50	0.72
153/M	0.5	too low	0.55	too low	0.53	5.34	0.5	4.16	0.50	32.70
154/D	0.55	too low	0.5	too low	0.53	2.38	0.5	4.92	0.50	0.42
154/F	0.55	too low	0.5	too low	0.53	17.7	0.5	0.17	0.50	1.57
155/D	0.55	too low	0.5	4.8	0.51	3.18	0.5	4.28	0.50	2.62
155/M	0.5	0.11	0.5	3.06	0.5	8.26	0.5	7.52	-	-

Table 9: 16S rDNA relative quantification in samples from the Solvay site.

Wells	**24 Mar 2015**	**27 Mar 2015**	**7 Apr 2015**	**23 Jun 2015**	**4 Nov 2015**
153/F	1.00	0.14	0.25	11665.82	66823.68
153/M	1.00	1.13	233004.78	66450.85	10998.35
154/D	1.00	5.06	7590.61	16270.83	6948.59
154/F	1.00	1.55	800199.39	2759.13	26295.05
155/D	1.00	134756.94	71220.26	76331.98	12309.66
155/M	1.00	61.82	91.77	82.14	not analyzed

Table 10: Organohalide-respiring bacteria and specific genes detection in samples of the Solvay site.

Wells	24 Mar 2015	27 Mar 2015	7 Apr 2015	23 Jun 2015	4 Nov 2015
153/F	Desulfitobacterium Dehalobacter	Desulfitobacterium		bvcA Desulfitobacterium Dehalobacter	bvcA Desulfitobacterium Dehalobacter
153/M			bvcA Desulfitobacterium Dehalobacter	bvcA Desulfitobacterium Dehalobacter	Desulfitobacterium Dehalobacter
154/D			bvcA Desulfitobacterium Dehalobacter	bvcA Desulfitobacterium Dehalobacter	bvcA Desulfitobacterium Dehalobacter
154/F			bvcA Desulfitobacterium Dehalobacter	Dehalobacter	bvcA Desulfitobacterium Dehalobacter
155/D		bvcA Dehalobacter	Desulfitobacterium Dehalobacter	Desulfitobacterium Dehalobacter	Desulfitobacterium Dehalobacter
155/M	Dehalobacter	bvcA Desulfitobacterium Dehalobacter	Dehalococcoides Desulfitobacterium Dehalobacter	Desulfitobacterium Dehalobacter	Not analyzed

3.2.4 Balassagyarmat, HU (Carbo-Iron®)

Water samples (500 mL) were obtained before Carbo-Iron® injection (10 Sep 2015), as well as 2 days (17 Sep 2015), 1 week (22 Sep 2015), 1 month (21 Oct 2015) and 3 months (9 Dec 2015) and 7 months (7 Apr 2016) after the injection. DNA was isolated from all samples, and qPCR with specific primer sets (Table 5) was performed to monitor changes in total bacterial biomass (16S rDNA), different organohalide-respiring bacteria and vinyl chloride reductase genes level (vcrA and bvcA).

Total bacterial biomass level increased after Carbo-Iron® injection in most of the wells, decreased only in well CMT3/2 and remained rather stable in well CMT3/3 (Fig. 36). After 3 months, it returned to levels similar to those prior to injection, and after 7 months it increased again in wells CMT2/2, CMT2/3 and CMT3/3. The evolution of DNA yield over the whole sampling period is presented in Fig. 37.

Vinyl chloride reductase (vcrA) gene was not detected in the sampled wells prior to Carbo-Iron® injection, but was detected in well CMT2/2 after 2 days, in well CMT2/3 after 1 month and in well 14/04 after 3 months. In well CMT3/2, vcrA gene was not detected throughout the whole monitoring period, and in well CMT3/3 its level increased after the injection, with a peak after 1 month (data not shown). A similar pattern was observed for the bvcA gene (data not shown).

The level of *Dehalococcoides* remained rather stable after the injection in wells CMT2/2, CMT2/3 and CMT3/3, and no impact of Carbo-Iron® was observed. In the remaining wells, *Dehalococcoides* were not detected before the injection and appeared 2 days (CMT3/2) and 3 months (14/04) after the injection.

The level of *Dehalobacter* was stable throughout the experiment in wells CMT2/2, CMT2/3 and CMT3/2, and decreased in well CMT3/3. A ten-fold increase in *Dehalobacter* was observed in well 14/04, followed by a decrease (Fig. 38).

The level of *Desulfitobacterium* increased after the injection in wells CMT2/2 and CMT2/3, decreased

in wells CMT3/3 and CMT3/2 and remained stable in well 14/04. Changes in monitored parameters in well 14/4 and CMT2/2 are shown as examples in Fig. 38 and 39.

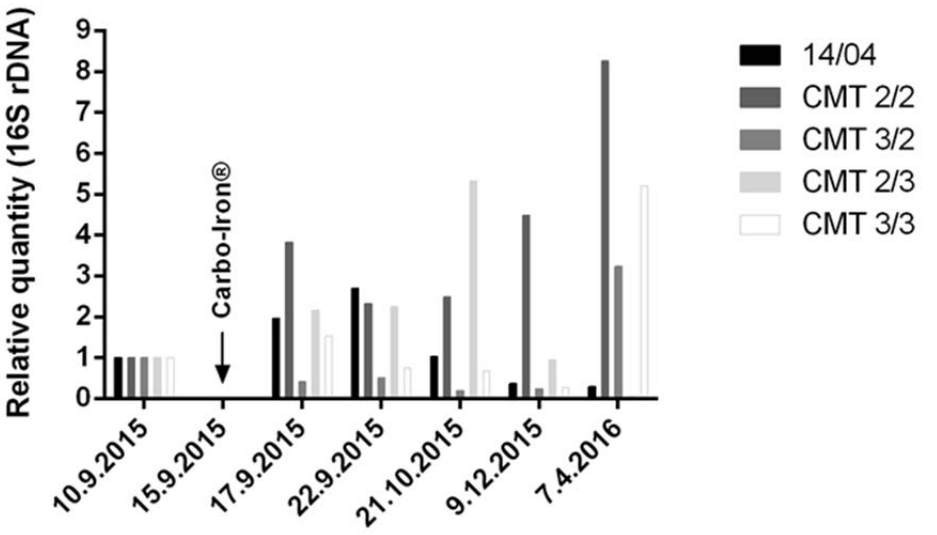


Figure 36: Relative quantification of 16S rDNA in Balassagyarmat samples.

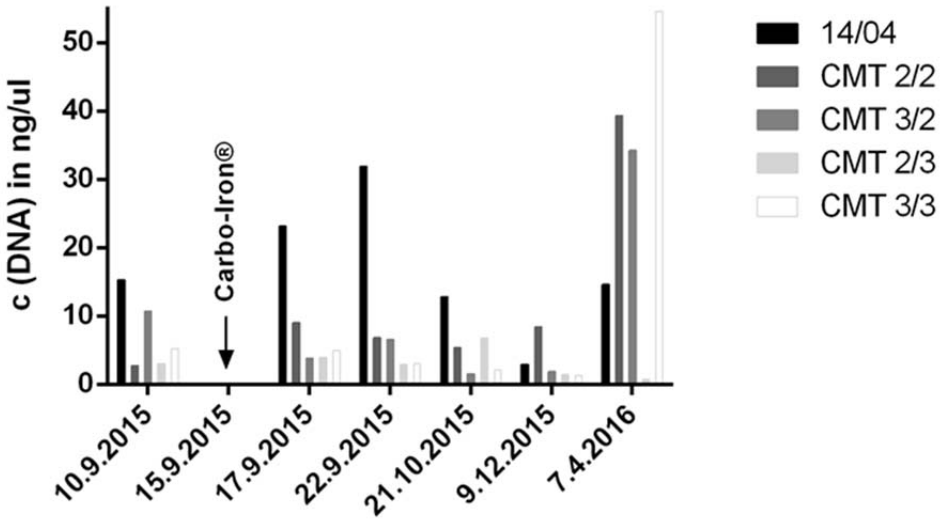


Figure 37: DNA yield from Balassagyarmat samples.

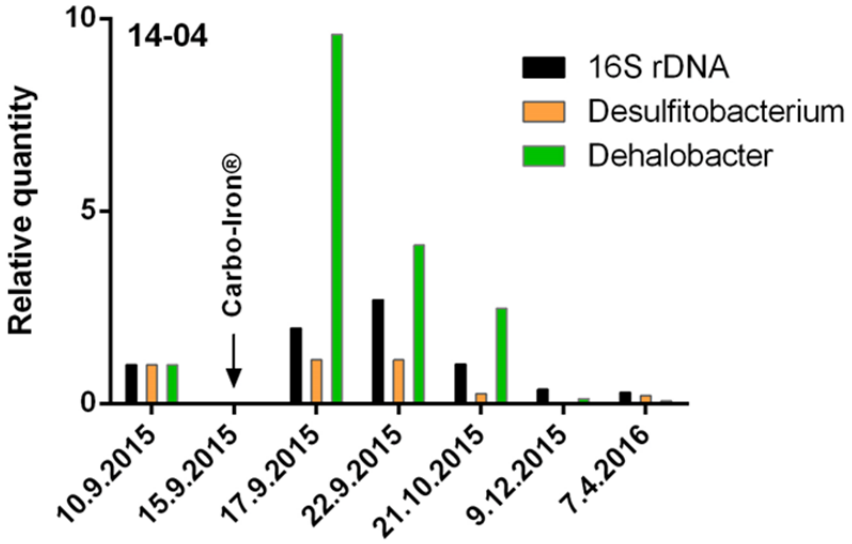


Figure 38: Relative quantification of monitored parameters in well 14/04. Vinyl chloride reductase genes and *Dehalococcoides* were not detected until 3 months after the injection.

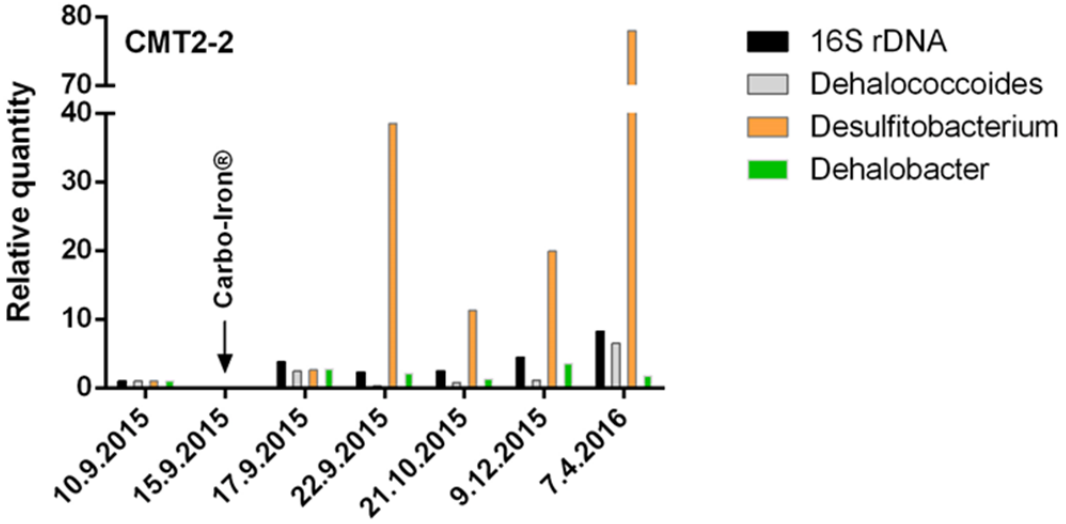


Figure 39: Relative quantification of monitored parameters in well CMT2/2. Vinyl chloride reductase genes were not detected right after the injection.

To conclude, harmful effects associated with the injection of Carbo-Iron® were not detected in water samples from Balassagyarmat. Moreover, vinyl chloride reductase genes vcrA and bvcA and *Dehalococcoides*, which were not detected prior to Carbo-Iron® application, appeared in most wells right after application. The reason could be the decrease in redox potential caused by Carbo-Iron® application, as a more reductive ORP is beneficial for the growth of organohalide-respiring bacteria (Dolinová et al. 2016). In addition, the total bacterial biomass increased in most of the monitored wells. This is an indication that other bacterial groups (such as sulfate or nitrate-reducers) in addition to bacteria utilizing chlorinated hydrocarbons were supported by the newly established conditions.

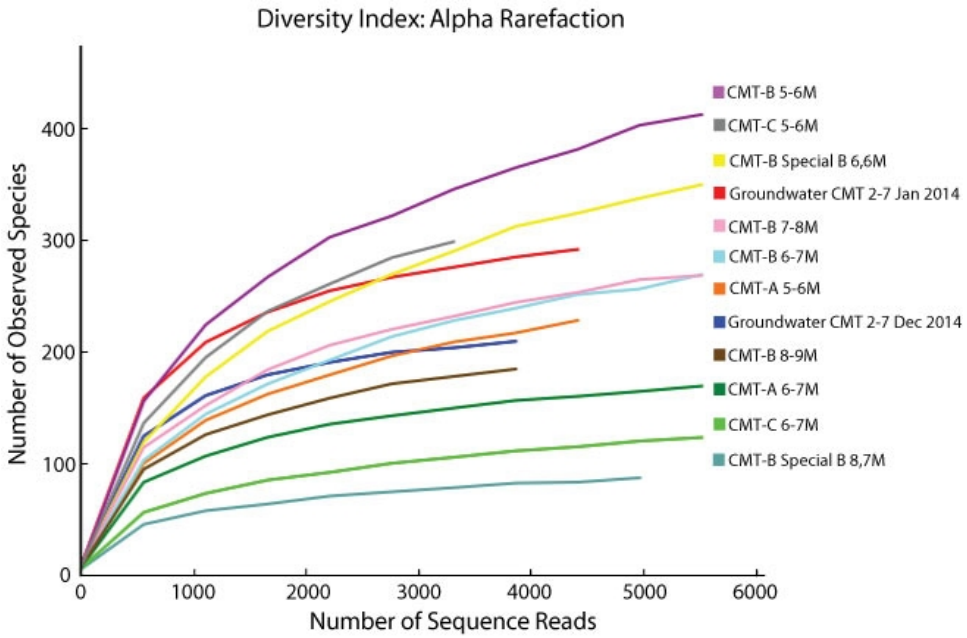
3.2.5 Leipzig, DE

The site is Celle, in Germany, and has been injected with Carbo-Iron®. It is not formally one of the NanoRem field sites, but contributes to our understanding of NP injection's effects on microbial community composition. More background information about the site can be found in Mackenzie et al. 2016.

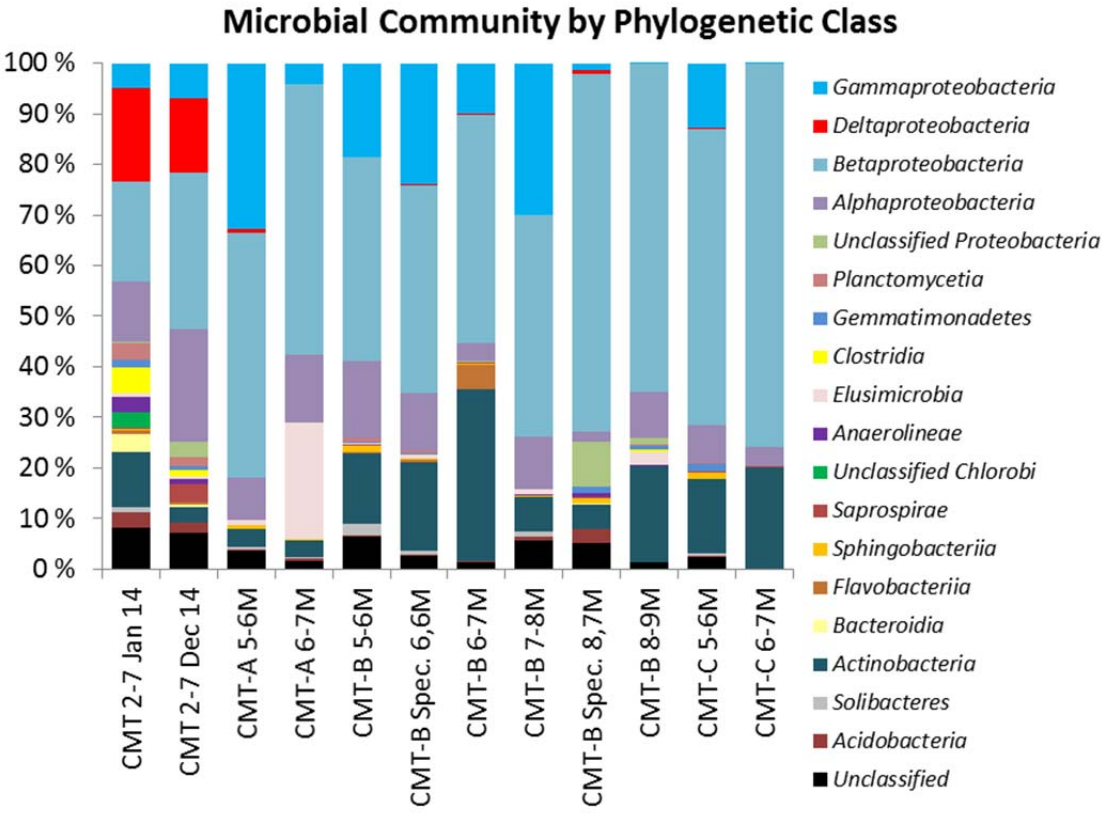
DNA in sediment samples from Leipzig was extracted and used to assess the microbial diversity index and microbial community composition to the class level (Fig. 40).

Table 11: Samples from Leipzig processed for microbial community composition analysis.

Sample no.	Sample type	Sampling date	Label	DNA Extracted	Sequenced
2	Groundwater - Cloudy	Jan-14	CMT 2-7 Jan14	Yes	Yes
3	Groundwater - Clear	Dec-14	CMT 2-7 Dec 14	Yes	Yes
4	Sediment	Dec-14	CMT A 5-6M	Yes	Yes
5	Sediment	Dec-14	CMT A 6-7M	Yes	Yes
6	Sediment	Dec-14	CMT B 5-6M	Yes	Yes
7	Sediment	Dec-14	CMT B 6-7M	Yes	Yes
8	Sediment	Dec-14	CMT B 7-8M	Yes	Yes
9	Sediment	Dec-14	CMT B 8-9M	Yes	Yes
10	Sediment	Dec-14	CMT Special B 8,7M	Yes	Yes
11	Sediment	Dec-14	CMT Special B 6,6M	Yes	Yes
12	Sediment	Dec-14	CMT C 5-6M	Yes	Yes
13	Sediment	Dec-14	CMT C 5-6M	Yes	Yes



(A)



(B)

Figure 40: Microbial analyses of sediment samples from UFZ, Leipzig (A) Sample diversity index, (B) 16S rRNA gene microbial community composition.

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