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Resilience of planktonic and biofilm cultures to supercritical CO_2

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ABSTRACT

Supercritical CO_2 has been shown to act as a disinfectant against microorganisms. These organisms have most often been tested in vegetative or spore form. Since biofilm organisms are typically more resilient to physical, chemical, and biological stresses than the same organisms in planktonic form, they are often considered more difficult to eradicate. It is therefore hypothesized that supercritical CO_2 (SC–CO_2) induced inactivation of biofilm organisms would be less effective than against planktonic (suspended) growth cultures of the same organism. Six-day old biofilm cultures as well as suspended planktonic cultures of *Bacillus mojavensis* were exposed to flowing SC–CO_2 at 136 atm and 35 °C for 19 min and slowly depressurized after treatment. After SC–CO_2 exposure, *B. mojavensis* samples were analyzed for total and viable cells. Suspended cultures revealed a 3 $\log_{10}$ reduction while biofilm cultures showed a 1 $\log_{10}$ reduction in viable cell numbers. These data demonstrate that biofilm cultures of *B. mojavensis* are more resilient to SC–CO_2 than suspended planktonic communities. It is hypothesized that the small reduction in the viability of biofilm microorganisms reflects the protective effects of extracellular polymeric substances (EPS) which make up the biofilm matrix, which offer mass transport resistance, a large surface area, and a number of functional groups for interaction with and immobilization of CO_2. The resistance of biofilm suggests that higher pressures, longer durations of SC–CO_2 exposure, and a quicker depressurization rate may be required to eradicate biofilms during the sterilization of heat-sensitive materials in medical and industrial applications. However, the observed resilience of biofilms to SC–CO_2 is particularly promising for the prospective application of subsurface biofilms in the subsurface geologic sequestration of CO_2.

1. Introduction

In medical practice, sterilization methods include steam, ethylene oxide, and gamma irradiation treatments. However use of these methods may not be the best practice for some implantable or electronic medical devices sensitive to water or high temperature [1]. Using pressurized CO_2 as a sterilant has several potential benefits over traditional sterilization techniques including low temperature, and being a non-toxic, chemically inert, non-flammable, dry sterilant, which induces limited polymer changes [1,2]. Carbon dioxide has a critical temperature of 31.5 °C and critical pressure of 73 atm. Supercritical CO_2 (SC–CO_2) has liquid-like density, a low viscosity ($3–7 \times 10^{-5}$ N s m^{-2}, between 73 and 148 atm, at 37 °C; [3]), a diffusivity about two orders of magnitude higher than typical liquids, and low surface tension, so it can quickly penetrate into complex cellular material.

Previous research has highlighted the use of SC–CO_2 as a disinfectant for vegetative state bacteria and fungi, as well as spores, which are a highly resistant dormant form of various bacilli and clostridia. Parameters that influence the efficacy of bacterial inactivation with SC–CO_2 are temperature, pressure, moisture content of the sample, processing time, degree of pressure cycling, and organism state [4]. Twenty-two tested vegetative species of microorganisms reported in the literature were completely deactivated at some combination of pressure and temperature in the presence of SC–CO_2 [1]. The use of SC–CO_2 in the food industry to reduce food spoilage in juice, milk and other products has been examined and promising results have been shown [5,6]. For example, a 5 $\log_{10}$ decrease in the number of viable (living) cells of *E. coli* 0157:H7, *S. typhimurium*, and *L. monocytogenes*, was achieved with high pressure CO_2 treatment in orange juice without deleterious effects to the juice [6].

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$SC–CO_2$ inactivation studies have also been performed on spore forming organisms including *Bacillus subtilis*, *Bacillus cereus*, and *Bacillus pumilus*. *B. pumilus* spores were reported to have a 0.58–3 $\log_{10}$ reduction in viable spore numbers with conditions of 50–80 °C and 271 atm over 4 h in the presence of water [7]. Other studies also reported rather little spore inactivation using $SC–CO_2$, and varying degrees of time and pressure necessary for inactivation are reported [1,8]. Because bacterial endospores are resistant to killing, due to a thick envelope and dehydrated state, combinations of high temperature, pressure, and extended treatment times are required to inactivate spores, especially to achieve a 6 $\log_{10}$ reduction in viable cells required for the United States Food and Drug Administration (FDA) approval of new sterilization technologies [1,7].

The effect of $SC–CO_2$ upon the viability of biofilms has received no attention to date. Biofilms are microorganism assemblages firmly attached to a surface, which form and are encased within self-produced extracellular polymeric substances (EPS), a hydrated matrix of mostly polysaccharides and proteins [9,10]. Biofilms are the predominant way of life for most microorganisms in the environment because matrix enclosed multi-cellular communities offer structural support, protection from physical, chemical and biological stresses, optimal location relative to substrates required for metabolic function, and symbiotic benefits in multi-species communities [9–12]. Biofilms are a common cause of infection, especially due to the adherence to implanted medical devices and because they are particularly resistant to antibiotics [13–16]. Diffusion limitations and antibiotic deactivation during penetration are possible mechanisms for the biofilm defense [16]. $SC–CO_2$ may offer a method to effectively sterilize biofilm communities in the medical and food industries although little is known about the resilience of biofilms to $SC–CO_2$, as most research into $SC–CO_2$–microbe interactions has been undertaken on suspended planktonic cultures.

$SC–CO_2$–microbe interactions are also of interest because of the proposed use of microorganisms for the subsurface geologic sequestration of CO_2. Engineered subsurface viable biofilms are being investigated as a means of plugging fractures in the $SC–CO_2$ receiving reservoirs and reducing upward leakage of $SC–CO_2$, and have been shown to cause up to a 99% reduction in permeability in a Berea Sandstone core when grown at high pressure (88 atm) and moderate temperature (≥32 °C) [17]. Controlled microbiological autotrophic consumption of CO_2 and microbially mediated precipitation of $CaCO_3$ [18] are other potential mechanisms in which microorganisms could enhance the subsurface sequestration of CO_2 [19].

In this study we examined the inactivation of *Bacillus mojavensis* biofilms and suspended planktonic growth cultures by $SC–CO_2$ under treatment conditions of 35 °C, 136 atm flowing CO_2, and a slow depressurization. Our objective was to determine how the number of total and viable cells in biofilm communities is affected by exposure to $SC–CO_2$ compared to suspended planktonic forms of the same organism. We hypothesized that EPS, which makes up the biofilm matrix, offers a protective environment for the microorganisms.

2. Materials and methods

2.1. Bacterial culturing and suspended culture growth

B. mojavensis was used as a model biofilm organism in this study. It was isolated from a Berea Sandstone core used for previous high pressure biofilm experiments at the Center for Biofilm Engineering, Montana State University (MSU), and identified using 16S rDNA sequencing [17]. When grown in a sand support matrix under low flow conditions, *B. mojavensis* forms thick biofilm cultures, which are characterized by a copious EPS matrix. *B. mojavensis* was originally isolated from desert soil, occurs singly or in short chains at a vegetative size of 2–4 μm long and 0.5–1.0 μm wide. It is a gram-positive organism, a central sporulator, and a nitrate reducer [20]. To generate a suspended culture for subsequent $SC–CO_2$ exposure, a frozen stock culture of *B. mojavensis* was warmed to room temperature and inoculated at a ratio of 1:100 into 100 mL autoclaved Brain Heart Infusion (BHI) + salt medium [18 g L^{-1} BHI (Oxoid, Lenexa, KS), 0.75 g L^{-1} NH_4Cl; 40 g L^{-1} NaCl, 3 g L^{-1} $NaNO_3$ (Fisher, Pittsburgh, PA)]. The culture was grown on a horizontal shaker at 150 rpm and incubated at 30 °C, transferred after 24 h into fresh medium at a dilution of 1:100, and incubated for another 16–18 h by which time a dense planktonic culture of approximately $3.2 \times 10^8 \pm 0.09 \times 10^8$ colony forming units per milliliter (CFU/mL) (=8.46 $\log_{10} \pm 0.23$) had developed. Aliquots (10 mL) of the culture were decanted into six 10 mL volume PEEK (polyetheretherketone) $SC–CO_2$ extractor cartridges from a SFX 220 Supercritical Fluid Extractor (ISCO, Inc., Lincoln, NE) (Section 2.3) and were capped. Each cartridge comprised of a tube with a stainless steel frit with a 0.5 μm pore size at each end, to contain the cartridge's contents while allowing $SC–CO_2$ to flow through. The same culturing conditions were used to generate an inoculum for biofilm growth (Section 2.2.).

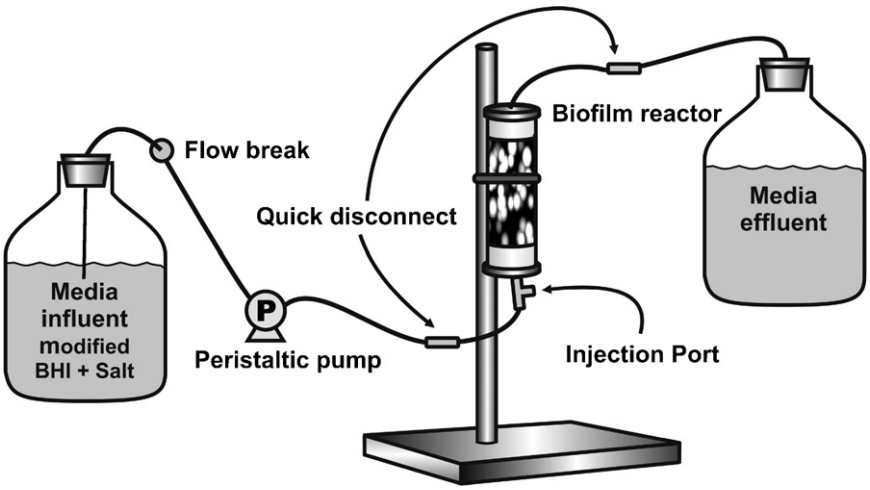

Fig. 1. Schematic of biofilm growth reactors used in this study.

2.2. Flow reactor and biofilm growth

In order to promote biofilm growth, *B. mojavensis* was grown under flow conditions in porous media columns (Fig. 1) housed in an incubator at 30 °C. Six SC–CO_2 extractor cartridges, the same as described in Section 2.1, were filled to the top with 70 g of 1–2 mm diameter sieved sand (JTL Gravel Pit, Belgrade, Montana). The sand was pre-washed with deionized water, and dried at 90 °C overnight, before being packed into the cartridges. The filled cartridges, fittings and tubing (Masterflex, Vernon Hills, IL; Peroxide-Cured Silicone, size 16) were autoclaved (20 min, 121 °C, 0.99 atm positive pressure). Media bottles (Pyrex, Lowell, MA) containing autoclaved BHI + salt medium were then attached to the flow system and medium was run through the cartridges in up flow orientation with a Masterflex peristaltic pump at 3 mL min^{-1} for 20 min, followed by 0.3 mL min^{-1} for 30 min to condition the system.

The columns were inoculated by injecting 3 mL of a culture of *B. mojavensis* (Section 2.1) after stopping media flow. The culture was injected via a port directly prior to the cartridge (Fig. 1). The culture was incubated overnight in the cartridge to allow the bacteria to attach to the sand before media flow was started at a flow rate of 0.3 mL min^{-1} and continuously run for 6 days to grow *B. mojavensis* biofilms. SC–CO_2 extractor cartridges containing the biofilm cultures were shipped overnight to Los Alamos National Lab (LANL) to undergo SC–CO_2 exposure. *B. mojavensis* suspended cultures (Section 2.1) were grown during the last 24 h of the biofilm growth period (Day 6), immediately before shipping.

2.3. Exposure to supercritical CO_2

Biofilm and suspended cultures were exposed to SC–CO_2 at the LANL Supercritical Fluids Facility using a SFX 220 Supercritical Fluid Extractor, a 260D syringe pump, and an SFX 200 Controller (ISCO, Inc., Lincoln, NE) (Fig. 2). Three biofilm replicates and three suspended growth replicates were subjected to flowing SC–CO_2 (Scott Specialty Gases, Plumsteadville, PA; 99.997% SFE Grade). Each cartridge was placed in the temperature controlled extraction unit at 35 °C, a temperature under which *B. mojavensis* is known to remain viable without any negative influence of temperature on viability [17]. The SC–CO_2 challenge was initiated with immediate pressurization to fill the cartridge with SC–CO_2, followed by a 1 min hold to stabilize the SC–CO_2 conditions at 136 atm. This pressure, which is well above the critical point for CO_2, was used to reduce the density and viscosity fluctuations that occur close to the critical point of SC–CO_2. After the 1 min hold, SC–CO_2 was allowed to flow at ~1 mL min^{-1} for 19 min. This duration was chosen to give ample time for deactivation by SC–CO_2. For example, planktonic cells of *B. subtilis*, which is a close relative of *B. mojavensis* and only distinguishable by differences in whole-cell fatty acid composition, divergence in DNA sequence, and resistance to genetic transformation between taxa, required a SC–CO_2 exposure of only 2.5 min at 7.4 MPa and 38 °C for complete deactivation of cells to occur [21–23]. At the end of the cycle the pressure was stepped down 17 atm every 15 s until 68 atm, after which depressurization to atmospheric pressure was allowed to proceed over 4 min. The effluent of each treatment from the SC–CO_2 depressurization line passed through a heated pressure restrictor capillary to avoid freezing upon depressurization. Effluent was captured in aerosol capture units, made from sterile serum bottles (Wheaton, Millville, NJ), rubber septa and crimp seals (Geo-Microbial Technologies, Ochelata, OK), and vented with 0.2 μm pore size polycarbonate syringe filters, to collect any cells which may have been purged from the reactor cartridge by SC–CO_2 through the 0.5 μm pore sized frit. The 0.5 μm pore size frits were chosen to retain most of the *B. mojavensis* cells (width ~0.5–1.0 μm) within the cartridge while allowing SC–CO_2 to flow through. Samples were then shipped back overnight to MSU for microbiological analysis.

Control samples of the biofilm and suspended cultures were generated to allow comparison with the SC–CO_2 exposed samples. A set of three biofilm and three suspended culture cartridges were shipped to LANL alongside the SC–CO_2 treatment samples. These cartridges were exposed to 35 °C, atmospheric pressure, flowing filter sterilized phosphate buffered saline (PBS) solution [NaCl = 40 g L^{-1} + KH_2PO_4 = 0.61 g L^{-1} + K_2HPO_4 = 0.96 g L^{-1} (Fisher, Pittsburgh, PA)] at the same flow rate as the SC–CO_2 (1 mL min^{-1}) to account for any hydraulic shear stress applied to the microorganism by the SC–CO_2 in the test experiments. The effluent was collected in the same sterile aerosol capture units. All samples and cartridges were then shipped back to MSU overnight for microbiological analysis.

2.4. Determining total and viable cells in biofilm and suspended cultures

Total and viable cells present in the biofilm and suspended cultures which had either been exposed to SC–CO_2 or PBS were determined approximately 12 h later at MSU, after overnight return

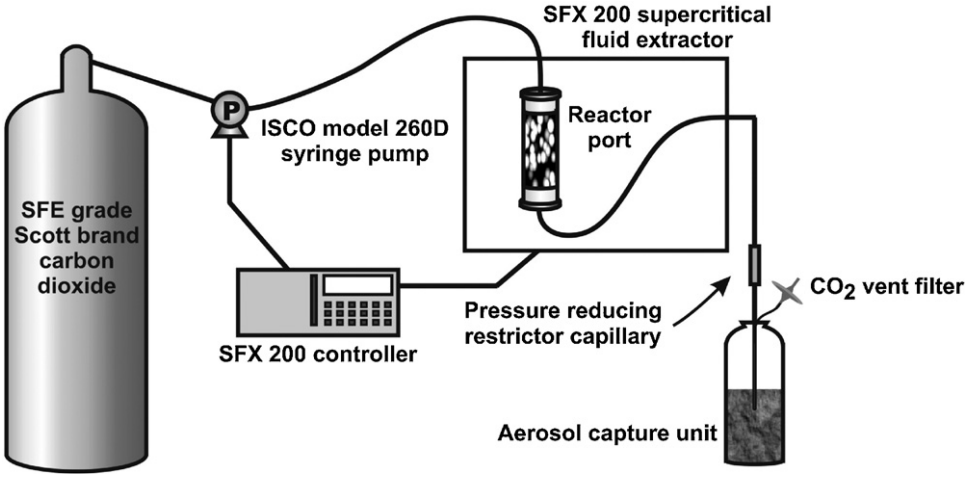

Fig. 2. SFX220 Supercritical Fluid Extractor schematic at the Los Alamos National Laboratory (LANL) Supercritical Fluids Facility.

shipping of the samples from LANL. Total cells are defined as the total number of cells present, and viable cells are those cells which are still physiologically active, and form a colony forming unit (CFU) when placed on a plate of growth medium. The extraction procedure for recovering cells from the SC–CO_2 cartridges used a modification of methods presented by Camper et al. [24]. The method was optimized to achieve maximum cell recovery and minimum difference between total and viable cell counts by replacing the Tris buffered Zwittergent 3–12, EGTA, and peptone solution described by Camper et al. [24] with PBS. The sand–biofilm matrices and the suspended cultures were transferred from the SC–CO_2 cartridges into sterile 50 mL centrifuge tubes. Cells remaining on the inside of the cartridge were recovered as completely as possible by pipetting 10 mL of PBS into the cartridge, reattaching the caps, and vortexing the cartridge. PBS addition and vortexing were repeated three times and each time the contents of the cartridges were transferred to the centrifuge tube. The biofilm samples were further processed to detach and breakup the biofilm from the sand. Each centrifuge tube containing biofilm was vortexed for 30 s, sonicated on ice for 1 min using a FS15 Fisher Scientific sonicator, and vortexed again for 30 s [24].

Viable cells were determined from the cell extracts after serial dilution in PBS solution. Sample aliquots were plated on BHI + salt agar [18 g L^{-1} BHI (Oxoid, Lenexa, KS), 0.75 g L^{-1} NH_4Cl; 40 g L^{-1} NaCl, 3 g L^{-1} $NaNO_3$ (Fisher, Pittsburgh, PA); Difco Granulated Agar (BD, Sparks, MD)] and incubated at 30 °C. Bacterial colony forming units were counted after 48 h on plates exhibiting between 30 and 300 colony forming units. Total cell counts were determined after 4′,6-diamidino-2-phenylindole [(DAPI), Roche Diagnostics, Indianapolis, IN] staining (final concentration = 0.01 mg mL^{-1}) from the appropriate serial dilution of the extracted cells, and counting of stained cells by epifluorescence microscopy using a Nikon Eclipse E800 microscope with 1000× magnification. The number of viable and total cells flushed from the SC–CO_2 cartridges during SC–CO_2 or PBS exposure and collected in the aerosol capture unit was also determined using plate and microscopic direct counts, respectively. Cell numbers are expressed as the total number of cells in each SC–CO_2 cartridge, including those flushed into the aerosol capture unit. The relative standard deviation of cell counts from replicate samples was always less than 6%.

2.5. Quantification of bacterial endospores

In order to estimate the proportion of spores in biofilm and suspended growth cultures immediately before the beginning of the SC–CO_2 challenges at LANL, triplicate cultures of suspended and biofilm cells were grown as described in Sections 2.1 and 2.2. Since all microbiological analysis had to be conducted at MSU, the cultures in the SC–CO_2 extractor cartridges were subjected to simulated overnight shipping, to simulate the conditions the treatment samples were exposed to during shipment to LANL. This was achieved by placing the samples in a cardboard box cushioned as if prepared for shipping and placing the box on a rotary shaker at 150 rpm. The box remained on the rotary shaker for four 15 min intervals. After each 15 min interval, the box was rotated four times 360 degrees and then dropped to the floor from bench top height (38 in.). The fraction of spores was determined by heating the cartridges at 80 °C for 10 min to kill vegetative cells as described by Venkateswaran et al. [25]. Total and viable cells after this treatment were determined as described above. The fraction of spores was operationally defined as colony forming units measured after the heat treatment [25].

2.6. Statistical analysis

The differences in total and viable cells between the SC–CO_2 exposed and the PBS exposed suspended and biofilm samples were determined from the $\log_{10}$ of cell numbers [26]. Such an approach distinguishes significant differences in cell numbers, and is applied in studies of cell viability reduction by antimicrobial agents and for FDA approval of sterilization techniques [27]. For the biofilm *or* the suspended cultures, the mean of the $\log_{10}$ cell numbers for the control samples ($\overline{C}$) and the mean of the $\log_{10}$ cell numbers for the SC–CO_2 test samples ($\overline{T}$) were calculated. Next, the variances of the $\log_{10}$ densities (S^2) were calculated for the PBS exposed control samples and the SC–CO_2 exposed test samples, which are needed

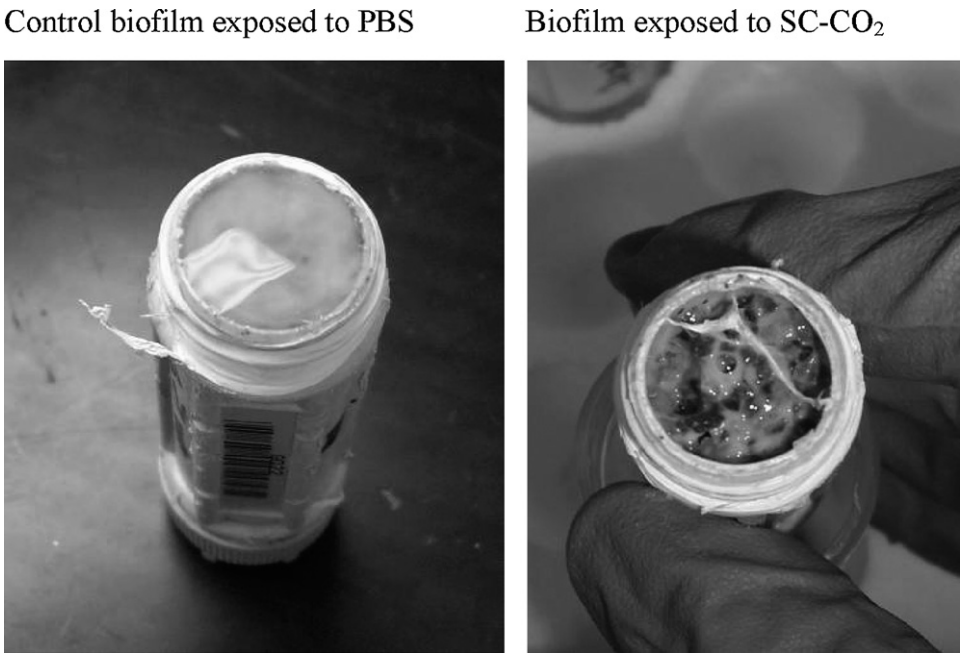

Fig. 3. Photos of SC–CO_2 extractor cartridges containing biofilm after exposure to PBS or SC–CO_2. PBS exposed biofilm remained very moist whereas the biofilm samples processed with SC–CO_2 lost a portion of their water content and became visibly desiccated.

to calculate the standard error (S.E.):

$$S^2 = \frac{(C_1 - \overline{C})^2 + (C_2 - \overline{C})^2 + (C_3 - \overline{C})^2}{N - 1}$$
$$S_T^2 = \frac{(T_1 - \overline{T})^2 + (T_2 - \overline{T})^2 + (T_3 - \overline{T})^2}{M - 1} \quad (1)$$

where C_1, C_2, and C_3 indicates the individual $\log_{10}$ of cell numbers recorded from the three individual SC–CO_2 extractor cartridges for the PBS exposed samples, with N = number of control samples (N = 3), and T_1, T_2, and T_3 indicate the individual $\log_{10}$ of cell numbers recorded from the individual SC–CO_2 extractor cartridges for the SC–CO_2 exposed samples, with M = number of test samples (M = 3).

The $\log_{10}$ reduction (LR) in cell numbers between the control and test was calculated according to:

$$\mathrm{LR} = \overline{C} - \overline{T} \quad (2)$$

The S.E. of the difference in cell numbers between the control and test samples was finally calculated according to:

$$\mathrm{S.E.} = \sqrt{\frac{S_C^2}{N} + \frac{S_T^2}{M}} \quad (3)$$

3. Results

3.1. Visual observations of suspended and biofilm cultures

Biofilm and suspended samples in the SC–CO_2 extractor cartridges which had been exposed to either PBS or SC–CO_2 were compared visually before processing for total and viable cell numbers (Fig. 3). The suspended samples which had been exposed to either PBS or SC–CO_2 both exhibited a visually indistinguishable, turbid, suspended culture. The PBS exposed biofilm samples exhibited wet mucoid biofilm, while the SC–CO_2 exposed biofilm samples, by comparison, appeared desiccated. Also, when the caps and frits were removed from the SC–CO_2 exposed cartridges, the biofilm–sand matrix expanded, and rose out of the cartridge. The suspended samples which had been exposed to either PBS or SC–CO_2 did not expand when the caps and frits were removed.

3.2. Total and viable cells in suspended and biofilm cultures

Results of the total and viable cell numbers in suspended and biofilm cultures which were exposed to either flowing PBS at 1 atm and 35 °C or SC–CO_2 at 136 atm and 35 °C are shown in Fig. 4. The total number of cells recovered from the suspended cultures was 11.3 $\log_{10}$ cells per sample for the PBS exposed cultures, and was 11.4 $\log_{10}$ cells per sample for the SC–CO_2 exposed sample. This difference of 0.1 $\log_{10}$ cells per sample was not statistically significant (S.E. = 0.19) demonstrating that cells were not being lost or lysed during SC–CO_2 exposure. However, the number of viable cells recovered from the PBS and SC–CO_2 exposed suspended cultures were significantly different. The PBS exposed suspended culture exhibited 9.0 $\log_{10}$ cells per sample, while the SC–CO_2 exposed suspended culture exhibited only 6.0 $\log_{10}$ cells per sample, a 3 $\log_{10}$ reduction in viable cells relative to the PBS control (S.E. = 0.14).

The total number of cells recovered from the biofilm cultures was 12.0 $\log_{10}$ cells per cartridge for the PBS exposed samples and 11.7 $\log_{10}$ cells per cartridge for the SC–CO_2 exposed sample. This difference of only 0.3 $\log_{10}$ cells per sample was not significant (S.E. = 0.31) demonstrating that, again, cells were not being lost or lysed during SC–CO_2 exposure. However, the number of viable cells recovered from the PBS and SC–CO_2 exposed biofilm cultures was significantly different. The PBS exposed biofilm culture exhibited 10.4 $\log_{10}$ cells per sample, while the SC–CO_2 exposed biofilm culture exhibited 9.3 $\log_{10}$ cells per sample, a 1 $\log_{10}$ reduction in viable cells relative to the PBS control (S.E. = 0.21), but far less than the reduction in viable cell numbers in the suspended cultures.

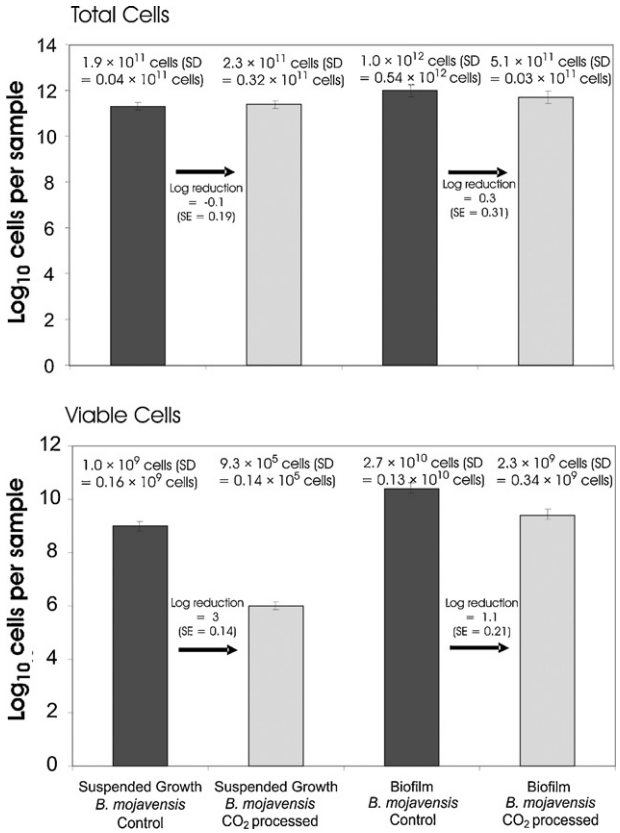

Fig. 4. Total and viable cell numbers in suspended and biofilm cultures of *B. mojavensis* when exposed to PBS (control) and SC–CO_2. Standard Deviation (S.D.) of cell numbers, and the Standard Error (S.E.) of the difference in $\log_{10}$ cell numbers between the control and test samples are also shown.

3.3. Spore numbers in suspended and biofilm cultures

The proportion of colony forming units determined from heat treated cultures, operationally defined as spores [25], was determined from triplicate samples (Fig. 5). In the suspended cultures, the number of colony forming units decreased from 6.7 $\log_{10}$ before heat treatment, to 2.5 $\log_{10}$ after heat treatments, indicating that approximately 0.006% of colony forming units in the suspended cultures were operationally defined spores. In the biofilm cultures the number of colony forming units decreased from 10.4 $\log_{10}$ before heat treatment, to 8.3 $\log_{10}$ after heat treatment, indicating that approximately 0.9% of colony forming units measured in the biofilm cultures were operationally defined spores. The total number of cells measured in these control experiments (Fig. 5) was on the same order as those in the main control and test experiments (Section 3.2; Fig. 4). These data indicate that less than 1% of the cells were present in spore form and colony forming units measured in these experiments were almost entirely due to the presence of vegetative (non-sporulated) cells. It also indicates that more than just spores survived in the SC–CO_2 exposed biofilm cartridges since a roughly two order of magnitude decrease in cell numbers would have been observed if only spores had survived. Analogously, the suspended culture spore fraction assessment indicates that a fraction of vegetative cells survived the SC–CO_2 treatment in the suspended cultures.

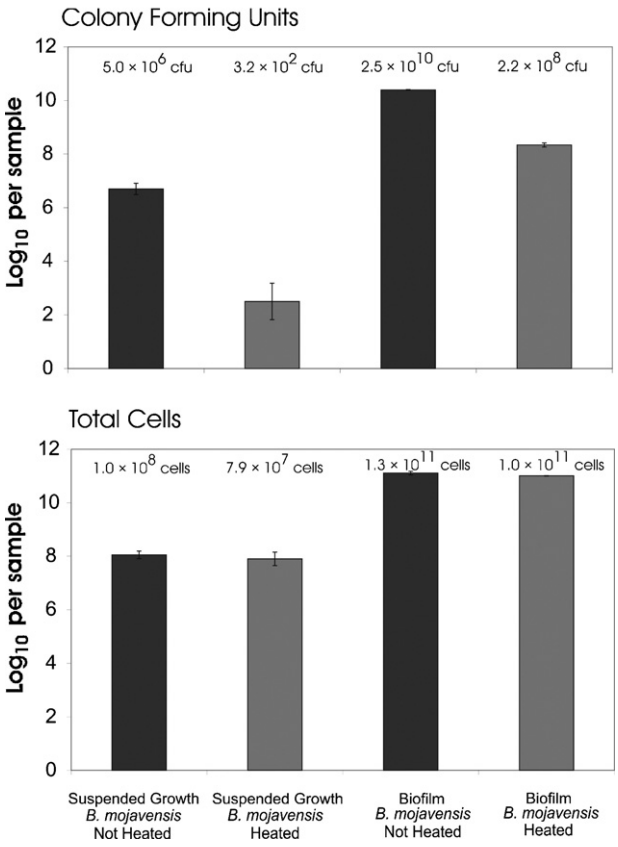

Fig. 5. Colony forming units (CFU) determined from plate counts and total number of cells from suspended and biofilm cultures of *B. mojavensis* before and after heat treatment. Colony forming units after heat treatment are operationally defined as spores [25].

4. Discussion

4.1. Reductions in cell viability due to SC–CO_2 exposure

Exposure of suspended cells of *B. mojavensis* to SC–CO_2 (136 atm, 35 °C) resulted in a far higher reduction in cell viability (3 $\log_{10}$ reduction) than for biofilm grown cells (1 $\log_{10}$ reduction in viable cells) (Fig. 4). Hence, the data presented here demonstrate that biofilms are more resilient to SC–CO_2 exposure than suspended cultures. While a 3 $\log_{10}$ reduction in cell viability is significant within the field of biomedical research and sterilization [26,27] it does not meet the target of a 6 $\log_{10}$ reduction required for FDA approved sterilization techniques.

The reduction in cell viability in suspended and biofilm cultures will be controlled by mechanical and physiological mechanisms of SC–CO_2 induced cell inactivation [1]. Physical cell rupture was the earliest proposed mechanism of deactivation during both pressurization [28] and depressurization [29,30]. However, a relatively slow depressurization rate was used during the research described here in order to reduce the suggested mechanical deactivation effect. The likely mechanisms of cell inactivation in the current study are the physiological effects of SC–CO_2, including low intracellular pH, enzyme denaturation, and CO_2 extraction of intracellular material [1]. Low intracellular pH, caused by the dissolution of CO_2 and the disassociation of carbonic acid within the cell, causes vital biological processes, such as glycolysis, to be inhibited [31], and can cause enzyme inactivation [32]. SC–CO_2 has also been shown to extract intracellular material and thus lead to cell inactivation [32], as, for example, demonstrated with the extraction of fatty acids [33]. The relative importance of cell rupture compared to physiology-induced inactivation is still poorly understood, widely debated, and is likely to be species specific [1].

4.2. Cell survival after SC–CO_2 exposure

Cell survival in both the biofilm and suspended cultures may firstly reflect an insufficient duration of SC–CO_2–microbe interaction for inactivation. Indeed, most published studies often report either a 'complete' kill of suspended cultures or at least a 5-$\log_{10}$ reduction in viable cell numbers under conditions similar to our experiments [1]. However, some suspended cultures of organisms including *B. cereus* and *Straphlococcus aureus* have shown only a 2–3 log inactivation at 34 °C, 202 atm, 0.6 h [21]. The slow depressurization cycle after SC–CO_2 treatment which we used, may also account for cell survival, allowing cells to survive better than in a previously reported experiment in which depressurization occurred in the matter of seconds to minutes [7]. Lastly, *B. mojavensis* cells may be particularly resistant to SC–CO_2. Being a gram-positive organism, the cells exhibit thick peptidoglycan layers (10–20 layers thick, as much as 90% of the cell wall) compared to gram-negative organisms, which have much thinner peptidoglycan layers (1–2 layers thick, only 10% of the cell wall) [34]. This makes gram-positive cells strong and robust, so they are less likely to be broken mechanically, and are less permeable than gram-negative cells [1,23].

The survival of spore forming bacteria, such as *B. mojavensis*, in the presence of SC–CO_2 is often contributed to the presence of endospores, which are extremely resistant to SC–CO_2 due to their thick envelope and dehydrated state. In the experiments described here, the fraction of spores was relatively low in both the biofilm and suspended cultures, although the proportion of spores was significantly higher in the biofilm (0.9%) than the suspended cultures (0.006%). However, the presence of spores alone was not sufficient to explain the apparent resilience of biofilm and suspended cells to SC–CO_2 exposure. If only spores had survived the SC–CO_2 exposure, an approximate decrease of 99% (or two orders of magnitude) and greater than 99.99% (or four orders of magnitude) of CFUs would have been observed for biofilm and suspended cultures, respectively. However, reductions of 90% (one order of magnitude) and 99.9% (three orders of magnitude) of CFUs were observed for biofilm and suspended cultures, respectively. Enhanced spore formation under high pressure has been suggested [4,8,35] and may explain some of the resilience of biofilm and suspended cells to SC–CO_2 exposure. This would require a one order of magnitude increase in spore numbers during the 19 min SC–CO_2 exposure in both the biofilm and suspended cultures. However, this phenomenon has only been observed under far higher pressures than used here (1000–6000 atm) and in the absence of SC–CO_2 [36], therefore it is hard to speculate whether spore formation during SC–CO_2 exposure was possible.

4.3. Resilience of biofilm to SC–CO_2 exposure

The greater survival in viable cell numbers in the biofilm cultures of *B. mojavensis* compared to that of the suspended culture suggests that the biofilm structure itself offers a protective barrier to SC–CO_2. The EPS matrix in biofilms has a varying composition, but predominately comprises of polysaccharides [34,37]. The interaction of pressurized CO_2 with polymers has received some attention in recent years due to the utility it may offer for modifying the physical properties of polymers, and thus for drug impregnation [38–40], pharmaceutical blending, and interfacial bonding [41,42], but there is still a major lack of knowledge of CO_2–biopolymer interactions [42]. However, general observations in this field allow

us to hypothesize the effect of SC–CO_2 upon polysaccharide dominated biofilms. SC–CO_2 is able to penetrate the polymer matrix easily, due to the low viscosity and low surface tension of SC–CO_2 [1]. Such penetration often results in swelling and plasticization of polymeric substances (increased softening and fluidity) by sorption of SC–CO_2 [42,43]. The expanding biofilm–sand matrix observed when the caps were removed from the CO_2 extractor cartridges (Fig. 3) confirms penetration and swelling of the biofilm. This effect increases with increasing pressure, but decreases with increasing temperature [42].

Biofilm communities may be protected from the degradative effects of SC–CO_2 by specific interaction between the CO_2 molecules and the biofilm matrix, resulting in immobilization or transformation. Biofilm EPS matrices are rich in carboxylic acids that contain carbonyl groups [44,45] suggesting that the carbon atom of CO_2 molecules could have a specific interaction with the electron lone pairs of the carbonyl oxygen in the biofilm EPS matrix. Specific interaction of electron lone pairs of the carbonyl oxygen with the carbon atom of the CO_2 molecule has been observed by FT-IR [46]. CO_2 molecules may also interact with other charged or uncharged functional groups. Such interaction may slow down the penetration or immobilize CO_2 molecules and reduce the inactivation effects of SC–CO_2 by inhibiting the generation of carbonic acid and the lowering of pH, enzyme denaturation, and CO_2 extraction of intracellular material [1,47]. While abundant carbonyl groups are not exclusive to EPS, and may also be found in the outer membranes of individual suspended cells, it is proposed that the EPS matrix offers a large surface area and high density of functional groups for interaction and immobilization of CO_2. Cells deep within the biofilm will be more protected from CO_2 molecules than those in the outer biofilm due to concentration gradients from the outer to inner biofilm. Indeed, the resistance of biofilms to antibiotics has been shown to reflect similar phenomena, where the slow or incomplete penetration, and the transformation or immobilization of the antibiotic protects cells deeper within the biofilm [13,14,16,48].

SC–CO_2 also appears to remove water from the biofilm, as was observed after SC–CO_2 exposure (Fig. 3). These observations are consistent with experiments in which injected SC–CO_2 desiccated a brine–rock system [49]. Desiccation of both brine and biofilm is consistent with the physical chemistry of the CO_2–H_2O system, which is known to be mutually soluble under a broad range of temperature, pressure, and salinity [50,51].

These data therefore indicate that biofilms of *B. mojavensis* are particularly resistant to the inactivation mechanisms of SC–CO_2 relative to suspended cells of the same culture. It was not possible with our experimental methods to generate the 6-$\log_{10}$ reduction required for FDA approval as a sterilization technique [1]. However, SC–CO_2 exposure for longer durations, and higher pressures may increase the reduction in cell viability, and should be investigated in future research efforts. Because of the low temperature required for supercritical conditions, this will be beneficial for the sterilization of biofilms in implantable or electronic medical devices sensitive to high temperature, and in food processing. Further studies are required to determine the resilience of biofilms to SC–CO_2 for a wide range of microorganisms.

The observed resistance of biofilm to SC–CO_2 could be particularly beneficial for the proposed use of microorganisms for the enhanced subsurface geologic sequestration of CO_2. Subsurface microorganisms are being investigated for plugging fractures in the SC–CO_2 receiving reservoirs [17], for consuming CO_2 via autotrophic carbon fixation, and for precipitating CO_2 in microbially formed $CaCO_3$ [18,19]. The data presented here suggest promoting biofilm over planktonic cell growth would be beneficial for these microbially mediated engineering approaches. Indeed, the current study demonstrates that approximately 90% of the *B. mojavensis* biofilm cells remain viable after the SC–CO_2 challenge, suggesting that the maintenance of viable subsurface biofilms in engineered subsurface geologic carbon sequestration strategies is possible.

5. Conclusions

A 19 min exposure to 35 °C, 136 atm supercritical CO_2 resulted in a 3 $\log_{10}$ reduction in viable *B. mojavensis* cell numbers of suspended cultures, but resulted in only a 1 $\log_{10}$ reduction in viable cell numbers from biofilm cultures. The presence of spores alone could not explain the difference in survival between the suspended and biofilm cultures. These data demonstrate that biofilm cultures are more resistant to the sterilization effects of SC–CO_2 than suspended planktonic cells. The biofilm–sand matrix expanded during SC–CO_2 exposure demonstrating that SC–CO_2 penetrated the biofilm matrix and caused swelling of the biofilm. It is hypothesized that the small reduction in the viability of biofilm microorganisms reflects the protective role of the biofilm EPS matrix, which offers a large surface area and high density of functional groups for slowing the penetration or immobilizing CO_2. Cells deep within the biofilm will be more protected from CO_2 molecules than those in the outer biofilm due to concentration gradients from the outer to inner biofilm. The resistance of biofilm to flowing SC–CO_2 at 136 atm and 35 °C for 19 min suggests that higher pressures, longer durations of SC–CO_2 exposure, and a quicker depressurization rate may be required to eradicate biofilms during the sterilization of heat-sensitive materials in medical and industrial applications. However, the observed resilience of biofilms to SC–CO_2 is particularly promising for the prospective application of subsurface biofilms in the subsurface geologic sequestration of CO_2. Further studies are required to investigate the mechanisms of biofilm resistance to SC–CO_2, particularly the interaction of CO_2 and EPS for a range of microorganisms.

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