Dielectrophoretic sample preparation for environmental monitoring of microorganisms: soil particle removal

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Abstract

Detection of pathogens from environmental samples is often hampered by sensors interacting with environmental particles such as soot, pollen or environmental dust such as soil or clay. These particles may be of similar size to the target bacterium, preventing removal by filtration, but may non-specifically bind to sensor surfaces, fouling them and causing artefactual results. In this paper we report the selective manipulation of soil particles using AC electrokinetic microfluidic systems. Four heterogeneous soil samples (smectic clay, kaolinitic clay, peaty loam, and sandy loam) were characterised using Dielectrophoresis to identify the electrical difference to a target organism. A flow-cell device was then constructed to evaluate dielectrophoretic separation of bacteria and clay in a continous flow through mode. The average separation efficiency of the system across all soil types was found to be 68.7% with a maximal separation efficiency for kaolinitic clay at 87.6%. This respresents the first attempt to separate soil particles from bacteria using dielectrophoresis, and indicate that the technque shows significant promise; with appropriate system optimisation, we believe that this preliminary study represents a golden opportunity to develop a highly effective sample processing system.

Introduction

There are myriad scenarios requiring that the environment be sampled in order to detect pathogenic organisms such as bacteria or viruses; this might be in airborne samples such as at airports in order to prevent the spread of highly contagious diseases; on the battlefield or in enclosed spaces to detect the use of bioweapons; or in food and hospital environments to detect the source of infections such as *Legionella*. Biosensor devices exist that can identify pathogens due to the interaction between organism and antibodies or similar surface-bound detection molecules, by capturing airborne samples into a liquid stream through the use of a cyclone capture device. However, such tests are often confounded by the presence of other particles in the environment with similar sizes (hence not removable by conventional filtration) and capable of non-specifically binding to the sensor, fouling it and in turn causing false positives whilst lowering sensitivity. An alternative, non-mechanical means of separation is therefore required in order to prepare the sample ahead of the detection stage.

Previous methods developed for the separation of bacteria from soils have been based on resins or blending-centrifugation procedures with a further use of density gradients or elutriation[1-5]. A common technique for releasing microbes from soil samples is the homogenization of the sample in 0.2% solution of sodium pyrophosphate and low speed centrifugation (≤600 x g) to remove soil particulates in what is known as a homogenization-centrifugation protocol. Physiological saline, diluted Winogradsky salt solution[1], TRIS buffer, sodium cholate solution, chelating agents, detergents and even pure water have all been used as a dispersion medium. Work conducted by Bakken[2] showed that the homogenization- technique was most effective when multiple steps were performed, with 10 - 20% bacterial extraction in a 1 step protocol, increasing to 50% with 4 steps and 75% with 8 steps from a clay loam. The remaining cells (> 1.9 µm diameter), after homogenization, were deposited by low speed centrifugation through a density gradient of 1.2 g/ml. This was indicative of bacterial cell adhesion to clay particles. Three possible reasons of bacterial attachment to soil particles have been identified[6]: 1) serendipity, 2) selective advantage for survival via organic and mineral nutrient uptake and 3) saturation of bacteria in porous gaps between soil aggregates. Hence, these attachments can be via a number of mechanisms including electrostatic attraction and sorption. Baath[7] demonstrated bacteria separated from a sandy loam after 8 cycles of homogenization-centrifugation displayed higher growth rates than bacteria separated after only just 1 cycle, further indicating tightly bound bacteria with soil may have nutritional benefits. Separation of bacteria from clay particles using Percoll (sucrose or Nycodenz can also be used) density gradient method (stepped and continuous) was described by Martin and Macdonald[8] and optimized by Macdonald[9]. Initial results of this one-step technique were deemed unsatisfactory as denser cells (e.g., endospores $\rho > 1.17$ g/cm^3) usually

sedimented with the clay particles below the Percoll. Thus, separation of the microorganisms from some smaller organic clay matter was much harder than the separation of higher density mineral materials from the microorganisms. Lindahl and Bakken[10] evaluated chemical and physical dispersion methods predominately used for extracting bacteria from soils. They concluded that chemical dispersion methods were inferior to physical methods (i.e., rotating rubber pestle or Waring blender) with ultra-sonication and shaking being highly inefficient dispersion methods. Also noted was that physical methods did not destroy the cells. On the other hand investigations conducted by Riis et al recommended a one-step extraction process through shaking and ultra-sonication and subsequent centrifugation at 100 x g for time critical processing[11]. Furthermore, to obtain only microscopic and fine particles, they suggested allowing the sample to stand for 5mins allowing larger particles to sediment. This method was found not to impede microbial counting techniques /investigations such as colony formation studies, assessment of biochemical activities or the enrichment or selection of particular microbes in dilute suspensions.

Whilst these methods are useful for laboratory-based analysis of samples, they are potentially very difficult to implement in a continuous-flow sampling process such as would be required for continuous monitoring in airports, hospitals and so on. In order to overcome this obstacle, an alternative approach is therefore required. Microfluidic devices have been employed in many similar scenarios in the last two decades and may have something to offer this application. One technology of growing interest which operates effectively on this scale is dielectrophoresis (DEP), a term used to describe the lateral motion of a polarisable particle in a non-uniform electric field. DEP has been employed in microfluidic systems to form an electrostatic separation system whereby particles are separated on the basis of the way in which they interact with the electric field, which in turn depends on their properties. It has been shown for numerous combinations of cells that it is possible to remove particles from a bulk flow as is passes the microelectrode structures or even deflect it towards different flow streams for spatial or temporal based fractionation.

When either a neutral or charged particle is subjected to a to a non-uniform electric field , the particle acquires an electric dipole; in a highly divergent electric field one side of the dipole will interact with a weaker electric field than the other side, resulting in a net translational force imparted on the particle (the dielectrophoretic force F_{DEP}). F_{DEP} is affected by many factors including the magnitude and gradient of the electric field, the volume of the particle (r^3) and the absolute permittivity (ε_m) of the medium in which the particles are suspended. This can be described by equation 1:

$$\langle F_{DEP} \rangle = 2\pi r^3 \varepsilon_m Re[K(\omega)] \nabla |E_{RMS}|^2 \qquad (1)$$

The interaction of the electrical properties of particle and medium are described by the real part of the Clausius-Mossotti factor ($Re[K(\omega)]$). This is a frequency-dependent quantity which has a range of -0.5 to 1 for spherical particles. A factor of less than 0 indicates a particle is directed away from high field intensities (negative DEP), while a factor greater than 0 indicates a particle is directed towards high field intensities (positive DEP). In addition to the frequency dependency, the complex permittivity (ε^*) and conductivity (σ^*) of both medium (i = m) and particle (i = p) contributes to the polarizability factor (Equation 2 and 3):

$$K(\omega) = \frac{\varepsilon_p^* - \varepsilon_m^*}{\varepsilon_p^* + 2\varepsilon_m^*} \quad (2)$$

where $\varepsilon_i^* = \varepsilon_i - j\frac{\sigma_i}{\omega}$ (3)

The electrical properties of bacteria have been studied by DEP on numerous occasions[12]. One of the first determined the electrical properties of a range of Gram positive and Gram negative microorganisms over a frequency range of 10 – 100 kHz, and was able to use this information to do batch separation of different species using an interdigitated castellated electrode array [13]. Further research into the potential use of DEP as a real time detection tool for microorganisms in biotechnology and environmental monitoring[14] have since been described; whilst other studies (e.g.[15]) have examined the potential of DEP for determining bacterial responses to antibiotics. Separation studies involving the application of DEP to bacteria have included using conductivity gradients to separate bacteria in DEP systems[16]; using high conductivity media and medium exchange to separate bacteria from cerebrospinal fluid[17]; insulator-based DEP (IDEP) to concentrate/separate live and dead bacteria in channels containing obstructions (which generate non-uniform fields) using direct currents[18,19]; collecting algae from environmental samples for analysis[20], separating bacteria from diesel particles[21]; tagging bacteria to alter their permittivities in a multi-target dielectrophoretic activated cell sorter[22]; and directing bacteria via pDEP and nDEP to regions of a biochip for assisted immuno-capture and detection of food-borne microorganisms[23]. More complex electrode geometries, coupled with other microscale technologies (i.e., magnetic, hydrodynamic etc.) are being designed and fabricated, mainly focused at present for biomedical/life science applications but with applicability to environmental monitoring automated lab on a chip systems[24-28].

Whilst the dielectric properties of soil/water composites have been measured in the past[29-32], manipulation of soils using AC electric fields in microflows has not been reported to our knowledge and the work described in the following sections will represent a novel approach to improve bacterial concentrations from contaminating soils using DEP in order to enhance, for example, downstream

biosensor detection limits. In this paper we present the separation of *B.atrophaeus* (analogous to *B.anthracis*) from four soil types using electric fields in a microfluidic system. In order to determine the parameters, required for electrical separation of bacterium and from soil types, dielectrophoretic characterization of each population was performed.

Materials and Methods

Materials

Four types of soil were obtained from DSTL stocks in 1.5ml vials; example images are shown in figure 1. Peaty loam (PL) is a dark to black colored coarse grained soil predominately composed of organic material, coarse sand, medium silt & fine clay particles in varying ratios. Sandy Loam (SL) is reddish-brown granular soil, supposedly composed of relatively equal fractions of sand (silica or SiO_2), silt and clay. Though in other compositions sand (~50%) is usually dominant at no particular grain size, with silt (~43%) and clay (<7%) making up the remaining fractions. Kaolinitic clay (KC) is light brown in appearance with coarse grains making up a significant proportion of the sample, probably due to residual organic matter from the soil. They tend to be mixtures of very fine grained minerals with different proportion of clay minerals in which kaolinite ($Al_2Si_2O_5(OH)_4$) is predominant. Smectic clay (SC) is also brown in appearance with similarities in texture to KC, though larger grain particles exist. Belonging to the smectite group include saponite and montmorillonite which can contain Fe, Ca, Mg, Na and hydrous aluminium silicates. Structurally the 2:1 silicate layers have a slight negative charge due to ionic substitutions in the octahedral and tetrahedral sheets. The bacterium *Bacillus atrophaeus* (ATCC® 9372™) was obtained from LGC Standards (Teddington, UK), and was cultured in Nutrient Broth #3 (BD 234000) overnight at 30°C.

Soil particle sizing

For each of the soil samples, 0.1 g was weighed out on a precision scale and mixed with 1ml distilled water. The mixtures were ultrasonicated for 30min in a water bath then analyzed using a Mastersizer particle analyzer (Malvern Instruments Ltd, UK). Each sample was added to a large continuous-stirred dispersion unit. A pump was used to continually transfer the particles + dispersant in to the optical flow cell. Optical measurements of the media without the soils were first conducted for calibration purposes. An obscuration value of >5% was needed to attain accurate measurements of the soil particles when in the laser's optical path. Measurements of the each of the samples were taken a minimum of 4 times for accuracy. The system is capable of measuring sizes in the range of 0.2 – 2000 µm, with settings constant across the soil samples such that dispersant (water) had a refractive index

of 1.33, a range lens of 300RF (Reverse Fourier) mm and a beam length of 2.40mm. Variation in obscuration between soil samples ranged between 5.3 to 25%.

Dielectrophoretic characterization

Test solutions consisted of distilled water, supplemented with potassium chloride (KCl) up to 7 different conductivities; range = 0.36 mSm^{-1} - 284.6 mSm^{-1}. Characterisation was performed using a DEPtech 3DEP dielectrophoresis analyser (Labtech, Uckfield, UK), and each soil sample was analyzed at 20 points over a frequency range of 10 kHz to 60 MHz for a period of 60seconds. Change in light intensity as a function of dielectrophoretic particle motion, per frequency point, was used to generate the DEP spectrum within different medium conductivities. 0.02 g of each soil sample was diluted in 1 ml of each of the prepared conductivities; to reduce the interference due to coagulated lumps of material, soil samples were filtered using a 40µm Nylon cell strainer (BD Falcon, 352340). To analyse the bacterial sample, 1 ml of the cultured suspension was centrifuged at 10000 rpm for 10mins and re-suspended in similar media to that used for soil for the DEP characterization, then mixed and centrifuged at 5000 rpm for 5 mins. This was repeated 3 times to obtain the required medium conductivity and to remove any debris. Particles were characterised at least 4 times.

Separation device

A flow cell was fabricated in gold deposited on microscope slides with a titanium seed layer (University of Sheffield, UK). Slides were cut to 36 x 26.5±0.5 mm, washed in ethanol and patterned by conventional photolithographic methods. Electrodes were of an interdigitated design with electrodes being 250 µm wide with an inter-electrode gap spacing of 50 µm. Coverslips coated in Indium tin oxide (ITO) (Delta Technologies, USA) were cut to 36 x 15 mm using a glass cutting machine to form a lid. 4 holes aligned with the bores of the top part of the flow-cell holder were drilled using a 1 mm diamond drill bit (Eternal Tools, UK). Using a Graphtec Craft Robo Pro vinyl cutter (MDP Supplies, UK), arrays of the gasket designed in CorelDraw were cut out of 150 µm thick double side adhesive sheets (Lohmann, UK). With total assembly time less than 5 minutes, used flow-cells were disassembled and cleaned according to a similar protocol by Čemažar et al.[33], and a new gasket was replaced for each experiment [**] . Tubing was manually flushed through with ethanol and distilled water, and air dried prior to reassembly. The microfluidic channel was 8 mm wide and 18 mm long. The ITO holes were carefully aligned with the gasket entry and exit regions then affixed to the patterned microelectrode array. Pressure applied to the sandwich by hand was sufficient to ensure the structure was tightly bonded. Manual flow of fluid from a 1 mm syringe was used to test flow resistance or leakage in the channel before placing it in a flow-cell holder and conducting DEP separation experiments.

Soil samples were double filtered in a 40µm Nylon strainer and then a Transwell 8µm pore size PET membrane (Corning Life Sciences, USA). The filtrates were washed in 30 ml of 15.5 mSm^{-1} KCl solution (@ 19.2 ± 0.2 ^{o}C). Final stock solution conductivity of the soils was measured after 3 washes and a particle count in a hemocytometer was performed for each filtered soil sample. Bacteria were resuspended into low-conductivity medium as outlined above. Before mixing the bacteria with the soils, each of the soils and bacteria samples were individually stained with Live/dead BacLight bacterial viability kit (Molecular Probes). Equal volumes (50 µl) of SYTO9 dye (3.3 mM) and propidium iodide (20 mM) were mixed through agitation in a microcentrifuge tube. 3 µl volumes of the resulting mixture was added to each of the samples and allowed to incubate in the dark for 20 minutes. Each stained sample was inspected under fluorescence microscopy at an excitation wavelength of 485 nm from a Mercury lamp attached to a Nikon microscope. This procedure was carried out to assess the influence of the dye on the soil sample as a potential technique for analyzing separated bacteria from soil particles in the effluent stream and whether the soils caused background-fluorescence.

Soil and bacteria were mixed at a volumetric ratio of 2:1 in a centrifuge tube and agitated before transferring the mixture into a 5ml syringe. A syringe pump operating at 1.02 ml hr^{-1} was used to flow the solution through the DEP flow-cell and the effluent stream was collected downstream in a receptacle. The tubing (PTFE, OD 1/16'', 0.3 mm ID) connecting the syringe and the inlet port was 32.5 cm in length while the exit length of the tubing was 16.5cm. Electric field distributions between coplanar electrodes spaced at 50 µm were model for 5 V_{pp} and 10 V_{pp} up to a maximum chamber height of 350 µm.

Results and Discussion

Analysis of soil separates

Figure 2 shows the representative size distributions of the four soil types. For PL, with an obscuration value of 22.9 ± 1.1 % across the repeats, a modal value of 166.8 µm at 5% volume concentration is measured from the graph above. The range was 69.2 µm (@ ~1%) to 878.67 µm (@ 0.2%). The large size particles scattering the light may contribute to the perceived absence of smaller sized particles which were confirmed present through optical microscopy. For SL, no aggregate formation was seen, indicating this soil is mineral rich. The large proportion of smaller particles were assumed to be silt (quartz & feldspar), which are typically smaller than sand (<50 µm according to USDA) though similar in size to clays. The larger size particles scattering the light may have contributed to the perceived absence of smaller sized particles which are confirmed present through optical microscopy. For KC,

the higher particle concentration seen is due to the low particle size distribution across the KC sample, with a modal value of 6.0 µm at 3.46% volume concentration. The range was 0.7 µm (@ 0.15%) to 92.7µm (@ 0.22%). A representative analysis of the SC's size distribution is also shown in Figure 2, with a modal value of 124.4µm at 4.5% volume concentration. The range was 13.1 µm (@ 1.3%) to 488.8 µm (@ 0.02%). Microscopic analysis of the raw samples showed a high degree of heterogeneity in particle shapes within a single batch.

Dielectrophoretic characterization

Soil samples were analysed using the 3DEP system with a well diameter approaching 1 mm. It was found that neither of the clay samples were able to provide useful DEP data at the lowest conductivity (0.36 mSm^{-1}), whilst the other two samples showed positive DEP at all frequencies. At higher conductivities (i.e. 105 mSm^{-1} and 284 mSm^{-1}), the spectra across all soil types were extremely noisy and scattered, hence no further investigation into higher conductivities were warranted.

In the intermediate conductivities, a common trend was found between the soils analysed, being a single dielectric dispersion at the lower end of the frequency spectra. In solutions with conductivity 9.6 mSm^{-1} and 35 mSm^{-1}, positive DEP occurred at frequencies between 10 kHz and 25 kHz, with a crossover (point at which no movement occurs) beginning at 40 kHz and stayed relatively constant throughout. Increasing the medium conductivity to 55.8 mSm^{-1} gave similar DEP trends, though the crossover was found at 62.4 kHz. Apart from smectic clay, all soil types at the stated conductivities were found to have a DEP force approaching zero at frequencies of around 100 kHz, while smectic clay displayed positive DEP up to ~10 MHz before tailing off to zero force. Sample single-run DEP spectra can be seen in figure 3. Characterization of bacteria using the 3D wells has been previously reported [34, 35]. A typical DEP response of *B. atrophaeus,* as characterized using the 3D wells, suspended in a medium conductivity of 35 mSm^{-1} is shown **Error! Reference source not found.**. A comparative analysis of the DEP spectra of the soils versus the bacteria shows that at lower frequencies negative dielectrophoresis influences bacteria up to ~ 1 MHz, where a transition occurred to positive DEP. A second cross over frequency (f_{x2}), indicating the presence of another dielectric dispersion, can be seen at approximately 10 MHz.

Separation of bacteria from soils in a DEP flow-cell

A clear distinction was found between the dielectrophoretic spectra of the soils and bacteria. At medium conductivities between 10 and 60 mSm^{-1}, with exception of smectic clay the soils exhibited pDEP at low frequencies (<100 kHz). Smectic clays displayed pDEP over a wider frequency range than

the other soils making frequency selection for separation operations ideally suited to a value within the range 100 kHz < $f_{separation}$ < 1 MHz. A medium conductivity of 18.6 ± 2 mSm^{-1} (measured when containing both soil and bacteria) was used for all cases; the electrodes were energized by a 10 V_{pp} signal at 30 kHz for all samples except smectic clay, where 300 kHz was used. In the experiment, 3 ml of soil and bacteria mixtures were pumped through the flow-cell at a volumetric flow-rate of 1.02 ml hr^{-1}; although this is low for real-world applications, the possibility of scalable DEP separation architectures (e.g. well-based devices[35, 36]) exist and could be exploited for this application at much higher flow-rates.

To attempt quantification of the separation efficiency, soils filtered through the 8 µm PET membrane were stained with BacLight bacterial viability to ascertain whether fluorescence / background fluorescence was present as a result of the particles within the sample. Apart from PL, where larger particles showed minimal fluorescence, most likely due to the organic material present, all other soil samples did not fluoresce. The stained bacteria sample gave off a distinctive green fluorescent signal when stained with BacLight indicating live bacteria were present.

At the applied electrical conditions, collection of soil particles pumped through the flow-cell was seen across all soil samples. Employing a strategy for collection of soil particles via pDEP and repelling bacteria from the electrodes via nDEP, particles were separated in a single inlet and single outlet flow through system. The separation efficiency of dielectrophoresis, defined as the removal of targeted particles from volumetric flow within the DEP flow-cell, based on the defined operational parameters for each soil type was calculated using equation 4.

$$Eff_{separation} = \frac{\%_{input} - \%_{output}}{\%_{input}} \, x \, 100 \qquad (4)$$

Figure 5 shows the proportions of soil particles trapped, the proportion of bacteria eluted and separation efficiencies achieved in the flow through process for the different soil samples. It can be seen that both clays (mineral rich) and peaty loam containing organic material achieved $Eff_{separation}$ > 75%. Kaolinitic clay showed the highest level of separation at $Eff_{separation}$ = 87.6%, while both smectic clay and peaty loam has comparable levels of separation at 75.5% and 80.4% respectively. Sandy loam showed the lowest level of separation efficiency at 31.4%. With the exception of sandy loam, separation efficiency was comparable to the current benchmark standards outlined in the Introduction.

One aspect of separation which is worthy of consideration is that of the case where bacteria adhere to the soil particles, and are thereby prevented from reaching a downstream sensor device by being

trapped at the DEP filtration stage. The hydrophobic and electrostatic properties of soil particles dictates the extent to which bacteria will or will not sorb to the particle surface. Charges associated with primary silicates (e.g., feldspar) are generally low. Charge increase for minerals such as kaolinites, with positive charges in the presence of iron and aluminium oxides (e.g., smectite) which have the capacity for anion exchange[6]. Charge differences between minerals and bacteria, and the crystalline structure of the mineral have an influence as to how bacteria interact with the mineral surface. This could be a reason to the low elution percentage of bacteria observed with kaolinitic clay with a low surface charge (Cmol kg^{-1} at pH 7) of -13 compared with smectite clays ranging between -21 to -127 [37]. Sandy loam which contains a significant proportion of silt and relatively low charge (-2) had a high level (78.8%) of bacteria elution. Furthermore, it is thought that electrostatic attraction between 2 negatively charged particles is a result of polyvalent cations providing a bridging capacity, coupled with weak electrostatic repulsion upon closer interaction resulting in adsorption due to hydrogen bonds and Van der Waal's forces (fluctuating dipole - induced dipole). Two important parameters which have been reported to influence bacterial adsorption with soil particles are the ionic strength of the medium and the particle distribution and size of the soils. Both factors tend to increase bacterial adsorption with increases in either ionic strength up to 0.1M (DVLO theory) or particle concentration coupled with smaller grained particles.

Conclusion

Separation of different soil particle types from bacteria using a dielectrophoretic clean-up microsystem has been demonstrated for the first time. All but peaty loam could be separated at efficiency equal to or exceeding the current gold-standard techniques; elimination of smectic clay required the use of a different energising frequency, but this could easily be accounted for through the use of either multiple stages or the application of multiple frequencies simultaneously. This would also be the case for the trapping of diesel particles already reported by the authors, which could be achieved simultaneously and with the same apparatus.

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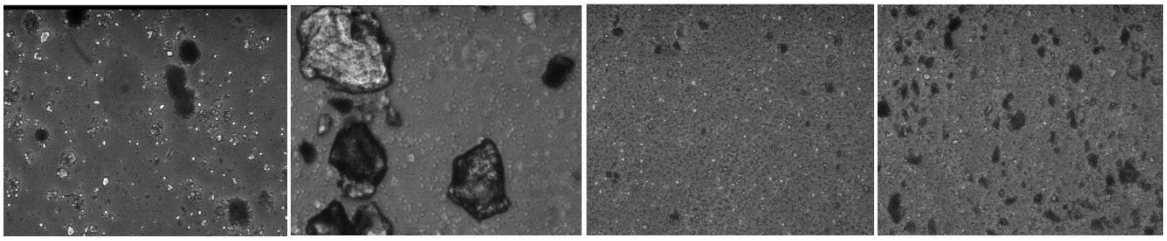
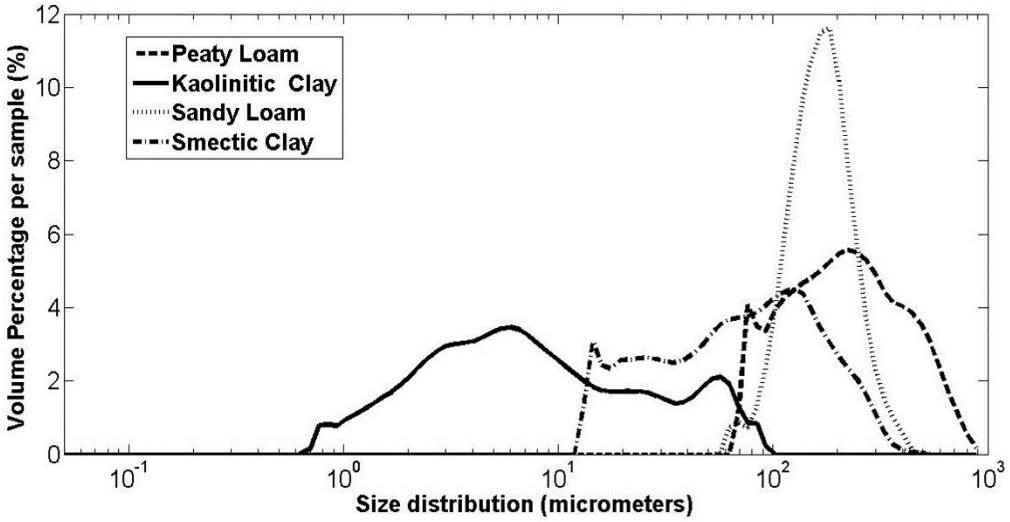
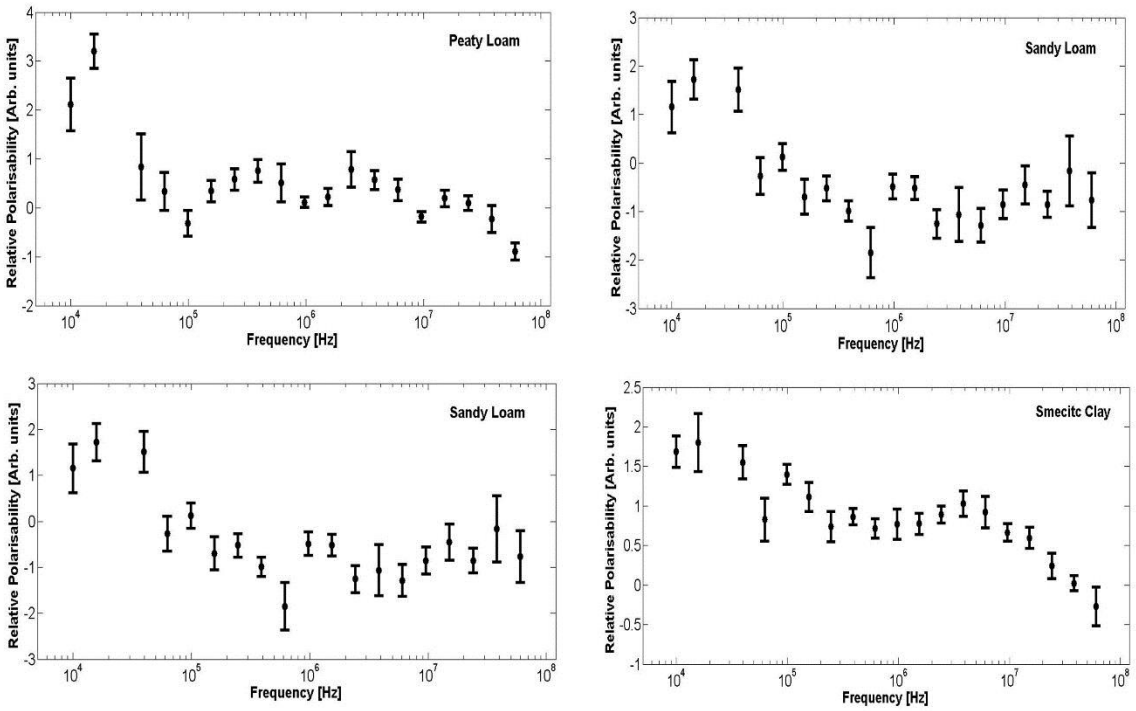
Figures



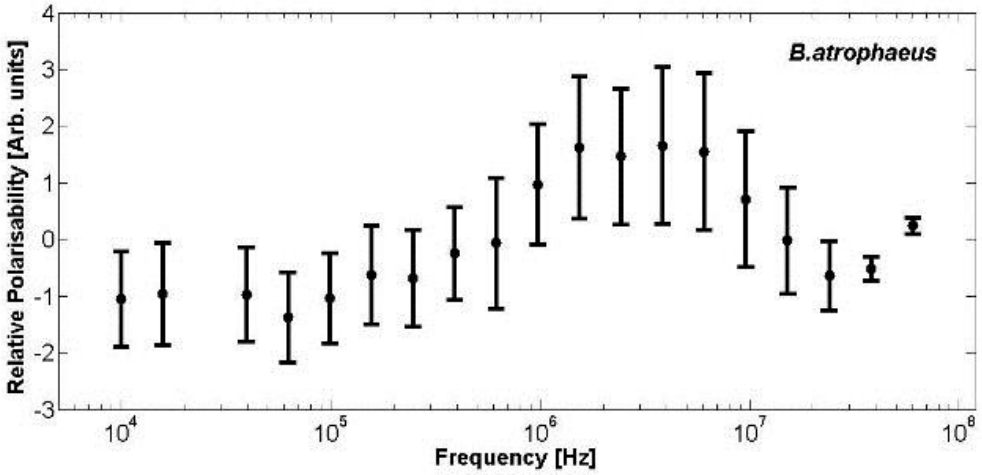
1. Microscopic images of the four clay times used in this study. Images were taken using a Nikon Eclipse E400 camera with x10 lens. Scale bar = 100 µm. (a) Peaty loam; (b) sandy loam; (c) kaolinitic clay; (d) smectic clay.



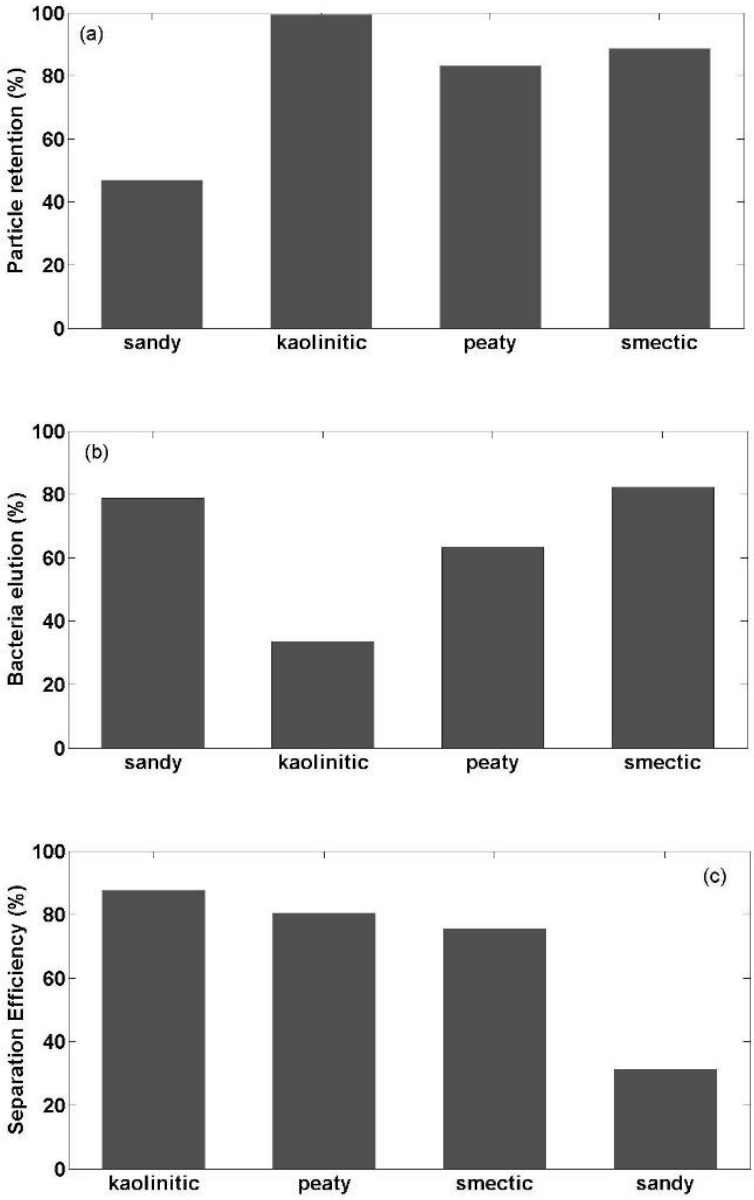
2. Size distribution as volume percentage of (a) peaty loam: (b) sandy loam (c) kaolinitic clay (d) smectic clay



3. Typical DEP spectra showing the DEP responses of the four clay types when suspended in a 56 mSm^{-1} solution. (a) Peaty loam; (b) sandy loam; (c) kaolinitic clay; (d) smectic clay. Bars indicate the range over which the light intensity varied across the 10 bands for each frequency, rather than error.



4. A typical DEP spectrum showing the response of *B. atrophaeus* in 35 mSm^{-1} solution. Bars indicate the range over which the light intensity varied across the 10 bands for each frequency, rather than error.



5. Results of DEP separation of bacteria from clay. (a) Proportion of clay particles retained (b) proportion of bacteria eluted (c) trapping efficiency.