Anaerobic *in situ* biodegradation of TNT using whey as an electron donor: a case study

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Contamination by 2,4,6-trinitrotoluene (TNT), an explosive extensively used by the military, represents a serious environmental problem. In this study, whey has been selected as the most technologically and economically suitable primary substrate for anaerobic *in situ* biodegradation of TNT. Under laboratory conditions, various additions of whey, molasses, acetate and activated sludge as an inoculant were tested and the process was monitored using numerous chemical analyses including phospholipid fatty acid analysis. The addition of whey resulted in the removal of more than 90% of the TNT in real contaminated soil (7 mg kg^{-1} and 12 mg kg^{-1} of TNT). The final bioremediation strategy was suggested on the basis of the laboratory results and tested under real conditions at a TNT contaminated site in the Czech Republic. During the pilot test, three repeated injections of whey suspension into the sandy aquifer were performed over a 10-month period. In total, approximately 5 m^3 of whey were used. A substantial decrease in the TNT groundwater concentration from the original levels (equalling 1.49 mg l^{-1} to 8.58 mg l^{-1}) was observed in most of the injection wells, while the concentrations of the TNT biotransformation products were found to be elevated. Pilot-scale application results showed that the anoxic and/or anaerobic conditions in the aquifer were sufficient for TNT bio-reduction by autochthonous microorganisms. Whey application was not accompanied by undesirable effects such as a substantial decrease in the pH or clogging of the wells. The results of the study document the suitability of application of whey to bioremediate TNT contaminated sites *in situ*.

Introduction

Contamination of water and soil with 2,4,6-trinitrotoluene (TNT), historically the most widely used explosive, is a serious and widespread environmental problem because of the toxicity, mutagenity [1–4] and teratogenity of this compound toward many organisms [5]. Disposal of munition wastes is a costly process usually entailing excavation of the contaminated material, dumping at specified landfill areas or consequent use of various methods leading to final decontamination. Incineration is the most effective remediation alternative, but the use of this method for polluted soils is expensive because of the costs of soil excavation, transport and the energy for incineration [6,7]. Several technologies have been proposed for the treatment of TNT in liquid wastes, for example, chemical reduction, thermal decomposition, subcritical water degradation, photocatalytic degradation [7] or advanced oxidation processes (using peroxide or ozone) and adsorption [8]. Biological removal of explosives has also been shown to be feasible in many studies using aerobic

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and anaerobic bacteria and fungi [7,9–11]. TNT can be transformed both aerobically and anaerobically and, in both cases, the initially formed products are typically the reduced amino derivatives 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene. Further degradation to the most reduced product, triaminotoluene (TAT), requires highly negative redox potential values [12]. Since the anaerobic process has certain advantages, such as rapid reduction at a low redox potential, it is therefore advantageous to study an anaerobic system for TNT degradation to obtain more efficient removal rates; in addition, azoxynitrotoluene products (arising from partly reduced forms of TNT under aerobic conditions) are then not formed [7].

The choice of the most suitable remediation strategy usually requires a site-specific approach. Technical and economic constraints often require the use of remediation without excavation. It is then necessary to select a decontamination technology that can be applied *in situ*. *In situ* biodegradation to eliminate TNT is based on recent microbiological research suggesting that explosive-degrading bacteria are widespread but require selected co-substrates as electron donors and anoxic conditions to completely transform most nitrosamine and nitroaromatic compounds. In the search for suitable co-substrates, Adrian *et al*. [13,14] mention the addition of H_2 or electron donors that produce H_2 as a useful strategy for enhancing the anaerobic biodegradation of explosives in contaminated groundwater and soil. In this study, ethanol, propylene glycol, butyrate or hydrogen gas were used as electron donors, while the authors avoided using media such as sludge, molasses, whey, etc., because of their undefined character. Numerous other potential electron donors were described in other studies. Pure substances including glucose, citrate [15], sucrose [16], oleic acid, dextrose, acetate [17] and lactate [18] were tested, as well as less definable substrates like corn syrup, emulsified oil [19], molasses [20] and, for example, guar gum [17]. Yeast extract and whey were used for anaerobic biodegradation by 1,3,5-trinitro-1,3,5-triazacyclohexane (RDX) [21,22] but no study to date has tested the usefulness of whey in TNT biodegradation.

In this study, the above mentioned principles were used to create a final remediation strategy usable for *in situ* remediation of a TNT-contaminated site. Since the economic aspect of the process is one of the key factors, the use of waste products from the food (processing) industry like whey or molasses as electron donors is advantageous and desirable. The complexity of these substrates enables their simultaneous utilisation as a nutrient source, which can also play an important role in the economy of the remediation process. The work was conducted to discover which substrate is the most suitable for actual application using semi-scale experiments in the laboratory. The usability of the selected substrate was then tested in a pilot-scale experiment to verify the proposed remediation strategy. Phospholipid fatty acid (PLFA) analysis was used to determine the effect of the chosen electron donor addition on bacterial community quantity and composition over time, providing an insight into microbial community dynamics and enabling evaluation of the studied procedure as a remedial strategy for contaminated aquifers. The main aim of the pilot test was therefore to demonstrate the successful scaling-up of the proposed method.

Materials and methods

Soil, groundwater and chemicals

The soil and groundwater used in this study were collected from the TNT-contaminated site in the Czech Republic. The soil collected for laboratory and semi-scale testing originated from the same area, where the subsequent pilot test was also conducted. For laboratory purposes, the soil was sieved (5 mm) to remove large debris such as leaves and wood and homogenized and the groundwater was thoroughly stirred before the input analyses. TNT concentrations in the soil and groundwater samples were found to be as high as 12 mg kg^{-1} and 10 mg l^{-1}, respectively. Liquid waste sludge was obtained from a municipal waste water treatment plant in Central Bohemia, Czech Republic. Whey originated from the dairy at Čejetičky, Czech Republic. Molasses was obtained from the distillery in Kolín, Czech Republic and acetate was provided by Chromservis (Czech Republic). Standards of 2,4,6-trinitrotoluene (TNT), 2,4-dinitrotoluene (24DNT), 2-amino-4,6-dinitrotoluene (2A46DNT), 4-amino-2,6-dinitrotoluene (4A26DNT), 2,4-diamino-6-nitrotoluene (24DA6NT) and 2,4,6-triaminotoluene (TAT) were purchased from Aldrich, Germany. All trace analysis quality or gradient grade solvents were purchased from Chromservis (Czech Republic).

TNT biotransformation with various electron donors (laboratory experiments)

Sterile 500 ml glass bottles with 120 g of the contaminated soil were completely filled with ground water originating from the same contaminated area. Whey, molasses and acetate were evaluated for their ability to support the anaerobic biodegradation of explosives by comparison with TNT removal in bottles containing a control not treated with an electron donor. These substrates were each added at 0.5% (v/v). The electron donors as waste products from the food industry (whey and molasses) were chosen intentionally for their low cost and their ability to simultaneously serve as a potential source of nutrients and micronutrients for degrading microbes, which is desirable from the technological and economical point of view. Each of the mentioned variations (control, molasses, whey and acetate addition) were also tested with extra addition of 1% (v/v) of liquid waste sludge. Three parallels of each microcosm were sealed and maintained under anoxic condition in the dark at 10°C, which simulated real conditions in the contaminated aquifer. Soil samples were taken periodically for analysis for explosives, their intermediates and PLFA analysis; aqueous samples were collected for ecotoxicological testing. ORP and pH were measured directly in the reaction mixture.

Semi-scale process

The influence of the initial substrate concentration on the transformation process and testing the scale-up of the process were the main aims of the semi-scale experiment. Contaminated soil (4 kg) and water (20 l) samples were incubated in 30 l hermetically closed polyethylene barrels equipped with a control valve and a vent for potential addition of the liquid substrates. All the samples including controls were realized in triplicate. Regular sampling was facilitated by a hermetically closable port with inner diameter of 5 cm at the bottom of each barrel. Whey, selected in the laboratory-scale experiment as the most suitable electron donor (see Results and Discussion), was added in three different input

concentrations: 0.25%, 0.5% and 1% (v/v). Naturally occurring biological processes established anaerobic conditions in the hermetically closed barrels. The barrels were maintained at 10°C. Soil samples were taken for identical analyses as in the laboratory experiment.

Pilot test

In order to demonstrate the suitability of the conditions found during the previous experiments, the pilot scale experiment was performed *in situ*. The pilot test was performed in the area of about 400 m^2 (20 m $\times$ 20 m) situated down-gradient to the former TNT production facility. The site geology comprises Quaternary fine grained sand underlain by Mesozoic siltstones at a depth of 3–4 m below ground level (bgl). The shallow phreatic aquifer is developed in highly permeable Quaternary sands. The depth to the groundwater level is approximately 1 m bgl. The groundwater is slightly acid (pH $\sim$ 6.5) with a high content of total dissolved solids (TDS $\sim$2000 mg l^{-1}) and elevated concentrations of sulphates (600–1200 mg l^{-1}). The redox potential (from 250 to 400 mV) indicates rather aerobic conditions. The initial concentrations of 2,4,6-trinitrotoluene (TNT) ranged from 1.49 mg l^{-1} to 8.58 mg l^{-1}.

The pilot test setup consisted of three injection wells (IN-1 to IN-3) for addition of whey, two background monitoring wells (MV-13 and MV-18) located up-gradient and four monitoring wells (MV-14 to MV-17) located down-gradient to the injection wells, see the site layout map in Fig. 1. All the low diameter wells (32 mm) were advanced to the depth of impermeable siltstone layer (3–3.5 m below ground surface) with a screen range of 2 meters. Whey suspension was delivered by the low pressure injection method. In total, 4.76 m^3 of whey was used during three repeated injections over a 10 month period (0.52, 0.35 and 0.55 m^3 for IN-1; 0.5, 0.4 and 0.35 m^3 for IN-2; 0.49, 0.85 and 0.75 m^3 for IN-3). The groundwater samples were collected in a dynamic regime with a peristaltic pump from the middle of the aquifer after purging out of three well volumes and immediately transported to a laboratory. The groundwater samples were analysed for contents of TNT and its transformation products 4A26DNT, 2A46DNT and 24DA6NT. In addition, the contents of total organic carbon

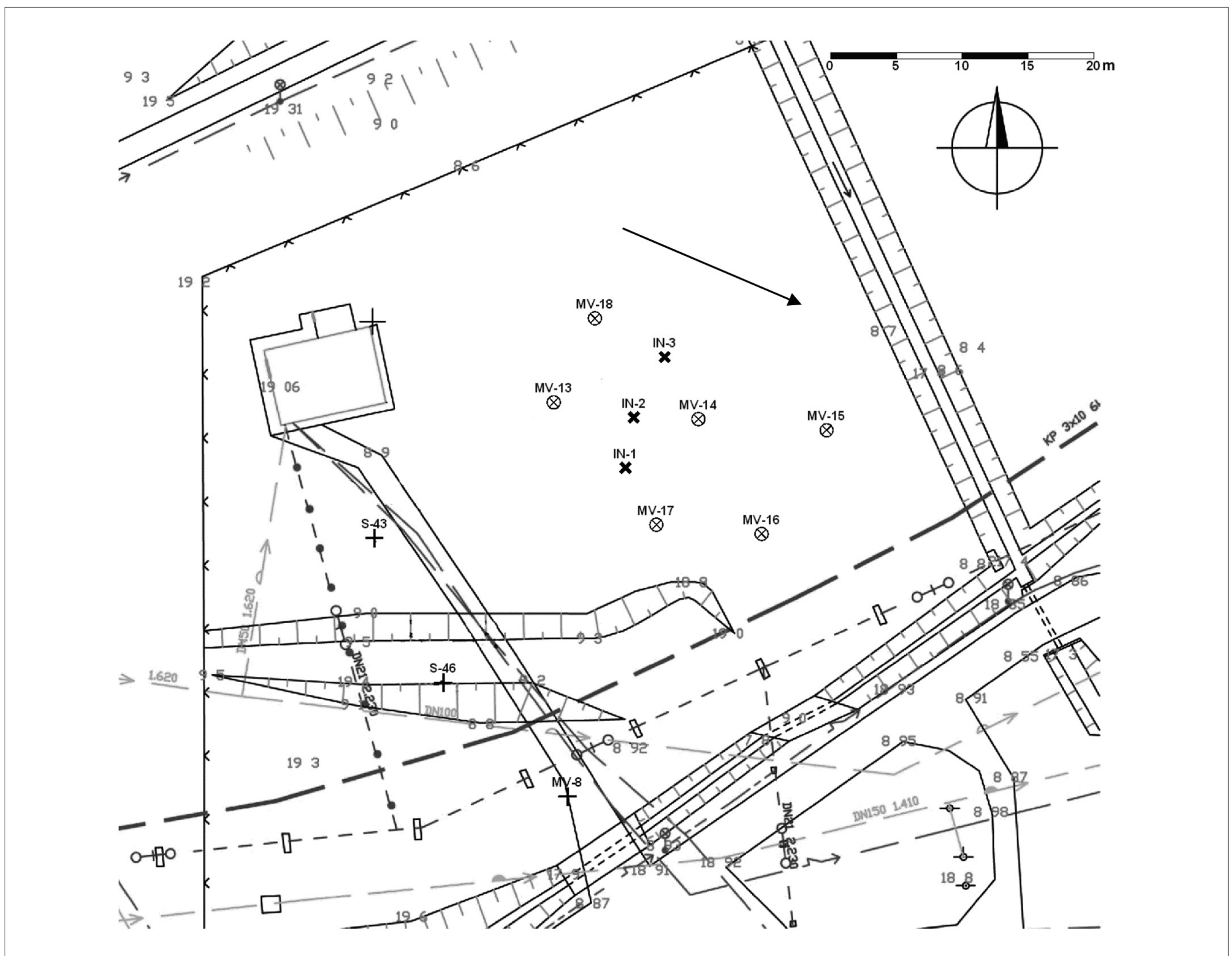

FIGURE 1

Arrangement of monitoring and injection wells in the field-scale pilot test (⊗ – monitoring well, X – injection well), the main direction of the groundwater flow is south-easterly as indicated by the arrow.

(TOC), volatile fatty acids (acetic, propionic, lactic and butyric acids) and basic inorganic constituents were monitored in selected wells. Parameters such as the redox potential, conductivity, pH and temperature were measured using a Multi 340i (WTW) multimeter during groundwater sampling.

Analytical methods

TNT, 24DA6NT, 2A46DNT, 4A26DNT and TAT were analyzed using an HPLC-UV system (Waters 2695 Separations Module) equipped with a diode-array detector (Waters 2996). Prior the analysis, the soil samples were centrifuged (2500 rpm, 5 min) and the soil was dried at ambient temperature (25°C). The samples were then extracted with methanol at 125°C and 2000 psi using a Dionex ASE200 extractor. The extracts were evaporated to dryness under a stream of nitrogen and redissolved in 1 ml of methanol for HPLC analysis. The groundwater samples were centrifuged (2500 rpm, 5 min) and directly submitted to analysis. All the samples were then injected into HPLC and the separation was performed using a Bridge C18 (3.5 μm, 250 mm × 4.6 mm) column at 35°C. A linear gradient from 20% to 100% of methanol over 20 min was employed. The second solvent was bidistilled water. This method was employed for analysis of TNT, 24DNT, 2A46DNT, 4A26DNT and 24DA6NT. TAT was analyzed using a different HPLC method with the Synergi RP-polar (4 μm, 250 mm × 4.6 mm) column. The binary mobile phase consisted of solvent A, methanol and solvent B; 50 mM ammonium acetate was employed with the following linear gradient program (min/%A) 0/5, 5/5, 10/90.

The soil samples for PLFA analysis were extracted with a mixture of chloroform–methanol–phosphate buffer (1:2:0.8) according to [23]. Phospholipids were separated using solid-phase extraction cartridges (LiChrolut Si 60, Merck), and the samples were subjected to mild alkaline methanolysis [24]. The free methyl esters of PLFAs were analysed by GC/MS (456-GC, SCION SQ mass detector, Varian, USA). The GC instrument was equipped with a split/splitless injector and a DB-5MS column was used for the separation (60 m, 0.25 mm i.d., 0.25 μm film thickness). The temperature program started at 60°C and was held for 1 min in the splitless mode. Then the splitter was opened and the oven was heated to 160°C at a rate of 25°C min^{-1}. The second temperature ramp was up to 280°C at a rate of 2.5°C min^{-1}; this temperature was maintained for 10 min. The solvent delay time was set at 8 min. and the transfer line temperature was set at 280°C. Mass spectra were recorded at 1 scan s^{-1} under electron impact of 70 eV, mass range 50–350 amu. Methylated fatty acids were identified according to their mass spectra using a mixture of chemical standards obtained from Sigma. Bacterial biomass was quantified as the sum of i14:0, i15:0, a15:0, 16:1ω7t, 16:1ω9, 16:1ω7, 10Me-16:0, i17:0, a17:0, cy17:0, 17:0, 10Me-17:0, 10Me-18:0 and cy19:0 ($PLFA_{bact}$). 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 16:1ω7, 18:1ω7, cy17:0, cy19:0, 16:1ω5. Anaerobic bacteria were quantified using cy17:0, cy19:0, and 18:1ω9 [25].

Volatile fatty acids (acetic, propionic, lactic and butyric acids) were determined using capillary electrophoresis with UV detection by ALS Czech Republic s.r.o. according to their internal accredited protocol. Dihydrogen and methane were determined using headspace analysis with 6820 gas chromatograph (Agilent, USA) equipped with thermal conductivity and flame ionization detectors. The separation was performed with HayeSep Q precolumn (Agilent) and with a combination of HP-PLOT Q and HP-Molsieve 5A (both 30 m, 0.53 mm i.d., 50 μm film thickness) columns according to Agilent Application Note 5988-9260EN.

Results and discussion

TNT transformation at laboratory scale, ecotoxicity and microbial community analysis

The addition of whey to the bottles filled with soil samples from an actual contaminated site enhanced the transformation of TNT by autochthonous bacteria as did the introduced bacterial culture present in the added waste sludge. The results are displayed in Fig. 2. All the chemical analyses were performed in triplicate and the data are displayed as means with standard deviations. Removal of TNT in the amended soil after 79 days of incubation was 91.7% and 90.4% following the addition of whey and whey with waste sludge, respectively. Negative values of ORP were obtained only under these conditions (see Fig. 2). Bacteria present in both the variants probably utilized oxygen, resulting in anaerobic conditions within a few days. Negative values of ORP appeared to be necessary for the satisfactory progress of transformation of explosives, in agreement with a previously published work [12]. Addition of molasses and molasses with waste sludge led to a lesser decrease in ORP values and lower TNT removal from the soil (68.4% and 75.6%, respectively). Similar results were achieved with acetate and acetate with sludge (72.7% and 71.7% decrease in TNT concentration). A relatively long treatment time was necessary for all the variations, possibly because of the temperature employed (10°C, simulating conditions in the aquifer) which corresponds to the full-scale anaerobic TNT biodegradation performed by [26]. A partial decrease in the TNT concentration in the non-sterile control sample (23.3%) demonstrated a certain natural biotransformation capacity of the treated soil. Addition of waste sludge without another electron donor source caused 32.6% elimination of TNT. Transient, insignificant formation of 4A26DNT, 2A46DNT and 24DA6NT (see the Supplementary Material Table 1) but no accumulation of TAT during the biodegradation process was observed and the pH value ranged between 6.4 and 7.3 in all the variations (data not shown).

Although the complex character of whey and molasses does not permit identification of the exact electron donors supporting the biodegradation activity [13], the use of the waste products from the food industry can be advantageous for the successful scale-up of the process. In addition, the application of whey was shown to be promising to follow-up testing under semi-scale and full-scale (pilot) conditions.

Our understanding of the changes in the microbial consortium during the treatment process is limited due to the fact that only a small fraction of environmental organisms can be cultivated in the laboratory. Therefore, it is necessary to use a cultivation-independent laboratory technique to identify the effect of the employed treatment method on the composition of the microbial community [21]. PLFA analysis is a useful method for characterization and quantification of the microbial community and its changes during the remediation process because of its relative simplicity and the absence of any kind of cultivation step [25]. The results of PLFA analysis of the laboratory scale experiment are shown in Table 1.

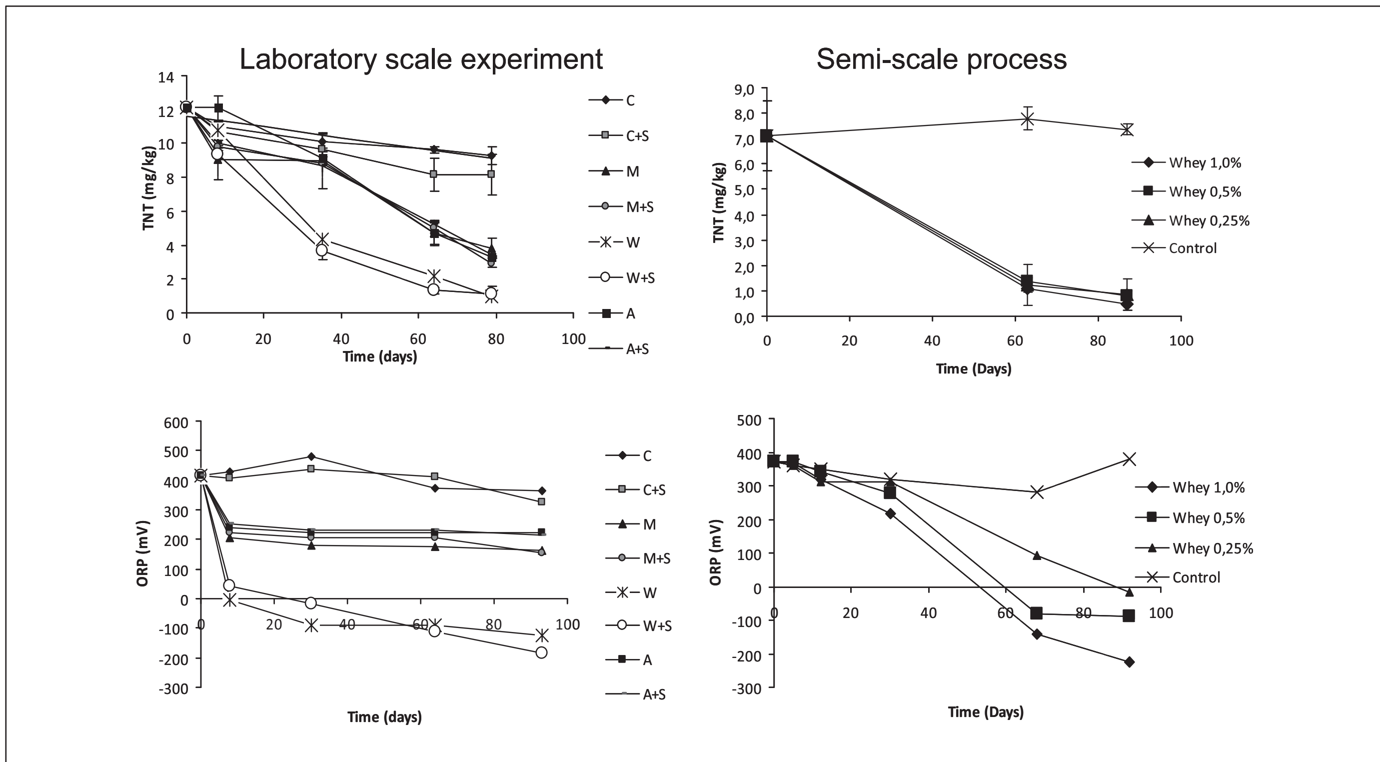

FIGURE 2

Biotransformation of TNT on the laboratory scale (left part) and ORP during the process (C – control sample, W – whey addition, M – molasses addition, A – acetate addition, S – waste sludge addition) and biotransformation of TNT in the semi-scale experiment (right part) with addition of various amounts of whey. The TNT concentrations represent the means of three parallel samples and the error bars are the standard deviations.

The results document that addition of all the substrates resulted in more rapid development of the bacterial populations compared to the respective controls. It is noteworthy that sludge caused a significant increase in the bacterial biomass in all the samples represented mainly by anaerobic bacteria; however, it did not have any substantial effect on the transformation efficiency. These findings correspond to the results of Fahrenfeld *et al.* [18]. Addition of whey probably enabled more rapid development of bacteria

TABLE 1

Development of the microbial community biomass (PLFA analyses) during TNT biotransformation process on the laboratory scale. The data represent the summary concentrations of characteristic fatty acids representing the respective groups of microorganisms

Day	Bacteria (ng g^{-1})	G+ bacteria (ng g^{-1})	G− bacteria (ng g^{-1})	Anaerobic bacteria (ng g^{-1})	Bacteria (ng g^{-1})	G+ bacteria (ng g^{-1})	G− bacteria (ng g^{-1})	Anaerobic bacteria (ng g^{-1})
	Control				**Control + sludge**			
0	667	64	220	159	141	17	68	55
27	426	34	196	242	12,144	443	988	4649
56	2137	12	178	1795	10,713	613	1496	6557
61	2500	12	162	2280	11,293	18	242	10,964
	Acetate				**Acetate + sludge**			
0	183	14	107	65	796	37	352	247
27	1543	46	407	854	8389	263	1258	5585
56	1956	12	155	1741	18,436	51	530	17,660
71	2396	0	183	2010	13,265	15	979	12,199
	Molasses				**Molasses + sludge**			
0	287	15	155	51	2213	137	875	396
27	1993	102	755	572	25,698	882	2979	16,275
56	1406	45	583	593	39,889	1029	4218	26,876
71	4051	9	437	3491	16,784	24	845	15,807
	Whey				**Whey + sludge**			
0	303	11	184	69	2587	70	1409	415
27	5240	128	2595	2081	8678	374	1724	4635
56	1969	19	1345	85	18,510	51	833	17,426
71	1347	4	123	1166	23,244	66	2633	20,423

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within the first 27 days of incubation compared with other treatment methods. However, taken together with the other results, ORP probably plays a more important role than the amount of biomass itself in the transformation of TNT. Despite the fact that the addition of waste sludge failed in terms of improvement of the transformation, these results also document that the amount of microbial biomass is a less important factor. On the other hand, the data from PLFA document in general that all the substrates were suitable for anaerobic microbial growth. In this way, PLFA analyses can also provide specific evidence about the potential toxicity of the environment [25]. We also performed a standard ecotoxicity test employing *Vibrio fischeri* according to ISO 11348-3 [27]; however, no specific trends were observed in inhibition of the bacterial strain. The data generally indicated that the treatment procedures did not cause any increase in the ecotoxicity after TNT transformation in variants with addition of acetate and molasses. Only whey addition slightly, but not-significantly ($P = 0.05$), increased the toxicity of the tested water (data not shown).

Semi-scale process and influence of whey dosage

The addition of whey in three different input concentrations (1.0, 0.5 and 0.25%, v/v) led to the removal of 92.8%, 89.6% and 91% of the TNT, respectively, within 87 days (Fig. 2). The partial decrease in the TNT concentration in the non-sterile control samples (20.6%) was in agreement with the results of the laboratory test. During the semi-scale process we also observed the transient formation of 4A26DNT, 2A46DNT and 24DA6NT without any TAT accumulation in all the tested variants. A small decrease in the pH values (between 6.1 and 7.1) was observed during the experiment (data not shown).

The minimal differences in the TNT transformation rates among the variants with three initial organic substrate concentrations suggest a broad range of possibilities for the remediation process. However, because of the potential risk of clogging of the wells during rapid microbial biomass generation and a pH decrease via short-chain fatty acid production during fermentation, a higher frequency of use of lower injected amounts of whey can be a suitable strategy preventing these undesirable effects.

The pilot test

We monitored lactic, acetic, propionic and butyric acids, hydrogen and methane formation following the injection of whey into the aquifer (Table 2). Monitoring of VFA production during pilot and full-scale applications is necessary to monitor the whey fermentation process and because of their potential ability to decrease the pH values, which can cause collapse of the entire biodegradation process [28]. While lactate indicated the presence of whey in the monitored well and corresponded to the TOC content (data not shown), gradual formation of the other acids has already been described as a side effect of added electron–donor oxidation during the anaerobic biotransformation processes [29,30]. It is noteworthy that these compounds together with hydrogen can also serve as electron donors and some authors concentrated on using VFA as a primary substrate for anaerobic biotransformation [31]. The data correspond to the VFA concentrations before and after the third (last) injection of whey at the pilot test site. After the whey injection, the total concentrations of all the monitored VFA in the groundwater of injection well IN-2 increased to a level exceeding $1\ g\ l^{-1}$. Lactic acid predominated one month after the whey injection; however, after other 2 months, the acetic acid concentration prevailed as a product of secondary fermentation. Production of VFA led to a temporary decrease in the pH below 6. Eleven months after the whey injection, the total content of VFA in the groundwater was minimal and the pH returned to the original levels. Only acetic acid was detected at an elevated concentration in the nearest monitoring well MV-14. These results demonstrate that cheese whey can play a role as an electron donor in the biotransformation process [21]. Moreover, cheese whey contains noticeable amount of nitrogen and phosphorus that can participate as nutrients in the process [32,33].

Biotransformation processes enhanced by the whey injection resulted in a decrease in the redox potential in the groundwater. From the original range of +250 to +400 mV, the redox potential gradually decreased in the injection wells to about +50 mV within three months after the 3rd injection of whey (Fig. 3). This period corresponded to the maximum concentrations of the

TABLE 2

Concentrations ($mg\ l^{-1}$) of volatile fatty acids in groundwater from injection well IN-2 and monitoring well MV-14 (ND – not determined)

Monitoring round	4 days before 1st whey injection	1 week before 3rd whey injection	1 month after	3 months after	7 months after	11 months after
Well IN-2 ($mg\ l^{-1}$)						
Acetic acid	<0.50	0.6	342	734	27.6	2.6
Propionic acid	<0.50	<0.5	<20	227	6.21	<0.50
Butyric acid	<0.50	<0.5	136	253	1.68	<0.50
Lactic acid	<0.50	<0.5	1380	40.4	<1	<0.50
pH	6.38	6.36	5.31	5.88	6.44	6.26
Methane ($\mu g\ l^{-1}$)	3.5	25.9	16.3	71.6	735.0	ND
Dihydrogen ($\mu g\ l^{-1}$)	<0.1	<0.1	<0.1	<0.1	<0.1	ND
Well MV-14 ($mg\ l^{-1}$)						
Acetic acid	ND	22.2	27.2	44.8	73.9	10.9
Propionic acid	ND	<0.5	<0.5	0.56	0.56	1.22
Butyric acid	ND	<0.5	<0.5	<0.5	<0.5	<0.5
Lactic acid	ND	<0.5	0.63	1.65	0.77	1.26
pH	ND	6.56	5.86	6.39	6.34	6.61
Methane ($\mu g\ l^{-1}$)	10.8	9.0	14.8	10.8	8.1	ND
Dihydrogen ($\mu g\ l^{-1}$)	<0.1	<0.1	<0.1	<0.1	<0.1	ND

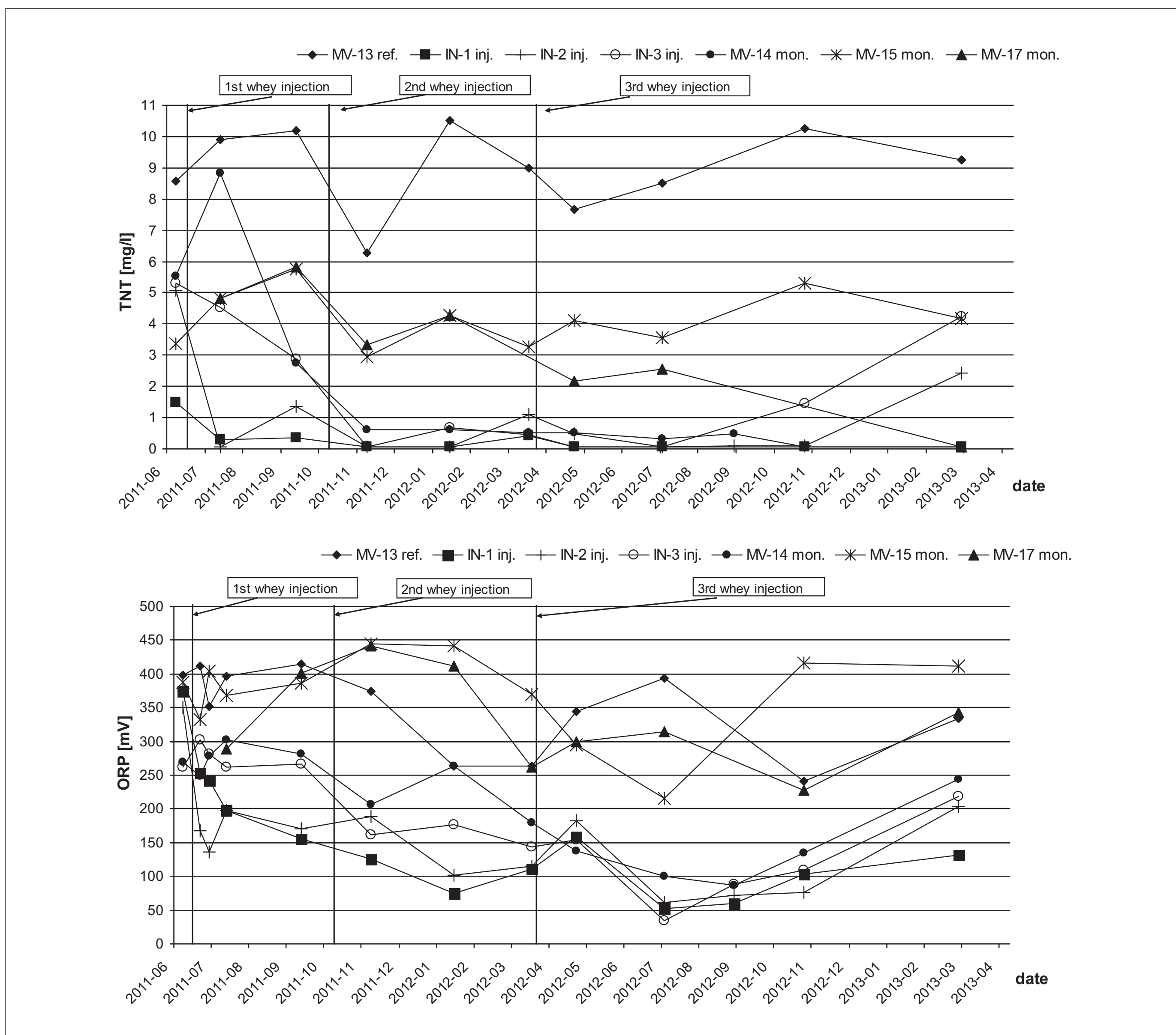

FIGURE 3

TNT concentrations and groundwater redox potentials in the groundwater (mg l^{-1}) during the pilot test in the injection and monitoring wells (TAT and 24DNT were not detected).

TNT transformation products (see below in Fig. 4). It should be noted that, after this period, the redox potentials began to return to their original values because of exhaustion of the injected organic substrate. Specific formation of methane in the injected area demonstrated that not only acidogenic and acetogenic fermentation (for which dihydrogen and VFA production is typical) occurred during the monitored period (Table 2). Also methanogenesis was observed, which proves reaching of strict anaerobic conditions in some parts of aquifer [34]. Any significant change in dihydrogen content was not observed. Probably, it was immediately consumed as an electron donor during TNT reduction [13] and/or methanogenesis. It is very likely that also oxic conditions played role in the biotransformation process due to complexity and inhomogeneity of the treated system. Azoxy-toluenes produced by spontaneous concentration of partially reduced TNT [35] or any other potential dead-end products were not monitored, nevertheless ecotoxicology data did not indicate any formation of toxic compounds typical for the strictly aerobic process.

Fig. 3 shows a rapid decrease in the TNT concentrations in the groundwater from injection wells IN-1 and IN-2 and a slower decrease in the TNT concentration in injection well IN-3. One month after the second whey injection, the TNT concentration in the groundwater from these wells dropped below the quantification limit of the method (0.1 mg l^{-1}), corresponding to a removal efficiency of more than 95%. The rebound in the TNT concentration especially at the end of 2012 and the beginning of 2013 was probably caused by depletion of the added organic substrate and the inflow of TNT-contaminated groundwater from the up-gradient area. A decrease in the TNT concentrations was also observed in the closer monitoring wells MV-14 and MV-17. On the other hand, the effects of whey injections were not detected in the groundwater of the more distant monitoring well MV-15. Differ-

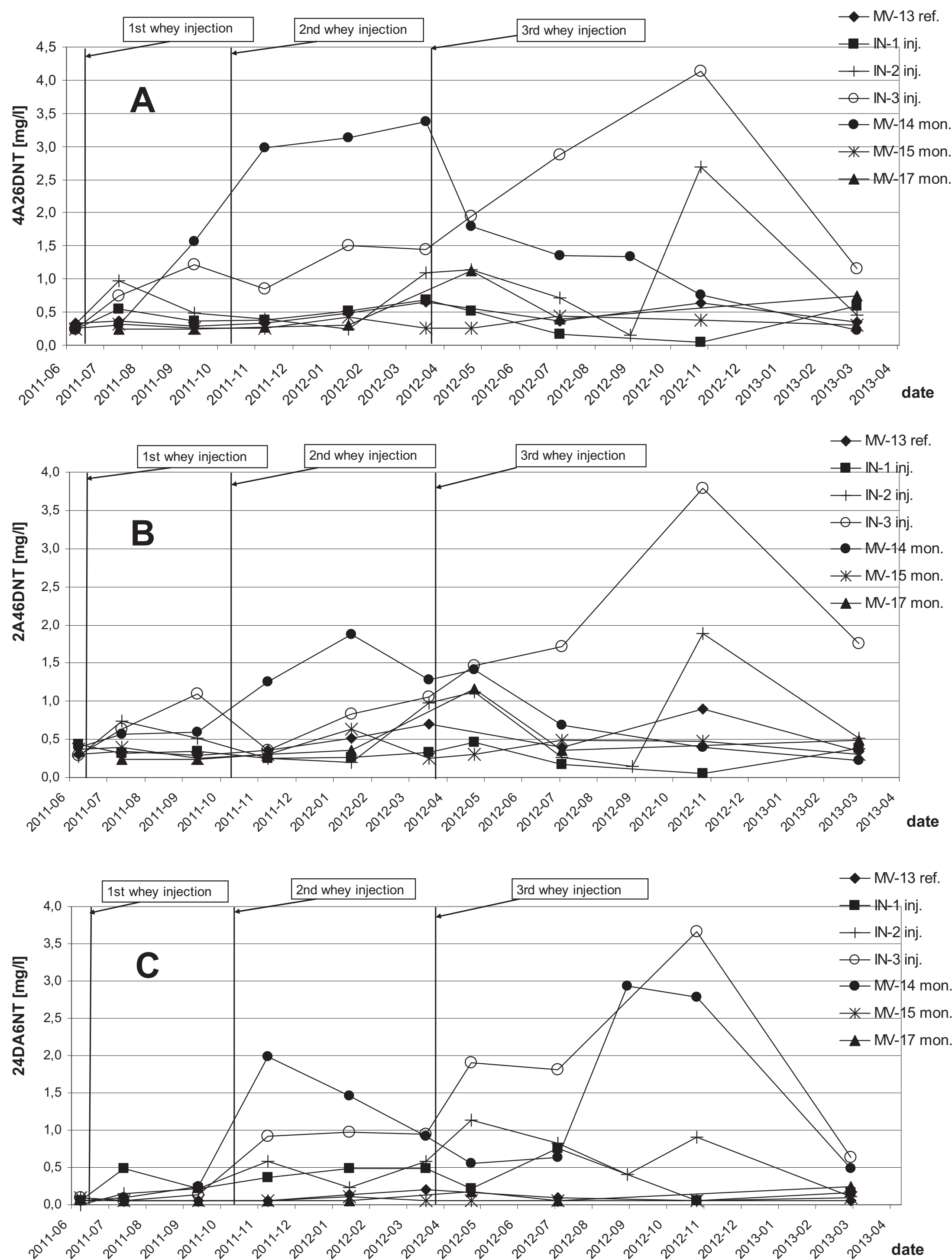

FIGURE 4

Concentration of 4A26DNT **(a)**, 2A46DNT **(b)** and 24DA6NT **(c)** in the groundwater

ent results were obtained for the concentrations of the TNT metabolites (4A26DNT, 2A46DNT and 24DA6NT) that were monitored in the groundwater (see Fig. 4). In injection well IN-3, the concentrations of all the three monitored TNT metabolites increased continuously and culminated seven months after the 3rd (last) whey injection whereas, in other injection wells (wells IN-1 and IN-2), the increase in the TNT metabolite concentrations was less substantial and/or fluctuated. A significant increase in the TNT metabolite concentrations was also recorded in well MV-14, the monitoring well closest to the injection wells.

Conclusions

The results of the study demonstrate that TNT is readily biodegradable under anaerobic conditions by autochthonous cultures

in the presence of whey as a suitable electron donor source. Molasses and acetate were generally less effective than whey. The conditions for the pilot test were partly optimized under laboratory conditions in the laboratory flasks and barrels and subsequently successfully applied in an actual contaminated aquifer using whey injection, which resulted in a decrease in the TNT concentration of more than 90%. Additional inoculation with allochthonous cultures using waste sludge did not have any effect on the efficiency of the remediation process. At the laboratory scale, the process was monitored using PLFA analysis and the results showed that the developed microbial biomass was not the major factor influencing the degree of TNT transformation. The ORP value, which was found to be the lowest for the whey reaction, probably played a more important role in the transformation process. Typical transformation products of TNT were monitored where no accumulation was observed under the laboratory conditions and a slightly elevated concentration was detected only in one of the injection wells. Generally, it is possible to conclude that the finally proposed method tested *in situ* is a suitable approach to remediate aquifers contaminated by TNT.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nbt.2015.03.014.

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