

# Biominalisation of metals in soil – effect of metal toxicity and precipitation as a protective mechanism

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## ABSTRACT

Biominalisation offers the potential for in-situ sequestration and subsequent reduction in the bioavailability of heavy metals and radionuclides in the subsurface environment. Calcium carbonate minerals are known to sorb and form solid solutions with a range of target elements, and are readily produced by the actions of common microorganisms on simple chemical precursors. The ability of a commonly used urea-degrading, calcium carbonate-precipitating bacterium, *Sporosarcina pasteurii*, to tolerate the presence of a model contaminant, strontium, was determined in aqueous solution, with reduction in growth only seen at concentrations of 10 mM. Its ability to remove strontium from solution via calcium carbonate precipitation was then determined, and here *S. pasteurii* was shown to be able to remove 99% (+/- 1%) strontium from solution at concentrations up to 30 mM. This suggests that biominalisation of metallic elements may afford a protective mechanism for the bacteria through providing a means to reduce the overall concentrations to tolerable levels. Finally, we explored the effects of ground conditions on mineralisation and strontium sequestration in different sand fractions (fine, medium and coarse), in a series of batch experiments. Almost all (97-99%) strontium present was removed from aqueous solution after three days, whereas no precipitation was observed in control samples over the same period. The amount of strontium removed increased with coarseness of sand grains under these static conditions, although over a very small range.

Keywords: Biominalisation, *Sporosarcina pasteurii*, Strontium, Urease, calcium carbonate

## 1. INTRODUCTION

The natural formation of calcium carbonate occurs both through abiotic and biotic processes. The process can be harnessed for engineering processes (again, biologically or otherwise) for various purposes, including soil cementation and precipitation and removal of a range of toxic or otherwise problematic chemical species. A common mechanism for engineering calcium carbonate precipitation is through microbial urea hydrolysis, ordinarily brought about by *urease* enzymes; bacteria with the ability to break down urea *via* this mechanism are widespread in the subsurface environment (Lloyd & Sheaffe, 1973). This, and the ability of calcium carbonate precipitation to co-precipitate a number of heavy metal species, including cadmium, lead and strontium, makes sequestration of such contamination by co-precipitation with this mineral an attractive technique (Fujita *et al.*, 2000).

Urea hydrolysis leads to elevated pH and an increase in carbonate ions, both of which increase the likelihood of metal carbonate precipitation (Gebrehiwet *et al.*, 2012). Forms of calcium carbonate, including calcite, may offer a stable environment over long periods depending on the local environment. The co-precipitation of heavy metals with calcium carbonate is controlled by a number of factors, including bacterial urease activity, metal ion concentration, and the precipitation rate (Lorens, 1981; Pingitore Jr and Eastman, 1986). Cells themselves often act as nucleation points for the formation of minerals due to their typically overall negative charge attracting metal ions and therefore creating a zone of high metal ion concentration (e.g. calcium). The continuous precipitation of calcium carbonate around bacteria can inhibit or reduce their ureolytic ability due to encapsulation of the cells, and so growth in cell numbers is necessary to maintain mineral production. In addition, release of ammonia and ammonium ions is of potential environmental concern and subsequent biological action may cause acidification and potential dissolution of the precipitate.

The size and morphology of precipitated calcium carbonate influences the mineral solubility and long-term immobilisation of co-precipitated contaminants. For example, strontium incorporation into a calcite lattice may result in a reduction in the durability of metal immobilisation by generating small and more soluble crystals (Mitchell and Ferris, 2006; Tobler *et al.*, 2011; Cuthbert *et al.*, 2012). Due to the inherent heterogeneity of subsurface environment, the spatial distribution of calcium carbonate precipitation within porous media remains highly variable, particularly at the microscopic scale. In

addition, there are temporal changes that occur due to the continual formation of calcium carbonate over time including chemical changes such as removal of mineral components (e.g. calcium) from solution and production of new materials such as ammonium. These conditions may also vary in the longer term, e.g. ammonium produced *via* urea hydrolysis may become oxidised leading to acidic conditions (Reed *et al.*, 2010). Physically the resulting precipitation can block pore throats, which leads to obstruction of the transport and mixing of delivered reactants (Gebrehiwet *et al.*, 2012). Fujita *et al.* (2010) found that urea hydrolysis processes *in situ* are not homogeneous, mainly due to the heterogeneous distribution of microorganisms. The heterogeneous distribution of activity in the subsurface environment may therefore lead to non-uniform mineralisation of target contamination, and so pose problems for design of biomineralisation treatment systems in particulate media.

Strontium has previously been a target for remediation *via* calcite biomineralisation (Fujita *et al.*, 2000; Fujita *et al.*, 2008; Fujita *et al.*, 2010; Achal *et al.*, 2012; Lauchnor *et al.*, 2013), due to its presence on certain sites as a radioactive heavy metal contaminant. It is not redox sensitive, and its availability in the subsurface environment is primarily affected by sorption and abiotic precipitation in high pH environment with some minerals. Studies have shown the ability of microorganisms to precipitate this metal as strontium carbonate (Newsome *et al.*, 2014). Precipitation of elevated levels of strontium in the subsurface environment as SrCO<sub>3</sub> can occur after an increase in groundwater pH due to oversaturation with carbonate. With low Sr concentration, the metal ions can be incorporated within the structure of calcium carbonate as a solid solution (Fujita *et al.*, 2004).

In this study the tolerance of *S. pasteurii* to strontium (Sr) as a representative heavy metal, and its ability to subsequently remediate this metal, is investigated. Firstly, the ability of *S. pasteurii* to tolerate Sr in aqueous growth medium was examined. Next, this is compared to the tolerance of the organism to strontium in aqueous media containing the necessary minerals for calcium carbonate precipitation and Sr co-precipitation *via* urea hydrolysis. Finally, the ability of this bacterium to induce calcium carbonate precipitation and co-precipitation of strontium in different sand fractions (fine, medium and coarse) was explored, as an initial study into the effects of ground heterogeneity on heavy metal biomineralisation.

## **2. METHODS**

### **2.1 Culture Media and Growth**

Due to its high urease activity in aqueous solutions *S. pasteurii* (National Collection of Industrial & Marine Bacteria, Aberdeen, UK; NCIMB8221 / ATCC6453), was used in this study. *S. pasteurii* is a widespread, gram-positive, endospore-forming soil bacterium. This strain was grown at 30°C for 24 hours in autoclaved Oxoid CM001 nutrient broth (13 g/L) amended with 20 g/L filter-sterilised urea (Fujita *et al.*, 2000). Bacterial pellets were harvested by centrifuging the grown cultures at 1450 RCF for 20 minutes, then the pellets were washed with phosphate-buffered saline (PBS: 8.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.6 mM NaH<sub>2</sub>PO<sub>4</sub>, 145 mM NaCl, pH 7.2). Cells were again pelleted by centrifugation prior to re-suspension in the working media defined below.

### **2.2 Tolerance of *S. pasteurii* to strontium in aqueous solution**

The minimum inhibitory concentration of strontium for *S. pasteurii* was determined. Bacteria were re-suspended in autoclaved nutrient broth (denoted medium M1, containing Oxoid CM001, 13 g/L). Six millilitres of this suspension was placed in sterile 10mL screw cap glass centrifuge tubes, and 2 mL of a sterile strontium chloride hexahydrate (SrCl<sub>2</sub>.6H<sub>2</sub>O) solution added aseptically to give a range of final metal concentrations (0, 0.1, 0.5, 1, 2, 5, 10 mM). The final bacterial optical density at 600 nm wavelength incident light (OD<sub>600</sub>) was 0.064 (equivalent to approximately 2x10<sup>6</sup> cells / mL). The pH was corrected to 6.5 in order to ensure solubility of the metal salt and each concentration was tested in triplicate. The range of strontium concentrations was specified according to preliminary tests to determine to what extent the bacteria can resist strontium. The tubes were incubated at 30°C for 72 hours, after which the OD<sub>600</sub> of the solution was measured by UV/visible spectrometry (Hitachi U1900).

### **2.3 Strontium Bioprecipitation in Aqueous Solution**

A urea-containing medium (denoted medium M2: Oxoid CM001 nutrient broth - 3 g/L; CaCl<sub>2</sub> - 50 mM; autoclaved prior to addition of 0.2µm filter-sterilised urea [to give 20 g/L], NH<sub>4</sub>Cl [to give 10 g/L] and

Na<sub>2</sub>CO<sub>3</sub> [to give 2.12 g/L] – Fujita *et al.*, 2000) was amended with strontium chloride as above to give a range of strontium concentrations (1, 3, 5, 10, 20, 30 mM). *S. pasteurii* was prepared as described above – killed-cell controls were prepared by autoclaving bacterial suspensions. Following pelletisation and washing, both live and killed cells were re-suspended in the urea/strontium media to give a final bacterial OD<sub>600</sub> of 0.111 (equivalent to approximately 5x10<sup>6</sup> cells / mL) and then 30 mL placed aseptically into 50 mL sterilised polypropylene tubes. The microcosms were incubated at 30°C for 7 days. Each concentration was again tested in triplicate. Strontium and calcium concentrations, and pH, were determined after 0, 1, 3 and 7 days. At each time a 5 mL aliquot was removed from each sample aseptically, then filtered (0.2 µm) to remove cells and any suspended precipitates. Half of the filtered sample was used to measure the pH. The rest was diluted as necessary using deionised water and analysed for strontium and calcium concentrations by ICP-OES (Optical Emission Spectrometer, Optima 2100 DV, Perkin Elmer).

## 2.4 Strontium Bioprecipitation in Soil

Leighton Buzzard sand (Hepworth Minerals and Chemicals Ltd - see Figure 1 for grain size distribution) was washed by soaking in 1M hydrochloric acid, then in 1M sodium hydroxide for 24 hours each with periodic stirring, to remove inorganic carbonate. Finally, the sand was rinsed with deionised water until it reached pH 7.0. It was then separated into three fractions by dry sieving (fine (63-300 µm), medium (300-425 µm) or coarse (600-1180 µm)). Sand fractions were autoclaved and dried at 30°C before use. Cultures of *S. pasteurii* prepared as above were suspended in medium M2 amended with 10 mM strontium. In all cases, 17 ml of the requisite medium containing suspended cells (equivalent to the pore volume) was added to sterile 50 ml polypropylene tubes prior to addition of 80g dry, autoclaved sand from individual fractions, which was wet-pluviated into the bacterial suspension to minimise air voids and encourage uniform bacterial distribution. This resulted in a final dry density of 1.65 g/cm<sup>3</sup> with final bacterial densities were calculated to be 2.55x10<sup>6</sup> cells per gram of sand. Multiple samples for each of the three sand fractions were prepared such that triplicate specimens could be destructively sampled at 3 and 7 days. Control samples were prepared in an identical fashion, but a urea-free medium (denoted M3), otherwise identical to M2, was used instead. The microcosms were then incubated at 30°C. At each sampling time, specimens were removed from the incubator and the solutions extracted under vacuum from the soil then filtered through a 0.2 µm membrane filter prior to testing of pH, strontium and calcium concentration as described above.

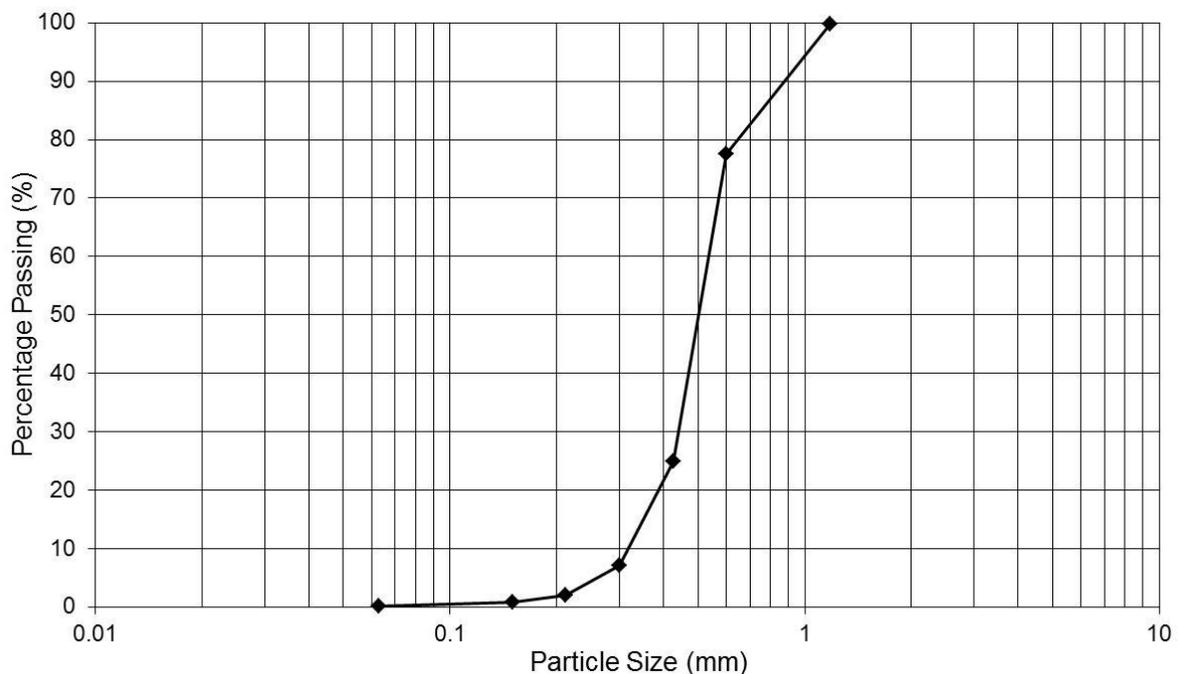


Figure 1. Particle size distribution for sand used (subsequently divided into fine, medium and coarse fractions as described).

### 3. RESULTS AND DISCUSSION

Figure 2 shows the tolerance of *S. pasteurii* to strontium in aqueous solution, presenting the OD<sub>600</sub> absorbance divided by the absorbance without strontium. Ruggiero *et al.* (2005) defined the toxic concentration of metals in solution as the concentration that inhibits the growth of a microorganism by >70% compared to controls. A concentration of 10 mM hindered growth of the bacteria (Figure 2); it is apparent that the minimum inhibitory concentration is between 5 and 10 mM.

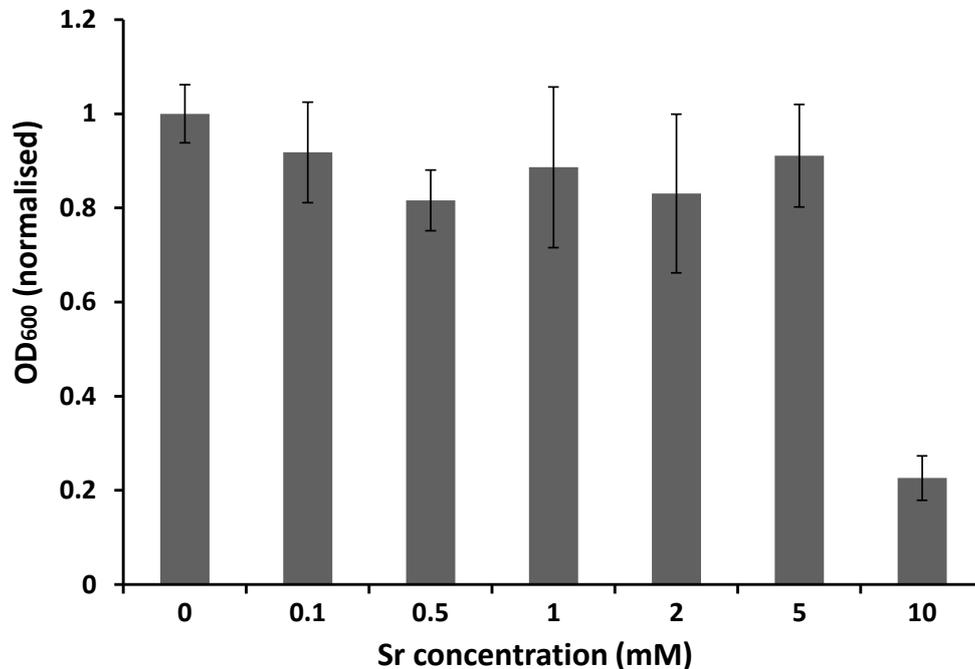


Figure 2. Response of *S. pasteurii* to a range of strontium concentrations ( $OD_{600} \text{ (normalised)} = OD_{600} \text{ at any Sr concentration} / \text{average } OD_{600} \text{ at zero Sr concentration}$ ). Error bars represent  $\pm 1SD$ ).

In the second part of this study, the ability of live *S. pasteurii* to induce calcium carbonate precipitation in the presence of a range of strontium concentrations (0-30 mM), and therefore co-precipitate strontium, was investigated. The changes in strontium and calcium concentrations, and pH, are shown in Figure 3. The pH increased over the testing period, reaching around 9.2 in all specimens by 7 days, thought to be due to the action of the *S. pasteurii* urease enzyme on urea. Calcium was rapidly removed in all specimens, with approximately 50% remaining in solution after one day and being undetectable after 3 days; this was concurrent with generation of white crystalline precipitates in each case. The decrease in concentration of calcium ions in solution was faster than that of strontium ions, with the amount lost in 24 hours greater than that of strontium by a factor of 1.45 ( $\pm 0.1$ ). Strontium was almost fully removed after 3 days, and not detectable after 7 days. In previous experiments, without strontium but otherwise identical, the majority of calcium ions were removed from solution in the first 24 hours with a pH value of up to 9.0. The delay of calcium removal in this experiment is thought to be related to the presence of strontium ions in solution. However, *S. pasteurii* cells were found to tolerate far higher Sr concentrations in this experiment with urea than in aqueous solution without it. It is therefore thought that the co-precipitation of Sr with calcium carbonate offered a mechanism of protection against metal toxicity.

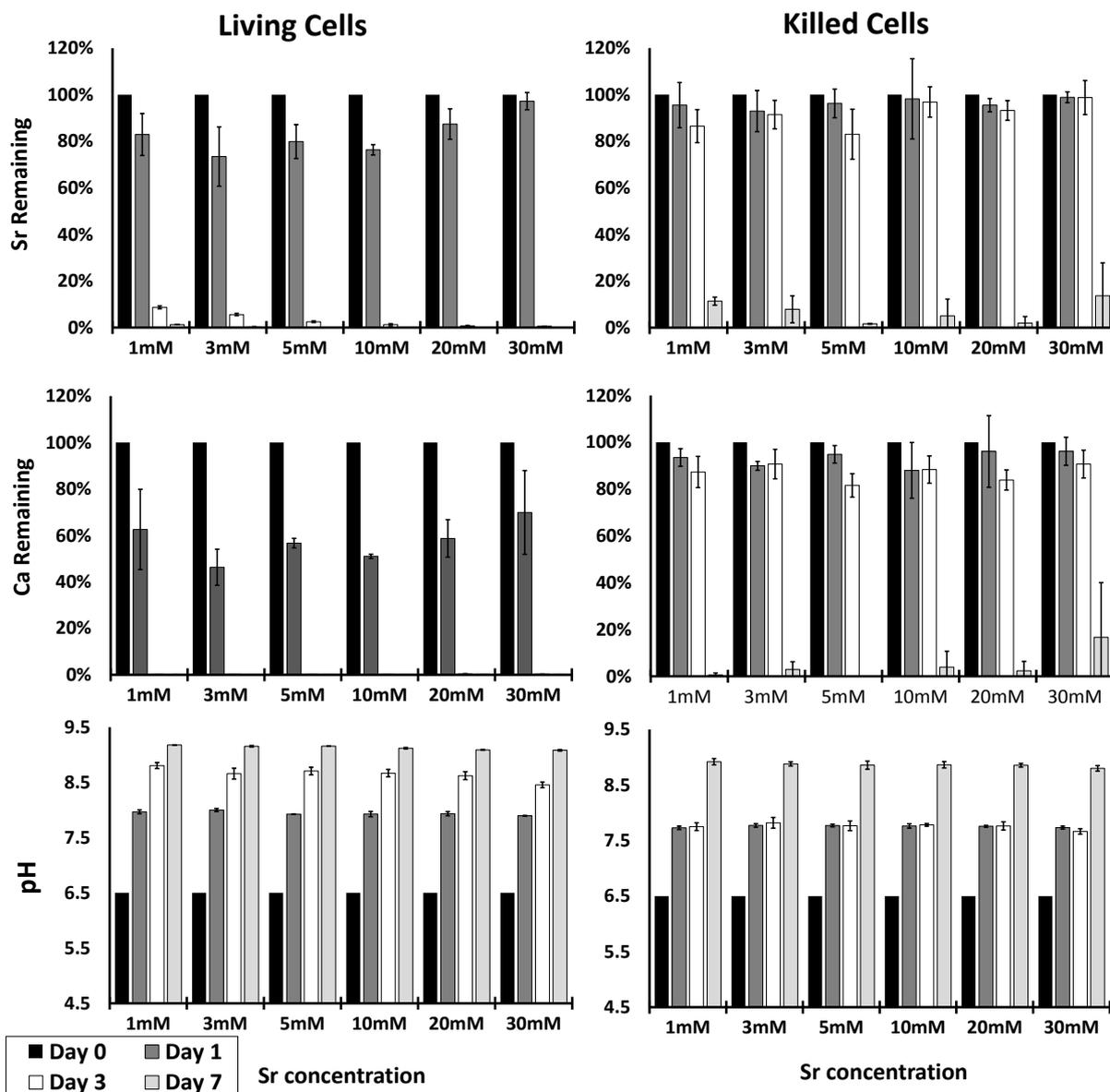


Figure 3. Response of Sr and Ca concentrations, and pH, to activity of *S. pasteurii* (error bars represent  $\pm 1SD$ ).

In killed-cell controls, pH had only slightly increased after three days and there was little loss of Ca or Sr from solution. The slight pH increase may be partially due to abiotic hydrolysis of urea in solution, which is slower than the biotic hydrolysis (United States Environmental Protection Agency, 2002), but by day 7 the pH had increased substantially – it is thought that the autoclave procedure used to sterilise samples in this part of the study was insufficient to deactivate bacterial spores, which then regenerated and had similar but delayed effects to live cells by day 7 (Stolp, 1988; Todar, 2008-12). The minimum inhibitory concentration in the presence of the urea medium has not been established, but appears to be greater than 30 mM for this system, more than three times the previous value.

Similar experimental data have been obtained from precipitation in sands (Figure 4). Without urea, there was little loss of Sr (around 20% after 3 and 7 days) whilst when urea was present removal was almost complete. Ca removal was very similar after 3 days, but reduced further to around 50% after 7 days, perhaps due to abiotic interactions with sand grains or cell surfaces. A slight increase in pH in no-urea controls may be due to chemical interaction with the sand grains. With urea present, pH increased to around 9. There was little discernible difference in pH change, or recovery of Sr or Ca, between sand grain sizes, with perhaps a small increase in Sr removal with increasingly coarse grain size. It is also shown that in particulate media, the presence of urea degradation allows survival at concentrations greater than the minimum inhibitory concentration seen in aqueous solution alone.

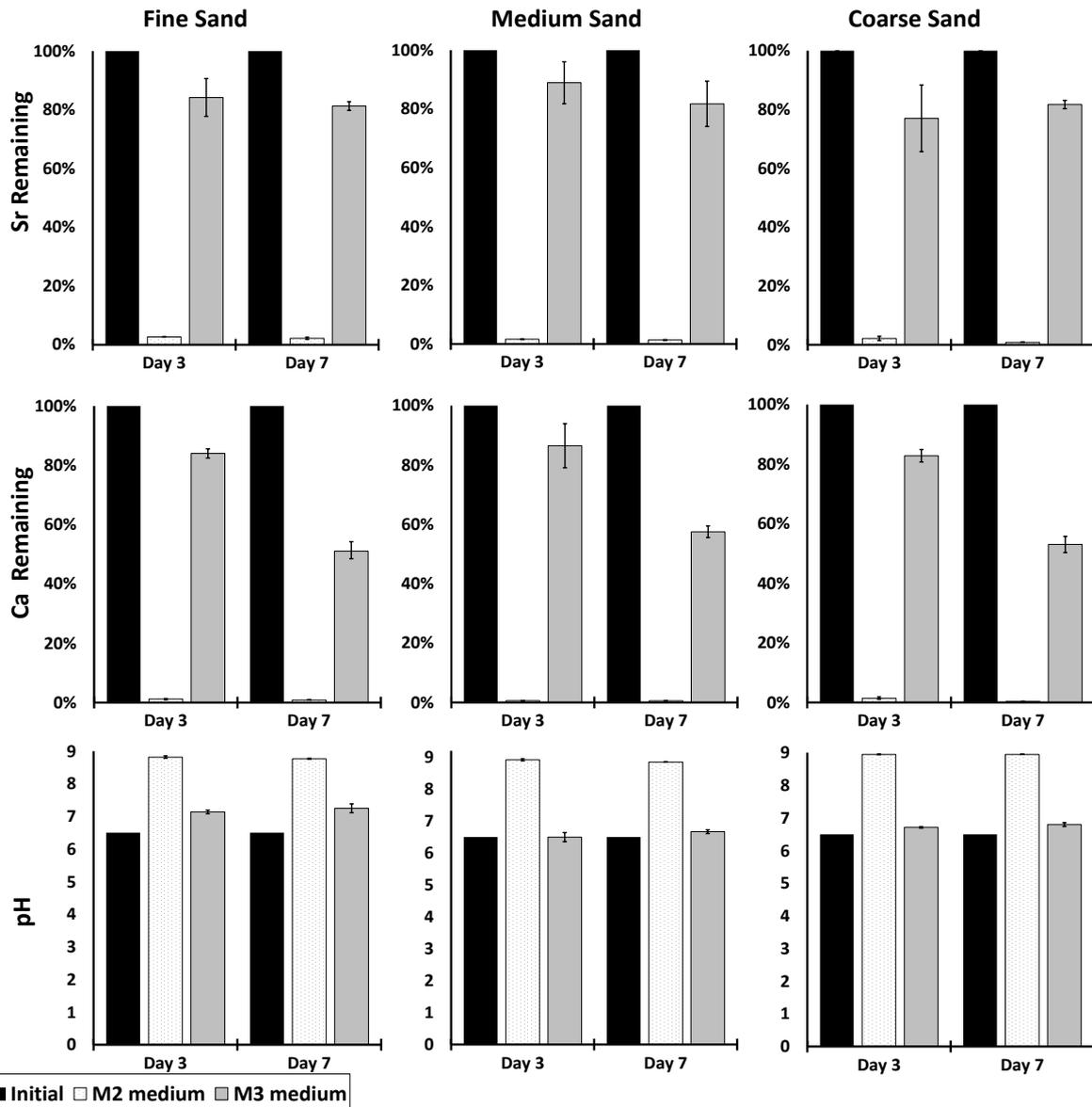


Figure 4. Response of Sr and Ca concentrations, and pH, to activity of *S. pasteurii* in soil pore fluid (error bars represent  $\pm 1SD$ )

It should be noted that these static experiments would not have been subject to the effects of heterogeneity through preferential flow or other dynamic effects, which will play a significant role in the heterogeneity of the delivery of subsurface remediation.

In the presence of urea, calcium and suitable nutrients, *S. pasteurii* will grow rapidly, altering the pH and causing calcium carbonate precipitation. However, their cell walls act as nucleation sites for calcium carbonate formation, and this in turn can lead to the bacteria becoming embedded within the solid matrix which is likely to kill cells by prevent access to nutrients and energy sources (Tobler *et al.*, 2011). However, due to the presence of a large number of cells, providing a small population remain following the precipitation of the mineral, the removal of metals from solution will enhance the environment for the remaining live cells. The technique provides a protective mechanism for the bacteria by reducing the overall metal concentrations to tolerable levels.

#### 4. CONCLUSION

The sequestering of heavy metals in the subsurface environment by bacterially induced calcium carbonate precipitation provides potential long-term removal of heavy metals in contaminated subsurface environments with simple requirements and potential for use in a wide range of

environments. It has been shown that provision of urea and other simple chemical species also offers bacteria that possess the urease enzyme a protective mechanism against metal toxicity. Urea degradation leads to an elevated pH and enhanced carbonate ion concentration in solution, making metal carbonate precipitation more likely. Initial precipitation reduces metal bioavailability, leading to enhanced bacterial growth and activity, which in turn causes further precipitation. A significantly increased minimum inhibitory concentration results, at least three times greater than the original value. A similar effect is seen in particulate media. However, no significant effect of particle size was seen, under the static flow conditions tested.

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