Supporting Information for 'Electrokinetic enhanced removal of toluene from physically heterogeneous media'

S.1.0 Materials and methods

S1.1 Inoculum growth on toluene under anaerobic conditions

The inoculum was grown under anaerobic conditions with toluene as the sole carbon source using a 50 mL aliquot of the initial R2A broth. This was centrifuged (10,000 rpm, 15 min) after which the supernatant was decanted and the pellet washed with a sample of the defined *T. aromatica* growth media. The washed re-suspended pellet was then added to a sealable flask containing 140 mL of defined media for anaerobic growth of *T. aromatica*. The defined media is composed of two solutions, A and B, that are adjusted to pH 7.2, autoclaved and then combined after cooling (Table 6.4). A sterile trace element and vitamin solution was added to the media once cooled (Table 6.3). Once the pellet of *T. aromatica* grown under aerobic conditions was transferred to the flask, the latter was capped and degassed using nitrogen. The degassing procedure consisted of inserting two needles aseptically through the vial cap: (1) a long needle (100 mm) that bubbled nitrogen from the base of the vial; and (2) a shorter needle (20-10 mm) to act as a vent. The inoculated flasks were then spiked with 7.4 µL of toluene that dissolved over several hours to give an equivalent concentration of 46 mg/L in the flasks. All 'Active' microcosms containing the inoculum were run concurrently with sterile 'Control' microcosms. Control microcosms contained sterile growth media prepared according to the same method for the active microcosms but omitting the inoculation step. A visible increase in the turbidity of the flasks containing the microbe was noted within one month of incubation. After this point a constant growth stock of *T. aromatica* was maintained by transferring 2 mL under anaerobic conditions to a fresh vial every 72 hours.

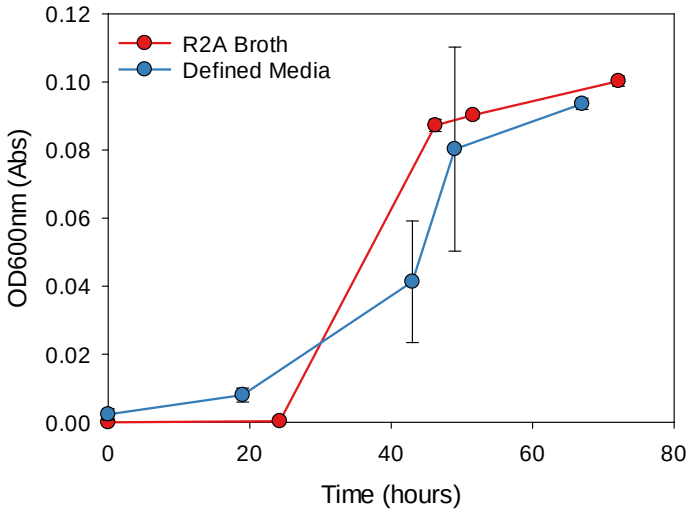

Figure S.1 Growth curves for *T. aromatica* in R2A broth and defined media (DM). An average value for the control vials was subtracted from the active vials.

T. aromatica growth (OD_{600nm}) under anaerobic conditions is shown in Figure 6.4, with the corresponding toluene and nitrate concentration for the same flasks in Figure 6.5. The exponential phase begins after 20 hours and coincides with a decrease in both the toluene and nitrate concentration. Analysis was done on aliquots of 2 mL extracted from the flasks and according to the methods outlined in Section 6.2.8. This demonstrates that the inoculum was actively degrading toluene using nitrate as an electron acceptor.

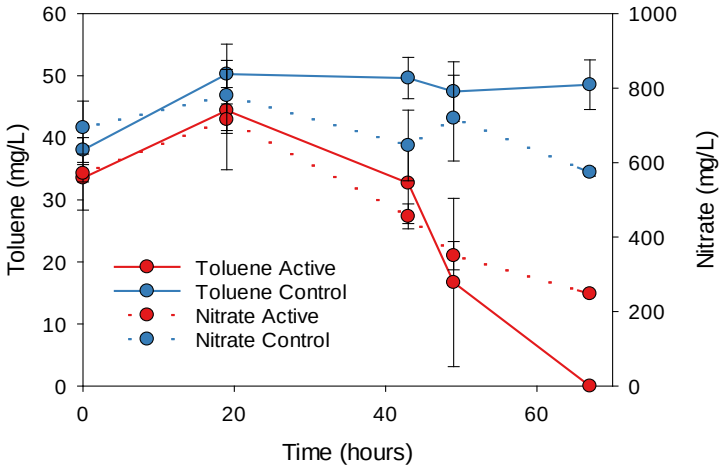

Figure S.2 Toluene and nitrate concentrations from the defined media growth experiment. Error bars represent one standard deviation from the mean of three replicates.

S.1.2 Trace element and vitamin solution properties

The components of the trace element and vitamin solution properties are given in Table 3

Table S.1 Composition of supplementary solutions for *T. aromatica* growth.

Trace Elements Solution		**Vitamin Solution***	
10 mL L^{-1}	HCl (25%; 7.7 M)	0.1 g/L	D-Biotin
1.5 g/L	$FeCl_2x4H_2O$	0.1 g/L	Choline Chloride
0.07 g/L	$ZnCl_2$	0.1 g/L	Folic Acid
0.1 g/L	$MnCl_2x4H_2O$	0.2 g/L	myo-Inositol
0.006 g/L	H_3BO_3	0.1 g/L	Niacinamide
0.19 g/L	$CoCl_2x6H_2O$	0.1 g/L	D-Pantothenic Acid•½Ca
0.002 g/L	$CuCl_2x2H_2O$	0.1 g/L	Pyridoxal•HCl
0.024 g/L	$NiCl_2x6H_2O$	0.01 g/L	Riboflavin
0.036 g/L	$NaMoO_4x2H_2O$	0.1 g/L	Thiamine•HCl
		8.5 g/L	NaCl

* Vitamin solution was obtained from Sigma Aldrich, UK No. (B 6891 BME)

S.1.3 High-K material consolidation method

The loose high-K material was consolidated using a shaker table at a frequency of 31 Hz, calibrated with a hand-held accelerometer. The test cells were filled with material in layers up to 3 cm and placed on the shaker table for intervals of 20s. This was repeated until the test cell was full, excess material removed, and the lid secured.

S.1.4 Preparation of inoculum for experiments

Preparation of an inoculated 10 L volume of synthetic groundwater for the bench-scale experiments included: (1) 17 x 140 mL anaerobic flasks containing degassed DM growth stock inoculated with 2 mL *T. aromatica* solution; (2) incubation of flasks for 48 hours at 30^{o}C to give an average of 0.20 Abs OD_{600nm}; (3) transfer of 40 mL to a falcon tube and centrifugation at 5000 rpm for 5 min; (4) decanting of supernatant and re-suspension of the pellet in a degassed 5 mL sample of sterile synthetic groundwater; (5) transfer of 5 mL concentrated *T. aromatica* to a carboy containing 10 L sterile and degassed synthetic groundwater, repeating steps 1-5 for remaining growth stock; and (6) storage of carboy at 5^{o}C until use in the experiments (up to 48 hours). The pelleting process created a dilution

factor between the defined media growth stock and the synthetic groundwater of 4.3. Samples of synthetic groundwater with and without *T. aromatica* were analysed for cell counts using epifluorescence microscopy. Samples were stained with Syto 9 (Invitrogen Ltd, UK), filtered and then viewed with an Olympus BX50WI Upright Fluorescence Microscope (Olympus OpticalCo. Ltd, London, UK) fitted with CoolSnap colour camera (Princeton Instruments, Buckinghamshire, UK). The synthetic groundwater containing the inoculum had 9.8 x 10^5 (± 4.5 x 10^5) cells/mL; the control had no observable cells.

S.1.5 Preparation of toluene stock

The concentrated toluene stock was made using a sterilised and degassed sample of synthetic groundwater within a 150 mL capped vial. An aliquot (96.4 µL) of pure toluene was added aseptically through the cap of the vial and allowed to dissolve completely over seven days at 10^{o}C. This was repeated for each rig setup. Samples were taken from the vials for GC-MS analysis before addition to the fine-grained sand and kaolin mix (Section 6.2.8).

S.1.5 Advective flux

The head difference is equivalent to a hydraulic gradient of 0.14 between the electrode chambers. Based on the hydraulic conductivity of the low-K zone this is equivalent to a flow rate of 1.2 mL/d and a solute flux (assuming a concentration of 10 g/L) of 0.077 g/d-m^2. This is three orders of magnitude lower than the predicted electromigration solute flux (assuming an effective ionic mobility of 6.5x10^{-9} cm^2/s-V and a voltage gradient of 50 V/m) of 28.1 g/d-m^2. Thus, the advective flux of nitrate is considered to be negligible during the experiment.

S.2.0 Results and Discussion

S.2.1 Toluene adsorption to bench-scale rig materials

Samples of acrylic and rubber were added to 20 mL vials containing water with four different concentrations (2.3, 4.6, 9.2 and 46 mg/L, respectively) of toluene. A series of control vials were included that contained no material. Each concentration was run in triplicate; in total 36 vials were used. Vials were sampled immediately after the materials were added and at 72 and 120 hours to determine when the toluene distribution was at equilibrium. The surface area of the acrylic and rubber sample was 1032 and 572 mm^2, respectively. Toluene concentrations were analysed using the GC-MS method specified in the main text and are shown in Figure S.3.

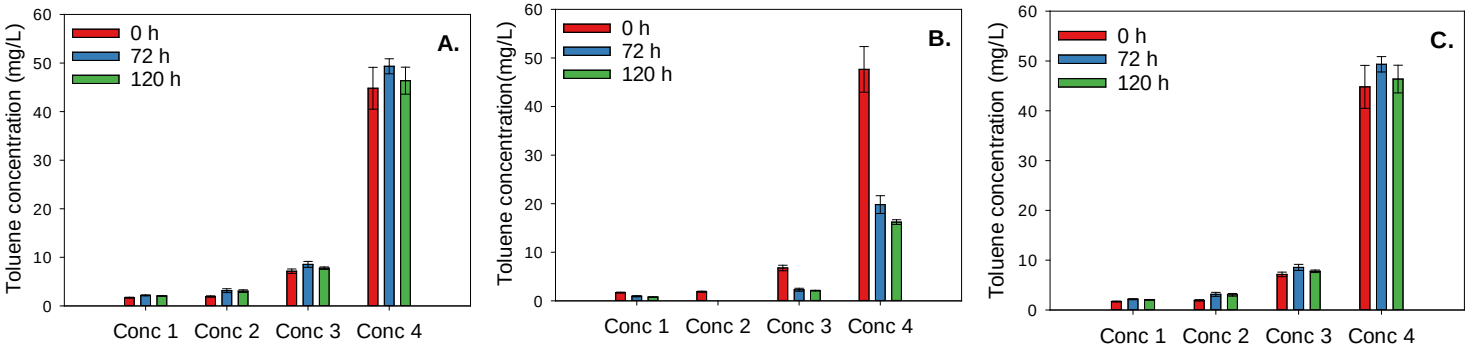

Figure S.3 Adsorption test for toluene onto materials used in rig construction. A, acrylic; B. rubber gasket; and C, no-material control. Error bars represent the standard deviation from mean of three replicates. Conc 1-4 represent toluene solutions containing 2.3, 4.6, 9.2 and 46 mg/L, respectively.

S.2.2 Toluene concentration in the high-K zone

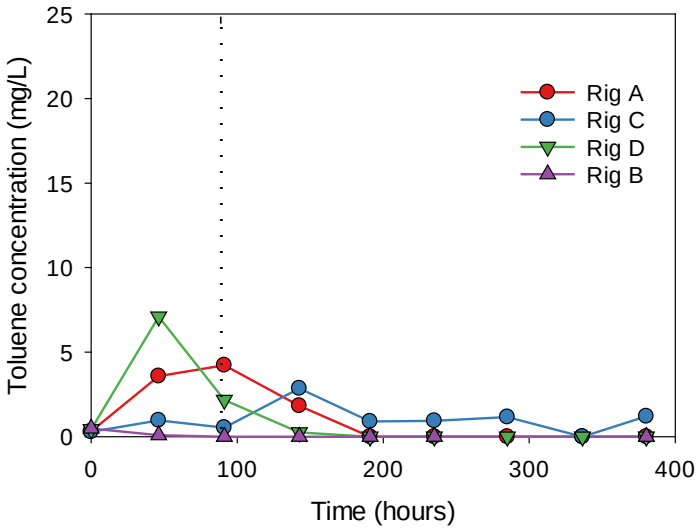

Figure S.4 toluene concentrations in the high-K zone for the different bench-scale rigs. Dashed black line indicates when the direct current is switched off in Rigs A, C and D.

S.2.2 DC swap between EK active and no-EK control rigs

Figure S.4 shows toluene concentration within the low-K zone for Rig B and C. In Rig B EK is applied to initiate electroosmosis and in Rig C, no EK is applied.

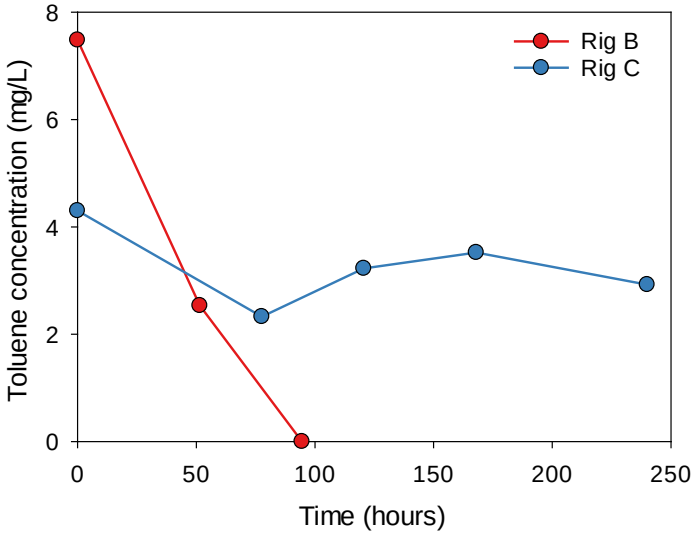

Figure S.5 Toluene removal from Rig B, EK applied and Rig C EK not applied

S.2.3 Carbon sources for nitrite production

Additional carbon sources known to be present that could account for the increase in nitrite, include the dilute vitamin solution (3.4 mg/L) and naturally occurring particulate organic matter in the sediment (2.7 mg/kg). To effectively assign a value for excess organic

matter it was assumed that the total mass is equivalent to the chemical structure CH_2O; this is equivalent to 20.9 mg and 0.09 mg in the rigs and microcosms, respectively. CH_2O can be oxidised to CO_2 via denitrification of nitrate to nitrite by the following reaction:

$$CH_2O + 2NO_3 \rightarrow 2NO_2 + CO_2 + H_2O$$

Equation 1

Based on this relationship, the total nitrite production in the rigs is equivalent to less than 100% of the organic matter present in the system (66, 75, 46 and 73% for Rig A-D, respectively). This implies that there is sufficient carbon other than toluene that can sustain nitrite production.

S.2.4 Potential limitations to enhanced biodegradation in bench-scale experiments

A low phosphate concentration in the pore fluid could explain the lack of enhanced biodegradation. The inorganic phosphate concentration in the synthetic groundwater should be 1290 mg/L. However, at the beginning of the bench-scale experiment the phosphate concentration was below detection limit. Similarly, in the microcosm experiment the phosphate concentration was relatively low at 60 mg/L. Phosphate could be consumed as a stress response by the microbes to the experimental conditions, leading to insufficient quantities being present once the nitrate is added to the system. A secondary microcosm study setup according to Section 6.2.7 confirmed that the inoculum cannot degrade toluene using nitrate when phosphate is absent. Future EK experiments using this inoculum should consider including phosphate as part of the amendment solution.

The pH of the pore fluid in EK-active systems is not anticipated to affect biodegradation. Values of pore fluid pH are plotted against the distance between electrodes for Rig D (Figure 7). At the beginning of the experiment pH in the low-K zone was 5.2 – 5.6, which is lower than the limit of toluene degradation by *T. aromatica* observed in the growth studies (pH 6.6). Over the first 91 hours of EK application the pH was elevated to between 7.2 and 6.4 in the low-K zone. This could be due to the electroosmotic flow of fluid from the anode chamber, where the pH over this time ranges from pH7.1 – 6.9. The pH is maintained above pH 6.6 in the low-K zone for the duration of the experiment.

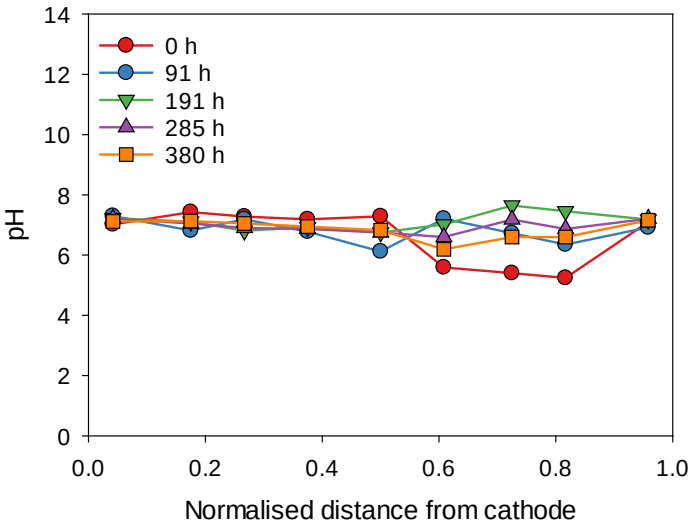

Figure 6 pH of pore fluid in the bench-scale experiments for Rig D.