Biodegradation (2010) 21:947–956
DOI 10.1007/s10532-010-9354-z

ORIGINAL PAPER

Development of a treatment solution for reductive dechlorination of hexachloro-1,3-butadiene in vadose zone soil

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Received: 3 November 2009 / Accepted: 30 March 2010 / Published online: 10 April 2010

Abstract The biodegradation of chlorinated organics in vadose zone soils is challenging owing to the presence of oxygen, which inhibits reductive dehalogenation reactions and consequently the growth of dehalorespiring microbes. In addition, the hydraulic conductivity of vadose zone soils is typically high, hence attempts to remediate such zones with biostimulation solutions are often unsuccessful due to the short residence times for these solutions to act upon the native bacterial community. In this study we have identified sodium alginate as a hydrogel polymer that can be used to increase the residence time of a nutrient solution in an unsaturated sandy soil. Additionally we have identified neutral red as a redox active compound that can catalyse the reductive dechlorination of the chlorinated organic hexachloro-1,3-butadiene by activated sludge fed with lactate and acetate. Finally we have shown that a nutrient solution amended with neutral red and sodium alginate can lower the redox potential and reduce hexachloro-1,3-butadiene concentrations in a contaminated vadose zone soil.

Keywords Soil bioremediation · In situ dechlorination · Alginate polymer · Hexachloro-1,3-butadiene · Biostimulation · Neutral red

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Introduction

Bioremediation of chlorinated compounds in anaerobic groundwater by reductive dechlorination is an established low cost remediation practice. However the treatment of vadose zone or unsaturated soils is more challenging due to the soil environment being predominantly aerobic with associated high redox potentials. This reduces the activity and therefore the abundance of dehalorespiring bacteria such that dechlorination rates are negligible (Ferguson and Pietari 2000).

The concentration of oxygen and the associated redox potential in vadose zone soils can be reduced by increasing soil moisture content, which serves both to displace air (21% oxygen) from the soil void space and enhance heterotrophic microbial activity provided electron donors and essential nutrients are not limiting (Ferguson and Pietari 2000). This activity consumes oxygen and other available electron acceptors such as sulfate, nitrate and ferric iron. The hydraulic conductivity of the vadose zone in sandy soils, however, is typically high and any attempt to increase the moisture content by addition of water to the soil results in short-lived saturation conditions. Increasing the viscosity of biostimulation solutions with water-soluble polymers is a promising approach to lowering the redox potential of the vadose zone environment such that reductive dechlorination rates are enhanced. Water-soluble hydrogel polymers such as carrageenan (Cassidy et al. 1997),

alginate (Gentry et al. 2004) and gellan gum (Moslemy et al. 2004) have been used previously to carry or immobilise bacterial cells but not to facilitate a reduction in soil redox potential.

On its own the use of hydrogels to retain soil moisture content does not resolve the limitation that dehalorespiring microbes are present at low abundance in the vadose zone. Whilst this could be relieved through bioaugmentation of the vadose zone with enrichment cultures containing dehalorespiring bacteria, such cultures are typically enriched from foreign environments and their performance can therefore not be guaranteed. The generation of enrichment cultures using inocula from a specific site requires long incubation periods and is costly.

The application of redox active compounds commonly referred to as electron shuttles has received relatively little attention in the development of technologies for enhancing biological remediation of chlorinated organics. Electron shuttles such as 9,10-anthraquinone-2,6-disulfonate or cyanocobalamin can be reduced by a broad spectrum of microbes and in turn abiotically reduce target chlorinated organics (Van der Zee and Cervantes 2009). In closed systems or environments in which shuttle movement is limited the abiotic reduction of a target pollutant returns the shuttle to the oxidized state such that it can serve once more as a recipient of reducing power generated by the catabolism of reduced carbon substrates by microbes. Rather than relying on a single respiratory group such as dehalorespiring bacteria, electron shuttles have the potential to direct the reducing power of a variety of microbes with different respiratory machinery towards dechlorination reactions. The utility of electron shuttles in the degradation of azo dyes is better established than it is for chlorinated organics (Van der Zee and Cervantes 2009).

Hexachloro-1,3-butadiene is a volatile colourless liquid used in the past as an insecticide, a heat transfer fluid or as a solvent in rubber production. The major sources of exposure for the general public are through consumption of contaminated fish or drinking water or inhalation of contaminated dust and air near point sources (Lecloux 2004). A large quantity (45,000 m^3) of hexachloro-1,3-butadiene contaminated soil is stockpiled on the Botany Industrial Park in Sydney, Australia.

In this study we have screened commercially available electron shuttles for their ability to catalyse the reductive dechlorination of hexachloro-1,3-butadiene by activated sludge fed with lactate and acetate. We have additionally tested a range of water-soluble, hydrogel polymers for their ability to facilitate the lowering of redox potential in a sandy soil through an increase in the viscosity and soil residence time of a nutrient solution composed of ammonium phosphate and a mixture of electron donors. Furthermore, we demonstrate a reduction in hexachloro-1,3-butadiene concentrations in a column containing sandy soil from a polluted site through application of the nutrient solution amended with the hydrogel alginate and the electron shuttle neutral red.

Materials and methods

Test for electron shuttle activity

Known redox active compounds were screened in triplicate assays for degradation of hexachloro-1,3-butadiene and production of C4 gases. The screen was carried out by transferring 5 ml aliquots of dewatered activated sludge resuspended in artificial wastewater media to 20 ml headspace vials (Sun Sri) (15 ml headspace) amended with 500 μM hexachloro-1,3-butadiene and 200 μM of the test compound. Activated sludge was collected from St Mary's Sewage Treatment Plant (Sydney, Australia) and stored without aeration at room temperature overnight. The activated sludge was dewatered by centrifuging 200 ml of activated sludge at 4°C for 10 min at 6000 × *g*. The media was purged with nitrogen for 30 min before 150 ml was used to resuspend the dewatered biomass in an anaerobe chamber. Per litre the media contained 0.2 g NH_4Cl, 0.15 g $CaCl_2{\cdot}2H_2O$, 0.33 g KCl, 0.30 g NaCl, 3.15 g $MgCl_2$, 1.26 g K_2HPO_4, 0.42 g KH_2PO_4, 260 mg $CoCl_2$, 5 g $C_3H_5NaO_3$, 2.87 g $C_2H_3NaO_2$, 0.25 g yeast extract and trace element and vitamin solutions (So and Young, 1999). Vials were sealed with pre-assembled silicon septa with aluminium crimp caps (Alltech) and incubated at 30°C for 28 days.

Negative controls lacking a test compound and positive controls containing cyanocobalamin were established as above. Gas samples taken from the headspace and solvent extracts of liquid samples were analysed as described below. The compounds tested were alizarin, 5-aminolevulinic

acid, anthraquinone-2-sulfonic acid, 9,10-anthraquinone-2,6-disulfonic acid, coenzyme Q_0, coenzyme Q_{10}, cysteine, emodin, flavin adenine dinucleotide, folic acid, hydroquinone, indigo, juglone, lawson, menadione, manaquinone, methyl viologen, neutral red, nile red, phenazine, phenol red, protoporphyrin IX, purpurin, resorcinol, riboflavin, sudan black B, tannic acid, thiamine, tiron, and trimethoprim.

Test for increasing viscosity and residence time

Polymers were tested for their ability to retard the movement of a nutrient solution through a sandy soil in 25 ml plastic tubes with a KimWipe tissue (Kimberly-Clark) packed under 30 g soil. A nutrient solution (5 ml) consisting of 9.3 g/l sodium lactate, 4.1 g/l of anhydrous sodium acetate, 6.7 g/l of diammonium hydrogen phosphate and amended with 200 μg/ml bromophenol blue and a test polymer was added to the top of the small soil column and incubated statically at room temperature until the solution broke through the column of soil as indicated by the tissue staining blue. The polymers tested were κ-carrageenan (Sigma-Aldrich), polyacrylamide emulsion (Sigma-Aldrich), sodium alginate Manugel DMB (ISP) and Manucol DMF (ISP). The soil used is a sandy soil with a bulk density between 1.3 and 1.6 g/ml.

Test for lowering redox potential

Perspex columns 35 cm in length and 5 cm in internal diameter were placed onto a bed of acid washed sand and 250 g of sandy soil was packed around an inverted Rowe Scientific IJ64 Ionode redox probe to a bulk density of 1.5 g/ml. The probe was connected to a data logger (TPS smartCHEM-CP) to continuously measure redox potential. The nutrient solution (170 ml) described above amended with 0.625% (w/v) Manugel sodium alginate was applied to the top of the column and allowed to percolate into the soil by gravity.

Test for hexachloro-1,3-butadiene degradation in soil

A perspex column (200 cm high and 20 cm in internal diameter) was placed in a 50 l bin containing a bed of sand and packed with 45 kg of soil (fine to loamy sand) taken from a contaminated site on the Botany Industrial Park, Sydney, Australia. Based on the soil type it is expected to have a water holding capacity of approximately 70 mm/cm depth of soil and soil porosity of approximately 25%. The soil reached to a height of 1 m in the column, with a void space of approximately 7 l and a column headspace of 31 l. During packing, three inverted Ionode redox probes (Ag/AgCl) were positioned at depths of 20, 50 and 80 cm in the soil and connected via the column head to a multi-channel redox meter and data-logger (TPS smartCHEM-I3 HB, Australia). Probe readings were converted to E_h values by adding 200 mV. Twelve litres of treatment solution (Manugel® DMB ISP alginate, 0.625%; $(NH_4)_2HPO_4$, 6.5 g/l; Newman Zone emulsified vegetable oil, 20 mg/l; Glucose, 10 g/l; Neutral red, 100 μM; Ethanol, 20 ml/l) was administered to the exposed surface of the soil in the column. The top of the column was sealed with a threaded aluminum lid containing two sample ports for 100 ml Tedlar gas-sampling bags, the contents of which were analysed weekly with respect to hydrocarbon gases.

Anion analysis

To quantify anions (chloride, nitrate and sulphate) in the soil, 10 g soil samples were extracted with 45 ml of deionised water at 60°C, vortexed for 30 s and shaken at 150 rpm for 30 min. Soil slurries were then centrifuged at 5000 × *g* for 15 min and supernatants filtered using 0.22 μm filters (MilliPore, USA). Anion analysis was conducted on a Dionex—ICS1000 Ion Chromatography System using an Ion pac AS14A, 4 × 250 mm analytical column with a conductivity detector.

Quantification of hexachloro-1,3-butadiene and breakdown products

Hexachloro-1,3-butadiene was extracted from cultures by placing 1 ml samples taken with a syringe into a glass vial, where an internal standard (500 μM 1,3,5-tribromobenzene) was added along with 2 ml of ethyl acetate and incubated on an orbital shaker for 4 h. Soil extractions involved incubating 5 g soil samples (wet weight) in 10 ml of ethyl acetate in a glass vial on an orbital shaker overnight. Subsamples (1 ml) of the organic solvent from culture or soil extracts were then transferred to glass gas

chromatographic (GC) vials (2 ml) using a glass pipette. GC/MS analysis was performed on a Hewlett Packard 5890 GC equipped with a model 5971 mass spectrometer (MS). The capillary column employed was a J & W DB-17MS 30 m × 0.25 mm (internal diameter) × 0.25 μm (film thickness). The inlet was operated at a split ratio of 2:1 and temperature was maintained at 300°C and a pressure of 8.0 psi. The column temperature was maintained initially at 50°C for 1 min and ramped to 250°C at 15°C/min with a constant carrier gas (He) flow of 1.73 ml/min. The MS was operated in scan mode and covered the mass range of 30–300 amu. The transfer line was maintained at 300°C. The concentration of hexachloro-1,3-butadiene was calculated by relating the ratio of the concentration of hexachloro-1,3-butadiene to 1,3,5-tribromobenzene found in the samples to a standard curve developed by relating the ratio of peak areas of a range of known quantities of hexachloro-1,3-butadiene with fixed quantities of 1,3,5-tribromobenzene.

To identify and quantify C4 gases, 100 μl headspace samples were injected manually into a Trace GC Ultra/DSQII (Thermo Scientific). The capillary column employed was a J&W GS-Gaspro 60 m × 0.32 mm (internal diameter) without stationary phase film (Agilent Technology). The inlet was operated in a split ratio of 5:1 and its temperature was maintained at 260°C. The column temperature was maintained at 50°C for a total of 7 min. A constant carrier gas (He) flow was maintained at 1 ml/min. The MS was operated in scan mode and covered the mass range from 30 to 80 amu. The transfer line between the GC and the MS was maintained at 300°C. Standard curves for 1,3-butadiene were developed by relating a range of known concentrations of the gas in a known headspace volume to the peak area measured from samples.

Denaturing gradient gel electrophoresis analysis of 16S rRNA genes

DNA was extracted from 0.5 g soil samples using the Wizard Genomic DNA extraction kit (Promega) and 100 ng was used as template in a polymerase chain reaction to amplify the V3 region of 16S rRNA templates using forward primer GC356F (5′-cgcccgccgcgccccgccccggcccgccgcccccgcccactcctacgggaggcagc-3′) and 519R (5′-gtattaccgcggctgctg-3′) as previously described (Manefield et al. 2002). The GC clamped products were separated on 10% (w/v) polyacrylamide gels with a 30–60% urea/formamide denaturing gradient as previously described (Whiteley and Bailey 2000). Denaturing gradient gels were cast and run using the CBS Scientific DGGE system at 60°C and 80 V for 16 h. Gels were stained with SYBR gold nucleic acid gel stain (Molecular Probes) and visualised by UV trans-illumination. Organisms represented in DGGE profiles were analysed by re-amplifying and sequencing bands of interest. Bands were excised from gels and eluted in water. Recovered DNA was re-amplified using primers 356F and 519R as above. Products were sequenced at the Clive and Vera Ramaciotti Centre for Gene Function and Analysis, Sydney on an Applied Biosystems capillary sequencer. Sequence homology was determined by BLASTN searches to determine the most similar sequences in the GenBank/EMBL database (Altschul et al. 1997).

Testing the impact of neutral red on the viability of cultivable microbes in soil

Contaminated sandy soil samples (10 g) taken from the Botany Industrial Park, Sydney, Australia, were placed in triplicate in 50 ml serum bottles with 25 ml of reverse osmosis water and mixed manually to make a slurry. Neutral red was added to serum bottles at concentrations of 10, 100 and 1000 μM. Control cultures were not treated with neutral red. Serum bottles were sealed with rubber septa and aluminium crimp caps, flushed with nitrogen gas for 2 min to reduce oxygen concentrations and incubated stationary at 30°C. Subsamples of the slurry (100 μl) taken over 27 days were diluted in Luria Bertani broth and spread onto Luria Bertani agar plates, which were incubated under aerobic conditions at 30°C for 20 h before enumeration of colony forming units.

Results

Neutral red catalyses the reductive dechlorination of hexachloro-1,3-butadiene

The recent discovery that the complete reductive dechlorination of hexachloro-1,3-butadiene can be catalysed by cyanocobalamin (James et al 2008) prompted us to screen known redox active compounds

for a cost effective alternative. Activated sludge samples were incubated anaerobically for 28 days in artificial wastewater media containing acetate and lactate as electron donors and 500 μM hexachloro-1,3-butadiene as an electron acceptor in the presence of test catalysts at a final concentration of 200 μM. Of the 30 commercially available redox active compounds tested only neutral red significantly reduced hexachloro-1,3-butadiene concentrations (t-value = −2.09, P-value = 0.05) and generated C4 gases butadiyne and butenyne (Fig. 1).

Hexachloro-1,3-butadiene concentrations in control cultures without amendment decreased by an average of 205 μM or 41% of the starting concentration. The lack of C4 gas production in these controls suggests this loss is due to adsorption of hexachloro-1,3-butadiene to the culture vessel and/or loss during extraction, although C4 gases may have been produced and then consumed in these cultures. An additional decrease in hexachloro-1,3-butadiene concentration of 126 μM or 25% of the starting concentration was observed in treatments containing neutral red. This is compared with the loss of 268 μM or 54% of the starting concentration of hexachloro-1,3-butadiene in treatments containing cyanocobalamin. These losses are exclusively electron shuttle dependent. We conclude that neutral red is approximately half as effective in reducing hexachloro-1,3-butadiene in this assay compared to cyanocobalamin. Additional experiments testing the impact of different concentrations of neutral red revealed that the catalysis was concentration dependent and that 100 μM was sufficient to accelerate hexachloro-1,3-butadiene reduction (data not shown).

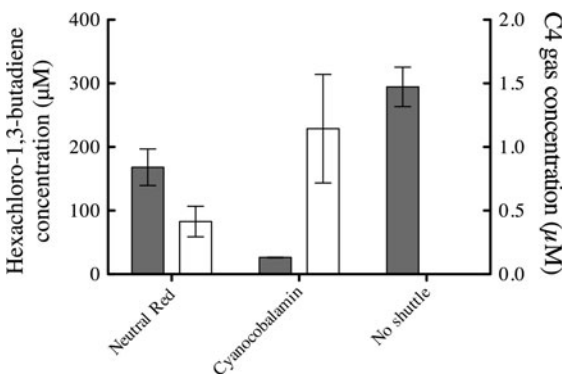

Fig. 1 Hexachloro-1,3-butadiene (*grey bars*) and C4 gas concentrations (*white bars*) in activated sludge cultures amended with neutral red, cyanocobalamin or no electron shuttle. Triplicate activated sludge samples were incubated anaerobically in artificial wastewater medium containing lactate, acetate and hexachloro-1,3-butadiene for 28 days. Control cultures containing no electron shuttles did not produce C4 gases. The C4 gases produced were butadiyne and butenyne. *Error bars* represent standard deviation

Alginate increases the viscosity and residence time of a nutrient solution in a sandy soil

Three hydrogels (polyacrylamide, κ-carrageenan and alginate) were assessed for their ability to reduce the percolation rate of a biostimulation solution containing ammonium phosphate, sodium lactate and sodium acetate through a sandy soil. This was carried out by timing the break through of the nutrient solution in 20 g soil samples in a 25 ml plastic tube. Carrageenan (0.3% w/v), polyacrylamide (10, 25 or 32% w/v) and Manucol sodium alginate (0.3, 1 and 2% w/v) resulted in residence times of less than 2 days, which was considered too short for an effective in situ biostimulation application.

In contrast, there was a strong relationship between the concentration of Manugel sodium alginate (hereafter alginate) and the residence time of the amended solution in the soil (Fig. 2). A sharp rise in residence time was observed with alginate concentrations between 0.5 and 0.7% (w/v). The kinematic viscosity for this concentration range was determined to be 8.1 and 16.6 cSt/s for the solutions containing 0.5 and 0.7% (w/v) alginate respectively. At concentrations above 0.7% (w/v) the alginate amended solution pooled at the soil surface for several hours. This delayed penetration of the soil surface is undesirable as consumption of carbon, nitrogen and phosphate in the treatment solution will commence before it has entered the soil.

Alginate facilitates a decrease in redox potential in a sandy soil

Preliminary tests were established to assess the impact of alginate and nutrients in an aqueous solution on the redox potential of a sandy soil. Small Perspex columns packed with 250 g of sandy soil mounted on a bed of acid washed sand treated with water, water amended with 0.625% (w/v) alginate, nutrient solution or nutrient solution amended with 0.625% (w/v) alginate. The columns were allowed to drain for 72 h

before soil was transferred to sealed containers and monitored for redox potential over 28 days. These preliminary tests revealed that both nutrients and alginate were required to decrease soil redox potential. The water treatment or treatments containing alginate or nutrient solution alone did not reduce the redox potential of the soil (data not shown).

To assess the impact of the nutrient solution amended with 0.625% (w/v) alginate on the redox potential of a sandy soil in an open-ended column, 170 ml was applied to a 250 g soil sample in a small Perspex column with a redox probe packed into the soil. Figure 3 shows that the redox potential within the column decreased rapidly once the nutrient solution containing alginate reached the redox probe 48 h after the experiment commenced, despite exposure of both ends of the column to air. The potential dropped to a minimum of approximately −300 mV after 8 days before climbing to a potential of 400 mV between the tenth and twelfth day of the experiment. It is not clear if the rise in potential resulted from a decrease in soil moisture content through evaporation or from exhaustion of the electron donors.

A nutrient solution amended with neutral red and alginate stimulates dechlorination of hexachloro-1,3-butadiene in a sandy soil

To test the utility of a nutrient solution containing alginate (0.625% w/v) and neutral red (100 μM) in the degradation of hexachloro-1,3-butadiene in a sandy soil, 12 l of the treatment solution was applied to a Perspex column (20 cm diameter; 2 m height) packed with 45 kg of sandy soil (1 m depth) excavated from a site contaminated with approximately 5 mg/kg (19 μmol/kg) of hexachloro-1,3-butadiene. The treatment solution had percolated into the soil within 12 h. Redox probes packed into the soil at depths of 20, 50 and 80 cm recorded a decrease in soil redox potential upon application of the treatment solution to the soil surface (Fig. 4).

At all three depths the redox potential dropped from just over 500 mV to approximately −210 mV within 1–3 days. The potential then rose at all depths to approximately −50 mV and diverged thereafter according to depth. Readings from the uppermost probe increased to approximately 350 mV over the next 2 weeks before climbing steadily towards the starting point of just over 500 mV after 56 days. Readings from the probe at 50 cm depth remained stable at approximately 0 mV for the second and third week after treatment addition before increasing thereafter reaching 200 mV after 56 days. Readings from the probe at 80 cm depth stayed between −40 and −120 mV for the duration of the experiment. There was a strong relationship between depth and redox potential, with increases in depth correlated to decreases in redox potential. The initial rapid drop in redox potential followed by an increase some days later is likely to reflect the oxidation of the readily accessible electron donors glucose and ethanol.

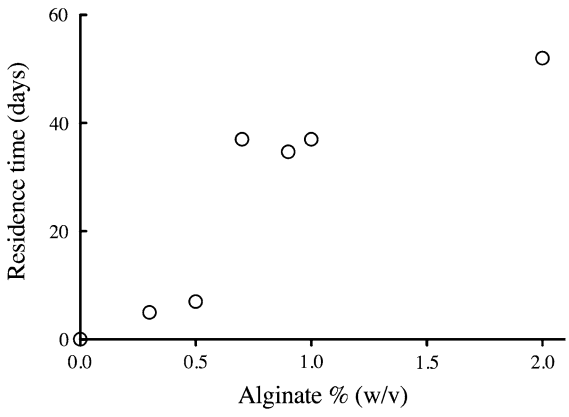

Fig. 2 Comparison of residence times for treatment solutions with different concentrations (w/v) of Manugel DMB sodium alginate in 20 g samples of a sandy soil at 25°C. The residence time of the solution without alginate was less than 1 day

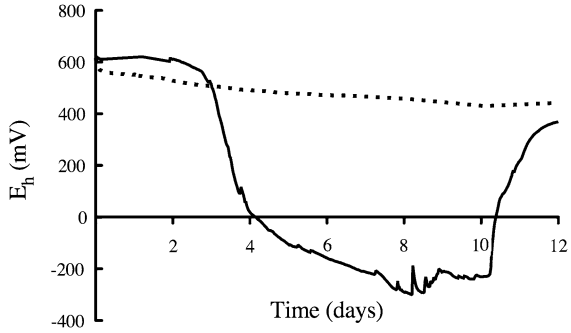

Fig. 3 The impact of 170 ml of nutrient solution containing 0.625% (w/v) alginate (*solid line*) on the redox potential of a sandy soil in an open-ended column over time. The control solution does not contain alginate (*dotted line*). The solution was applied to a 250 g soil sample in an open-ended column standing on a bed of sand. The redox potential was monitored continuously over 12 days with an inverted redox probe incorporated into the soil column

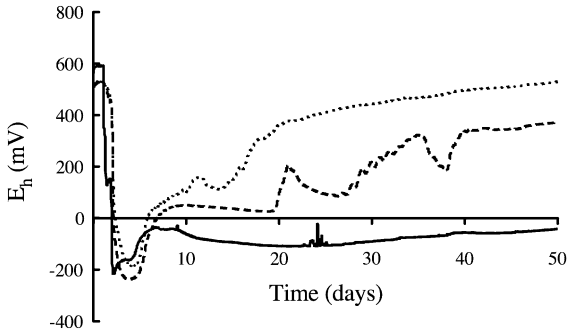

Fig. 4 The redox potential of soil measured at depths of 20 cm (*dotted line*), 50 cm (*dashed line*) and 80 cm (*solid line*) in a 1 m soil column upon application of 12 l of nutrient solution containing 0.625% (w/v) alginate and 100 μM neutral red to the soil surface. A rapid decrease in redox potential was observed at all depths and the redox potential rose thereafter to degrees according with depth

The moisture content of the soil at the beginning of the experiment was approximately 10% (w/w). After 56 days the soil moisture content at the soil surface and 20 cm below the soil surface was approximately 14% (w/w). Soil samples from 50, 80 and 100 cm below the soil surface had moisture contents of approximately 18, 24 and 29% (w/w) respectively, indicating a positive relationship between moisture content and depth. This data shows that low redox potentials are associated with elevated moisture content and that the treatment solution sustains elevated soil moisture content over 56 days.

At the beginning of the experiment, the average hexachloro-1,3-butadiene concentration in the soil, as determined from five randomly taken samples, was 4.1 mg/kg or 15.6 ± 3.7 μmol/kg. Extracts from soil samples taken at different depths after 56 days had reduced hexachloro-1,3-butadiene concentrations (Fig. 5). At the top of the column the hexachloro-1,3-butadiene concentration had been reduced to 1.2 mg/kg (4.4 ± 0.86 μmol/kg) or to 28% of the starting value and at the bottom of the column the pollutant concentration had dropped to 0.6 mg/kg (2.2 ± 0.68 μmol/kg) or to 14% of the starting value. Based on five samples taken from different depths, the treatment decreased the hexachloro-1,3-butadiene concentration by an average of 12.3 μmol/kg soil, representing a total loss of approximately 556 ± 131 μmol out of 700 μmol (79%) in 56 days.

The headspace of the column was monitored for the appearance of completely dechlorinated breakdown products of hexachloro-1,3-butadiene over time. After 7 days of incubation the concentration of C4 gases (sum of butadiyne and butenyne quantified against a 1,3-butadiene standard curve) in the headspace was 25.4 ± 12.7 μM. After 14 days incubation the C4 gas concentration in the headspace had decreased to 14.1 ± 2.5 μM. After 21 days, no C4 gases could be detected in the headspace. The fact that less C4 gas was detected in the second week compared to the first and that no C4 gas was detected in the headspace after 3 weeks suggests that a burst of dechlorination activity took place in the first week and that dechlorination activity was greatly decreased thereafter. The activity observed is likely to have occurred between 24 and 72 h when the redox potential was at its lowest. Furthermore, it appears that the C4 gases generated were either being consumed or adsorbed in the soil or possibly lost from the column, which was intentionally not sealed at the base. The 25.4 μM C4 gas concentration observed in the headspace after 1 week represents 660 μM of C4 gas (31 l headspace + 7 l void space − 12 l treatment solution). Taking into account the standard deviation, this corresponds well with the 556 ± 131 μM of hexachloro-1,3-butadiene removed. Because C4 gas standard curves prepared with or without an aqueous phase showed no statistically significant differences (data not shown), C4 gas dissolved in the aqueous phase was not taken into

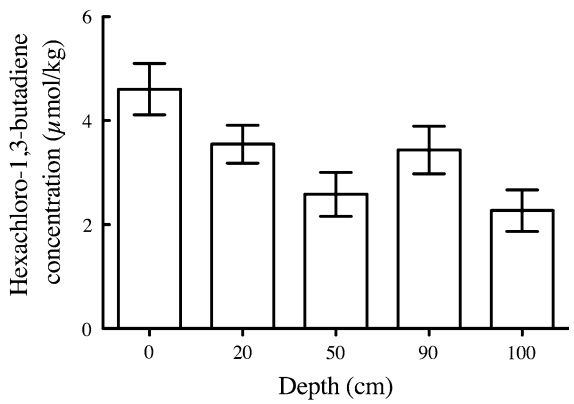

Fig. 5 Hexachloro-1,3-butadiene (HCBD) concentration at specific depths in a 1 m soil column 56 days after application of 12 l of nutrient solution containing 0.625% (w/v) alginate and 100 μM neutral red to the soil surface. The hexachloro-1,3-butadiene concentration in the soil at the start of the experiment was ~19 μmol/kg. Triplicate soil samples were analysed from each depth. *Error bars* represent standard deviation

account when calculating the C4 gas quantity in the column.

Impact of the treatment solution on the microbial community indigenous to a sandy soil

Given that there is no information in the literature on the toxicity of neutral red to microbes, the impact of this redox active compound on the viability of cultivable microbes in soil was assessed. Soil samples were made into slurries, treated with 10–1000 μM neutral red and incubated anaerobically for 28 days. Samples taken at 0, 4, 6, 21 and 28 days were diluted and plated for enumeration of colony forming units. There were no statistically significant differences in the viability of cultivable microbes at any of the time points between any of the concentrations of neutral red tested (Fig. 6).

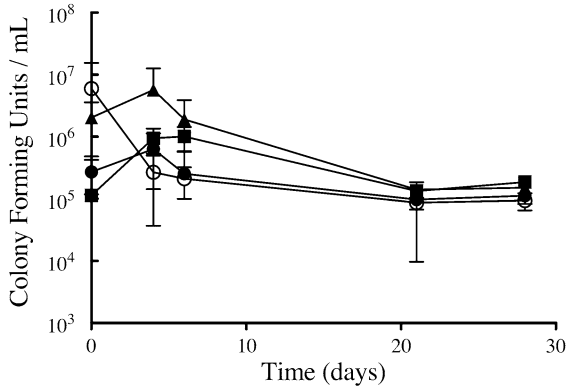

Fig. 6 The impact of neutral red at concentrations of 0 μM (*triangles*), 10 μM (*open circles*), 100 μM (*closed circles*) and 1000 μM (*squares*) on the viability of cultivable microbes in a sandy soil under anaerobic conditions over 28 days. Values presented are the average of triplicate treatments. *Error bars* represent standard deviation

To assess the impact of the treatment solution on the composition of the bacterial community indigenous to the sandy soil investigated, DGGE soil community fingerprints were generated from different depths in the column after 56 days. Figure 7 shows that the communities at different depths were clearly distinct from each other. The bacterial lineages detected by sequencing DGGE bands, represent the *Gammaproteobacteria*, *Firmicutes*, *Alphaproteobacteria*, *Bacteroidetes* and *Betaproteobacteria*, with the *Gammaproteobacteria* (x5) and *Firmicutes* (x4) sequences being more common. All sequences had close relatives that had previously been retrieved from soil. Sequences retrieved from 80 and 100 cm below the soil surface were from denitrifying Gram negatives (*Pseudomonads*), denitrifiying Gram positives (*Bacilli*) or fermentative Gram positives (*Clostridia*). Sequences retrieved from 50 cm below the soil surface were a mixture of denitrifiers, fermenters and aerobes indicating that at this depth there were microenvironments supporting both anaerobic and aerobic energy generation. All sequences retrieved from the soil surface were derived from organisms with aerobic respiratory systems from the *Xanthomonadaceae* family.

Discussion

In this study we have identified a water soluble hydrogel polymer (alginate) and an electron shuttle (neutral red) to increase the residence time of a nutrient solution in a vadose zone soil and catalyse the transfer of electrons from indigenous microbes to hexachloro-1,3-butadiene respectively. Water soluble polymers have been used in bioremediation applications previously. Specifically, carrageenan (Cassidy et al. 1997) and gellan gum (Moslemy et al. 2004) have been used to encapsulate bacteria in microbeads for delivery into contaminated aquifers. Alginates have also been used in bioremediation to immobilize bacteria in the vicinity of the rhizosphere to enhance metal uptake by plants (Gentry et al 2004) and more recently in electro-bioremediation applications (Shi et al 2008). To the best of our knowledge the present study is the first application of alginate to retard the movement of nutrient solutions in soil.

Alginates are polysaccharide based hydrogel polymers with much lower gelation temperatures than carrageenan or gellan gum. As a consequence, alginate solutions less than 1% (w/v) are typically liquids at room temperature. The gelling properties of alginate are related to the interactions between carboxylic acid moieties and divalent cations such as calcium that are common in soil (Tommasina et al 2007). These properties make alginate an ideal vehicle for retarding the movement of biostimulation solutions in soil. Of the two alginates tested, the one with a higher ratio of D-mannuronic acid to L-guluronic acid (Manugel) was superior, possibly because it generates a more elastic and viscous rather than brittle gel (Sriamornsak and Sungthongjeen 2007).

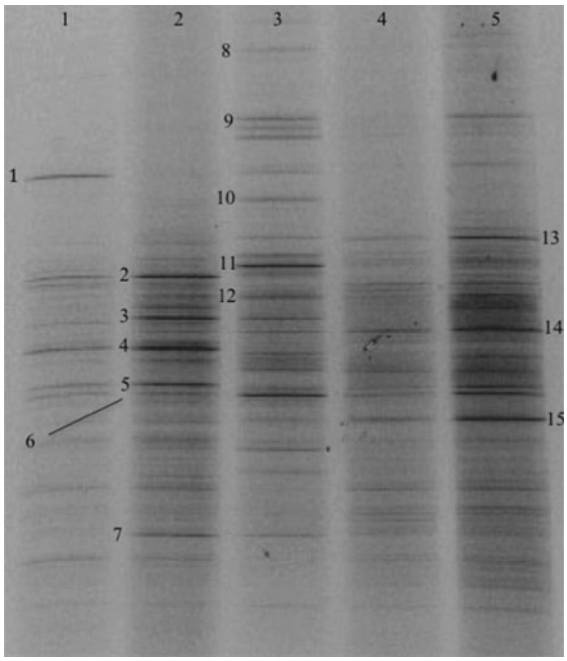

Fig. 7 Microbial community fingerprints (denaturing gradient gel electrophoresis) from soil samples taken at depths of 100, 80, 50, 20 and 0 cm from the soil surface (*Lanes* 1–5 respectively) of the 1 m soil column. Bands were cut, purified and identified by sequencing the approximately 200 base pair fragment as most closely related to *Pseudomonas pseudoalcaligenes* (Band 1), *Bacillus niacini* (Band 2), a *Bacillus* sp. (Band 3), a *Clostridium* sp. (Band 4), *Clostridium acetobutylicum* (Band 5), *Sinorhizobium meliloti* (Band 6), a *Sporolactobacillus* sp. (Band 7), *Sphingobacterium multivorum* (Band 8), *Pseudomonas aeruginosa* (Band 9), a *Lachnospiraceae* sp. (Band 10), a *Massilia* sp. (Band 11), a *Spingomonas* sp. (Band 12), a *Rhodanobacter* sp. (Band 13), a *Dyella* sp. (Band 14) and a *Xanthomonadeaceae* sp. (Band 15). No sequences were retrieved from lane 4

Of the redox shuttles tested, neutral red was the only compound that catalysed the reductive dechlorination of hexachloro-1,3-butadiene in the presence of activated sludge and electron donors. Neutral red is a synthetic phenazine derivative with a midpoint potential of −325 mV (H^+/H_2). This is the lowest potential of the compounds tested and similar to that of nicotinamide adenine dinucleotide, which transfers electrons from the citric acid cycle to the start of the electron transport chain. Because of this similarity, it is not thermodynamically possible for microbes to generate energy from the respiration of neutral red. We therefore speculate that the reduction of neutral red occurs through direct interactions with low potential intracellular components such as nicotinamide adenine dinucleotide rather than with reduced components of the electron transport chain.

The quantity of C4 gases produced in the soil during the first week of the experiment corresponded well with the quantity of hexachloro-1,3-butadiene lost from the soil. It was also observed that C4 gas concentrations decreased after production suggesting that they were being consumed, adsorbed or escaping from the system. Whilst we have found that 1,3-butadiene can be converted to 1-butene by activated sludge under anaerobic conditions (data not shown), this does not explain the loss of C4 gases from the soil system, because 1-butene was not detected amongst the C4 gases produced.

The bacterial community composition of the soil treated with the nutrient solution amended with alginate and neutral red reflected the different redox potentials at different depths. At 80 cm below the soil surface the potential was approximately −50 mV after 56 days, which is low enough for fermentation and nitrate reduction to have been taking place until the end of the experiment (Cronk and Fennessy 2001). Sulfate reduction is not likely to have been taking place in any part of the column outside of the 3 day window in which redox potentials were below −100 mV in the first week (Engler and Patrick 1973). In agreement with these observations the nitrate levels dropped with depth (Table 1). The soil

Table 1 Chloride, nitrate and sulfate ion concentrations at different depths in the soil column ten weeks after the treatment solution was added

Depth from soil surface (cm)	Chloride (mg/kg)	Nitrate (mg/kg)	Sulphate (mg/kg)
0	23.9 (±0.5)	669.6 (±24.8)	24.8 (±0.9)
20	30.2 (±1.4)	259.2 (±8.6)	29.7 (±1.4)
50	27.0 (±1.4)	10.8 (±0.9)	26.6 (±2.7)
80	28.8 (±1.8)	10.4[a]	34.7 (±1.8)
100	50.9 (±2.3)	Not detected	62.1 (±4.5)

[a] Nitrate was not detected in two out of three replicates

contained 13.1 ± 1.4 mg/kg chloride, 513 ± 6.3 mg/kg nitrate and 83.3 ± 1.4 mg/l sulfate at the beginning of the experiment. Whilst sulfate levels were lower at the end of the experiment, there was no trend with depth. The observation of known fermentative (*Clostridium*) and nitrate reducing (*Pseudomonas*, *Sinorhizobium* and *Bacillus*) bacterial lineages 80–100 cm below the soil surface is in good agreement with the conditions at this depth. At a depth of 80 cm a sequence affiliated with the microaerophilic *Sporolactobacillus* genus was observed, potentially indicating limited oxygen infiltration to this depth.

The redox potential of the soil 20 cm from the surface increased above 330 mV during the third week of the experiment suggesting that aerobic respiration could have taken place at this depth during the remaining 7 weeks (Cronk and Fennessy 2001). The detection of three sequences affiliated with oxygen respiring members of the *Xanthomonadaceae* family within the gammaproteobacterial phylum above this depth is in accordance with this observation. At a depth of 50 cm below the soil surface the redox potential remained below 250 mV until day 30 and below 330 mV until day 40. By the end of the experiment the presence of sequences from nitrate reducing *Pseudomonas* species, fermentative *Lachnospira* or *Lachnobacterium* species and aerobic *Sphingobacterium*, *Sphingomonas* and *Massilia* species could be detected reflecting the variable redox conditions. From the data presented it is not clear which organisms were primarily responsible for supplying reducing power for the reduction of hexachloro-1,3-butadiene, but it is likely to have been more than one metabolic group.

These laboratory experiments provide evidence to suggest that it is possible to formulate a biostimulation solution to accelerate the reductive dechlorination of chlorinated organics in aerobic vadose zone soils. Whilst the approach is yet to be tested in the field and has not been applied to other chlorinated organics besides hexachloro-1,3-butadiene, it has the potential to enable the reduction of pollutant concentrations in vadose zone soils below regulatory limits without the need to excavate. Further, increasing the residence time of nutrient solutions in the vadose zone decreases the risk of feeding nutrients into receiving water bodies. Future directions include field testing, formulation optimization and an exploration of delivery options including soil wetting prior to application.

Acknowledgements This work was supported by the Environmental Biotechnology Cooperative Research Centre and Orica Australia Pty Ltd. Mike Manefield, Lachlan Yee and Matthew Lee were supported by postdoctoral fellowships. Vibeke Aagaard and Angela Johnstone were supported by student stipends.

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