

Department of Civil and Environmental Engineering

University of Strathclyde

The Potential for Microbially Induced Carbonate Precipitation to Support Wellbore Sealing and Zonal Isolation in the Hydrocarbon Industry



by

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requirement for the degree of

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Declaration

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A handwritten signature in blue ink, reading "Fabian Steinacher". The signature is fluid and cursive, with the first name "Fabian" and last name "Steinacher" clearly distinguishable.

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Date:

28th of June 2021

To Martina

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Abstract

Today many countries have set ambitious climate goals towards a carbon net-zero future to reduce the impact of climate change on society and our planet. Global targets in greenhouse gas emissions have led to new innovation in research and practice in the fields of civil and geotechnical engineering developing alternative low carbon technologies that are resilient to climate change. This PhD thesis explores the potential for deployment of one such technology in the field of petroleum engineering. The PhD explores the low carbon biomineral sealing technology, Microbially induced carbonate precipitation (MICP) which uses the bacteria *Sporosarcina pasteurii* to precipitate calcium carbonate and evaluates its potential for sealing wellbores in the hydrocarbon industry.

Three experimental campaigns were conducted in this thesis. In the first, the potential for bacteria growth using an alternative yeast-based nutrient media was explored. *S. pasteurii* is commonly grown using brain heart infusion which is an expensive beef product with a high carbon footprint. The second campaign explored the effect of increased temperature and pressure on bacteria ureolysis. To apply MICP in the oil industry, the bacteria must be able to withstand elevated temperature and pressure combinations found at-depth. A small number of experiments to determine the potential for thermal ureolysis at much higher temperatures were also conducted. Finally, in the third campaign, experiments were conducted to seal sandstone well-cores at three temperature and pressure combinations found in the North Sea.

The experimental results presented in this thesis demonstrate the following key points:

(1) *S. Pasteurii* have the potential to grow in alternative low-cost, low-carbon, yeast-based waste-stream products (2) pressure alone has no significant impact on ureolytic activity (3) temperature does affect ureolysis. The optimal temperature for high and sustained ureolytic activity was around $T = 60^{\circ}\text{C}$ with ureolytic activities around 13 mM urea hydrolysed/min (4) at 70°C and above MICP is not applicable (5) thermally decomposed urea in a pilot experiment showed visible thermal degradation of urea above $T = 120^{\circ}\text{C}$, hence, for high temperatures thermally induced carbonate precipitation might be a feasible alternative (6) bio-grouting of heterogenous sandstone well-cores under *in-situ* wellbore pressure and temperature conditions could establish a CaCO_3 biomineral seal with permeability reductions of 5 to 6 orders of magnitude - reaching a minimum value of $k = 4.0 \text{ E-18 m}^2 (4 \text{ }\mu\text{D})$ after 18 MICP treatment

cycles (7) two fundamental nucleation processes in the formation of CaCO_3 biominerals were identified; random nucleation followed by chaotic growth and highly ordered spherulitic growth.

The results from this thesis provide new insights into the potential for MICP as a novel sealing technology within the hydrocarbon industry. Results show that MICP could be successfully deployed at depths up to ~1120 m (depending on the local geothermal gradient). MICP it is far more penetrable than traditional cements, as a result it may prove highly valuable for sealing well cement sheaths, fractured caprocks and as an additional wellbore barrier element (WBE) to seal production and stimulation horizons prior to plugging and abandonment.

List of Publications

Fabian Steinacher, Gráinne El Mountassir and Rebecca J. Lunn (2018) Bio-grout as a potential sealing technology for oil & gas wellbores: A new alternative for hydrocarbon leakage mitigation; Book of Abstracts, Interpore 10th Annual Meeting and Jubilee, New Orleans, United States; 14-17th May, 2018.

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Contents

Declaration	iii
Acknowledgements.....	v
Abstract.....	ix
List of Publications	xi
1 Introduction.....	1
1.1 Background	1
1.2 Research objectives	4
1.3 Thesis outline.....	5
2 Ureolytic carbonate precipitation: the process, influencing factors & potential for subsurface applications.....	6
2.1 Introduction	6
2.2 Naturally occurring carbonate precipitation	7
2.2.1 Abiotic carbonate precipitation	7
2.2.2 Biotic carbonate precipitation	7
2.3 Microbially induced carbonate precipitation via ureolysis.....	10
2.3.1 Ureolytic bacteria	11
2.3.2 Bacterial growth	11
2.3.3 Ureolytic activity.....	12
2.3.4 Enzyme kinetics.....	13
2.3.5 CaCO ₃ saturation.....	15
2.3.6 Nucleation processes and theory.....	16
2.4 Surface/ near-surface applications of MICP	18
2.5 Potential for use of MICP in hydrocarbon context.....	19
2.5.1 Provision of bacteria.....	19

2.5.2	Permeability reduction in unconsolidated soil systems	20
2.5.3	Permeability reduction in porous rock.....	20
2.5.4	Permeability reduction in fractured rock.....	21
2.5.5	Permeability reduction in cementitious material.....	22
2.5.6	MICP field trials for fracture sealing or in hydrocarbon wells	23
2.5.7	Influence of downhole conditions (elevated pressure/elevated temperature) ...	24
2.5.8	Thermally induced carbonate precipitation (TICP).....	26
2.6	Conclusion.....	26
3	Newly developed alternative low-cost & low-carbon feedstock for upscaling the growth of <i>Sporosarcina pasteurii</i> for field-scale applications	28
3.1	Abstract	28
3.2	Introduction	29
3.3	Experimental Methodology	31
3.3.1	Experiment 1: Growth media screening.....	31
3.3.2	Experiment 2: Generation of growth curves.....	33
3.3.3	Experiment 3: Enzyme kinetics	34
3.3.4	Determination of mass of carbonates precipitate	36
3.3.5	Characterisation of calcium carbonate biominerals via XRD.....	36
3.4	Experimental Results.....	37
3.4.1	Experiment 1: Growth media screening.....	37
3.4.2	Experiment 2: Growth curves of <i>S. pasteurii</i> via plate counting	38
3.4.3	Experiment 3: Investigation of the enzyme kinetics	38
3.4.4	Comparison of precipitated carbonate biominerals.....	40
3.4.5	Characterisation of calcium carbonate biominerals	41
3.5	Discussion.....	44
3.6	Conclusions	47

4	Investigation of the effect of temperature and pressure on whole <i>Sporosarcina pasteurii</i> cells and their implications on ureolytic activity.....	49
4.1	Abstract	49
4.2	Introduction	50
4.2.1	Effect of temperature and pressure on the urease enzyme	50
4.2.2	Thermally induced ureolysis	52
4.3	Materials and Methods	53
4.3.1	Bacteria, enzyme, and cultivation	53
4.3.2	Measurement of ureolytic activity	53
4.3.3	Temperature exposure experiments	54
4.3.3.1	Constant temperature.....	54
4.3.3.2	Ramping temperature.....	54
4.3.4	Pressure exposure experiments	56
4.3.4.1	Constant pressure.....	56
4.3.4.2	Ramping pressure	56
4.3.5	Combined pressure and temperature exposure experiment	56
4.3.5.1	Experimental setup of high-pressure and temperature core-holder	56
4.3.5.2	Combined pressure and temperature exposure experiment	58
4.4	Experimental Results	59
4.4.1	Temperature exposure experiments	59
4.4.1.1	Bacterial suspension exposure to constant temperatures	59
4.4.1.2	Plant-based urease exposure to constant temperatures.....	60
4.4.1.3	Bacterial suspension exposure to ramping temperature	61
4.4.2	Pressure exposure experiments	62
4.4.2.1	Bacterial suspension exposure to constant elevated pressures.....	62
4.4.2.2	Bacterial suspension exposure to ramped fluid pressure	63
4.4.3	Combined pressure and temperature exposure experiment	64
4.4.3.1	Bacterial suspension exposure to combined pressure & temperature ..	64
4.5	Discussion.....	66
4.5.1	Influence of temperature.....	66
4.5.2	Influence of pressure	66
4.5.3	Influence of combined pressure and temperature	67

Investigations beyond urease enzyme inactivation:.....	67
Thermally induced ureolysis	67
4.6 Materials and Methods	67
4.6.1 Reaction media and high-pressure and high-temperature vessel.....	67
4.6.2 Temperature exposure.....	68
4.6.3 Method development for the detection of urea via HPLC analysis.....	69
4.6.3.1 Method 1.....	69
4.7 Experimental Results.....	70
4.7.1 Thermal ureolysis for selected temperature range.....	70
4.8 Discussion.....	74
4.8.1 Thermally induced ureolysis.....	74
4.8.2 Treatment strategies for TICP.....	74
4.9 Conclusions	74
5 Mechanistic insights into the formation of CaCO₃ biominerals under <i>in-situ</i> wellbore pressure and temperature conditions.....	76
5.1 Abstract	76
5.2 Introduction	77
5.3 Materials and Methods	79
5.3.1 Sandstone well-core selection.....	79
5.3.2 Experimental setup.....	82
5.3.2.1 Bio-grout experimental setup of HPHT core-holder.....	82
5.3.3 Bacterial strain and cultivation.....	84
5.3.4 Cementation solution.....	85
5.3.5 Permeability measurement	86
5.3.6 Effluent sampling and biomineral characterisation	87
5.3.7 Micro xCT analysis of CaCO ₃ distribution in the sandstone cores	88
5.3.8 Scanning Electron Microscopy on polished blocks.....	89
5.3.8.1 Sample preparation for Scanning Electron Microscopy (SEM)	89
5.3.8.2 Tungsten Filament Scanning Electron Microscopy (W-SEM)	90

5.3.9 High resolution Scanning Electron Microscopy of biominerals.....	90
5.3.9.1 Cryo-Focused Ion Beam/Field Emission Gun-Scanning Electron Microscopy	90
5.3.9.2 Sample preparation for cryo-FIB/FEG-SEM	90
5.3.9.3 Sample preparation for cryo-fracturing	91
5.3.9.4 Cryo-FIB/FEG-SEM setup	91
5.4 Experimental Results.....	91
5.4.1 Permeability and porosity reduction under changing p, T conditions	91
5.4.2 Effluent biomineral characterisation.....	96
5.4.3 Micro xCT analyses of CaCO ₃ distribution in sandstone cores.....	100
5.4.4 Tungsten Filament Scanning Electron Microscopy (W-SEM)	104
5.4.5 Cryo-FIB/FEG-SEM	107
5.4.5.1 Surface and solution nucleation processes	107
5.5 Discussion.....	111
5.5.1 Reduction of permeability	111
5.5.2 Spatial distribution of calcium carbonate.....	112
5.5.3 Biomineral attachment and mineral growth.....	113
5.5.4 Calcium carbonate crystal polymorphism/pseudomorphism	113
5.6 Conclusions	115
6 Conclusions and Recommendations.....	116
6.1 Conclusions	116
6.1.1 Optimisation of the growth performance of <i>S. pasteurii</i> with a new low- cost/low-carbon nutrient media	117
6.1.2 Contributions to existing knowledge	118
6.1.3 Investigation of the effect of temperature and pressure on whole <i>S. pasteurii</i> cells and their implications on metabolic activity.....	118
6.1.4 Contributions to existing knowledge	119
6.1.5 Mechanistic insights into the formation of biominerals <i>under in-situ</i> wellbore pressure and temperature conditions.....	120
6.1.6 Contributions to existing knowledge	121
6.2 Implications for the oil industry	122
6.3 Recommendations for future work.....	127

Bibliography	129
List of Tables.....	145
List of Figures	147
Appendix A: Supplementary information to Chapter 3	154
Appendix B: Supplementary information to Chapter 4.....	157
Appendix C: Supplementary information to Chapter 5.....	158

Chapter 1

Introduction

1.1 Background

The United Kingdom Continental Shelf (UKCS) and the Norwegian Continental Shelf (NCS) have been providing Europe with energy security for well over 50 years and currently produce approximately 3.49 million barrels of oil equivalent a day to meet the power demand of the continent’s population and industry (UK Oil and Gas Authority, 2020; Norwegian Petroleum Directorate, 2021). Many oil & gas assets currently in production, are reaching their economic limit and need to be decommissioned. It is forecasted that over the next decade more than >1600 hydrocarbon wells will be decommissioned in the UK, with associated costs of up to £15.1 billion (Oil & Gas UK, 2020). Globally, decommissioning activities are estimated to cost £67 billion over the period 2019-2029 (Oil & Gas UK, 2019).

Operators have a responsibility to prevent the migration of hydrocarbon fluids between formations, and ultimately ensure the isolation of former producing zones from the surface or seabed. The quality of a wellbore, and reducing the risk it poses to the environment, is described as maintaining well integrity. This is explained in the Norwegian industry-standard NORSOK D-010 Rev. 5 as “*the application of technical, operational and organisational solutions to reduce the risk of uncontrolled release of formation fluids throughout the life cycle of a well*” (NORSOK Standard D-010 Rev.5, 2020). Well-operators in the UK have a legal duty to prevent any “*unplanned escapes of fluids from the well*” (The Offshore Installations and Wells (Design and Construction, etc.) Regulations 1996). This duty spans all stages of a well’s lifecycle from construction and operation through to abandonment and beyond. Due to the age of infrastructure on the UKCS and NCS, many wells pose a high risk of uncontrolled discharge of

hydrocarbons to the environment; fifty years ago, well construction standards were much lower than today.

Current information on the well integrity status of offshore wellbores is sparse since incidents are often unreported or not publicly disclosed. The Norwegian Petroleum Safety Authority (PSA) conducted a study on the Norwegian Continental Shelf (NCS) in 2008. This study revealed that 18%, 75 out of 406 wells, had some form of well integrity weakness or uncertainty (Vignes and Aadnøy, 2010). A subsequent examination of >1700 wellbore barriers (cement and tubings) found failures in ~25% of wells (Vignes and Aadnøy, 2010). An earlier study on the UK Continental Shelf (UKCS) found well shut-down rates of more than 10% (Davies *et al.*, 2014) due to ‘structural integrity issues’ in the well construction.

Failures in well integrity are often associated with the integrity of the cement bond used to seal the borehole annulus. A typical hydrocarbon wellbore consists of multiple steel casing strings with a variety of diameters starting from the largest, the conductor pipe with ~20-inch diameter, and ending with the smallest, usually a 9 5/8 inch in diameter standard casing string. In each borehole section (surface, intermediate through to production) a casing will be set in the drilled borehole and cemented by displacing cement from the surface to the bottom of the well, via the inner casing, which is eventually pushed into the annulus (the gap between the casing and the rock). Return flow of cement via the annulus indicates the annular gap is filled with cement (Dusseault, Gray and Nawrocki, 2000). The purpose of the cement is to create a bond between the casing and the formation and provide zonal isolation (prevent vertical fluid migration).

Inadequate well construction, as well as shear displacement on geological features like faults/fractures, can result in poor cement bonds that lead to well-bore leakage (Dusseault, Gray and Nawrocki, 2000; Watson and Bachu, 2009; Van Der Tuuk Opedal *et al.*, 2014). Inadequate construction includes: formation damage (caving), casing centralisation, no adequate drilling mud removal (Van Der Tuuk Opedal *et al.*, 2014), incomplete cement placement (pockets), inadequate steel – cement – formation bonding, excessive free water, cement sheath shrinkage and finally contamination of cement by mud or formation fluids (Kosinowski and Teodoriu, 2012). During operation, events like hydraulic fracturing, well testing, enhanced oil recovery, depletion, and workover can challenge well integrity by cracking the cement-casing/cement-rock interface or fracturing the cement (Kosinowski and Teodoriu, 2012) (Fig. 1.1). Last but not least, damage can also occur from CO₂ injection which chemically

reacts with the cement through carbonation and from sulfate or acid attack, depending on the injected media composition.

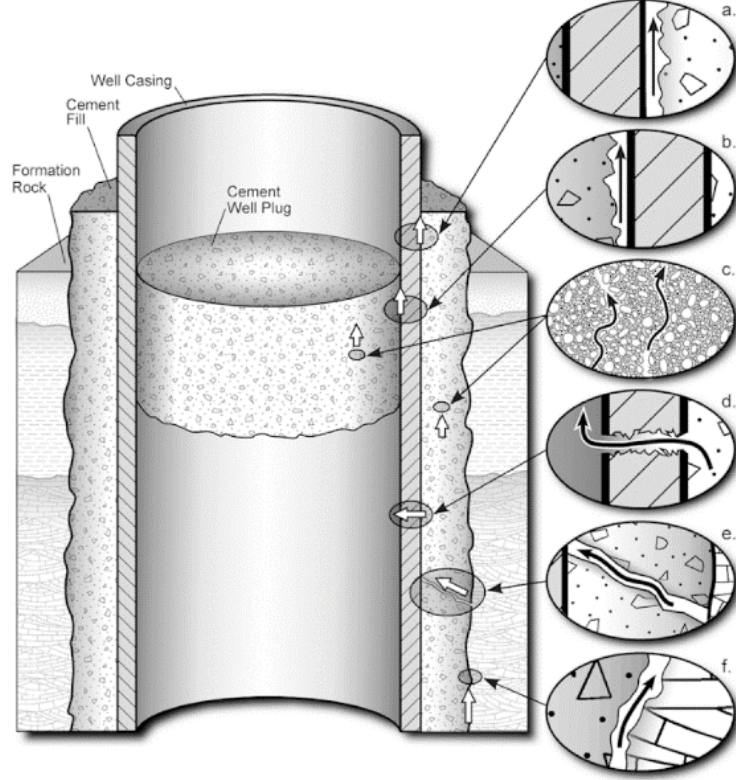


Figure 1.1: Multiple routes for fluid migration from a cemented wellbore. Starting at the top with (a) between the cement – casing interface; (b) the cement plug and casing or production tubing; (c) in the cement matrix of the well plug; (d) through casing, tubing corrosion; (e) compromised or inadequate cement sheath; and (f) at the cement – geological formation interface. Figure reprinted from Celia *et al.* (2005).

Fluid migration pathways in the annular space of the wellbore are hard to detect, costly to repair and often need sophisticated equipment to fix. Commonly repair materials are cement or resins (Todorovic *et al.*, 2016). The penetrability of cements into fine cracks is limited by the particulate size. If there are larger defects in the cement sheath present, a so-called squeeze job is needed (Rike and Rike, 1982). Depending on the size of the defect, high pressures squeeze the cement which can lead to fracturing of the damaged area, rather than sealing.

In addition to migration pathways within the cement annulus, and at the cement/steel, steel/cement or cement/rock interfaces, leakage pathways also have the potential to form within the cement matrix in the well plug itself, as well as due to corrosion of the casing (Celia *et al.*, 2005). In order to reduce the potential for fluid migration along the leakage pathways outlined,

one potential strategy to achieve zonal isolation may also include reducing the permeability of the former production zone (e.g., sandstone formation) in the region close to the well.

The research presented in this PhD thesis investigates the application of microbially induced carbonate precipitation (MICP) for sealing fluid migration pathways in and around the wellbore. The research was partially funded by the Oil & Gas Technology Centre (OGTC). This thesis investigates the application of MICP technology in a high-pressure, high-temperature environment to determine the conditions under which MICP could be deployed in an oil and gas context.

1.2 Research objectives

The specific research objectives of this thesis were to:

1. Optimise the growth performance of *S. pasteurii* with a new low-cost/low-carbon nutrient media and assess performance based on biomass and enzyme kinetics.
2. Investigate the influence of temperature and pressure on the ureolytic activity of *S. pasteurii* and hence determine the depth range over which MICP might be applicable for wellbore sealing.
3. Investigate the sealing potential of MICP over a range of pressure and temperature combinations consistent with subsurface conditions in the North Sea.
4. Investigate the influence of temperature and pressure on the mineralogy and morphology of carbonate crystals produced via MICP and determine how these may influence the success of the biogrout as a future sealing technology.
5. Outline potential application of microbially induced carbonate precipitation for establishing environmental barriers in subsurface applications based on the results from MICP sealing ability under simulated depth conditions.

This dissertation presents experimental research at laboratory scale and proof of concept studies simulating downhole conditions of a North Sea wellbore and investigates the conditions under which MICP could be deployed in the oil and gas sector to prevent hydrocarbon leakage.

1.3 Thesis outline

This dissertation is organised in 6 chapters:

Chapter 2 is a literature review on ureolytic carbonate precipitation: the process, influencing factors & potential for subsurface applications.

Chapter 3 is organised in the form of a paper manuscript: *Newly developed alternative low-cost & low-carbon feedstock for upscaling the growth of Sporosarcina pasteurii for field-scale applications*: It investigates growth performance and enzyme kinetics for a range of different growth media . Furthermore, it compares the costs and environmental footprint of MICP treatment vs traditional cementitious grouting.

Chapter 4 is organised as a paper manuscript: *Investigation of the effect of temperature and pressure on whole S. pasteurii cells and their implications on ureolytic activity*: it presents experiments on the performance of the urease enzyme with increasing temperatures and pressures. It also explores application of thermally induced carbonate precipitation for very high temperature subsurface environments.

Chapter 5 is organised as a paper manuscript: *Mechanistic insights into the formation of carbonate biominerals under in-situ wellbore pressure and temperature conditions*: this paper investigates the potential of MICP to seal sandstone reservoirs at selected pressure and temperature conditions based on the results from *Chapter 4*.

Chapter 6 summarises the results from previous *Chapters* and discusses implications of the research findings for application in the oil & gas industry.

Chapter 2

Ureolytic carbonate precipitation: the process, influencing factors & potential for subsurface applications

2.1 Introduction

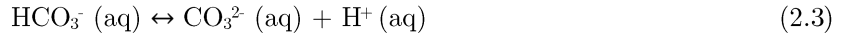
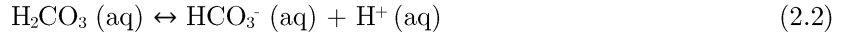
Bacteria are incredibly diverse in the Earth's biosphere and have adapted to survive in almost every possible environment, so long as they have sufficient access to water. Bacteria can be found in abundance in environments as extreme as geothermally active areas with temperatures close to 100°C (Stetter, 1996), deep sea environments with pressures beyond 100 MPa and in very acidic or high salinity environments (Kleinmann, Crerar and Pacelli, 1981; Antón *et al.*, 2002). Microorganisms play an important role in many geochemical processes on the Earth's surface. For example, in the weathering process of rocks due to organic acid excretion or biogeochemical cycling through cell metabolic activity (i.e. reduction of iron and sulfate)(Nealson, 1997; Lin *et al.*, 2018). One of the most fascinating processes is the ability of bacterial surfaces to act as reactive interfaces for ion exchange within a microenvironment and for mineral precipitation (Lowenstam, 1981; Beveridge and Fyfe, 1985). This ability of bacteria to act as surfaces for mineral precipitation under certain environmental conditions is known as biomineralisation (Lowenstam, 1981). This review presents first a discussion of naturally occurring carbonate biomineralisation, followed by the process of microbially induced carbonate precipitation (MICP) via ureolysis and a description of the various factors which can influence precipitation. Next, a brief overview of the range of surface engineering applications that the biotechnology MICP has been investigated for to date.

Finally, previous work which indicates the potential for MICP to be deployed within a hydrocarbon context i.e., to contain and prevent hydrocarbon contamination from former producing zones reaching the biosphere is also presented.

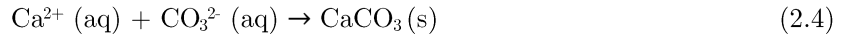
2.2 Naturally occurring carbonate precipitation

2.2.1 Abiotic carbonate precipitation

Carbonates are one of the most commonly occurring minerals on the surface of the Earth. Calcium carbonate dissolution in natural environments occurs when atmospheric CO_2 dissolves in water facilitating the production of carbonate ions (Eqs. 2.1-2.3) (Appelo and Postma, 2005; Forbes, Radha and Navrotsky, 2011). At surface or near surface conditions calcium carbonate is water insoluble unless carbon dioxide is present and carbonic acid is formed, as in Eq. (2.1). Hydrogen ions (H^+) dissociate from carbonic acid forming bicarbonate ions Eq. (2.2) and subsequently carbonate ions Eq. (2.3).



These carbonate ions in solution can then react with calcium ions present to form calcium carbonate, which may precipitate out of solution, depending on pH and temperature conditions:



In nature, calcium carbonate crystal growth occurs in four different crystal polymorphs, where the first is a carbonate precursor: amorphous calcium carbonate (ACC), vaterite, aragonite and calcite.

2.2.2 Biotic carbonate precipitation

The precipitation of calcium carbonates in nature can be an entirely abiotic process as described in Eqs. (2.1-2.4) alternatively the process may be “controlled”, “influenced” or “induced” by microorganisms.

Biologically-controlled mineralisation occurs where microorganisms, including algae or protozoa intracellularly produce CaCO_3 which is then transported to the cell surface to support the structure of the organism (e.g., calcite coccolith formation) (Westbroek *et al.*, 1986; Gadd, 2010). These microorganisms produce an organic tissue that forms a framework into which ions of the microenvironment are actively introduced. With the result that the microorganisms exert a high degree of control over mineral growth and crystallography (Lowenstam, 1981).

Biologically-influenced mineralisation is a passive process where mineral precipitation occurs due to the presence of microbial cell surfaces or extracellular polymeric substances (EPS) produced (Dupraz and Visscher, 2005; Decho, 2010; Benzerara *et al.*, 2011).

In contrast in *biologically-induced mineralisation* (Lowenstam, 1981) the microorganisms contribute to changing the local geochemistry of the microenvironment which triggers the mineralisation process. Although, the microorganism involved in the reaction has little to no control over the crystallography and architecture, (unlike in biologically-controlled systems) (Lowenstam, 1981). One manner in which biologically-induced mineralisation can occur is via enzymes produced by microorganisms; in the case of ureolytic microorganisms, it is the urease enzyme which can act as a catalyst for carbonate precipitation (Mobley and Hausinger, 1989; Hammes *et al.*, 2003).

One of the main differences between abiotic and biotic calcium carbonate precipitation is that in the case of abiotic precipitation the reaction may be very slow whereas for biologically-induced mineralisation with microorganisms that are capable of producing certain enzymes through metabolic activity, the reaction rates are faster since the enzyme is acting as the reaction catalyst, but also because of the increase in alkalinity induced by their metabolic activity (urea hydrolysis) (Hammes *et al.*, 2003; Chahal, Rajor and Siddique, 2011). Microbial metabolisms in biofilms and microbial mats and their associated microenvironments are the so-called “alkalinity engine” and affect the calcium saturation index (Dupraz and Visscher, 2005). The chemical and mineralogical composition of carbonate precipitates produced by microorganisms is very diverse and depends on the community species and environmental conditions present (Dupraz and Visscher, 2005).

Indeed, in nature many carbonate deposits are of biogenic origin (Lowenstam, 1981), occurring in all environments terrestrial, marine, lacustrine, fluvial and desert and on a massive scale.

The white cliffs of Dover on the Southcoast of England are an example of massive carbonate deposits; a significant proportion of these carbonates are of biogenic origin (Fig. 2.1) (Gadd, 2010).



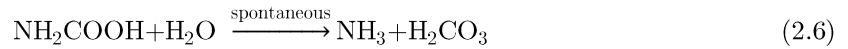
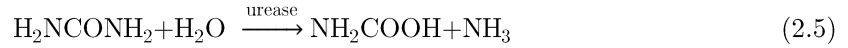
Figure 2.1: The white cliffs of Dover (UK), one of the biggest biogenic carbonate deposits in the form of chalk (Photograph taken by author)

There is evidence that biotic carbonate precipitation can and does occur naturally at depth in rock formations, for example: Pedersen *et al.* (1997), based on stable isotope analysis, presented evidence for the biogenic origin of calcite within a granodiorite fracture sampled from 207 m depth. However microbial abundance, diversity and activity decreases with depth due to limited oxygen and nutrient availability. This thesis focuses on microbially induced carbonate precipitation via injected ureolytic microorganisms (i.e., bioaugmentation) as a means of engineering the biomineralisation process to occur in a much shorter time frame compared to natural biotic mineralisation. This thesis investigates the potential of MICP as a subsurface technology for sealing fluid migration pathways in downhole oil and gas contexts. Microbially induced precipitation and the factors controlling this process are described in Section 2.3. Section 2.4 briefly outlines surface applications of MICP that have been investigated to-date followed by Section 2.5 which details previous work related to the potential deployment of MICP in the deep subsurface.

2.3 Microbially induced carbonate precipitation via ureolysis

In the last two decades microbially induced carbonate precipitation has been extensively studied and developed as a promising biotechnology for use in various engineering disciplines including environmental, geotechnical, and structural engineering. In particular, it is MICP via ureolysis using microorganisms that produce intracellular urease (Ferris *et al.*, 1996; Stocks-Fischer, Galinat and Bang, 1999; DeJong, Fritzges and Nüsslein, 2006) that has been the most studied to date.

In principle the hydrolysis of urea produces out of a non-ionic substrate (urea) ionic compounds according to equations (Eqs. 2.5-2.10) (Stocks-Fischer, Galinat and Bang, 1999).) Urea is hydrolysed by the enzyme urease into carbamic acid and ammonia (Eq. 2.5). This further hydrolyses spontaneously (Eq. 2.6) into ammonia and carbonic acid. From there, water deprotonates and protonates the ammonia producing ammonium and hydroxide anions (Eq. 2.7). Bicarbonate ions with the additional hydroxide anions shift the equilibrium towards carbonate ions.



If the microenvironment around the bacterial cells is supersaturated with respect to calcium ions, then calcium carbonate precipitation is promoted (Eq. 2.10):



Biom mineralisation is a complex process and is influenced by many factors including the microorganism employed (species), its ureolytic activity, availability of nucleation sites, environmental factors (temperature, pressure, and geochemistry of the microenvironment including salinity and pH), the treatment strategy employed (i.e., bacterial density, urea and calcium concentration, flow-rates, residence times) (Hammes *et al.*, 2003). These factors can influence the form, amount, and spatial distribution of the resulting biomineral precipitate.

Note, ureolysis-driven carbonate precipitation can also be thermally induced where the application of heat acts as a catalyst for ureolysis instead of microbial enzymes. This thesis predominantly focuses on MICP, however thermally induced carbonate precipitation (TICP) is also investigated in *Chapter 4*.

2.3.1 Ureolytic bacteria

Bacteria species which are able to utilise urea as a nitrogen source are called ureolytic bacteria. Many researchers have investigated the naturally occurring soil microorganism *Sporosarcina pasteurii* either as ATCC 11859 or DSMZ, type-33 (formerly known as *Bacillus pasteurii*). *S. pasteurii* is a ureolytic, non-pathogenic strain with the capability to produce large amounts of intracellular urease (Ferris *et al.*, 1996; Stocks-Fischer, Galinat and Bang, 1999; DeJong, Fritzges and Nüsslein, 2006) compared to other ureolytic strains in this category.

2.3.2 Bacterial growth

Bioaugmentation relies upon growth of microorganisms externally prior to injection into the target application zone. Commonly in MICP bacteria are grown to a target bacterial density (number of cells per volume of solution). This is often measured in terms of optical density of the bacterial suspension at a wavelength of 600 nm. Growth of the bacteria (including the nutrient sources), represents a substantial proportion (10-60%) of the overall financial expenditure of MICP as an engineering technology (Kristiansen, 2006). In the last two decades, various international research groups have investigated alternative nutrient sources for batch cultivation of *S. pasteurii* for microbial precipitation of CaCO_3 . This has included investigation of several nutrient media utilizing laboratory/analytical grade reagents such as Brain Heart Infusion (BHI), Nutrient Broth, Yeast extract, tryptic soy broth (Stocks-Fischer, Galinat and Bang, 1999; Fujita *et al.*, 2004; Van Paassen *et al.*, 2010; Mortensen *et al.*, 2011; Tobler *et al.*, 2011). Researchers have also investigated amending analytical grade nutrient broths with urea, NaHCO_3 and/or NH_4Cl (Stocks-Fischer, Galinat and Bang, 1999; Whiffin, 2004; Van Paassen *et al.*, 2010), although direct amendment with NH_4Cl is not considered desirable and could lead to potential ecological issues in surface and groundwater streams (Ip, Chew and Randall, 2001; Van Paassen *et al.*, 2010). More recently, researchers have investigated the use of waste streams for use in the cultivation of *S. pasteurii*, including Lactose Mother Liquor, Corn Steep Liquor, backstrap molasses, brewery waste yeast extracts, (Achal *et al.*, 2009; Cuzman *et al.*, 2015).

Achal *et al.* (2009) focussed on evaluating urease activity and resulting engineering properties for MICP treated sands using Lactose Mother Liquor compared to other standard media. Cuzman *et al.* (2015) also used a growth media arising from dairy waste but presented no comparison of the performance of the bacteria with those cultivated via other growth media. Most reported formulations for *S. pasteurii* growth media in the literature, however, still use laboratory-grade reagents (e.g. NH_4^+ salts) (Ebigbo *et al.*, 2012; Williams, Kirisits and Ferron, 2016). Field-scale applications of MICP will require growth media that is low cost and low carbon. *Chapter 3* investigates the growth behaviour of *S. pasteurii* in a range of different media and provides a comparison of cost and carbon emissions associated with selected media.

2.3.3 Ureolytic activity

The rate at which *S. pasteurii* can hydrolyse urea is termed the ureolytic activity. In this thesis, ureolytic activity is determined by measuring the change in electrical conductivity (EC) over time due to the gain in ionic products, as urea is broken down into ammonium and carbonate ions. That infers that the change in electrical conductivity (EC) over time is proportional to the enzyme present in the hydrolysis of urea (Hanss and Rey, 1971; Lawrence and Moores, 1972; Grunwald, 1984). The change in electrical conductivity over time is then converted into the amount of urea hydrolysed per unit time (mM urea hydrolysed/min/OD) by multiplying with a conversion factor that was experimentally investigated by endpoint calibration experiments where various known urea concentrations are hydrolysed by purified jack-bean meal (JBM) urease or by bacterial produced urease (from *S. pasteurii*) until no further change in electrical conductivity was seen.

The ureolytic activity can also be normalised by the optical density to the bacterial suspension to give the specific ureolytic activity (k_{urea}) (Eq. 2.11):

$$k_{\text{urea}} = \frac{\text{CF} \times \Delta \text{EC}}{\Delta t \times (\text{OD}_{600}) \times \text{dilution factor}} \quad (2.11)$$

where CF represents the conversion factor from the EC measurement (mS/cm) to (mM urea hydrolysed/min) (Calibration relationship developed by Whiffin (2004): urea hydrolysed (mM) = 11.11 x change in electrical conductivity (mS/cm)) and is normalised by the optical density of the bacterial or enzyme suspension measured photospectrometrically at a wavelength of $\lambda = 600$ nm. The dilution factor accounts for the addition of 3 ml of bacterial suspension to 27 ml of 1.11 M urea in the measurement.

In MICP literature, ureolytic activity values range from 0.5 - 60 mM urea hydrolysed/min. Specific ureolytic activities in the range of 0.79 to 29 mM urea hydrolysed/min/OD are common (Whiffin, 2004; Harkes *et al.*, 2010; Van Paassen *et al.*, 2010; Tobler, MacLachlan and Phoenix, 2012; Minto *et al.*, 2016; Terzis and Laloui, 2018).

2.3.4 Enzyme kinetics

Former studies on biologically-induced mineralisation have shown that the urease enzyme is capable of increasing the ureolysis rate up to 10^{14} times compared to uncatalysed reaction rates (Mitchell and Ferris, 2005). Furthermore, the enzyme is the rate limiting step in MICP, since ureolytic activity is directly related to microbial growth and survival of the cell. Once the cell dies, the enzyme may still be present, but activity may decrease. Furthermore, when the bacterial cells become entombed into calcite no urea diffusion may occur anymore and therefore also the ureolytic activity may cease (Stocks-Fischer, Galinat and Bang, 1999). Bacterial concentration, solution pH, as well as saturation conditions have a big influence on the kinetics of urea hydrolysis (Ferris *et al.*, 2004; Dupraz *et al.*, 2009; Tobler *et al.*, 2011).

The hydrolysis of urea in MICP involves a single enzyme catalysed reaction as described in Mobley and Hausinger (1989); Krajewska (2009); Siddique and Chahal (2011); Burbank *et al.* (2012). Once the kinetic parameters are known a model can be established. Several models have been developed that describe the enzyme kinetics during ureolysis. Up to now, researchers mainly focused on enzyme kinetics for urease from jack bean meal (JBM), which incorporates the whole complexity of pH, temperature dependence and ammonium concentration (Moynihan *et al.*, 1989; Qin and Cabral, 1994; Fidaleo and Lavecchia, 2003; Krajewska, Van Eldik and Brindell, 2012). Ebigbo *et al.* (2012) pointed out that these models might not be applicable to whole bacteria cell systems. The reason is that when whole ureolytic bacteria cells constitutively produce the urease enzyme (Mobley, Island and Hausinger, 1995), the uptake of urea into the cell of *S. pasteurii* is dependent upon diffusion across the cell membrane driven by a concentration gradient compared to a pure enzyme system where this step does not exist (Lauchnor *et al.*, 2015). Whole cell kinetics studies investigated so far have typically assumed zero or first-order (Eq. 2.12) ureolysis rates and Michaelis-Menten enzyme kinetics (Eq. 2.13) with respect to urea concentration (Ferris *et al.*, 2004; Okwadha and Li, 2010; Tobler *et al.*, 2011; Cuthbert *et al.*, 2012):

First-order model:
$$r_u = -k_1[\text{urea}] \quad (2.12)$$

In (Eq. 2.12), r_u describes the first **order** ureolysis rate in $[\text{mol l}^{-1} \text{ h}^{-1}]$, whereas k_1 is the first order ureolysis rate constant in $[\text{h}^{-1}]$ and $[\text{urea}]$ is the urea concentration in $[\text{mol l}^{-1}]$.

Michaelis-Menten model:
$$r_u = -\left(\frac{v_{\max}[\text{urea}]}{k_m + [\text{urea}]}\right) \quad (2.13)$$

One of the most used models for describing enzyme kinetics is the Michaelis-Menten kinetics model (Eq. 2.13, Fig. 2.2). Again, r_u describes the ureolysis rate in $[\text{mol l}^{-1} \text{ h}^{-1}]$ and the righthand side of the Michaelis-Menten equation is described by two important parameters, (1) the maximum rate constant $v_{\max}$, which is the maximum reaction rate an enzymatic reaction can have, (2) the Michaelis Menten constant k_m (half saturation constant) as the concentration where the enzymatic reaction is at half of the maximum rate (Cornish-Bowden, 2004).

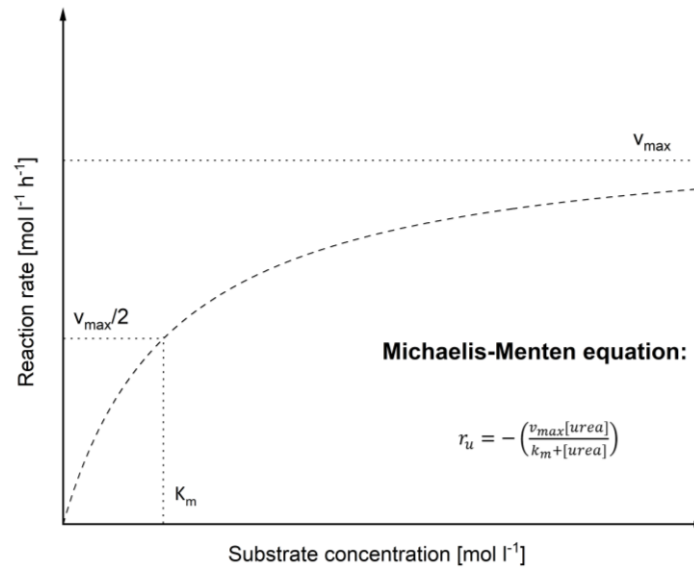


Figure 2.2: This graph describes the Michaelis-Menten kinetics model. Where r_u is defined as the ureolysis rate in $[\text{mol l}^{-1} \text{ h}^{-1}]$ and the righthand side of the Michaelis-Menten model is described by two important parameters, (1) the maximum rate constant $v_{\max}$, which is the maximum reaction rate an enzymatic reaction can have, (2) the Michaelis Menten constant k_m (half saturation constant) as the concentration where the enzymatic reaction is at half of the maximum rate.

Lauchnor *et al.* (2015) investigated whole cell kinetics of *S. pasteurii* with suspended cell experiments to investigate the influence of (1) cell concentration, (2) urea concentration, (3) ammonium concentration and finally (4) pH on the enzymatic reaction. Concluding from this

study it was found that the bacterial cell and urea concentration had the most significant impact on the enzyme kinetics. Up to a k_m value of 330 mM urea concentration, the reaction rate could be described properly by first order reaction kinetics and above a k_m value of 330 mM with zero order reaction kinetics (Lauchnor *et al.*, 2015), i.e. above a certain concentration the ureolysis reaction rate is constant and the urea concentration is no longer the limiting factor.

Other studies on whole cell kinetics studies on *S. pasteurii* cells have reported half saturation constants, k_m of 44.1 mM in Mahanty, Kim and Kim (2014) and a k_m of 722.6 mM in Kantzas *et al.* (1992), twice the value in the study of Lauchnor *et al.* (2015). Enzyme kinetics studies using pure jack bean urease have determined k_m values as low as 17 mM and 26.2 mM (Stocks-Fischer, Galinat and Bang, 1999; Bachmeier *et al.*, 2002). The much higher values of k_m determined in the whole cell studies is due to the lower affinity of the urease in the whole cell kinetics study due to the need for urea to be transported across the cell membrane into the interior of the cell (Jahns *et al.*, 1988). Low affinity means the enzyme requires a higher concentration of its substrate (i.e., urea) to achieve v_{max} .

In this thesis, the Michaelis-Menten model is used to compare ureolysis reaction kinetics for *S. pasteurii* grown in different growth media (*Chapter 3*).

2.3.5 CaCO₃ saturation

Calcium carbonate precipitation is dependent on the saturation state (Ω_{CaCO_3}) of the environment with respect to CaCO₃ and is defined in Eq. (2.14) (Dupraz *et al.*, 2009):

$$\Omega_{CaCO_3} = \frac{\{Ca^{2+}\}\{CO_3^{2-}\}}{K_s} \quad (2.14)$$

where Ca^{2+} and CO_3^{2-} are the ion activities and K_s represent the temperature dependent solubility constant. The saturation index (SI) is calculated as (Eq. 2.15):

$$SI = \log_{10}(\Omega_{CaCO_3}) \quad (2.15)$$

An $SI > 0$ indicates that the solution is oversaturated, and precipitation is thermodynamically favoured whereas an $SI < 0$ indicates that the solution is undersaturated, i.e., dissolution of CaCO₃ is thermodynamically favoured. An $SI = 0$ indicates that the solution is saturated, i.e., in equilibrium with the solid phase. In practice, mineral precipitation or dissolution begins when

a critical saturation state (Ω_{critical}) or saturation index, SI_{critical} , is reached, i.e., it is dependent on the degree of supersaturation or undersaturation of the solution.

In classical nucleation theory (CNT) the critical saturation state (Ω_{critical}) is related to the nucleation free energy barrier (Stumm and Morgan, 1995) which needs to be surmounted to initiate carbonate precipitation. Furthermore, the critical saturation state may be dependent on the metabolic pathway of the bacterial strain (in our case ureolysis), the presence of organic macromolecules i.e. extra polymeric substances (EPS) (Rodriguez-Navarro *et al.*, 2007). In literature a variety of saturation states have been reported based on batch experiments, ranging from $\Omega = 12 - 436$ (Ferris *et al.*, 2004; Mitchell and Ferris, 2005, 2006; Dupraz *et al.*, 2009; Tobler *et al.*, 2011).

2.3.6 Nucleation processes and theory

Mineral nucleation is the first step in the formation of a new crystalline solid phase from solution and provides a site around which particles assemble as the crystal grows. For calcium carbonate precipitation two nucleation theories have been developed: the *classical nucleation theory* (CNT) and the *non-classical nucleation theory*. Classical nucleation theory was established in the early 1930s by Becker and Doering (1935) based on the ideas by Gibbs *et al.* (1878). CNT differentiates between homogenous nucleation (Fig. 2.3b) which occurs in solution and heterogeneous nucleation (Fig. 2.3c) which occurs on surfaces (imperfections or microorganisms) (Cheong, Gaskell and Neville, 2013). CNT explains that there is a higher Gibbs free energy barrier for homogeneous nucleation (ΔG_{Hom}) to overcome for homogeneous nucleation compared to heterogeneous nucleation (ΔG_{Het}) (Fig. 2.3d) due to the large surface to volume ratio (Zhao *et al.*, 2018) of the small nuclei formed in solution. Whereas in the case of heterogeneous nucleation, for example in the case of MICP, the bacterial cell acts to reduce the activation energy barrier that needs to be overcome by acting as a nucleation site (Ferris *et al.*, 2004; Gadd, 2010).

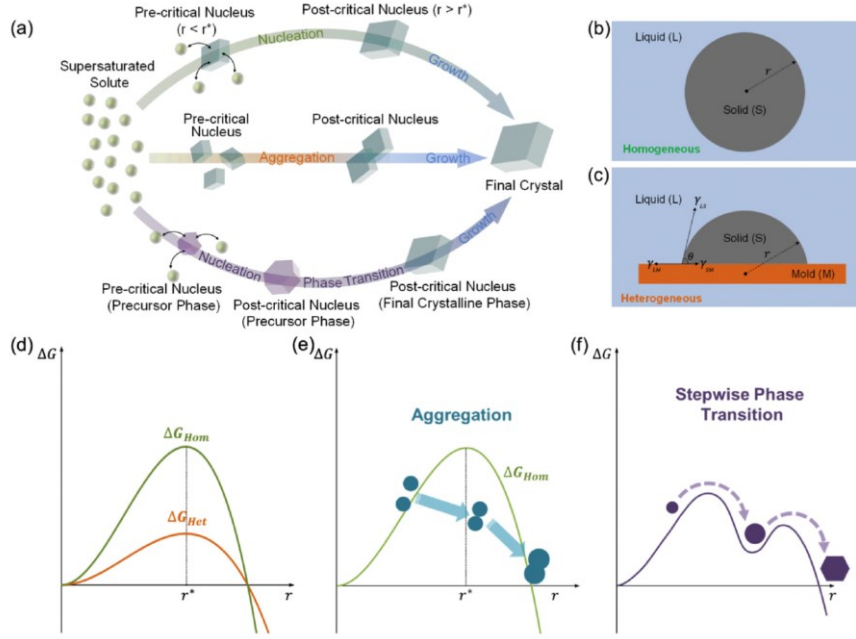


Figure 2.3: Schematic of homogenous (from solution) crystallisation pathways for CaCO_3 . (a) the upper part shows the classic nucleation theory, and the middle and bottom paths show non-classical nucleation behaviour i.e., pre-cluster nuclei aggregation and stepwise phase transition. (b, c) is showing homogenous and heterogenous nucleation based on the classical nucleation theory. (d) the change of free energy during homogenous and heterogenous nucleation. (e, f) non-classical nucleation including pre-cluster nuclei aggregation and stepwise phase transition illustrated by Zhao *et al.* (2018).

In contrast, non-classical nucleation theory is described by two nucleation pathways namely as cluster/nuclei aggregation (Fig. 2.3e) and stepwise phase transition (Fig. 2.3f). Where in pre-cluster nuclei aggregation the collision of nuclei establishes bigger aggregates by binding them together and forming a larger stable aggregate (Fig. 2.3e) and in stepwise phase transition (Fig. 2.3f) through surface dissolution and recrystallisation (e.g., vaterite to calcite). In the last decade, research has identified that the crystallisation process of calcium carbonate follows non-classical nucleation processes (Gebauer and Cölfen, 2011). This is because many phase transition paths can be involved i.e., transitioning from hydrous phases such as amorphous calcium carbonate (ACC) to anhydrous phases including crystalline vaterite, aragonite and calcite (Fig. 2.3f). This can occur either through surface dissolution and reprecipitation or solid state transformation (Gower, 2008).

In summary, the reaction sequence of microbially induced carbonate precipitation via ureolysis consists of three stages. First, a certain level of oversaturation with regards to CaCO_3 needs to be reached, followed by nucleation at critical saturation state (Ω_{critical}) and finally crystal growth

on the newly created nuclei. The activation energy required for nucleation is typically greater than that for crystal growth, such that calcium carbonate precipitation proceeds more rapidly once calcium carbonate is already present within the system (e.g., Rodriguez-Blanco, Shaw and Benning, 2011; El Mountassir *et al.*, 2014).

2.4 Surface/ near-surface applications of MICP

In the last two decades microbially induced carbonate precipitation (MICP) via the metabolic pathway of ureolysis has been studied and tested extensively for a variety of surface and near-surface engineering applications. These include: (1) co-precipitation of heavy metals or radionuclides in environmental remediation (Warren *et al.*, 2001; Mitchell and Ferris, 2005; Fujita *et al.*, 2008; Kurmaç, 2009), (2) ground improvement, in particular MICP has been investigated to increase compressive strength, shear strength and stiffness of geomaterials (DeJong, Fritzges and Nüsslein, 2006; Whiffin, Van Paassen and Harkes, 2007; Harkes *et al.*, 2010; Van Paassen, 2011), (3) to reduce wind or water-induced erosion of sandy soils (Gomez *et al.*, 2015; Hamdan and Kavazanjian, 2016; Salifu *et al.*, 2016), (4) permeability reduction in rocks and fractures (Minto *et al.*, 2016; Tobler *et al.*, 2018), and (5) for repair of construction materials (deteriorated concrete surfaces and preservation of building surfaces) (De Muynck *et al.*, 2008; Van Tittelboom *et al.*, 2009; Minto *et al.*, 2018).

Much of the literature to date on the deployment of microbially induced carbonate precipitation in various engineering applications has focused on controlling/optimising the variables influencing MICP e.g., bacterial density, ureolytic activity, calcium chloride and urea concentration, treatment strategy (e.g., injection flow rates, number of injection cycles) to achieve desirable hydraulic and mechanical outcomes. There are many reviews covering surface/near-surface applications in more detail e.g., (Dejong *et al.*, 2013; Wang *et al.*, 2017; El Mountassir *et al.*, 2018).

The next section focuses on previous research which is directly relevant to the deployment of MICP as a technology for sealing leakage pathways in oil and gas contexts.

2.5 Potential for use of MICP in hydrocarbon context

Previous studies from Mitchell *et al.* (2009); Cunningham *et al.* (2011, 2013) have investigated engineering the growth of biofilms as a potential technology to reduce permeability in the subsurface (i.e., bio-clogging). One of the most significant advantages of using microbes in the subsurface is the excellent penetrability of the bacterial suspension which is due to the small size of bacterial cells ($\sim 0.5\text{--}1.0\text{ }\mu\text{m}$) and the low viscosity of the suspension (aqueous or growth media). The result is that bacterial suspensions can access small aperture fractures (<100 microns) and small pore throats that traditional cementitious grouts could not penetrate. However, biofilms consist of colonies of bacterial cells (Mitchell *et al.*, 2013), and if they are cut off from their nutrient supply, they might die or enter a dormant (starvation) phase. As such promoting MICP using ureolytic bacteria to promote conditions favourable for precipitation of CaCO_3 biominerals provides the opportunity to establish a biogrout mineral seal, with far superior durability and mechanical properties to that of a seal composed solely of biological mass (i.e., bio-clogging).

This thesis focuses on the application of microbially induced carbonate in oil & gas well decommissioning in order to prevent hydrocarbons from former production zones reaching the seabed or ground surface. As outlined in *Chapter 1*, there is the potential for leakage pathways to exist/form in all the materials (rock, cement, steel) which surround and comprise the wells, and at the interfaces between these materials. Here findings from investigations on MICP for reducing permeability in unconsolidated porous media and consolidated porous media, as well as for fractured media and in degraded cement is presented. Finally, work on the influence of higher temperatures and pressures on MICP relevant to the hydrocarbon context is also presented.

2.5.1 Provision of bacteria

MICP treatment requires the presence of bacteria, and the addition of urea and a calcium source (e.g., calcium chloride). In early MICP research, experiments mainly focused on treating unconsolidated soils (sands) in column experiments. Ferris *et al.* (1996) mixed the microorganisms with sand *ex-situ* and then once packed into the column, a continuous gravitational flow of cementing solution was applied (i.e., nutrient broth, urea, ammonium

chloride, sodium bicarbonate and calcium chloride in distilled water). Ferris *et al.* (1996) demonstrated that after injecting 50 - 100 pore volumes of cementing solution the sand column permeability could be reduced by >90% from an initial value of 2500 mD.

Since pre-mixing of bacteria is not possible *in-situ*, there are in general two approaches to providing bacteria, (1) injecting bacteria and then growth nutrients to stimulate the development of a biofilm (Mitchell *et al.*, 2009; Cunningham *et al.*, 2011, 2013) followed by repeated injections of urea and calcium chloride and (2) injecting bacteria only (no growth media) followed by urea and calcium chloride in repeated cycles (i.e., including repeated injections of bacteria (Tobler *et al.*, 2011, 2018; Minto *et al.*, 2016). Studies using both approaches are outlined below. In this thesis the second approach has been adopted.

2.5.2 Permeability reduction in unconsolidated soil systems

As calcium carbonate is precipitated within a porous medium, pore-space reduces and thus can lead to changes in permeability. Early research mainly focused on sand column experiments and achieved up to 90 - 100% of permeability reduction after MICP treatment compared to initial permeability (Ferris *et al.*, 1992; Gollapudi *et al.*, 1995; Tobler, Maclachlan and Phoenix, 2012). Cunningham *et al.* (2011), Ebigbo *et al.* (2012) and Phillips *et al.* (2013) all investigated permeability reduction in sand packed columns (61 cm long and 2.54 cm in diameter) and reported permeability reductions in the range of 61 – 99%. Results revealed that non-equimolar concentrations of CaCl_2 /urea (1.25/0.33 M) led to limited carbonate deposition due to the limited production of CO_3^{2-} ions from ureolysis. Phillips *et al.* (2013) demonstrated that the use of equimolar cementing solutions of 0.33/0.33M CaCl_2 /urea and yielded higher CaCO_3 precipitation.

2.5.3 Permeability reduction in porous rock

Nemati and Voordouw (2003) investigated enzymatic precipitation of CaCO_3 using pure urease enzyme in Berea sandstone cores and reduced from 394 – 31 mD which corresponds to a permeability reduction of 92%.

Cunningham *et al.* (2013) demonstrated for the first time biomineralisation in sandstone cores at high pressure conditions. The permeability of the Berea sandstone cores was reduced from 37 mD - 0.3 mD at high fluid pressure (7.5 MPa and 25°C).

In another study, Tobler *et al.* (2018) investigated MICP injection strategies (with variations in bacterial suspension concentration ($OD_{600} = 0.15, 0.5, 1.0$) and treatment cycles (11 – 20) to obtain a homogenous $CaCO_3$ distribution along Bentheimer sandstone cores. Permeability reduction of three orders of magnitude were achieved with reductions from 2400 mD – 1.2 mD. The most uniform calcite distribution along the core length was achieved using a bacterial concentration of $OD_{600} = 1.0$ and then dropped to 0.5 once a backpressure of 15 kPa was reached. Although this strategy improved uniformity, it required significantly more treatment cycles to achieve a target permeability.

Minto *et al.* (2017) investigated biomineral seal longevity by applying six treatment cycles of bio-grout with bacterial concentrations in the range of $OD_{600} = 0.71 - 0.90$ to Berea sandstone cores. An initial reduction in permeability from 886 mD to 39.6 mD at 8.96 MPa fluid pressure and 50°C was achieved before two dissolution treatment cycles with HCl (pH = 3.02 (cycle 1) and pH = 1.01 (cycle 2)) were applied to investigate the seal longevity under acidic conditions. Dissolution acted to slightly increase the permeability: final permeabilities ranged from 47.6 mD – 66.2 mD.

2.5.4 Permeability reduction in fractured rock

Tobler *et al.* (2018) experimentally investigated MICP in fractured granite cores (36 mm diameter and 36 mm length). An artificial fracture surface was created for each core with an aperture of ~300 μm . After 17 treatment cycles fracture transmissivity was reduced 4 orders of magnitude. Microstructural analysis revealed that more than >67% of the fracture were filled with $CaCO_3$. Tobler *et al.* (2018) also demonstrated that the MICP increased the shear strength of the fractured cores, and that this was dependent on the proportion of the fracture area that had calcite bridged from one fracture surface to the other.

Phillips *et al.* (2013) investigated the suitability of MICP in the application of carbon capture and storage (CSS). They sampled a sandstone core from the Boyles sandstone formation, Alabama, USA. The cored sandstone sample had diameter of 74.3 cm and height of 37.5 cm.

A hole with 5.1 cm diameter in the middle of the rock was drilled to install a packer at a height of 7.6 and 15.2 cm from the bottom of the core. The sample was pressurised with water at 800 kPa pressure for 1.75 hr before the formation breakdown pressure was reached and a horizontal fracture was initiated. Fracture permeability was estimated at 23000 mD initially and reduced to 0.1 mD (5 orders of magnitude reduction) after 23 days. Further increase of the pressure from 800 kPa to 3 MPa re-fractured the sample, and first breakthrough could be seen after 1.6 hr. The new initiated fracture permeability was measured to be 2400 mD and this was subsequently resealed via MICP treatment, achieving 0.1 mD in only 5 days at atmospheric conditions.

In a second mesoscale fracture sealing experiment Phillips *et al.* (2015) simulated downhole conditions with a confining pressure of 4500 kPa simulating a depth below ground surface (bgl) of 457 m. After 28 pulses and 21 days treatment the fracture permeability was reduced from 26,000 mD to 40 mD.

Minto *et al.* (2016) demonstrated experimentally the sealing of an artificial fracture at close to field scale (3.1 m²) between a granite surface and polycarbonate surface (to enable imaging). In this study fracture transmissivity was reduced in three treatment cycles under constant flow conditions from 1.71E-05 to 8.75E-09 m²/s (over three orders of magnitude). This setup enabled to capture CaCO₃ precipitation via time-lapse photography. Minto *et al.* (2016) demonstrated that by controlling flow rate and hence fluid velocity they could inhibit bacterial attachment and calcium carbonate precipitation close to the well.

Permeability reduction of up to 5 orders of magnitude have been demonstrated in rock fractures, however the time required could be considering to be a limit to its potential uptake in hydrocarbon well operations/plugging procedures.

2.5.5 Permeability reduction in cementitious material

Ramachandran, S. K., Ramakrishnan, V., & Bang, (2001) and Achal, Mukerjee and Sudhakara Reddy (2013) investigated the remediation of cracks and fissures in concrete and cement mortar (Ordinary Portland Cement) with MICP. Results showed an increased resistance to absorption of water and chloride attack in the treated samples compared to untreated samples.

Recently, Kirkland *et al.* (2019) conducted a study sealing fluid migration channels in cement. An artificial channel was created by putting two half cut cement cylinders together in a reactor style setup establishing a void with measurements of 5 cm in length times 0.25 cm wide and 0.05 cm deep. MICP treatment was conducted using a constant flow rate of 1 ml/min and under atmospheric pressure conditions. A *S. pasteurii* inoculum of 60 ml was injected into the reactor followed by a 4 hr no flow period. Next, 120 ml of a nutrient solution was injected to promote biofilm growth of the ureolytic bacteria. 15 hr later the reactor was flushed with brine (NaCl, 24g/l) to prevent bio-clogging at the injection point. In a final step, 4 pulses of a 30 ml of cementation media (nutrient solution amended with 49 g/l CaCl_2) were injected with a 1 hr static no flow period. The reactor was reinoculated every night. In total 22 cementation pulses were applied. Initial permeability was reduced by three orders of magnitude from $5.6 \times 10^5 \text{ mD}$ to 263 mD. X-ray CT scanning was performed before and after treatment. It was observed in a replicate sample that an increased flow rate at the cementation stage (from 1 ml/min to 2 ml/min) with more cementation pulses enhanced the spatial distribution of CaCO_3 in the void.

2.5.6 MICP field trials for fracture sealing or in hydrocarbon wells

Cuthbert *et al.* (2013) carried out one of the first field trials of MICP treatment in fractured rock (dacite) sealing a 670 μm fracture at a depth of 25 m (bgl) in Leicestershire, UK. The aim was to establish a fracture seal with an extent of several m^2 using an injection and extraction borehole. Cross-hole hydraulic testing was conducted via two additional boreholes with a packer configuration and indicated a 99% reduction in fracture transmissivity close to the injection well and a 33% reduction at a distance of 2 m from the well (equivalent to a fracture aperture of around 90 μm).

Phillips *et al.* (2016) conducted an MICP in a field trial at the Gorgas well, in Jasper, Alabama, USA. Target depth was at 340 m depth, where a horizontal fracture was initiated in a very low permeable sandstone ($\sim 3 \text{ mD}$). Well fall-off pressure was reduced from 30% (initial test) down to 7% (post treatment). A well fall-off pressure transient test in simple terms consists of two parts: (1) fluid is injected at a constant rate to build up pressure, (2) then the well is shut-in, and the pressure decline is measured. With a MICP seal the backflow to the wellbore is restricted (it is known as skin) and leads in a reduction in the fall-off pressure. After well testing the sealed fracture was refractured and a higher fracture initiation pressure was observed. It is

unknown if the MICP seal itself was broken or a weaker area around the mineral seal was fractured.

Kirkland *et al.* (2020) conducted a field trial on the Rexing #4 well near Cynthiana, Indiana, USA. The well was formerly used as a water injector to enhance oil recovery towards a production well. This well was of particular interest due to having problems of maintaining the injection pressure. Water sweeps, the injected water that is supposed to displace hydrocarbons from an injection well to a production well, seemed to be inefficient due to a hydraulic connection from the target zone to a thief zone (high permeable formation) at 698 m depth. It was thought that this was due to a compromised well cement sheath that was identified via well logging. The thief zone was remediated with MICP and the injectivity reduced by 70%. Furthermore, the well was put back into service and could maintain the water injection pressure as before and is now back in operation. The injection tubing that was pulled out indicated the presence of hydrocarbons with carbonate precipitates. This is likely to happen due to injection close to an oil-bearing zone and indicates that MICP can indeed occur in the presence of hydrocarbons.

2.5.7 Influence of downhole conditions (elevated pressure/elevated temperature)

In contrast to surface/near-surface applications of MICP, the application of MICP for sealing in hydrocarbon wells and production/thief zones or for sealing fractures in caprocks, would take place under elevated confining and fluid pressures as well as elevated temperatures depending on the target depth. For example, in a North Sea offshore wellbore, the apparent geothermal gradient is $\sim 4.6 - 5.2^{\circ}\text{C}/100\text{ m}$ (personal communication, OGTC, 2018).

Most studies on the effect of temperature and pressure on ureolytic activity have been conducted using purified jack-bean meal urease (*Canavalia ensiformis*), rather than *S. pasteurii* cells. Bacterial cell concentration (biomass) affects ureolytic activity and studies from Van Paassen (2009) indicate a significant increase of ureolytic activity with increasing temperature. A rise of 10°C in temperature between 5 and 35°C let the ureolytic activity increase by a factor of 3.4 (Van Paassen, 2009). Whiffin (2004) reported a linear increase in specific ureolytic activity in

the temperature range of 25 to 60°C. Both studies were exposing the bacterial suspension to temperature for a very short exposure time of 5 min to measure enzyme activity.

Illeova *et al.* (2003) investigated the inactivation of jack-bean urease with injection flow calorimetry in the temperature range $T = 55 - 87.5^{\circ}\text{C}$. Temperature in this range had as expected a negative impact on the activity of the enzyme (e.g. at $T = 55^{\circ}\text{C}$ and an exposure time of 50 hr the activity had reduced by almost 50% whereas at $T = 87.5^{\circ}\text{C}$ complete activity was lost in an exposure time of less than 1 hr). Feder *et al.* (2021) similarly showed that at $T = 50^{\circ}\text{C}$ jack-bean urease became $\geq 50\%$ inactivated after 37.3 hr whereas at 80°C $\geq 50\%$ inactivation was reached in only 5.2 min.

Feder *et al.* (2021) investigated jack-bean urease for use at higher temperatures claiming that *S. pasteurii* does not grow at temperatures above $T = 40^{\circ}\text{C}$ (Skorupa *et al.*, 2019). This indeed could inhibit growth of ureolytic bacteria *in-situ* (i.e., bio-stimulation) or in the context where ureolytic bacteria are injected and then biofilm growth is promoted over a period of days at elevated temperatures. However, for bio-augmentation strategies, where *S. pasteurii* cultures are cultivated *ex-situ* (and *in-situ* growth is not required), sufficient ureolytic activity at higher temperatures may still remain to trigger MICP. For this reason, it is important to understand how the ureolytic activity of *S. pasteurii* cells is influenced by temperature and exposure time.

Krajewska *et al.* (2012) investigated the effect of temperature and pressure on jack-bean urease in a temperature range of 15 - 35°C and pressures up to 132 MPa. This was performed with a stopped-flow spectrometer, whereas the pressure related measurements were conducted in a high-pressure stopped-flow reactor (van Eldik *et al.*, 1993). The pressure dependent measurements were the first ever reported for jack bean urease (Krajewska *et al.*, 2012). Results in this study showed that temperature enhanced the activity of urease in the range of 15 - 35°C . Pressure tested in the range from $p = 5 - 132$ MPa at $T = 25^{\circ}\text{C}$ reduced the reaction rate of the enzyme but surprisingly not the half saturation constant k_m and therefore the binding of urea to the active site on the urease enzyme is not affected by pressure. Note also, that the reaction rate was only very slightly reduced in the range 5 - 40 MPa.

Experiments by Mitchell *et al.* (2013) were conducted in synthetic fractures, a polystyrene rod was prepared with 6 x 1 mm glass capillary tubes running through the core. The aim of the experiments was to investigate if ureolysis could occur under conditions relevant to a carbon

capture and storage reservoir. Pressure was at 7.5 MPa and temperature at 32°C (conditions where CO₂ is supercritical) and salinity was provided with a synthetic NaCl brine (NaCl rich 162 g l⁻¹ TDS 1.5 M, used by Kaszuba, Janecky and Snow, (2003)). Continuous effluent samples were analysed for CFUs and NH₄⁺ and confirmed urea hydrolysis was occurring.

2.5.8 Thermally induced carbonate precipitation (TICP)

Given that inactivation of the urease enzyme will occur at some temperature threshold, temperature is a limiting factor in the use of MICP in the deep subsurface. Field trials of MICP have been conducted at wellbore depths ranging from 310 – 699 m (Phillips *et al.*, 2016; Kirkland *et al.*, 2020). These depths are quite shallow in comparison to wellbore depths of several kilometres on the UK continental shelf (UKCS). In the case of higher wellbore depths, the subsurface temperature based on the geothermal gradient (°/km) will be the limiting factor for the application of MICP. Research by Phillips *et al.*, (2017) investigated thermally induced carbonate precipitation as an alternative carbonate precipitation mechanism for higher temperatures. They investigated the rate of ureolysis at temperatures up to T = 140°C and reported that urea decomposition was increased at a certain threshold temperature close to T = 120°C.

2.6 Conclusion

Most of the laboratory based experimental studies on MICP are still using microbiological grade reagents. In order to make the upscaling of MICP economically feasible and to contribute to ambitious net zero carbon targets then any growth media used in field-scale applications should be low-cost and low-carbon. *Chapter 3* investigates a range of growth media and investigates the influence of alternative growth media on reaction kinetics and on the biomineral precipitates formed.

As outlined in this literature review, there is an extensive body of previous work, which has demonstrated successful permeability reduction in porous and fractured rock, and fractured cements. Thus, there is clear potential for MICP to be used to seal migration pathways in and around hydrocarbon wells. However much of this laboratory work has been conducted at standard atmospheric pressures and ambient temperature, with few studies investigating MICP

under a select simulated set of pressure and temperature conditions. For example: Mitchell *et al.* (2013) investigated MICP in a synthetic porous media at a fluid pressure of 7.5 MPa and temperature of 32°C; whereas Phillips *et al.* (2015) investigated fracture sealing at 4.5 MPa confining pressure.

In order to apply MICP to a hydrocarbon context, a better understanding of the influence of pressure and temperature on the ureolytic activity of *S. pasteurii* is required. To date this, has mostly been investigated for jack-bean urease. In this thesis the influence of temperature and pressure on the ureolytic activity of whole cells is systematically investigated in *Chapter 4*, in order to determine what are the limiting conditions for the application of MICP in the subsurface, and whether alternatives can be used at higher temperatures (i.e., TICP).

Although at a few selected conditions of elevated pressure and temperature conditions, MICP has been successfully demonstrated, it remains unclear how temperature and pressure influence the permeability reduction that biomineralisation can achieve, the amount and spatial distribution of calcium carbonate precipitated, and the morphology and polymorphs of the biominerals created. A multi-scale investigation addressing these unknowns at *in-situ* wellbore/reservoir conditions is presented in *Chapter 5*.

Chapter 3

Newly developed alternative low-cost & low-carbon feedstock for upscaling the growth of *Sporosarcina pasteurii* for field-scale applications

3.1 Abstract

Bio-grouts have recently been developed for a range of applications including to improve the engineering behaviour of soils, rock and concrete, for stone conservation and for bioremediation. To date, the most studied bio-grout is microbially induced carbonate precipitation via ureolysis. However, microbiological grade nutrient media are typically used to grow the urease producing bacteria; these are expensive and have a high carbon footprint. Here, we investigate the growth and enzyme reaction kinetics of *Sporosarcina pasteurii* cultivated using a plant-based, low-cost media, derived from autolysed waste yeasts, and compare this to Brain Heart Infusion (BHI), the leading microbiological grade nutrient medium. Metabolic activity of *S. pasteurii* grown in the yeast medium resulted in similar reaction kinetics without significantly impacting on the quantity and mineralogical composition of calcium carbonates precipitated. Further, utilising the yeast media reduces upscaling costs by a factor of ~10 compared to BHI and substantially reduces carbon emissions.

3.2 Introduction

Microbially induced carbonate precipitation (MICP) is a natural process that plays a vital role in the cementation and deposition of carbonates in natural systems, including in lakes, oceans, caves, aquifers, soils and sediments (Riding, 2000; Banks *et al.*, 2010; Chou *et al.*, 2012). Increasingly microbially induced carbonate precipitation is being investigated as a means of engineering modifications to the hydraulic and mechanical behaviour of porous and fractured media, for example: to stabilise soils, to seal rock fractures, to repair cracks in concrete, to enhance oil recovery and for stone conservation (DeJong, Fritzges and Nüsslein, 2006; Sen, 2008; Bang *et al.*, 2010; Minto *et al.*, 2016, 2018; El Mountassir *et al.*, 2018; Tobler *et al.*, 2018). Furthermore, MICP is also being investigated for sequestration of heavy metals for bioremediation (Achal *et al.*, 2012). In ground engineering, microbially induced carbonate precipitation (MICP) is an attractive alternative technology which can be used in applications where conventional cements are not suitable, either due to their low penetrability and/or the high pressures required for their injection which restricts their deployment near-surface (Cunningham *et al.*, 2011). In comparison to conventional ground improvement techniques MICP may also result in reduced environmental impacts and a smaller carbon footprint (Chang, Im and Cho, 2016).

MICP via ureolysis is the most extensively studied microbial process to-date for enhancing the performance of geomaterials. The most widely investigated bacteria for MICP is *Sporosarcina pasteurii* (former *Bacillus pasteurii*) (Yoon *et al.*, 2001), due to its rapid microbial growth, and the large quantity of intracellular urease (Ferris *et al.*, 1996; Stocks-Fischer, Galinat and Bang, 1999; DeJong, Fritzges and Nüsslein, 2006) it produces and the resulting enzymatic activity to hydrolyse urea (Sumner and Myrbäck, 1951). This enzymatic activity leads to the production of ammonium and carbonate ions, and a concurrent increase in the pH of their microenvironment (Eq. 3.1) (Ferris *et al.*, 2004). In the presence of a calcium source, e.g., calcium chloride (CaCl_2), the increase in carbonate concentration and pH result in the precipitation of calcium carbonate minerals (Eq. 3.2).



In principle the biotechnology works by injecting a microbial solution in a first step, followed by the injection of a cementation solution (i.e., calcium source). Within porous or fractured media, the precipitation of calcium carbonate minerals fill pores and fractures with positive implications for the mechanical and hydraulic properties of the media (Tobler *et al.*, 2018).

For MICP to be widely adopted in engineering practice, it must be financially viable. Growth of the bacteria (including the nutrient sources), represents a substantial proportion (10 - 60%) of the overall financial expenditure of MICP as an engineering technology (Kristiansen, 2006). In the last two decades, various international research groups have investigated alternative nutrient sources for batch cultivation of *S. pasteurii* for microbial precipitation of CaCO_3 . This has included investigation of several nutrient media utilising laboratory/analytical grade reagents such as Brain Heart Infusion (BHI), Nutrient Broth, Yeast extract-amended with ammonium, tryptic soy broth (Stocks-Fischer, Galinat and Bang, 1999; Fujita *et al.*, 2004; Van Paassen *et al.*, 2010; Mortensen *et al.*, 2011; Tobler *et al.*, 2011). Researchers have also investigated amending analytical grade nutrient broths with urea, NaHCO_3 and/or NH_4Cl (Stocks-Fischer, Galinat and Bang, 1999; Whiffin, 2004; Van Paassen *et al.*, 2010), although direct amendment with NH_4Cl is not considered desirable and could lead to potential ecological issues in surface and groundwater streams (Ip, Chew and Randall, 2001; Van Paassen *et al.*, 2010). More recently, researchers have investigated the use of waste streams for use in the cultivation of microbes such as *S. pasteurii*, including Lactose Mother Liquor, Corn Steep Liquor, backstrap molasses, brewery waste yeast extracts, (Achal *et al.*, 2009; Cuzman *et al.*, 2015; Williams, Kirisits and Ferron, 2016; Joshi, Goyal and Reddy, 2018). Achal *et al.* (2009) focussed on evaluating urease activity and resulting engineering properties for MICP treated sands using Lactose Mother Liquor compared to other standard media. Cuzman *et al.*, (2015) also used a growth media arising from dairy waste, but presented no comparison of the performance of the bacteria with those cultivated via other growth media.

If MICP is to be used extensively as a commercial biotechnology, it is crucial to reduce the cost and the environmental impact of the growth process without reducing the efficiency of the MICP processes. Here, we investigate six different nutrient media. One of these was selected for further analysis: a yeast-based growth media (Marmite) alongside a conventional animal-based media (BHI). This study provides a comprehensive comparison of these two media including investigation of growth behaviour, enzyme kinetics and characterisation of

the carbonate minerals precipitated via X-ray diffraction. Furthermore, a comparison of costs and embedded carbon is also presented. The results provide insight into the advantages of using a yeast-based media in comparison to an animal-based media for large-scale MICP field applications.

3.3 Experimental Methodology

In order to identify non-animal based, low-cost, low-carbon nutrient media that perform similarly to standard microbiological laboratory-grade nutrient media we performed three sets of experiments. In **Experiment 1**, the growth performance of *S. pasteurii* over 24 hrs was characterised for six distinct nutrient media; 3 animal-based and 3 plant-based. Optical density measurements at a wavelength of 600 nm (OD_{600}) and the cost of the media per litre, were used to select the best performing of the plant-based and a conventional animal-based nutrient media for detailed comparison in Experiments 2 and 3. **Experiment 2** consisted of microbial plate growth experiments to evaluate the growth rate and generation time of *S. pasteurii* for the selected plant-based and conventional animal-based nutrient media. Plate counting was used to enumerate viable bacterial colonies as colony-forming units per ml (CFU/ml), and OD_{600} was also measured to establish the relationships between CFU/ml and OD_{600} specific to these media. In **Experiment 3** cell suspension reactions were conducted for the two selected media, for a comparison of the respective enzyme kinetics, efficiency of urea hydrolysis, and the quantity and composition of the biominerals precipitated was determined using X-ray powder diffraction (XRD).

3.3.1 Experiment 1: Growth media screening

In Experiment 1, the growth performance of *S. pasteurii* in six different nutrient media (3 animal-based and 3 plant-based) was evaluated over a time interval of 24 hr. To provide a baseline, the growth media formulation was initially selected for LMG – 139 (Media 1, Table 3.1) using the specification for *S. pasteurii* in the Handbook of Microbiological Media (Atlas, 2010). Then, for each of the other five media (Media 2-6, Table 3.1) the carbon source in Media 1 was replaced by the same mass of an alternative carbon source. The six different media are: LMG-139, Lab-Lemco Beef Extract, Bacteriological Yeast Extract, Tryptone, Marmite and Brain Heart Infusion (Table 3.1).

In Experiment 1, the ureolytic strain *Sporosarcina pasteurii* (DSMZ strain type 33) was revived from a fresh frozen -80°C glycerol stock culture and initially grown on solid brain heart infusion (BHI) agar (47 g/l) with the addition of urea (20 g/l) by aseptic filtration technique with a Millipore 0.45 µm filter after autoclave sterilisation of the agar. Each nutrient media was prepared in triplicate (volumes of 50 ml) without pH adjustment, alongside one additional uninoculated (blank) sample, and sterilised by autoclaving at 121°C for 15 min. After autoclaving, urea (20 g/l) was added by aseptic filtration to each media. Next, one single colony of *S. pasteurii* from the established agar plates was inoculated into the liquid growth solutions by transferring it from the agar plate to the growth solution in a sterile Erlenmeyer flask with a plastic loop and shaken to mix uniformly. Finally, the inoculated triplicates were auto-pipetted into a 96 well-template and incubated at 30°C in a temperature-controlled Biotek well-plate reader. The optical density (OD₆₀₀) of each bacterial suspension was measured in hourly time-steps against the blank sample (uninoculated media, Table 3.1) over a 24-hour period, using a Biotek well-plate reader.

Table 3.1 Composition of the six nutrient media studied

Experiment 1: Different tested nutrient media						
Sample Nr.	Amount of samples $V_t = 50$ ml in triplicates	Media	Constituents	Concentration (g/l)	Product	Cost/Litre (£/l)
1	3	LMG-139	Bacteriological Peptone	5.00	P5905 Sigma	3.36
			Bacteriological Yeast Extract	2.00	Y1625 Sigma	
			Lab-Lemco Beef Extract	1.00	Thermo Scientific LP0029B	
			Sodium Chloride	5.00	S1679 Sigma-Aldrich	
			Urea	20.00	U5378 Sigma	
2	3	LLB	Lab-Lemco Beef Extract	8.00	Thermo Scientific LP0029B	3.35
			Sodium Chloride	5.00	S1679 Sigma-Aldrich	
			Urea	20.00	U5378 Sigma	
3	3	YE	Bacteriological Yeast Extract	8.00	Y1625 Sigma	2.38
			Sodium Chloride	5.00	S1679 Sigma-Aldrich	
			Urea	20.00	U5378 Sigma	
4	3	TT	Bacteriological Tryptone (veg.)	8.00	T9410 Sigma-Aldrich	2.44
			Sodium Chloride	5.00	S1679 Sigma-Aldrich	
			Urea	20.00	U5378 Sigma	
5	3	MM	Marmite	8.00	Unilever, UK	1.13
			Sodium Chloride	5.00	S1679 Sigma-Aldrich	
			Urea	20.00	U5378 Sigma	
6	3	BHI	Brain Heart Infusion	8.00	53286 Sigma-Aldrich	4.51
			Sodium Chloride	5.00	S1679 Sigma-Aldrich	
			Urea	20.00	U5378 Sigma	

To compare the efficacy of samples, the efficiency yield was introduced, as defined by (Eq. 3.3), to create a selection criterion for the various nutrient media, based on their growth yield and their cost/litre:

$$\text{Efficiency yield} = \frac{\text{Biomass produced at time step } (\text{OD}_{600} \times 100)}{\text{Cost of growth media } (\frac{\text{€}}{\text{l}})} \quad (3.3)$$

OD_{600} values are multiplied by a factor of 100 to re-scale.

3.3.2 Experiment 2: Generation of growth curves

To establish growth rates based on viable cell counting, an inoculum from the same *S. pasteurii* stock culture described in Experiment 1 was grown in 50 ml of Brain Heart Infusion broth (37 g/l) with the aseptic addition of urea (20 g/l). 50 Erlenmeyer flasks were prepared with 50 ml of either BHI or MM broth (as detailed in Table 3.1), 48 of which were inoculated and used for hourly sampling up to 24 hours, and two uninoculated flasks which served as the controls. The individual flasks were liquid inoculated at 1% (vol/vol) with 500 μl from the inoculum solution and incubated at 30°C in an orbital shaker at 115 RPM. Each hour, 100 μl of the culture was retrieved from a flask and transferred into Eppendorf vials filled with 900 μl 1.0 M Phosphate-buffered saline (PBS) solution to avoid early lysing of the bacterial cells (Van Paassen, 2009). Optical density was measured on the remainder of the culture in that flask in triplicate. Next, a series of multiple dilution steps from 10^{-1} to reach a final dilution of 10^{-7} were performed. After dilution, the Eppendorf vials were vortexed, and 100 μl of each were auto-pipetted onto BHI agar plates and spread with a plastic cell spreader in triplicates for each dilution. The spread-plated agar plates were incubated for 24 hr at 30°C and colony counts were performed afterwards. The calculation of viable colonies grown was calculated using (Eq. 3.4):

$$\frac{\text{CFU}}{\text{ml}} = \frac{\text{Colony count}}{\text{Sample volume} \times \text{Dilution}} \quad (3.4)$$

The generation time in the exponential phase of the growth curve between four and ten hours was then calculated using (Eqs. 3.5-3.6) (Powell, 1956):

$$\text{Growth rate } r = \frac{\log N_t - \log N_0}{0.301 \times t} = \left[\frac{\text{generations}}{\text{hr}} \right] \quad (3.5)$$

where N_t is defined as number of viable cells generated at time point t in the exponential phase, and N_0 is the number of viable cells generated at the onset of the exponential phase (end of lag-time), t is the time interval in hr.

$$\text{Generation time } g = \frac{1}{r} = \left[\frac{\text{mins}}{\text{generation}} \right] \quad (3.6)$$

3.3.3 Experiment 3: Enzyme kinetics

In Experiment 3, enzyme kinetics were established based on an elemental analysis for the two selected media, BHI and Marmite (Appendix A, S1.1). Biological reagents have no single molecular structure and are generally an unspecified mixture of known compounds. Consequently, molecular weight is not as informative as a measure of the carbon or nitrogen available in the media. In order to compare carbon and nitrogen availability, an elemental analysis of each growth media was conducted to determine the mass of total carbon and nitrogen (S1). Then the total carbon and nitrogen, including urea, was benchmarked against commercially available Brain Heart Infusion media with a C:N ratio of 1.33 and the composition of the Marmite-based media was adjusted to achieve the same C:N ratio (Table 3.2).

Table 3.2 Broth preparation for Experiment 3

Experiment 3: Enzyme kinetics with selected nutrient media									
Sample Nr.	Amount of samples	Media	Constituents	Elemental analysis		Total Carbon and Nitrogen		C:N ratio	Concentration
	$V_t = 50$ ml in duplicates			TC (%)	TN (%)	TC (g/l)	TN (g/l)	(-)	
1	2	BHI	Brain Heart	37.2	10.9	13.76	4.03	1.33	37.00
			Infusion						
			Urea	19.9	46.6	3.98	9.32		20.00
2	2	MM	Marmite	28.9	7.4	13.76	3.52	1.33	47.60
			Urea	19.9	46.6	4.30	10.07		21.60

The BHI broth (37 g/l) or the yeast-based Marmite (MM) growth medium (47.6 g/l) was then dissolved in distilled deionised water. The pH of the Marmite media was adjusted to 7.4 ± 0.2 with drops of a 4 M sodium hydroxide solution (NaOH) to have the same initial pH as BHI. Each broth media was sterilised by autoclaving at 121°C for 15 min. Subsequently, urea was added by aseptic filtration: 20 g/l for BHI and at 21.6 g/l for MM. Both growth media were loop inoculated (culture provided from BHI agar plates), and the culture was grown at 30°C in an incubator shaker at 115 RPM for 20 hrs. Bacteria from both media were gram-stained and were gram-positive (Appendix A, S2). Finally, the bacterial suspensions were produced by ‘harvesting’ the bacteria from the growth media by centrifuging (at 6000 RPM for 8 min), decanting the supernatant, and resuspending the cell pellets with either Phosphate-

buffered saline (PBS) or sterilised tap water, to achieve an optical density (OD_{600}) = 1.0. For the kinetics experiment, it was considered important to resuspend the centrifuged bacterial cells in 1.0 M PBS solution in order to keep the bacterial cells viable and to avoid osmotic shock (Van Paassen, 2009); sterilised tap water was used for the investigation of the biomineral precipitates, to avoid the formation of calcium phosphates.

A series of substrate-dependent reactions were conducted on centrifuged bacterial suspensions, grown in commercial BHI medium and the alternative nutrient media Marmite, with an OD_{600} of 1.0, to determine the enzyme kinetics. Since urea is a non-ionic compound, bacterial ureolysis results in dissociation into ammonium and carbonate ions which increases the electrical conductivity of the aqueous solution. Therefore, electrical conductivity (EC) measurements were monitored over a time interval of one hour, at 0, 1, 2, 3, 4, 5, 6, 10, 15, 25, 40 and 60 min, to calculate the reaction rates of the enzyme (r_u) for a range of substrate concentrations [urea] (6 per media; starting at 333 mM urea concentration in 333 mM increments until 2000 mM). The reaction rate is calculated based on the slope (k_{EC}) of the measured electrical conductivities in the first five-minute time interval (linear section of EC) for each substrate concentration (Eqs. 3.7-3.8).

$$k_{EC} [\text{urea}]_i = \frac{\Delta EC}{\Delta t} \quad (3.7)$$

$$r_u [\text{urea}]_i = k_{EC} [\text{urea}]_i \quad (3.8)$$

The reaction rates determined experimentally were fitted with the Michaelis-Menten enzyme kinetics model described by (Johnson and Goody, 2011) in (Eq. 3.9):

$$r_u = - \left(\frac{r_{u\max} [\text{urea}]}{k_m + [\text{urea}]} \right) \quad (3.9)$$

$$\frac{1}{r_u} = \frac{k_m}{r_{u\max} [\text{urea}]} + \frac{1}{r_{u\max}} \quad (3.10)$$

The Michaelis-Menten enzyme kinetics model is a function of substrate concentration [S] and is defined by two parameters: (1) $r_{u\max} = v_{\max}$, which is the maximum reaction rate and (2) the Michaelis-Menten constant (k_m), which is the concentration of the substrate required to reach half of the maximum reaction rate ($r_{u\max}/2 = v_{\max}/2$). These parameters are usually calculated from the Lineweaver-Burk transformation (Lineweaver and Burk, 1934) which represents a linearisation of the Michaelis-Menten hyperbolic curve plotted as $(1/r_u)$ vs $(1/[\text{urea}]_i)$ as represented in (Eq. 3.10). The intercept on the y-axis is equal to $(1/r_{u\max})$, and

the intercept on the x-axis is equal to $(-1/k_m)$. The model parameter k_m was used to define the shape of the model curve (Eq. 3.9) and is used to compare the enzyme kinetics of the two selected growth media in terms of their enzyme affinity to the substrate.

3.3.4 Determination of mass of carbonates precipitate

Four different precipitation experiments were performed in triplicate to compare the mass and types of the precipitated biominerals from: (1) bacteria centrifuged from BHI and Marmite broth, to roughly mimic the sequence of injections with the cementing solution likely replacing much of the microbial growth media, and (2) bacteria not separated from BHI and Marmite growth media (as a semi control to the centrifuged experiments). For the centrifuged bacterial suspensions, the optical density was adjusted to 1.0 by dilution with sterile tap water to have the same biomass concentration in each suspension, whereas in the case of the non-centrifuged samples, the turbidity of the bacterial suspension was for BHI ($OD_{600} = 1.36$) and MM ($OD_{600} = 1.26$) prior to addition of the cementing solution.

6 ml of cementing solution (1.4 M equimolar urea/ $CaCl_2$) and 6 ml of bacterial suspension (either centrifuged or with broth from BHI and MM media) was added to 15 ml centrifuge tubes, all in triplicate and then placed on an orbital shaker at 115 RPM with a temperature control at 20°C. After 72 hr the biominerals were collected from the liquid media by centrifugation with an Eppendorf 5804 R Centrifuge at 6000 RPM for 8 min. The supernatant was decanted, and the collected sediment pellet was resuspended in sterilised tap water, vortexed and re-centrifuged, and the supernatant was decanted to remove any remaining urea and $CaCl_2$. The resulting precipitates were air-dried at 37°C in a Gallenkamp incubator for 72 hr after which the collected precipitates were weighed to determine the mass of the biominerals and for characterisation via X-ray diffraction.

3.3.5 Characterisation of calcium carbonate biominerals via X-ray powder diffraction analysis

To determine the nature of the precipitated biominerals, X-Ray Powder Diffraction (XRD) analysis was carried out on the dry powdered solid samples, using an X-ray Diffractometer (Bruker D8 Advance) with a $Cu K_\alpha$ shutter tube generating X-rays of wavelength 1.5406 Å (0.15406 nm). Patterns were collected over the 2θ scan range 5 - 80° using a step size of 0.02°, a scan speed of 0.6 s/step and a 0.6 mm X-ray slit size to reduce the divergence of the X-ray

beam. Samples were mounted on a flip stick and were continuously rotated during XRD pattern collection. Initial data evaluation was performed with DiFFRAC EVA, and quantitative information on the ratios of calcite and vaterite was obtained through a Rietveld refinement with TOPAS using the ‘cifs’ (crystallographic information files) for calcite (Markgraf and Reeder, 1985) and vaterite (Kamhi, 1963).

3.4 Experimental Results

3.4.1 Experiment 1: Growth media screening

Figure 3.1 presents the results of the growth media screening experiments. There are relatively small differences in the growth of the bacteria over 24 hr as shown by the values of OD_{600} in Fig. 3.1a. Figure 3.1b presents the efficiency yield (determined from Eq. 3.3) of the six different nutrient media over a 24 hr time period. The alternative nutrient media Marmite (amended with sodium chloride and urea) showed a considerably higher efficiency yield (followed by bacteriological yeast extract) compared to the other nutrient media investigated, this is due to its lower cost. For this reason, the Marmite-based media was selected for further study. BHI, although one of the least cost-efficient growth media Fig. 3.1b, was selected for further study as it has the highest growth rate Fig. 3.1a and is commonly used as the nutrient source for *S. pasteurii* growth in MICP laboratory and field tests (Minto *et al.*, 2016; Tobler *et al.*, 2018).

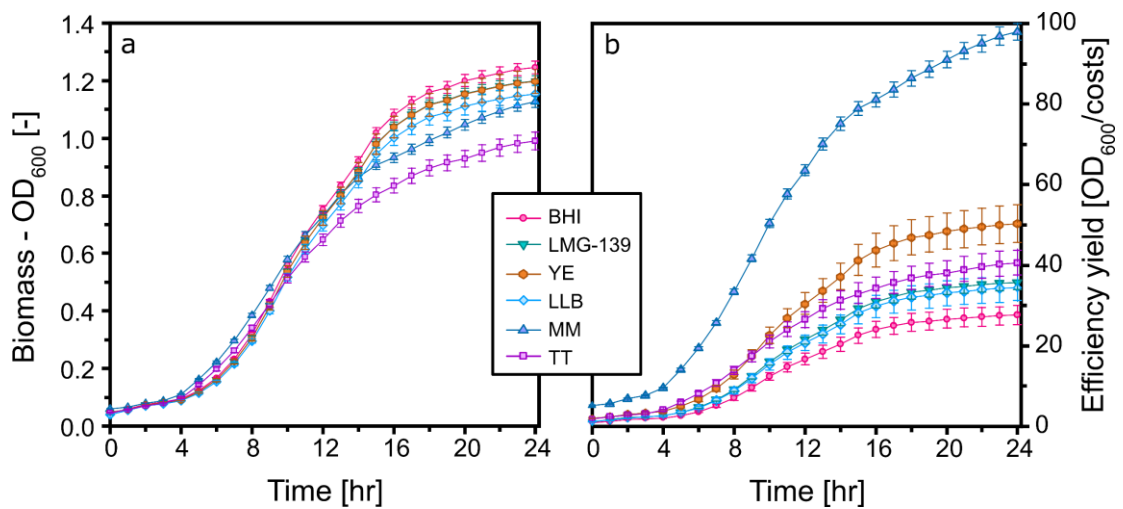


Figure 3.1. Summary of the growth media screening experiments, (a) OD_{600} and (b) the efficiency yield over a 24-hour period. The error bars represent the average $\pm$ SD of n = 6 replicates.

3.4.2 Experiment 2: Growth curves of *S. pasteurii* via plate counting and optical density

Figure 3.2 shows the growth curves of *S. pasteurii* grown in BHI and Marmite broth (a) and the relationship between colony forming units and optical density (OD_{600}) for cells grown from these media on BHI agar (b). The mean growth rate r was calculated for the exponential phase of the growth curve according to Eq. 3.5 and 3.6 and resulted in $r = 0.58$ and 0.54 generations/hr for BHI and MM, respectively, which corresponds to a generation times of $g = 103$ and 110 min/generation. The growth curves in Fig. 3.2a indicate almost similar bacteria reproduction during the early exponential phase, while the Marmite medium seems to move quicker into the late exponential growth phase, peaking at a lower biomass production after 24 hr when compared to the BHI. It is evident in Fig. 3.2b that there is a linear relationship between the number of colony forming units and OD_{600} between $0.4 - 1.0$ OD_{600} with an $R^2 > 0.99$ for both BHI and MM. This indicates that there is no significant difference growing *S. pasteurii* in the Marmite broth.

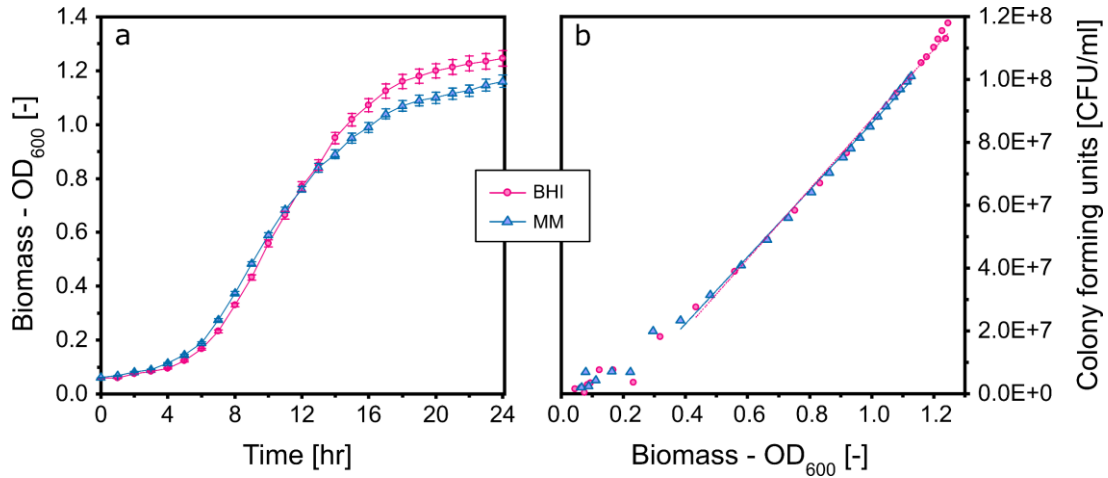


Figure 3.2. Growth curves of *S. pasteurii*, (a) in BHI and Marmite broth; b) linear relationship between CFU/ml vs OD_{600} for BHI and Marmite broth. The error bars represent the average $\pm$ SD of (a) $n = 6$ and (b) $n = 3$ replicates.

3.4.3 Experiment 3: Investigation of the enzyme kinetics

Figure 3.3a presents the rate of ureolysis against urea concentration for bacteria grown in BHI and Marmite broth. The enzyme kinetics plot indicates that, as expected, the rate of ureolysis

increases with increasing concentration of urea until the point of enzyme saturation is reached, when the maximum rate of reaction (r_{umax}) is achieved regardless of the increasing substrate concentration. These results are fitted with the Michaelis-Menten model using the corresponding k_m and r_{umax} values for each nutrient media, which were derived using the Lineweaver-Burk transformations, presented in Fig. 3.3b. The data show a good fit to the Michaelis-Menten model indicating that the enzyme reaction rates for the bacteria grown in BHI media are higher at low substrate concentrations, due to a higher substrate affinity compared to the bacteria grown in Marmite. For BHI, the half-maximum saturation constant results in $k_m = 310$ mM and for Marmite 572 mM. This is in line with studies conducted on whole-cell kinetics for cells grown in BHI (Lauchnor *et al.*, 2015; Minto, Mountassir and Lunn, 2019). A summary of the fitted model parameters is presented in detail in Table 3.3.

Table 3.3 Results of the enzyme kinetics of BHI and MM medium. All the experiments on kinetics were conducted in duplicates $n = 2$

Experiment 3: Results of enzyme kinetics of growth media					
Media	Slope = k_m/r_{umax}	y-axis intercept	k_m (mM)	$r_{\text{umax}}/2$ (mM/min)	r_{umax} (mM/min)
(Lineweaver-Burk Plot)					
Brain Heart Infusion	54.39(9)	0.175(6)	310(4)	2.85(1)	5.70(2)
Marmite	89.23(6)	0.156(4)	572(9)	3.20(2)	6.41(4)

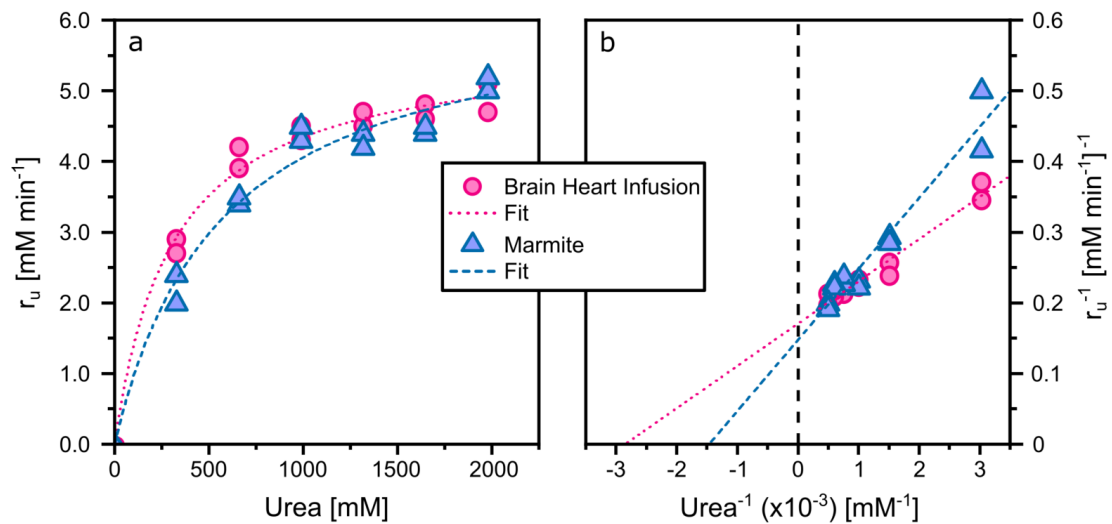


Figure 3.3. Summary of the enzyme kinetics experiment, (a) the hyperbolic Michaelis-Menten Fits and (b) the linearised version of the Michaelis-Menten curve is represented as the Lineweaver-Burk plot. In the case of Marmite and Brain Heart Infusion media, the maximum reaction rates are very similar. Dotted and dashed lines indicate the curve fits.

3.4.4 Comparison of precipitated carbonate biominerals

The chemical reaction and precipitation of carbonate biominerals using the bacteria cells in the broth, for both Marmite and BHI, started immediately upon addition of the urea and calcium chloride for all experiments in which the bacteria were not centrifuged (i.e., regardless of the growth media used). This occurs since much of the urea (20 g/l) in the growth media has already been hydrolysed and, hence, the pH is already at levels suitable for CaCO_3 precipitation. In comparison, bacterial suspensions that were centrifuged showed a distinct delay (on average 5 min) to the onset of precipitation. This occurs because the urea molecules first need to diffuse through the cell membrane before hydrolysis can occur (Lauchnor *et al.*, 2015).

Figure 3.4 shows the experimentally determined mass of precipitates, compared to the theoretical possible mass gain, after 72 hours for the four experimental conditions. For 6 ml of a 1.4 M equimolar cementing solution and 6 ml of bacterial suspension, this equates to a theoretical maximum achievable weight gain of 0.84 g CaCO_3 . The dry mass of the precipitates reveals that a similar amount of biomineral precipitation occurred with all four of the bacterial solutions (Fig. 3.4). It should also be noted that the presence of the broth contributes significantly to the final total mass as even in the control series where the broth suspensions were not inoculated with bacteria but cementing solution was added, a weight gain of 0.1 – 0.3 g is observed (Fig. 3.4). Visual observations concluded that the presence of the nutrient media simply accelerates the calcium carbonate precipitation process but does not greatly alter the final total mass of precipitates (Table 3.4).

Table 3.4 Results of the mass difference of the precipitates from BHI and MM medium non-centrifuged and centrifuged. All the precipitation experiments were conducted in triplicates $n = 3$

Experiment 3: Final total mass of biominerals precipitated				
Sample Nr.	Sample Name	Mass of biominerals (g)		% difference in mass
		non-centrifuged bacteria	centrifuged bacteria	
1	BHI_1	1.061	1.000	6.10
	BHI_2	1.063	1.013	4.94
	BHI_3	1.059	1.025	3.29
2	MM_1	1.100	1.073	2.43
	MM_2	1.087	1.069	1.66
	MM_3	1.090	1.065	2.32

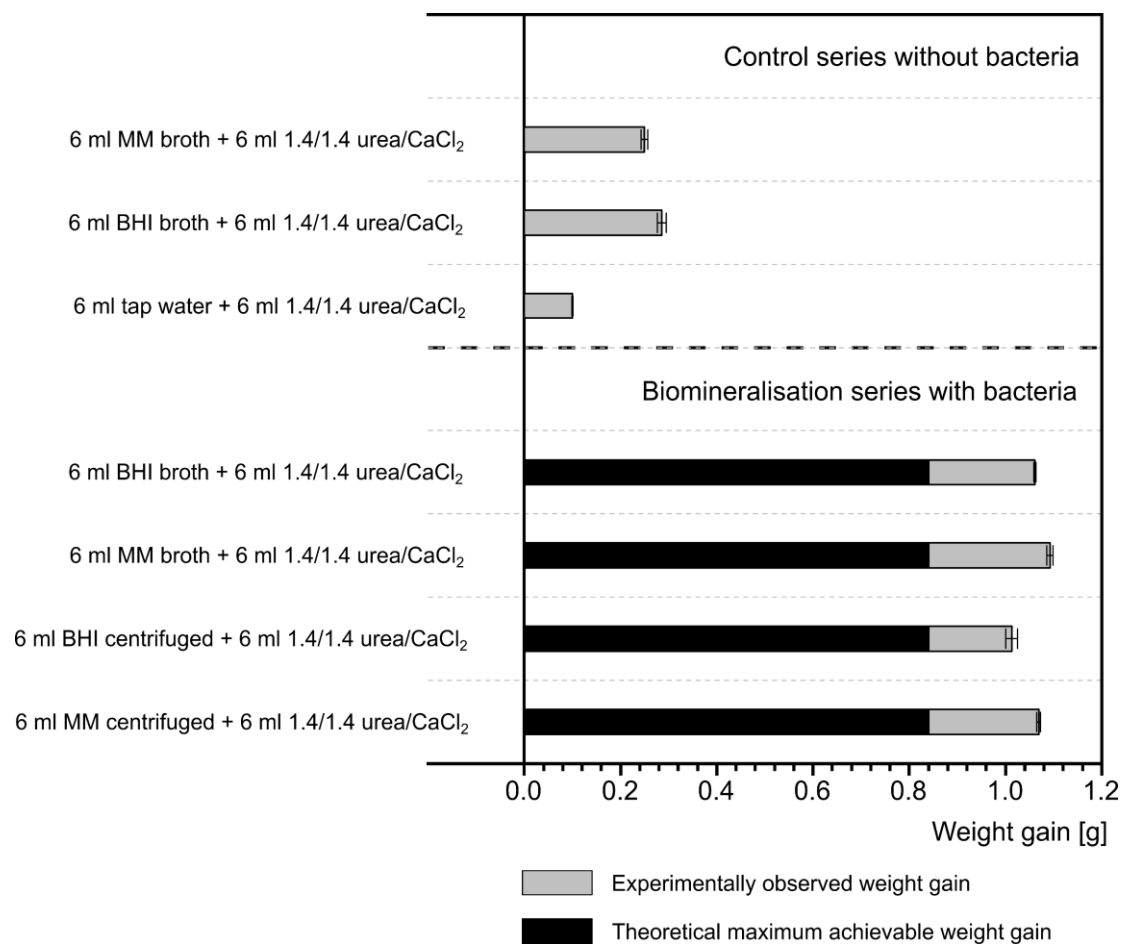


Figure 3.4. Comparison of the dry weight CaCO_3 precipitated from centrifuged/non-centrifuged *S. pasteurii* bacterial suspension (grey) compared to the theoretical possible weight gain (black). The error bars represent the average $\pm$ SD of $n = 3$ replicates.

3.4.5 Characterisation of calcium carbonate biominerals

Results of the XRD analysis for biominerals precipitated with Brain Heart Infusion and Marmite as growth media are shown in Fig. 3.5 and Table 3.5. Calcium carbonate precipitation occurred in all of the tested samples. When the microbes were separated from the media (BHI) centrifuged and MM centrifuged, Fig. 3.5) and resuspended in sterilised tap water prior to adding the urea and cementing solutions, calcite was the only detectable biomineralisation product (Table 3.5). When the urea and cementing solutions were added to the media (BHI broth and MM broth, Fig. 3.5), calcite and vaterite were both detected as the final biomineralisation product with 52.9% calcite and 47.1% vaterite in BHI broth and 1.9% calcite and 98.1% vaterite in MM broth (Table 3.5). These observations suggest that the media used influences the final biomineralisation polymorph and that the plant-based media

MM has a preferred tendency to preferentially form vaterite, the metastable form of calcium carbonate. The difference in the final polymorph in the BHI and MM broth experiments, additionally indicates that the BHI broth seems to have less influence on the associated calcium carbonate crystal polymorph when compared to Marmite media.

Under standard conditions, the Ostwald step rule would predict the crystallisation sequence (Radha *et al.*, 2010), describing how the transformation from a non-crystalline to crystalline carbonate polymorph proceeds. Starting at the lowest thermodynamically state amorphous calcium carbonate (ACC), proceeding into the higher thermodynamically state vaterite, (then to aragonite under some conditions) and finally into calcite (Radha *et al.*, 2010; Rodriguez-Blanco, Shaw and Benning, 2011; Bots *et al.*, 2012). The presence of vaterite in the experiments with growth media, conforms to the crystallisation of ACC following the transformation sequence from ACC to calcite, via vaterite at low temperatures ($<30^{\circ}\text{C}$) (Bots *et al.*, 2012); whereas without any microbial growth media (BHI centrifuged and MM centrifuged), the microbial biomineralisation process reached the final stable calcium carbonate polymorph. Biologically precipitated calcium carbonate, in the presence of growth media, has been observed to produce metastable vaterite by other authors, particularly in the presence of organic molecules (Guan *et al.*, 2018). Where a large variety of organic and inorganic compounds are involved (Tobler *et al.*, 2015), both the kinetics of recrystallisation and the transformation sequence of ACC to calcite can be significantly affected. As described above, the nutrient media did not appear to significantly impact on the quantity of biomineral formation after 72 hours. In addition, with the detection of (traces of) calcite in all XRD patterns (Figure 3.5), we conclude that the nutrient media only impacts on the kinetics of recrystallization and not on the efficiency of the biomineralisation process.

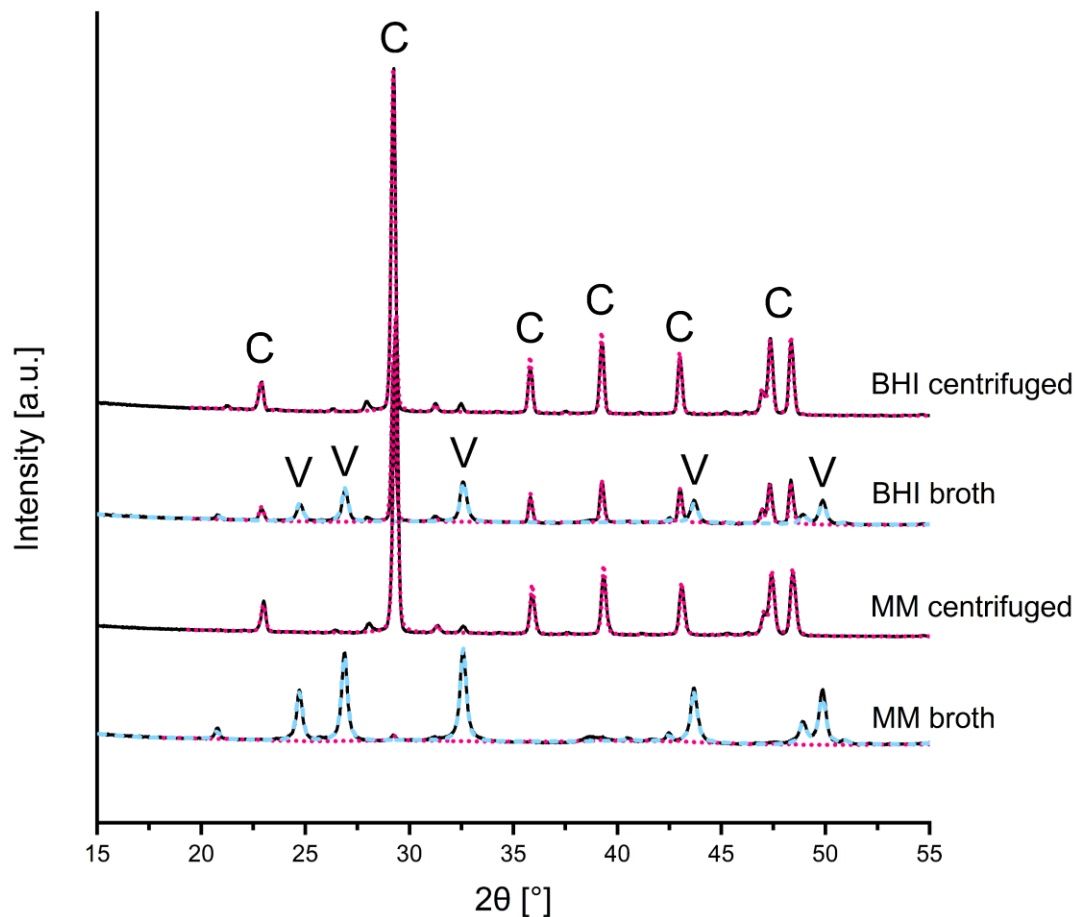


Figure 3.5. XRD patterns of calcium carbonate biomaterials and crystallisation products from top to bottom: (a) rhombohedral calcite; (b) mixed hexagonal vaterite with rhombohedral calcite; (c) rhombohedral calcite; (d) hexagonal vaterite without distinctive calcite peak at $2\theta = 29^\circ$. Dotted and dashed lines are Rietveld refinement results for calcite (magenta) and vaterite (light blue)

Table 3.5 Results of the semi-quantitative Rietveld refinements with TOPAS, on the phase composition of the precipitated carbonate biomaterials, the values between brackets are the uncertainties on the last digit of the weight percentage as determined by TOPAS

Experiment 3: Rietveld refinement results of biomaterials precipitated			
Sample Nr.	Reaction medium	Biomaterials detected	Weight percentage
1	BHI centrifuged	Calcite	100.0
2	BHI broth	Calcite	52.9 (3)
		Vaterite	47.1 (3)
3	MM centrifuged	Calcite	100.0
4	MM broth	Calcite	1.9 (2)
		Vaterite	98.1 (2)

3.5 Discussion

Our results show that Marmite is a promising plant-based media for the growth of *S. pasteurii* for MICP applications, producing similar growth rates as commercial BHI. Further, the alternative media did not result in significantly different quantities of biominerals precipitated. This suggests that from a biotechnological point of view, marmite could successfully be utilized within MICP engineering strategies.

However, for use of MICP within industry, it is important not only to consider the efficacy of the growth medium but also its cost and carbon footprint. Costs were estimated based on the treatment of 1 m³ of sand (Table 3.6) treated with 6 cycles of MICP using the approach of Minto *et al.* (2017). To estimate the costs, we assumed that each cycle comprises one injection of one pore volume of bacterial suspension, followed by injection of one pore volume of the cementing solution at a concentration of 1.4 M Urea/1.4 M CaCl₂. Comparison is made with the cost and carbon footprint of cement jet grouting; this was based on the assumption that only one pore volume is required. Cost comparison is only based on the material costs and does not include costs for the equipment needed for grouting. Unit costs for the biological and chemical reagents are given in the supplementary information (Appendix A, S3). The total costs of BHI broth with cementing solution is estimated at £5,440 in comparison to Marmite with the Urea/CaCl₂ cementing solution at £571, for the treatment of 1 m³ of sand (Table 3.7).

This highlights the importance of developing alternative nutrient media for cost reduction of MICP processes and subsequently for their utilisation within industrial applications. For large field applications, where extensive volumes of soil must be treated, this would represent a very significant cost saving. Further, Marmite is a commercial brand, hence, it is highly likely that the costs could be further reduced by identifying key elements for bacterial growth and developing a bespoke ‘Marmite-like’ mix. Traditional cement jet grouting was estimated to lead to costs of ~£94 (Gallagher, Spatari and Cucura, 2013) for the treatment of 1m³ sand.

Table 3.6 Assumptions of MICP and cement jet grout treatment volumes for field-scale applications

Assumptions for field-scale applications			
Porous Media	Volume (m³)	Porosity, Φ (-)	Pore volume, PV (m³)
Fine-grained sand	1.00	0.25	PV = Φ x V_t 0.25
	Injection cycles	Total pore volume (m³)	Total treatment volume (m³)
<i>Biogrout</i>			
Bacterial suspension	6.00	1.50	3.00
Cementing solution	6.00	1.50	
<i>Cement jet grout</i>			
Cement slurry (CEM I)	1.00	0.25	0.25

Table 3.7 Cost breakdown for upscaling to field-scale application and comparison

Cost breakdown for upscaling biogrout to field-scale applications compared to cement jet grouting							
Biogrout							
Bacterial suspension	Constituents	Concentration (g/l)	(£/l) lab-grade	(£/l) non-lab grade	(£/m³) field-scale	Costs for 6 injection cycles (£)	Costs (£)
BHI	Brain Heart Infusion	37.0	3.60	-	3596.40	5394.60	5399.00
	Urea	20.0	0.91	0.003	2.94	4.40	
MM	Marmite	47.6	0.35	-	350.34	525.50	529.91
	Urea	21.6	0.98	0.003	2.94	4.40	
Cementing solution							
1.4/1.4 M	Urea	84.1	3.83	0.012	12.34	18.52	41.35
	CaCl₂	154.1	0.89	0.015	15.23	22.84	
Total costs Biogrout with BHI nutrient media							5440.36
Total costs Biogrout with MM nutrient media							571.26
Cement jet grout							
Cement slurry	Constituents	Density (kg/m³)			(£/m³) field-scale	Costs for 1 injection cycle (£)	Costs (£)
Cement	CEM I	3150			384.55	96.14	96.14
Total costs Cement jet grout							96.14

Table 3.8 Greenhouse gas emission estimates/comparison per m³ biogrout and traditional cement jet grout

Greenhouse gas emissions (GHG) estimates for biogrout and cement jet grout			
<i>Biogrout</i>			
Bacterial suspension	Constituents	(kg/m ³)	(kg CO ₂ eq/kg constituent)
BHI	Brain Heart Infusion	37.0	16.00
	Urea	20.0	0.42 (Wood and Cowie, 2004)
MM	Marmite	47.6	0.36
	Urea	21.6	0.42
Cementing solution			
1.4/1.4 M	Urea	84.1	0.42
	CaCl ₂	154.1	0.89
Total kg CO ₂ eq/m ³ Biogrout with BHI nutrient media			767.54
Total kg CO ₂ eq/m ³ Biogrout with MM nutrient media			198.80
<i>Cement jet grout</i>			
Cement slurry	Constituents	Density (kg/m ³)	(kg CO ₂ eq/kg constituent)
Cement	CEM I	3150.0	0.86
Total kg CO ₂ eq/m ³ Cement jet grout			2709.00

The construction industry must reduce carbon emissions in order to meet national targets for net-zero carbon. In the UK and worldwide, the construction sector is estimated as being responsible for up to 7% of carbon emissions (Hertwich and Peters, 2009; Benhelal *et al.*, 2013). A major component of this is the ubiquitous use of cement in the construction industry. Replacing cement-based ground improvement applications by alternative methods, such as MICP will have a positive impact. The carbon footprint for both nutrient media are estimated here (Table 3.8) based on lifecycle assessments (LCA) for the production of greenhouse gases (GHG) per kilogram of beef (United Kingdom) – 16 kg CO₂ eq/kg (Schwarzer, 2012) and liquid yeast extract (EU yeast producers) – 0.363 kg CO₂/kg (PwC and COFALEC, 2012); which are the dominant components of BHI and Marmite. Urea and calcium chloride for the Urea/CaCl₂ cementation solution were estimated based on LCA conducted from fertiliser and chemical industry production. For urea, this value was 0.42 kg CO₂ eq/kg (Wood and Cowie, 2004) and calcium chloride with 0.89 kg CO₂ eq/kg (Winnipeg, 2012). Portland cement (CEM I) produced in the UK was estimated with emissions of 0.86 kg CO₂ eq/kg (GOV.UK., 2018). These figures are based only on greenhouse gas emissions associated with

the production of these materials and do not include any emissions associated with transport of materials to site, nor emissions associated with the injection of the grouts into the ground.

These figures show that the alternative plant-based nutrient media has the potential to reduced CO₂ emissions by ~75% when compared to the standard laboratory BHI media and by >90% when compared to cement grouts. The comparison of these carbon budgets highlights that MICP based on waste stream products (e.g., Marmite) has the potential to significantly contribute to much lower greenhouse gas emissions within the construction sector. Costs for such technologies are likely to reduce further as demand increases and optimisation of individual components occurs within the supply chain.

3.6 Conclusions

We have demonstrated that Marmite can be used as a nutrient media to grow *S. pasteurii* for MICP applications and that its performance is comparable to lab-grade growth media, such as BHI. Growth rates and generation times are in a very close agreement, as confirmed by the linear relationship presented between colony-forming units and optical density. Enzyme kinetics indicate that for Marmite, the initial reaction rate is lower, due to a slightly higher Michaelis-Menten constant, but with further progress, reaches almost the same reaction rate at maximum substrate concentrations.

Biom mineral precipitation experiments show that similar quantities of biominerals are precipitated, regardless of the growth media. XRD analyses also illustrate that when the bacteria were separated from the growth media, calcite formed regardless of the growth media used. For experiments where the growth media is present during the biomineralisation process, a combination of calcite and vaterite formed, with a higher percentage of vaterite being precipitated in the presence of the Marmite.

The cost analysis illustrates that the use of Marmite results in a cost almost 10 times lower than that of BHI broth, which could be a significant factor in making MICP commercially viable for large field-scale applications. Furthermore, based on available estimates of the embedded carbon in each of the nutrient broths and in the Urea/CaCl₂ cementing solution, it is anticipated that the yeast-based nutrient medium would produce ~75% less carbon emissions/kg compared to the BHI broth, and >90% less carbon emissions when compared to

conventional cement jet grouting. We propose that Marmite is a favourable new low-cost, low-carbon nutrient media suitable for MICP applications and that its considerably lower cost may facilitate the uptake of MICP as a low-carbon alternative to cement within the construction industry.

Chapter 4

Investigation of the effect of temperature and pressure on whole *Sporosarcina pasteurii* cells and their implications on ureolytic activity

4.1 Abstract

Microbially induced carbonate precipitation is currently being investigated for potential application in sealing leakage pathways in hydrocarbon wells in order to isolate former production zones from the seabed/ground surface as part of plugging and abandonment operations. Given that temperatures and pressures increase with depth in the subsurface, it is critical to understand how these elevated conditions influence ureolytic activity. In this study, we investigate the influence of temperature and pressure on whole *S. pasteurii* cells. Batch experiments were conducted within a temperature range of $T = 20 - 90^{\circ}\text{C}$ and pressures from $p = \text{atm} - 15.0 \text{ MPa}$. High specific ureolytic activity $k_{\text{urea}} = 15.9 \text{ mM urea hydrolysed/min/OD}$ was reached and maintained around the optimum temperature of the urease enzyme ($T = 60^{\circ}\text{C}$). Whereas bacterial suspensions exposed to temperatures above 70°C exhibited very high activity close to $k_{\text{urea}} = 17.9 \text{ mM urea hydrolysed/min}$ but activity significantly declined with exposure time. Elevated fluid pressure resulted in a slight increase of activity compared to atmospheric conditions. At a simulated depth of 1000 m ($p = 10.0 \text{ MPa}$, $T = 54^{\circ}\text{C}$) values of $k_{\text{urea}} = 13.9 \text{ mM urea hydrolysed/min/OD}$ were determined whereas at 1500 m ($p = 15.0 \text{ MPa}$, $T = 79^{\circ}\text{C}$)

activities were less than < 1.0 mM urea hydrolysed/min/OD. It is apparent that temperature is the limiting factor with respect to the application of MICP at depth. An alternative to microbially induced carbonate precipitation for higher temperatures, thermally induced carbonate precipitation, was investigated in the range $T = 120^\circ - 180^\circ\text{C}$. This study indicates that the deployment of MICP in the subsurface is limited to depths corresponding to temperatures $\leq 70^\circ\text{C}$. Thermally driven ureolysis has been shown here to be an alternative to microbially driven ureolysis for applications at higher temperatures.

4.2 Introduction

Microbially induced carbonate precipitation (MICP) is a process that has been widely investigated for ground engineering (DeJong, Fritzges and Nüsslein, 2006; Whiffin, Van Paassen and Harkes, 2007; Van Paassen *et al.*, 2010), environmental engineering (Fujita *et al.*, 2008; Gomez *et al.*, 2015; Hamdan and Kavazanjian, 2016) and structural engineering applications (Ramachandran, S. K., Ramakrishnan, V., & Bang, 2001; Achal, Mukerjee and Sudhakara Reddy, 2013). MICP uses ureolytic bacteria cells, i.e., bacteria which produce the urease enzyme. Urease is a bi-nickel metalloenzyme (Dixon *et al.*, 1975) that can catalyse the hydrolysis of urea (Krajewska, 2018) (Eqs. 4.1 - 4.2), producing ammonium and carbonate ions (Eq. 4.1). In a calcium rich environment, the nucleation of calcium carbonate is promoted on the surface of the bacteria cells (Stocks-Fischer, Galinat and Bang, 1999).



4.2.1 Effect of temperature and pressure on the urease enzyme

Here we focus on the potential deployment of MICP for sealing leakage pathways in hydrocarbon wells in order to isolate former production zones from the seabed/ground surface as part of plugging and abandonment operations. Given that temperatures and pressures increase with depth in the subsurface, it is critical to understand how these elevated conditions influence ureolytic activity. To date, most studies on the effect of temperature and pressure on ureolytic activity have been conducted using purified jack-bean meal (JBM) urease (*Canavalia ensiformis*), rather than the native urease from ureolytic bacteria i.e., *S. pasteurii* cells.

Illeova *et al.* (2003) investigated the inactivation of jack-bean urease with injection flow calorimetry in the temperature range $T = 55 - 87.5^{\circ}\text{C}$. Temperature in this range had as expected a negative impact on the activity of the enzyme (e.g. at $T = 55^{\circ}\text{C}$ and an exposure time of 50 hr the activity had reduced by almost 50% whereas at $T = 87.5^{\circ}\text{C}$ complete activity was lost in an exposure time of less than 1 hr). Feder *et al.* (2021) similarly showed that at $T = 50^{\circ}\text{C}$ jack-bean urease became $\geq 50\%$ inactivated after 37.3 hr whereas at 80°C $\geq 50\%$ inactivation was reached in only 5.2 min.

Feder *et al.* (2021) investigated jack-bean urease for use at higher temperatures claiming that *S. pasteurii* does not grow at temperatures above $T = 40^{\circ}\text{C}$ (Skorupa *et al.*, 2019). This indeed could inhibit growth of ureolytic bacteria *in-situ* (i.e. bio-stimulation) or in the context where ureolytic bacteria are injected and then biofilm growth is promoted over a period of days at elevated temperatures. However, for bio-augmentation strategies, where *S. pasteurii* cultures are cultivated *ex-situ* (and *in-situ* growth is not required), sufficient ureolytic activity at higher temperatures may still remain to trigger MICP. For this reason, it is important to understand how the ureolytic activity of *S. pasteurii* cells is influenced by temperature and exposure time.

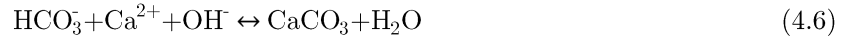
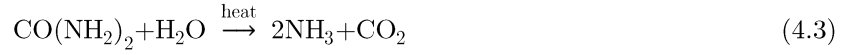
Krajewska *et al.* (2012) investigated the effect of temperature and pressure on jack-bean urease in a temperature range of $15 - 35^{\circ}\text{C}$ and pressures up to 132 MPa. This was performed with a stopped-flow spectrometer, whereas the pressure related measurements were conducted in a high-pressure stopped-flow reactor (van Eldik *et al.*, 1993). The pressure dependent measurements were the first ever reported for jack bean urease (Krajewska, Van Eldik and Brindell, 2012). Results in this study showed that temperature enhanced the activity of urease in the range of $15 - 35^{\circ}\text{C}$. Pressure tested in the range from $p = 5 - 132$ MPa at $T = 25^{\circ}\text{C}$ reduced the reaction rate of the enzyme but surprisingly not the half saturation constant k_m and therefore the binding of urea to the active site on the urease enzyme is not affected by pressure. Note also, that the reaction rate was only very slightly reduced in the range 5 - 40 MPa.

The studies reported above (Illeová *et al.*, 2003; Krajewska, Van Eldik and Brindell, 2012; Feder *et al.*, 2021) have reported the effects of temperature and pressure independently. In the case of applying microbial induced carbonate precipitation via bacterially produced urease from *S. pasteurii* in deep geological sections of a wellbore, it is of importance to demonstrate the performance of the bacterial strain under relevant downhole temperatures and pressures. Therefore, we investigate the influence of elevated temperatures and pressures individually and

in combination. These experiments are conducted in order to scope out the potential depths of deployment of MICP in a wellbore context.

4.2.2 Thermally induced ureolysis as an alternative for temperature beyond enzyme inactivation

It is known that urease enzyme inactivation does occur at some threshold temperature ($\sim 80^\circ\text{C}$) (Feder *et al.*, 2021). In this study we investigate thermal hydrolysis of urea at temperatures $> 80^\circ\text{C}$. Similar to MICP, thermal ureolysis results in the breakdown of urea into ammonium and carbonate ions resulting in a rise in pH, such that when a calcium source is supplied, calcium carbonate precipitation can occur (Eqs. 4.3 – 4.6):



The specific objectives of this experimental study were to: (1) investigate the influence of exposure to (a) constant elevated and (b) increasing temperatures on the ureolytic activity of bacterially produced urease by *S. pasteurii* and purified Jack-bean urease (*Canavalia ensiformis*), (2) investigate the influence of exposure to (a) constant elevated and (b) increasing fluid pressure on ureolytic activity of *S. pasteurii*. Conditions of increasing temperature and pressure are investigated to simulate fluids travelling downhole and injection at depth. (3) investigate the ureolytic activity of *S. pasteurii* under combined temperature and pressure conditions for assigned depths in a hydrocarbon wellbore; and (4) explore thermal ureolysis as an alternative to urease enzyme catalysis for deep subsurface temperature and pressure conditions.

4.3 Materials and Methods

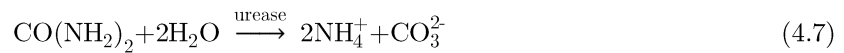
4.3.1 Bacteria, enzyme, and cultivation

Bacterial temperature and pressure exposure studies were conducted with the gram-positive ureolytic soil strain *S. pasteurii* (DSMZ, type 33) and for the enzyme exposure experiments with Jack-bean meal urease (Thermofisher Scientific, 99.9% purity). *S. pasteurii* were grown in Brain Heart Infusion broth (37.0 g/l BHI broth) with 20 g/l of urea supplement under sterile conditions in an incubator shaker for 24 hr at 30°C. The readily grown bacteria in late exponential growth phase were separated by centrifugation at 6000 RPM for 8 min, washed twice and re-suspended in sterile tap water to achieve a final optical density (OD₆₀₀) of 1.0. The OD₆₀₀ was measured spectrophotometrically at a wavelength of $\lambda = 600$ nm, using 1 cm path length cuvettes. This corresponds approximately to 8.6×10^7 CFU/ml calculated based on the linear relationship of *S. pasteurii* OD₆₀₀ to CFU/ml at low optical densities by Ramachandran, Ramakrishnan & Bang, (2001).

As a comparison, Jack-bean meal (JBM) was prepared at a concentration of 2.7 g/l of Jack-bean meal, in order to achieve the same ureolytic activity at $T = 20^\circ\text{C}$ and atmospheric pressure as the bacterial suspension at an an OD₆₀₀ = 1.0 (2.77 mM urea hydrolysed/min/OD). JBM was added to distilled deionised water into a beaker with a lid on a stir-plate and mixed at 150 RPM overnight (16 hr). Afterwards, the urease suspension was filtered through sterile 0.2 μm Millipore filters (Merck) prior to the experiment to remove non-dissolvable Jack-bean meal residue that could interfere with the conductivity measurement.

4.3.2 Measurement of ureolytic activity

The hydrolysis of urea is catalysed by the urease enzyme and produces out of a non-ionic substrate ionic compounds according to the following equation (Eq. 4.7) (Mitchell *et al.*, 2010):



The gain in ionic products, in this case through the breakdown of urea into ammonium and carbonate ions, creates an increase in the solution conductivity. That infers that the change in

electrical conductivity (EC) over time is proportional to the enzyme present in the hydrolysis of urea (Hanss and Rey, 1971; Lawrence and Moores, 1972; Grunwald, 1984).

The change in electrical conductivity over time is then converted into an amount of urea hydrolysed per unit time (mM urea hydrolysed/min/OD) by multiplying with a conversion factor that was experimentally investigated by endpoint calibration experiments where various known urea concentrations are hydrolysed by purified JBM urease or by bacterial produced urease (from *S. pasteurii*) until no further change in electrical conductivity was seen.

The ureolytic activity can also be normalised by the optical density to the bacterial suspension to give the specific ureolytic activity (k_{urea}) (Eq. 4.8):

$$k_{\text{urea}} = \frac{\text{CF} \times \Delta \text{EC}}{\Delta t \times (\text{OD}_{600}) \times \text{dilution factor}} \quad (4.8)$$

where CF represents the conversion factor from the EC measurement (mS/cm) to [mM urea hydrolysed/min) (Calibration relationship developed by Whiffin (2004): urea hydrolysed (mM) = 11.11 x change in electrical conductivity (mS/cm)) and is normalised by the optical density of the bacterial suspension measured photospectrometrically at a wavelength of $\lambda = 600$ nm. The dilution factor accounts for the addition of 1 ml of bacterial suspension to 9 ml of 555 mM urea in the measurement.

4.3.3 Temperature exposure experiments

4.3.3.1 Constant temperature

The prepared bacterial and enzyme suspensions were prepared in triplicate in 15 ml centrifuge tubes and were exposed to constant temperatures of $T = 20^\circ\text{C}$ (baseline temperature), 40°C , 60°C , 80°C and 90°C in a digital water bath with a Mettler Toledo InLab Pt1000 temperature probe ($20 - 150^\circ\text{C}$) for fixed exposure durations of 15 min, 30 min, 1 hr and 2 hr.

4.3.3.2 Ramping temperature

For the temperature ramping experiment, the water bath temperature was calibrated for each temperature to reach the target temperature ($T = 60^\circ\text{C}$, 70°C , 80°C and 90°C) in 30 min (Fig. 4.1). Bacterial suspensions in triplicate in 15 ml centrifuge tubes were ramped to the

temperature in 30 min and held constant for an additional 30 min to simulate transportation from surface to depth & injection (personal communication, OGTC 2018).

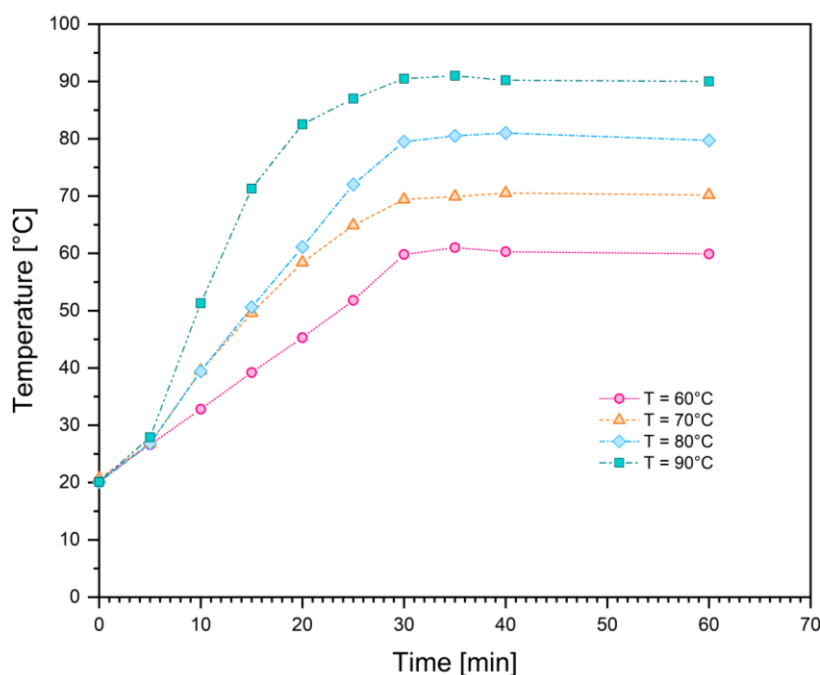


Figure 4.1. Ramping profiles of the water bath temperature. For every individual temperature exposure experiment the heating rate was adjusted to reach the target temperature ($T = 60^{\circ}\text{C}$, 70°C , 80°C and 90°C) in $t = 30$ min with an additional 30 min of a hold period to simulate transport from surface to depth and injection.

After exposure to temperature (either constant or ramped followed by constant temperature), the bacterial or enzyme suspensions were returned to laboratory standard temperature conditions by placing them into a water bath ($T = 20^{\circ}\text{C}$). Specific ureolytic activity was then determined by adding 1 ml of the bacterial or enzyme suspension to 9 ml of a 555 mM urea solution leading to a final urea concentration of 500 mM. Electrical conductivity was measured over a time interval of 5 min. The change in electrical conductivity over time was then converted to urea hydrolysed per unit time and optical density using equation (4.8).

4.3.4 Pressure exposure experiments

4.3.4.1 Constant pressure

The prepared bacterial suspensions (in triplicate) were injected into a modified Hassler-type stainless steel core-holder (Fig. 4.2). A PTFE liner was manufactured out of a PTFE rod, into which a hole was drilled from top to bottom, with a total volume of $V = 13$ ml of the injection fluid. To ensure that the hole within the PTFE liner was filled properly the sampling port was opened and injection proceeded until the bacterial suspension was observed to exit at the outlet. The outlet valve was then closed, followed by the 3-way valve in the line sequence to allow the fluid pressure to build up. The bacterial suspensions were exposed to constant pressures of $p = 1.0$ MPa, 5.0 MPa, 10.0 MPa and 15.0 MPa at standard laboratory temperature: $T = 20^\circ\text{C}$ for exposure durations of 15 min, 30 min, 1 hr and 2 hr.

4.3.4.2 Ramping pressure

In the pressure ramping experiment the highest pressure: $p = 15.0$ MPa was selected, and the fluid pressure was increased in 30 min to the target pressure and then held constant for an additional 30 min to simulate transportation from surface to depth & injection (personal communication, OGTC 2018). Pressure ramping was only conducted on one sample due to the complexity of ramping pressure using a constant pressure pump.

After exposure to pressure (either constant or ramped followed by constant pressure), the bacterial suspensions were returned to laboratory standard atmospheric conditions ($p = \text{atm}$, $T = 20^\circ\text{C}$) by decreasing the fluid injection pressure in steps and then opening the outlet valve of the sampling port. Specific ureolytic activity was then determined as described in section 4.3.2.

4.3.5 Combined pressure and temperature exposure experiment

4.3.5.1 Experimental setup of high-pressure and temperature core-holder

A schematic of the experimental setup used for this experiment is shown in Fig. 4.2. For the pressure and combined pressure and temperature exposure experiments a PTFE liner was manufactured out of a PTFE rod with a hole drilled through it from inlet to outlet with an

internal volume of $V = 13$ ml. The setup enabled recording of fluid pressure at the inlet and outlet of the core. Continuous fluid injection is achieved with a constant pressure Cole Parmer Masterflex Ultra-High-Pressure dual-piston pump (max. flow rate 12 ml/min, max. operating pressure 124 MPa) and the core outlet pressure is regulated with a Swagelok Model 1000D backpressure regulator. Aqueous injection fluids can be injected to the maximum pressure rating of the Hassler type core-holder of 15 MPa and maximum operating temperature of 90°C. Confining pressure is applied with an Enerpac P141, single-speed, hydraulic hand pump and was set for all experiments to $p = 15.0$ MPa (2175 psi).

The injection fluid passes from the high-pressure dual-piston pump through high-pressure injection lines (stainless steel) with an OD of 1/16", through a water bath heat exchanger that then connects to the high-pressure high-temperature stainless steel Hassler-type core-holder. Fluids can exit the core-holder for sampling at the sampling port or via the backpressure regulator into a waste reservoir. A fluid line that bypasses the core-holder is installed for servicing the pump at regular intervals during the experiment. This line is connected via two 3-way valves either side of the core-holder. The high-pressure and high-temperature (HPHT) core holder and the injection lines are thermally insulated to reduce the heat loss from the injection fluid.

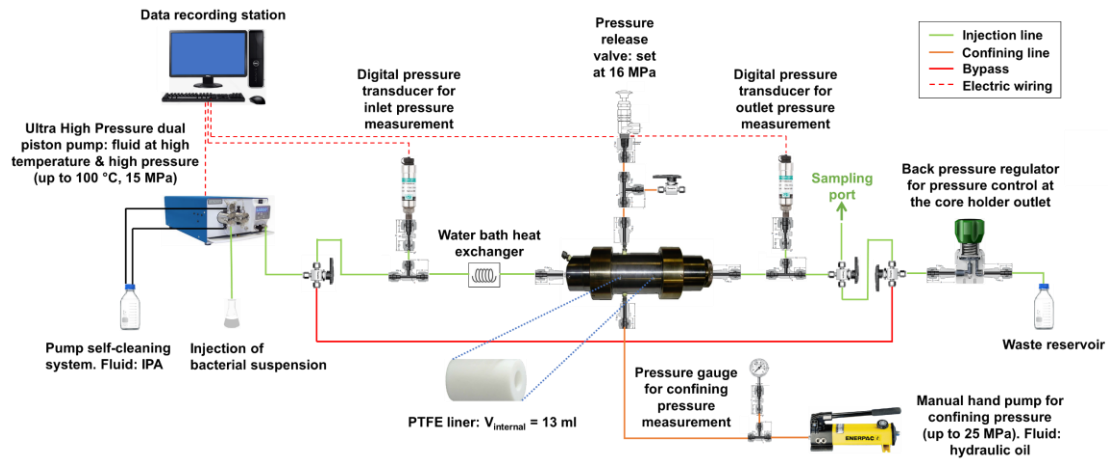


Figure 4.2. Schematic of the experimental setup of the pressure and combined pressure and temperature exposure experiments. The fluid pathway is indicated by the green line starting at the high-pressure pump passing a water bath heat exchanger reaching the inlet of the Hassler type core-holder with the PTFE liner and leaves at the outlet via the sampling port or backpressure regulator to a waste reservoir. The solid red line indicates a fluid bypass line to service the pump and backpressure regulator. The red dashed lines indicate the data logging system consisting of two pressure transducers at the inlet and outlet side of the core holder, as well as a data connection with the high-pressure pump for flow-rate monitoring and pressure setting connected to a data recording station.

4.3.5.2 Combined pressure and temperature exposure experiment

For a given depth, the likely formation temperature was determined based on the geothermal temperature gradient below the North Sea of $\sim 4.92^{\circ}\text{C}$ per 100 m depth and an average North Sea temperature of 5°C (Eq. 4.9):

$$\begin{aligned} \text{Formation temperature} &= (\text{Wellbore depth} \times \text{geothermal temperature gradient}) \\ &+ \text{average North Sea temperature} = [^{\circ}\text{C}] \end{aligned} \quad (4.9)$$

Given the wellbore depth, the fluid pressures (pore pressures) at the calculated injection depths were calculated assuming hydrostatic pressure (Eq. 4.10):

$$p_{\text{pore}} = \rho_w g h = [\text{Pa}] \quad (4.10)$$

Thus, to simulate well depths of 1000 and 1500 m, prepared bacterial suspension were exposed to a combination of pressure and temperature of $p = 10.0 \text{ MPa}$ at $T = 54^{\circ}\text{C}$ and $p = 15.0 \text{ MPa}$ at $T = 79^{\circ}\text{C}$. Pressure and temperature were maintained for a duration of 1 hr. Fluids were heated to target temperature via hotplate and passed a water bath heat exchanger to maintain the fluid temperature. All the high-pressure injection lines were insulated.

After exposure to combined pressured and temperature, bacterial suspensions were retrieved via a sampling port on the experimental setup and returned to laboratory standard temperature conditions ($T = 20^{\circ}\text{C}$) by placing them into a water bath. Specific ureolytic activity was then determined as described previously.

4.4 Experimental Results

4.4.1 Temperature exposure experiments

4.4.1.1 Bacterial suspension exposure to constant temperatures

Figure 4.3 presents the results of the bacterial suspensions after exposure to different constant temperatures for up to 2 hr. The baseline specific ureolytic activity of the bacterial suspensions at room temperature 20°C was 2.8 mM urea hydrolysed/min/OD. Similar activity was observed after exposure to 40°C, even up to 2 hr duration. As the temperature increased to 60°C, specific ureolytic activity was increased to 13.0 mM urea hydrolysed/min/OD after 60 min exposure and it decreased slightly to 10.8 mM urea hydrolysed/min/OD after 120 min exposure. Exposure to a higher temperature is increasing ureolytic activity before prolonged duration acts to reduce it below that of the baseline activity (at 20°C). A similar trend was observed at temperatures of 80°C and 90°C, where at low exposure durations of 15 min and 30 min the bacterial suspensions showed maximum specific ureolytic activity. Longer exposure durations to the high temperatures led in the case of 80°C to zero ureolytic activity after an exposure time of 2 hr. Whereas at 90°C, the ureolytic activity was ultimately reduced to zero after an exposure of only 30 min. This is due to the fact that the urease enzyme is a protein and as the environmental conditions are altered (i.e., at higher temperatures) the typical lock and key configuration between the substrate and the enzyme cannot be achieved anymore, urease is getting denatured and thus losing its ability to catalyse ureolysis. Furthermore, Fig. 4.3 illustrates that the optimum temperature for urease is around $T = 60^{\circ}\text{C}$, where enhanced ureolytic activity could be maintained for a longer duration.

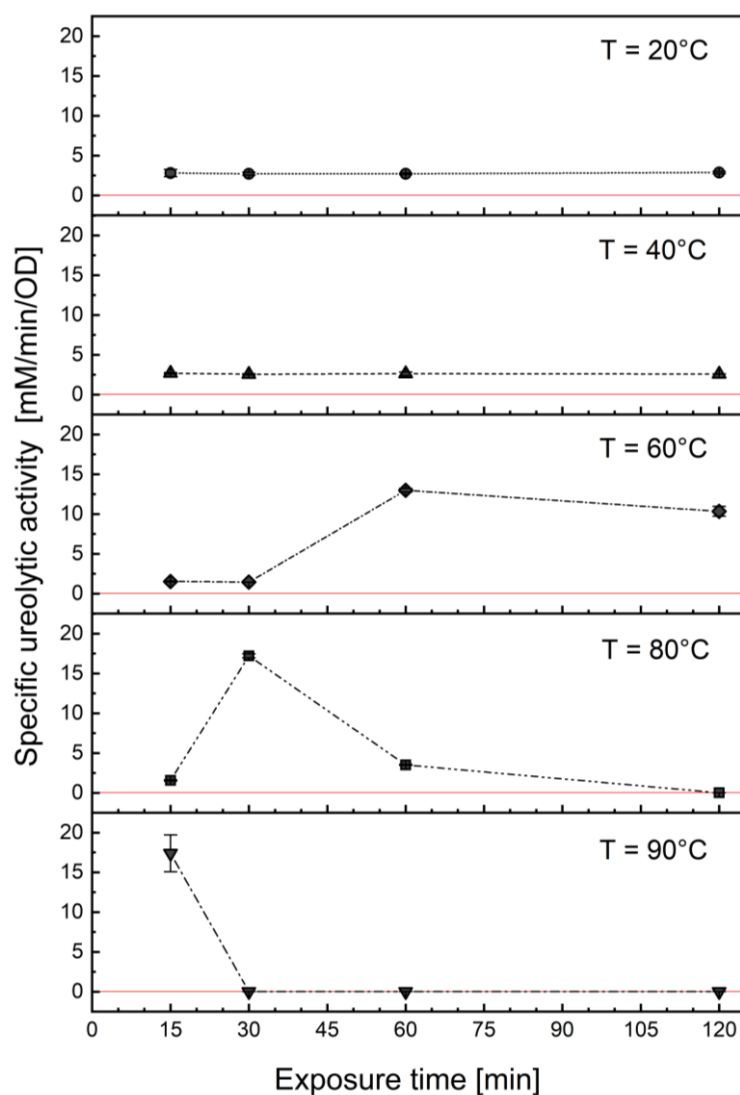


Figure 4.3. Constant temperature exposure results: Specific ureolytic activity for bacterial suspension exposed to different temperatures, from top to bottom: $T = 20 - 90^{\circ}\text{C}$ and for different durations: $t = 15 - 120$ min. All experiments were conducted in triplicate. Where error bars cannot be detected they might be hidden by the datapoint.

4.4.1.2 Plant-based urease exposure to constant temperatures

Figure 4.4 presents the results of the JBM suspensions after exposure to different fixed temperatures. At 40°C and 60°C the specific ureolytic activity remains similar to that of the baseline (at 20°C) regardless of exposure duration. However, on increasing temperature further to 80°C for 30 min leads or 90°C for 15 min, the activity is reduced almost immediately to zero, indicating deactivation of the enzyme. These results suggest that the plant-based urease enzyme is more sensitive to temperature and deactivation than the bacterial produced urease enzyme. Therefore, further investigations will focus primarily on the use of bacterial urease.

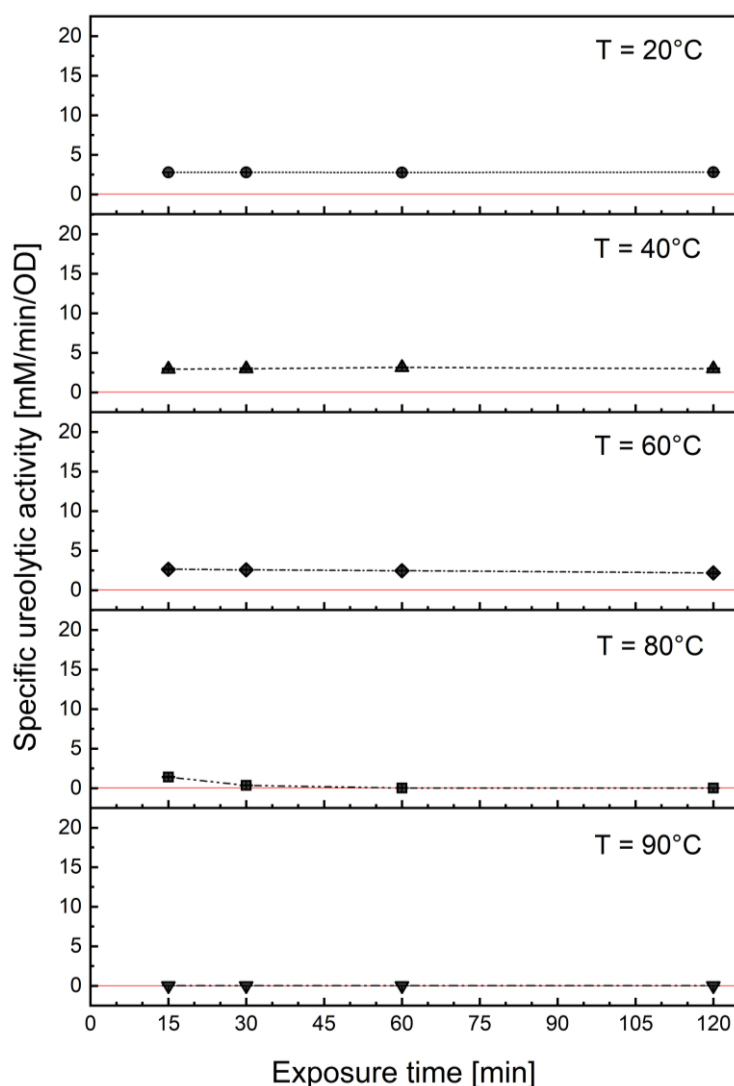


Figure 4.4. Constant temperature exposure results: Specific ureolytic activity for Jack-bean urease suspension exposed to different temperatures, from top to bottom: $T = 20 - 90^{\circ}\text{C}$ and for different durations: $t = 15 - 120$ min. All experiments were conducted in triplicate. Where error bars cannot be detected they might be hidden by the datapoint.

4.4.1.3 Bacterial suspension exposure to ramping temperature

Experimental results presented in Fig. 4.5 indicate that after ramping from ambient laboratory conditions to 60°C in 30 min and further maintaining the temperature at 60°C for another 30 min the specific ureolytic activity increased. However, at 70°C after ramping the activity is at 14.8 mM urea hydrolysed /min/OD, and after an additional 30 min at 70°C the activity is reduced to 0.06 mM urea hydrolysed/min/OD. For the ramping temperatures 80°C and 90°C tested no activity was observed, even after only 30 min of ramping to the target temperature. This indicates that the threshold for use of MICP may be limited to temperatures $\leq 70^{\circ}\text{C}$.

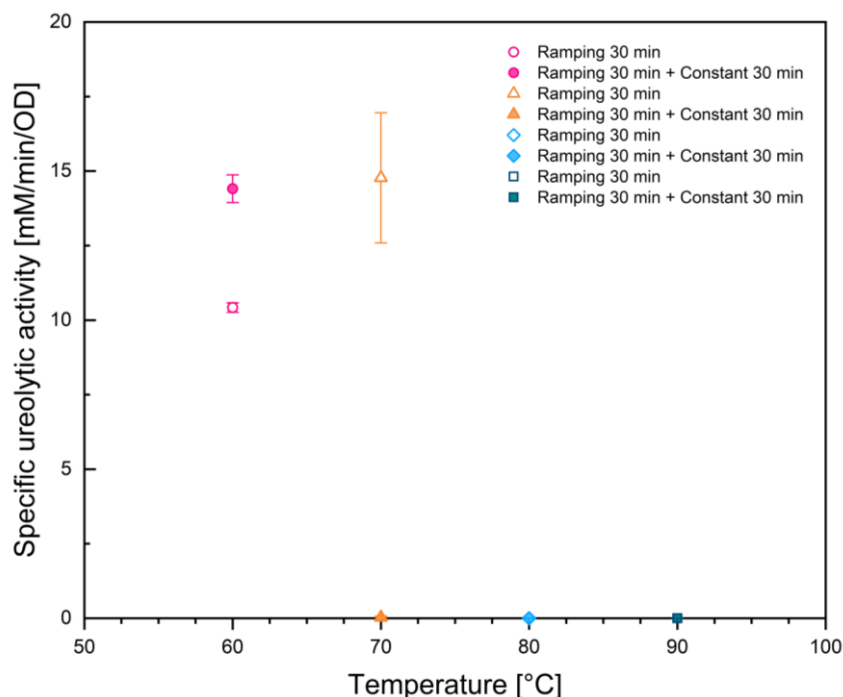


Figure 4.5. Temperature ramping and hold experimental results: Specific ureolytic activity after ramping to different temperatures in 30 min and additional hold of the temperature for 30 min. All experiments were conducted in triplicate. Where error bars cannot be detected they might be hidden by the datapoint.

4.4.2 Pressure exposure experiments

4.4.2.1 Bacterial suspension exposure to constant elevated pressures

Fig. 4.6 presents the results of the specific ureolytic activity of the bacterial suspensions after exposure to different constant pressures (all conducted at $T = 20^{\circ}\text{C}$). For the entire fluid pressure range investigated 1.0 – 15.0 MPa, the specific ureolytic activity was slightly higher than the baseline at atmospheric pressure conditions even up to 2 hr duration. At each pressure tested, a slight increase was observed in ureolytic activity after 30 min exposure, and thereafter activity decreased slightly with increasing exposure duration. Maximum ureolytic activity was observed at 1.0 MPa after 30 min exposure (3.77 mM urea hydrolysed/min/OD). As pressure was increased to 5.0 MPa, 10.0 MPa and 15.0 MPa lower ureolytic activity (after 30 min) in the range from 3.26 - 3.66 mM urea hydrolysed/min/OD was observed. This is in line with findings from Krajewska, Van Eldik and Brindell, (2012) stating that increased pressures are reducing the reaction rate of the enzymatic reaction. Effluent samples were collected after depressurisation of the core holder and bacterial cell damage was observed at higher exposed

pressures (Appendix B, S1). It is unfortunately unclear if this had happened during the experiment or as a side effect of the depressurisation of the core-holder.

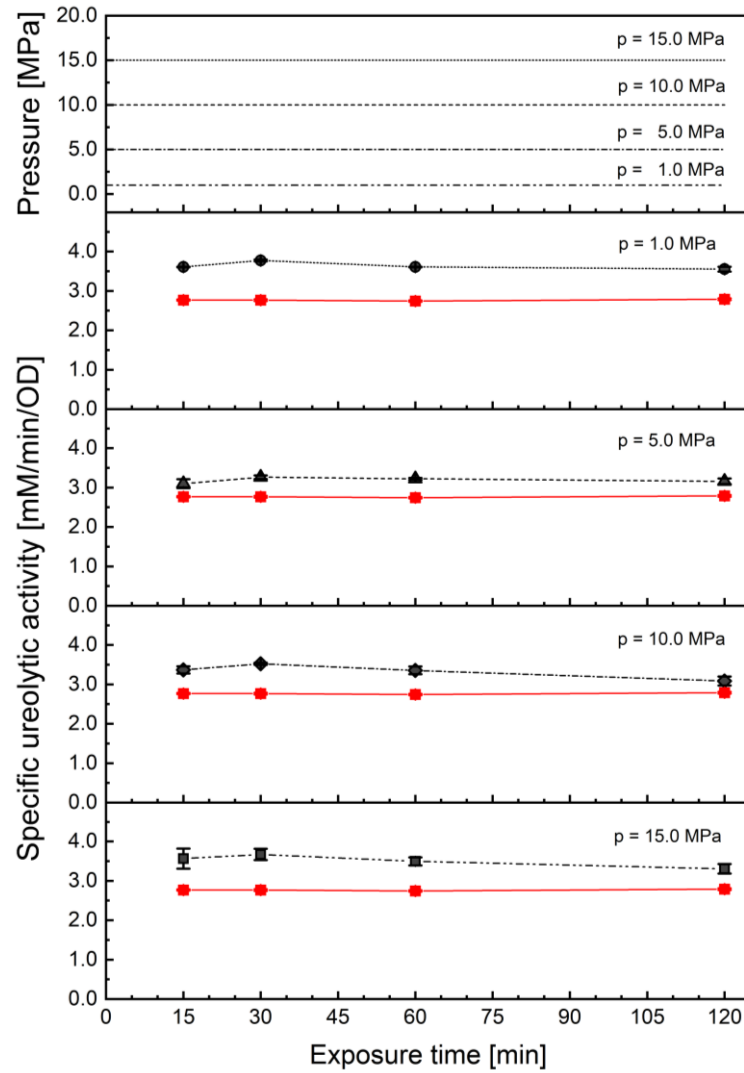


Figure 4.6. Constant elevated pressure results: Specific ureolytic activity for bacterial suspension exposed to different pressures and for different durations. The red datapoints are presenting the results of the baseline temperature exposure experiment ($T = 20^\circ\text{C}$ and $p = \text{atm}$). All experiments were conducted in triplicate. Where error bars cannot be detected they might be hidden by the datapoint.

4.4.2.2 Bacterial suspension exposure to ramped fluid pressure

Figure 4.7 presents 4 datapoints, in pink are bacterial suspensions which were ramped to 15.0 MPa in 30 min and then depressurised. In blue are bacterial suspensions which were ramped to 15.0 MPa in 30 min and then the fluid pressure was held constant at 15.0 MPa for a further

30 min before depressurising. These are compared with bacterial suspensions which were held constant at 15.0 MPa for 30 min (orange triangle) and 60 min (black square). It appears that there was a slight increase in specific ureolytic activity after ramping the pressure to 15.0 MPa in 30 min and maintaining the pressure constant for further 30 min the activity increased to 3.49 mM urea hydrolysed/min/OD (compared to 3.27 mM urea hydrolysed/min/OD after ramping only for 30 min). After ramping the pressure to 15.0 MPa for 30 min and holding the pressure constant for an additional 30 min at 15.0 MPa the activity showed exactly the same activity as when bacterial suspensions were held constant at 15.0 MPa for 60 min.

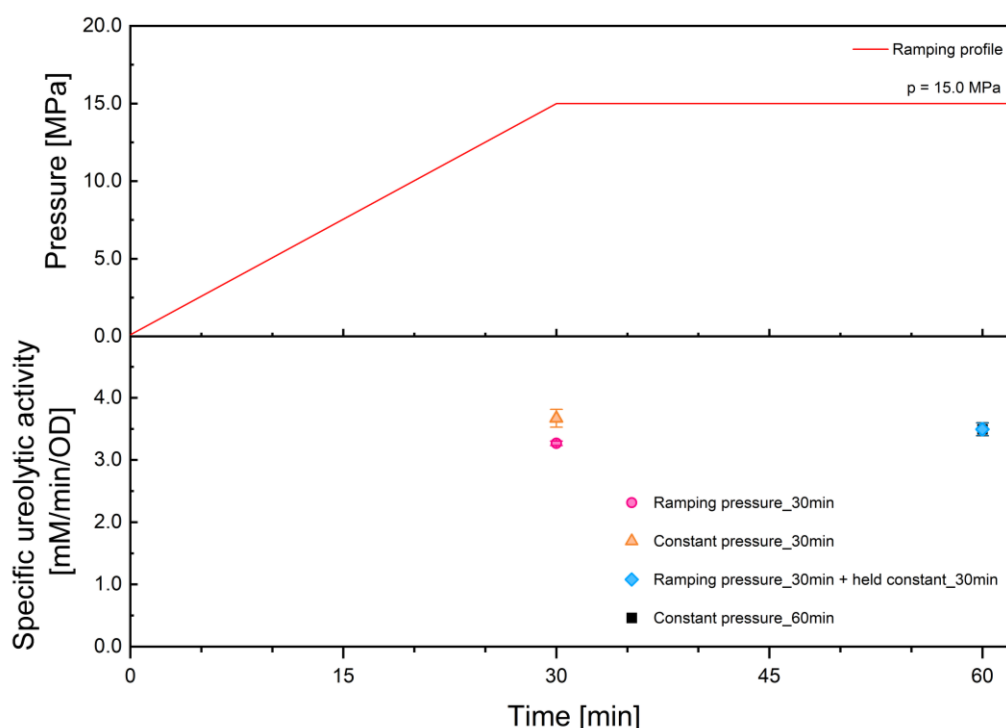


Figure 4.7. Specific ureolytic activity after ramping fluid pressure to 15.0 MPa in 30 min plus holding the pressure constant for further 30 min. All experiments were conducted in triplicate. Where error bars cannot be detected they might be hidden by the datapoint.

4.4.3 Combined pressure and temperature exposure experiment

4.4.3.1 Bacterial suspension exposure to combined pressure and temperature

In this section we present the results of the bacterial suspensions after exposure to combined pressures and temperatures relating to wellbore depths of 1000 and 1500 m (Table 4.1).

Table 4.1 Simulated pressure and temperature combinations for the bacterial suspension at well depth

Simulated <i>in-situ</i> wellbore pressure and temperature conditions		
Simulated depth [m]	Pore pressure [MPa]	Formation temperature [°C]
1000	10.0	54
1500	15.0	79

Figure 4.8 presents the specific ureolytic activity of bacterial suspension which were exposed to the target pressure and temperature conditions for 60 min. At a simulated depth of 1000m, (10.0 MPa fluid pressure and a temperature of 54°C) after 60 min of exposure, enhanced specific ureolytic activity is observed of 13.19 mM hydrolysed/min/OD (above the baseline of 2.8 mM urea hydrolysed/min/OD (red)). This is due to being near to the optimum temperature where the urease enzyme has its highest activity ($T_{\text{optimum}} = 60^{\circ}\text{C}$) (Krajewska, Van Eldik and Brindell, 2012; Feder *et al.*, 2021). At a simulated depth of 1500 m (a pressure of 15.0 MPa and 79°C), bacterial suspensions showed a significant drop in activity (12.71 mM urea hydrolysed/min/OD) related to temperature denaturation of the enzyme and possible pressure destruction of the bacterial cell wall.

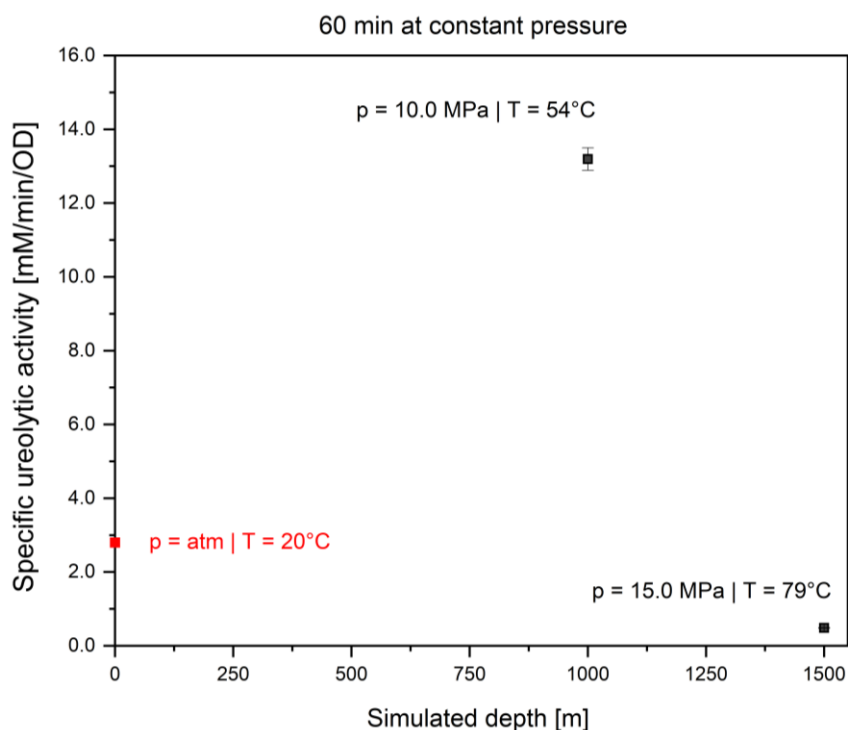


Figure 4.8. Specific ureolytic activity after combined pressure and temperature exposure. All experiments were conducted in triplicate. Where error bars cannot be detected they might be hidden by the datapoint.

4.5 Discussion

4.5.1 Influence of temperature

The current batch experiments conducted at elevated *constant temperature* on bacterial and enzyme suspensions found that temperature affects the activity of the urease enzyme. Temperatures close to $T = 60^{\circ}\text{C}$ were found to be the most effective ones leading to higher specific ureolytic activities than measured under the baseline conditions (20°C and atmospheric pressure) and could were shown to be maintained up to 2 hr. This finding is in agreement with (Krajewska, Van Eldik and Brindell, 2012; Feder *et al.*, 2021) which showed that the optimum temperature of the urease enzyme is $T = 60^{\circ}\text{C}$. At higher temperatures, high ureolytic activities could be achieved in this study, but inactivation of the enzyme occurred within short exposure durations. Results from the jack-bean meal urease, show more rapid inactivation of the enzyme at higher temperatures. This suggests that in the whole-cell experiments, it is the bacterial cell that may be protecting the urease enzyme such that activity is not straight away completely inhibited. Although, the results from bacterial urease from some published studies are consistent (Whiffin, 2004; Van Paassen, 2009) but limited due short exposure times (~ 10 min for each temperature tested). For JBM urease a possible explanation might be that the amount of urease available in jack-bean meal is limited compared to bacterial urease that is produced by a living organism through constitutive expression (Moblely and Hausinger, 1989) along its lifetime. *Temperature ramping* surprisingly had a more significant effect on the inactivation of the enzyme. Comparison with the fixed temperature exposure result of bacterial suspension at $T = 60^{\circ}\text{C}$ for 30 min showed an almost 5-fold increase in activity over the same time period, however after holding the temperature constant for another 30 min led to a similar activity. That infers that ramping the temperature is less lethal to the whole bacterial cell and its enzyme. While exposure to temperatures above $T = 70^{\circ}\text{C}$ might led to structural integrity issues of the cell wall and furthermore the cell protection of the enzyme is compromised, and inactivation is occurring.

4.5.2 Influence of pressure

The results of the effect of *constant elevated pressure* on *S. pasteurii* cells shows that there had occurred a slight increase in specific ureolytic activity (baseline experiment was executed at

earlier day). Over a 2 hr exposure duration, this did not lead to a significant increase or decrease in activity. The ureolytic activity seemed not to vary much with pressure, nor depend on the duration of exposure to a given pressure. Results from the ramping pressure experiments were comparable with the constant pressure experiments.

4.5.3 Influence of combined pressure and temperature

It is evident from the above results that temperature had the most significant impact on the ureolytic activity. This was confirmed in the combined pressure and temperature experiment where bacterial suspensions exposed to $T = 54^{\circ}\text{C}$ and $p = 10.0\text{ MPa}$ for 60 min exhibited an activity of $k_{\text{urea}} = 13.9\text{ mM urea hydrolysed/min/OD}$, similar to the activity achieved in suspensions exposed only to $T = 60^{\circ}\text{C}$ for 60 min ($13.0\text{ mM urea hydrolysed/min/OD}$). At the temperature and pressure combination of $T = 79^{\circ}\text{C}$ and $p = 15.0\text{ MPa}$, temperature had the most significant impact and led to a very low ureolytic activity ($0.48\text{ mM urea hydrolysed/min/OD}$).

Investigations beyond urease enzyme inactivation:

Thermally induced ureolysis

Given the results obtained, demonstrate urease inactivation in <60 mins at 70°C , thermally induced ureolysis was also investigated as a potential means of triggering carbonate precipitation at elevated temperatures. In comparison to bacterial ureolysis, where bacterial urease results in the breaking down of urea into ammonium and carbonate ions, in thermal ureolysis, the reaction is catalysed by temperature. For this experiment four elevated temperatures ($T = 120^{\circ}\text{C}$, 140°C , 160°C and 180°C) were considered.

4.6 Materials and Methods

4.6.1 Reaction media and high-pressure and high-temperature vessel

A 1.0 M urea stock solution (initial $\text{pH} = 6.7$) (ACS grade, 99.9% purity, Thermo Fisher Scientific) was used in this experiment. A high-pressure and high-temperature cell (HPHT)

(PARR 4750) with a maximum pressure and temperature rating of $p_{\max} = 200$ bar and $T_{\max} = 350^{\circ}\text{C}$ was used in this experiment as shown in Fig. 4.9. In order to avoid corrosion of the vessel, a PTFE hollow cylinder ($d_m = 3.2$ cm, $l = 11.18$ cm) and lid was manufactured, which could be inserted into the PARR HPHT vessel. For each test, a volume of 20 ml of the urea stock solution was placed into the PTFE cylinder, based on the calculation of a maximum allowable liquid volume of $V_{\text{MAWL}} = 25.85$ ml at $T_{\max} = 350^{\circ}\text{C}$ from the PARR 4750 (handbook).

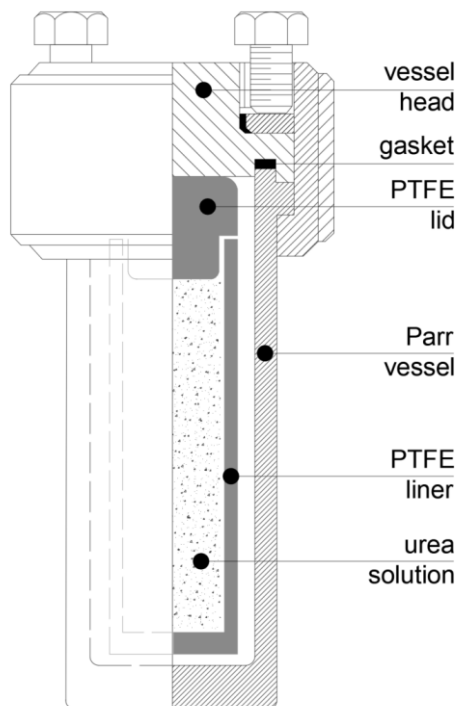


Figure 4.9. Schematic of the high-pressure, high-temperature vessel (PARR 4750). The vessel was modified with a PTFE liner with lid to avoid contact of the pressure and temperature exposed fluid within the stainless-steel vessel.

4.6.2 Temperature exposure

The temperature exposure was conducted in a muffle furnace (Nabertherm L 3/12) for temperatures of $T = 120^{\circ}\text{C}$, 140°C , 160°C and 180°C each tested for fixed exposure times of $t = 4$ hr, 8 hr, 12 hr, 16 hr, 20 hr and 24 hr.

After temperature exposure the high-pressure and high-temperature cell was cooled down in an ice bath for 2 hr. In a next step, the pressure vessel was opened, pH and electrical conductivity

were measured, and samples were transferred into 50 ml centrifuge tubes and stored in the fridge at $T = 4.0^{\circ}\text{C}$.

4.6.3 Method development for the detection of urea via HPLC analysis

The most common approach in measuring urea is the detection of ammonium after urea hydrolysis. This involves colour forming reactions, enzymatic and colorimetric methods (e.g., Modified Nessler method for ammonium) (Cunningham *et al.*, 2011). Other techniques (e.g., Jungs assay for the detection of urea) (Jung *et al.*, 1975) uses noxious reagents (e.g., sulfuric and boric acids). High performance liquid chromatography (HPLC) can also be used to measure urea concentration. However, use of C18 HPLC columns surfaces are populated with silanol functional groups (Si-OH) that are slightly acidic, and can interact with positive charged ions from bases. This can lead to unreliable retention and poor peak shape, if the surfaces of the Type-C columns used are populated by silicon hydride functional groups. To overcome this problem a HPLC analysis was carried out using a HPLC column with Type-C silica bonded phases (i.e., a Cogent Bidentate HPLC column) that can retain high concentrations of polar compounds (e.g., urea).

In this section, a method for the detection of urea with High Performance Liquid Chromatography (HPLC) is presented as *Method 1*. Before running the calibration and analysing the temperature treated urea samples, the High Performance Liquid Chromatography (HPLC) system was purged to remove any air from the system, then the column was conditioned with the mobile phase either at 100% ultrapure water (UPW) (*Method 1*) for 30-45 min to assure that the HPLC backpressure is stable (no leaks or entrapped air).

4.6.3.1 Method 1

Urea standards were prepared in triplicate in a concentration range of $C = 0.1\text{ M}$, 0.25 M , 0.50 M , 0.75 M and 1.00 M and then were diluted 100 times with the mobile phase and filtered through a $0.22\text{ }\mu\text{m}$ Millipore PTFE filter (Merck) into chromatography crimp neck vials. Next, the standards were eluted in an isocratic run with 100% ultrapure water (UPW) as the mobile phase at a flow rate of 0.5 ml/min on a HPLC system: Dionex 3000 (ThermoFisher Scientific) with a Cogent Bidentate C18 | $4.6 \times 150\text{ mm}$ | $4.0\text{ }\mu\text{m}$, $100\text{ }\text{\AA}$ column with an Ultimate 3000 variable wavelength detector at a wavelength of $\lambda = 210\text{ nm}$. The sample injection volume was

10 μ l and the column backpressure was maintained at 96 bar. The standards were injected from lowest to highest concentration and always with blank samples in between.

In a final step, the heat-treated urea samples were diluted and prepared via the same procedure as the sample standards for the calibration curve and run on the HPLC system to determine the remaining urea concentration after thermal degradation in the samples. The change in peak area (mAU*min) for urea was then related via the established standard calibration curve to the remaining urea concentration in solution.

4.7 Experimental Results

4.7.1 Thermal ureolysis for selected temperature range

This section presents the results of High Performance Liquid Chromatography analyses on thermally decomposed 1.0 M urea solutions. The calibration curve in Fig. 4.10 c (*Method 1*) shows an excellent linear straight-line relationship between peak height area and urea standard concentration with an R^2 of 0.994.

Figure 4.10 a,b present chromatograms for 120°C and 140°C heat treated samples and indicate thermal decomposition of urea (chromatograms of 160°C and 180°C are not shown). *Method 1* as presented by the chromatograms in (Fig. 4.10 a,b) showed excellent peak shape with a retention time of around $RT = 3.60$ min.

Thermal urea hydrolysis was observed to start to be effective at a temperature of 140°C (peak area decreased substantially and no remaining urea was found in the samples after 20 hr. The samples treated with the highest treatment temperature of 160°C and 180°C were decomposed even faster with no urea remaining in solution after 12 and 6 hr respectively (Fig. 4.11).

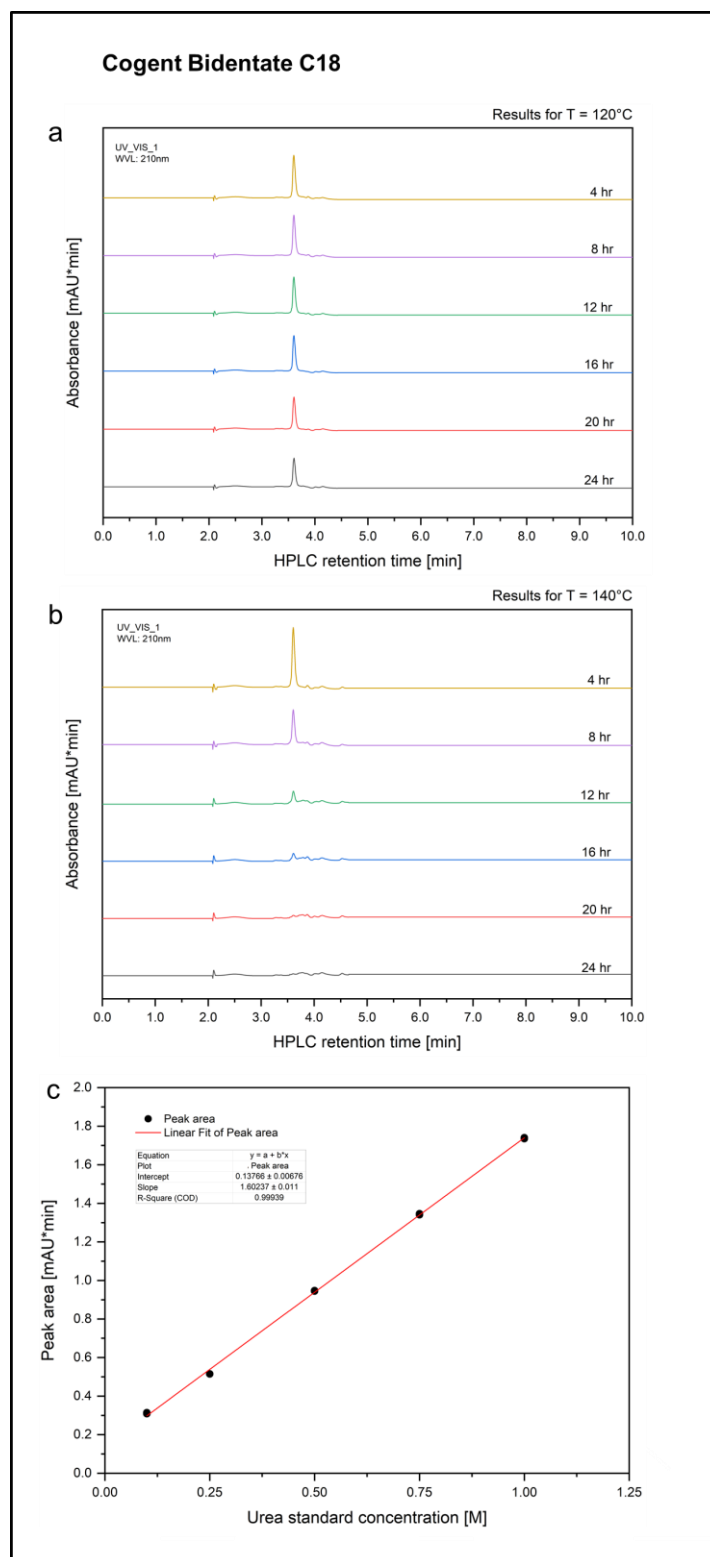


Figure 4.10. This figure presents HPLC chromatograms and the corresponding calibration curve. All heat-treated urea samples were run in triplicate with a blank sample in between. (a-b) shows the results from thermally decomposed urea at 120°C and 140°C run over a Cogent Bidentate C18 column with 100% UPW as mobile phase ($RT_{urea} = 3.60$ min). At 140° a substantial drop in peak area of urea was observed indicating that urea is getting decomposed. (c) is the corresponding calibration curve.

Figure 4.11 presents the results from the chromatograms calculated as remaining urea concentration against duration of heating applied. Heat treatment of the urea with 120°C showed some decomposition, but was slow with 70% of the initial urea concentration still remaining after 24 hr. At a temperature of 140°C, >40% of the initial concentration has degraded within 8 hr and >80% by 12 hr. At 160°C heat treated urea samples showed very rapid decomposition, with complete degradation < 12 hr, and at 180°C in less than < 8 hr.

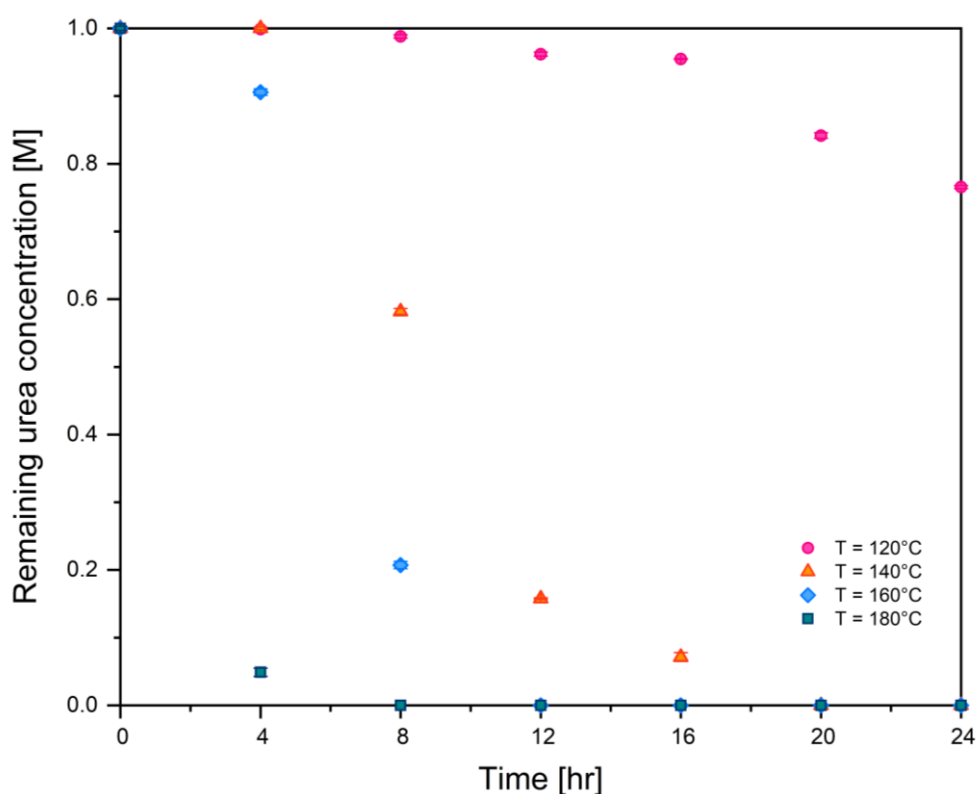


Figure 4.11. This graph is presenting the results of the thermal decomposition of a 1.0 M urea solution measured with a Cogent Bidentate C18 HPLC column (best peak shape and retention time). The remaining urea concentrations in solution are calculated based on the detected peak area (urea peak) from the chromatograms that are related to concentrations via the established calibration curves (Fig. 4.10c). All the samples were run in triplicate and where error bars cannot be detected they might be hidden by the datapoint.

Figure 4.12 presents solution pH measured over time of a 1.0 M urea solution exposed to temperatures of 120, 140, 160 and 180°C. These are compared to a 1.0 M urea solution hydrolysed by a 1.0 OD₆₀₀ *S. pasteurii* bacterial suspension (black datapoints). Figure 4.12 confirms that the pH change achieved due to ureolysis via microbial ureolysis is similar to that achieved via thermal treatment at temperatures $\geq 140^\circ\text{C}$. Similar endpoint pH levels with similar range (9.46 – 9.63) were achieved for all treatment methods after 24 hr. pH change in the solution thermally treated at 120 °C progressed much more slowly than for the higher temperatures or for the bacterially-driven hydrolysis, confirming the results presented in Fig. 4.11.

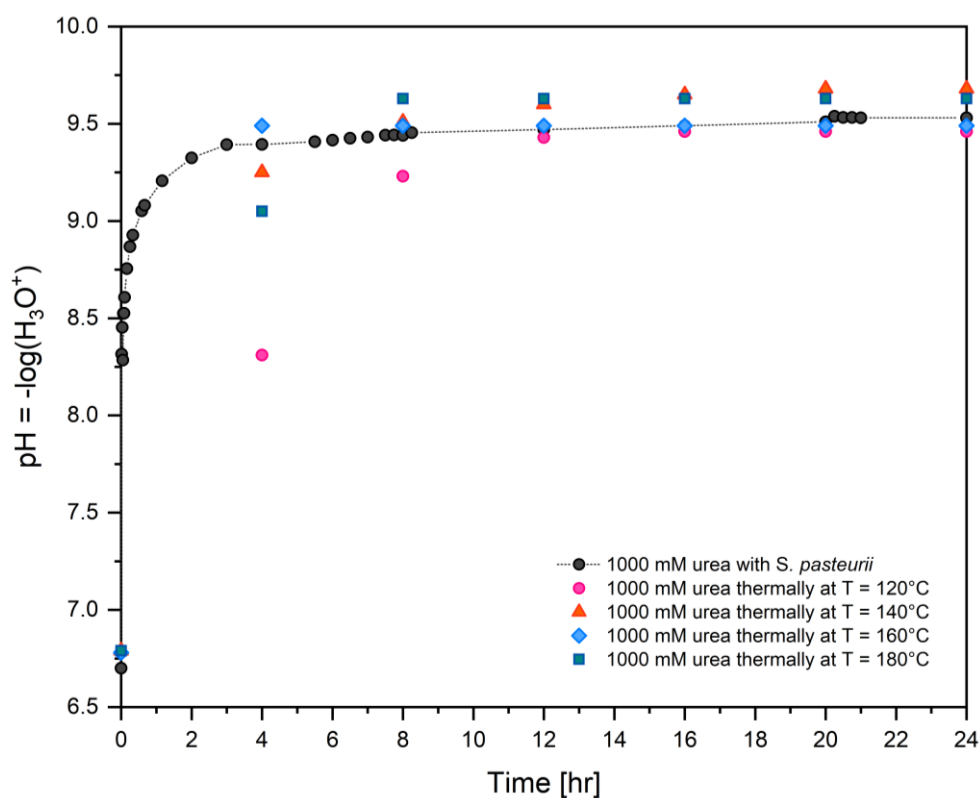


Figure 4.12. This graph is showing a comparison between the measured solution pH over time of a 1000 mM thermally hydrolysed urea (at 120, 140, 160 and 180°C) and a 1000 mM urea solution hydrolysed by a 1.0 OD₆₀₀ *S. pasteurii* bacterial suspension (black datapoints).

4.8 Discussion

4.8.1 Thermally induced ureolysis

The results of the study on thermal ureolysis suggest that this technology might be feasible to use for High-pressure and High-temperature environments above $T = 120^{\circ}\text{C}$. The findings of the current study are consistent with preliminary results presented by MSU Montana (Phillips *et al.*, 2017) where researchers investigated thermal ureolysis up to $T = 130^{\circ}\text{C}$. Here we have shown the potential of thermal ureolysis to drive thermal induced carbonate precipitation up to temperatures of $T = 180^{\circ}\text{C}$. It appears that thermal ureolysis can reach similar reaction rates to engineered microbial ureolysis (*S. pasteurii* with high OD) at temperatures $>140^{\circ}\text{C}$. Based on the geothermal gradient below the North Sea, thermally induced carbonate precipitation could be considered for deployment at depths > 2744 m. There are currently 227 HPHT deep wells in the North Sea with depths $>2500\text{m}$ (Glass, 2005).

4.8.2 Treatment strategies for TICP

Further work would be required to develop appropriate treatment strategies for TICP deployment at depth. Where a staged injection approach has often been adopted in MICP as bacteria can become encased in calcite cutting themselves off from urea supply, this may not be necessary in TICP where the ureolysis is being driven purely by temperature. Furthermore, TICP would require fewer treatment solutions to be injected (only urea and CaCl_2) compared to MICP which would also require a microbial suspension to be injected. The benefit of TICP in deep HPHT wellbores would be the fact that thermal hydrolysis would proceed as long as urea was available to be decomposed.

4.9 Conclusions

The influence of temperature and pressure on the ureolytic activity of bacterial suspensions of *S. pasteurii* was investigated. Experimental results showed that elevated temperatures had a much greater impact on the ureolytic activity of bacterial suspensions than elevated pressures. Optimum working temperature for maximum ureolytic activity was found to be $\sim T = 60^{\circ}\text{C}$. Exposure to higher temperatures resulted in high ureolytic activity that decreased rapidly when

exposed for longer periods of time. In addition, temperature ramping experiments showed that the threshold of inactivation of the urease enzyme is around $T \sim 70^{\circ}\text{C}$. Elevated pressures did not significantly impact the ureolytic activity of *S. pasteurii*.

Results on thermal ureolysis indicate that the decomposition of urea becomes more efficient (similar to microbial ureolysis) at $T > 140^{\circ}\text{C}$. These results indicate that thermal ureolysis could be used to induce carbonate precipitation at depths and areas where the temperature is above the inactivation threshold of the urease enzyme.

In terms of recommendations for depths where MICP and TICP can be deployed it has to be considered that the geothermal gradients vary around the world. Our depth recommendations are based on an average geothermal gradient of $4.92^{\circ}\text{C}/100\text{ m}$ for the North Sea/Europe. Our results indicate that MICP could be deployed as a sealing technology up to depths of 1118 m (i.e., $T \leq 60^{\circ}\text{C}$). Whereas TICP could be deployed in HPHT deep wellbores at depths $> 2744\text{ m}$ (i.e., at $T > 140^{\circ}\text{C}$).

Chapter 5

Mechanistic insights into the formation of CaCO_3 biominerals under *in-situ* wellbore pressure and temperature conditions

5.1 Abstract

Microbially induced CaCO_3 precipitation (MICP) has shown potential as an emerging biotechnology for sealing compromised well cement sheaths and modifying the permeability of rock formations. However, little is known about the application of MICP at high pressures and temperatures typical of *in-situ* wellbore conditions in the hydrocarbon industry. In this study, MICP sealing experiments were conducted at high temperatures and high pressures to consider its potential for well sealing and zonal isolation in a hydrocarbon context. Experiments were conducted using *S. pasteurii* and a cementing solution containing urea and calcium chloride under three different pressure and temperature combinations: ambient conditions ($p = \text{atm}$, $T = 20^\circ\text{C}$) and conditions typical of the North Sea at wellbore depths of 914 m (9.14 MPa , $T = 50^\circ\text{C}$) and 1118 m ($p = 11.18 \text{ MPa}$ and $T = 60^\circ\text{C}$) respectively. MICP treatments were conducted on sandstone cores from the Dunlin oilfield in a high-pressure and high-temperature core-holder. Permeability reduced exponentially in all cases reaching a value of less than $k = 5.0 \text{ E-}17 \text{ m}^2$ (0.05 mD) after 18 treatment cycles (9 consecutive days) for all pressure and temperature combinations. The rate of permeability reduction in early cycles increased with

increasing temperature.

Microstructural X-Ray CT analyses of the bio-grouted cores at the core-scale revealed that CaCO_3 biominerals had precipitated throughout the well-cores. Small-scale sedimentary features caused variations in the calcium carbonate precipitation, specifically the presence of high porosity laminae coincided with zones of increased calcium carbonate precipitation.

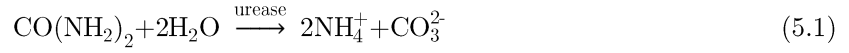
Results from sub-core scale imaging analysis (cryo-FIB/FEG-SEM) under fully saturated conditions of the rock found that two nucleation processes occurred: most calcite crystals nucleated on grain surfaces, but some appeared to have nucleated in solution. Spherulitic CaCO_3 growth with progressing addition of carbonate layers was observed at all temperatures. At the highest pressure and temperature combination only ($p = 11.18$ MPa, $T = 60^\circ\text{C}$) these spherulites showed overgrown faceted crystals. This type of crystal development can result from variations in the saturation index (promoting more crystal growth than nucleation). The presence of these rough faceted crystals could explain the more rapid permeability drop in early cycles that was observed with increasing temperature. The research presented here suggests that MICP may be a promising future well-sealing technology for *in-situ* applications where the temperature does not exceed 60°C .

5.2 Introduction

The UK oil and gas authority forecasts that over the next decade, >1600 hydrocarbon wells will be decommissioned in the UK, with associated costs of up to £15.1 billion (Oil & Gas UK, 2020). Globally, decommissioning activities are estimated to cost £67 billion over the period 2019 - 2029 (Oil & Gas UK, 2019). Consequently, there is a drive for new plugging and abandonment technologies that can ensure long-term isolation of former production zones from the seabed/ ground surface. Well abandonment is conducted in three phases: (i) Production zones of reservoirs are first isolated, (ii) isolation of the intermediate zones (from the reservoir to the well-top) and (iii) the top-hole equipment and conductor are removed entirely. The greatest cost in plugging and abandonment operations is the requirement for a fixed drill rig. Thus, any development that would enable rig-less plugging and abandonment, would be highly advantageous (e.g., using a drillship and coiled tubing). Microbially induced carbonate precipitation (MICP), a low viscosity and low solid content grout (the only solids component

are the bacteria cells), is one such potential technology.

Microbially induced carbonate precipitation with the ureolytic bacteria *Sporosarcina pasteurii* has been studied extensively and proposed for trapping contaminants (i.e., radionuclides) (Fujita *et al.*, 2004), soil strengthening (Whiffin, Van Paassen and Harkes, 2007), embankment erosion control (Salifu *et al.*, 2016), reducing well leakage (Phillips *et al.*, 2013), sealing fractures in rocks (Minto *et al.*, 2016) and for microbially enhanced oil recovery (Wu *et al.*, 2017). Microbially induced carbonate precipitation uses the ability of ureolytic bacteria to produce an enzyme that accelerates a biochemical reaction able to hydrolyse urea Eq. (5.1) and creates conditions, when exposed to a high calcium saturated environment, for the precipitation of calcium carbonate Eq. (5.2) (Mitchell *et al.*, 2010):



Studies have demonstrated that permeability reduction via microbial induced carbonate precipitation is possible at various scales from laboratory to field-scale trials in porous and fractured media (Ferris *et al.*, 1996; Van Paassen *et al.*, 2010; Tobler, McLachlan and Phoenix, 2012; Cuthbert *et al.*, 2013; Phillips *et al.*, 2013; Sham *et al.*, 2013; Minto *et al.*, 2016, 2017).

This paper presents the first experiments to investigate the effects of temperature and pressure on the formation of calcium carbonate crystals precipitated during MICP treatment. Three sandstone rock cores from a North Sea offshore well were subjected to the same MICP treatment process, using atmospheric conditions ($p = \text{atm}$, $T = 20^\circ\text{C}$) and pressure and temperature combinations; $p = 9.14, 11.18 \text{ MPa}$ and $T = 50, 60^\circ$, which correspond to depths beneath the North Sea of 914 and 1118 m (2999 and 3668 ft below msl).

The specific objectives of this experimental study were to: (1) investigate permeability reduction in sandstone well-cores at *in-situ* wellbore pressure and temperature conditions as a result of applying a staged MICP treatment strategy, (2) investigate the influence of temperature and pressure on the spatial distribution of calcium carbonate precipitated and (3) gain mechanistic insights into the formation of calcium carbonate biominerals under subsurface wellbore pressure and temperature conditions with various state-of-the-art imaging technologies from macro (core-scale) to sub-micron-scale (sub-core scale).

5.3 Materials and Methods

5.3.1 Sandstone well-core selection

In order to investigate whether reservoir quality sandstone well-cores can be sealed using MICP, well-cores from the ‘Brent group’ of the Dunlin-Field in the UK Block 211/23a on the UK Continental Shelf (UKCS) (provided by Fairfield Energy) (Fig. 5.1a) were selected based on information from accompanying well logs (Caliper, Gamma Ray, Resistivity, Neutron Density and Neutron Porosity) (Fig. 5.1b).

Four 3.5” (88.9 mm) $\frac{1}{2}$ cut - well-cores of the ‘Upper Ness’ formation from wellbore DA-18 (211/23-A10) (Fig. 5.1a, blue frame) were selected from the interval (11155 – 11165 ft TVD) (Fig. 5.1b). These were taken from the top of the ‘Upper Ness’ formation which is a moderate-to-well sorted coarse-grained sandstone with continuous up dip tilted laminations and moderate hydrocarbon stains (211/23-core 3, 211/23-core 4, 211/23-core 5 and 211/23-core 6). The $\frac{1}{2}$ cut well-cores were sub-cored for the experiments to establish cores with dimensions of 1.5” (36 mm) in diameter with a corresponding length of 3.0” (77 mm). Average porosity was 24.8% with a corresponding average permeability of 3594 mD.

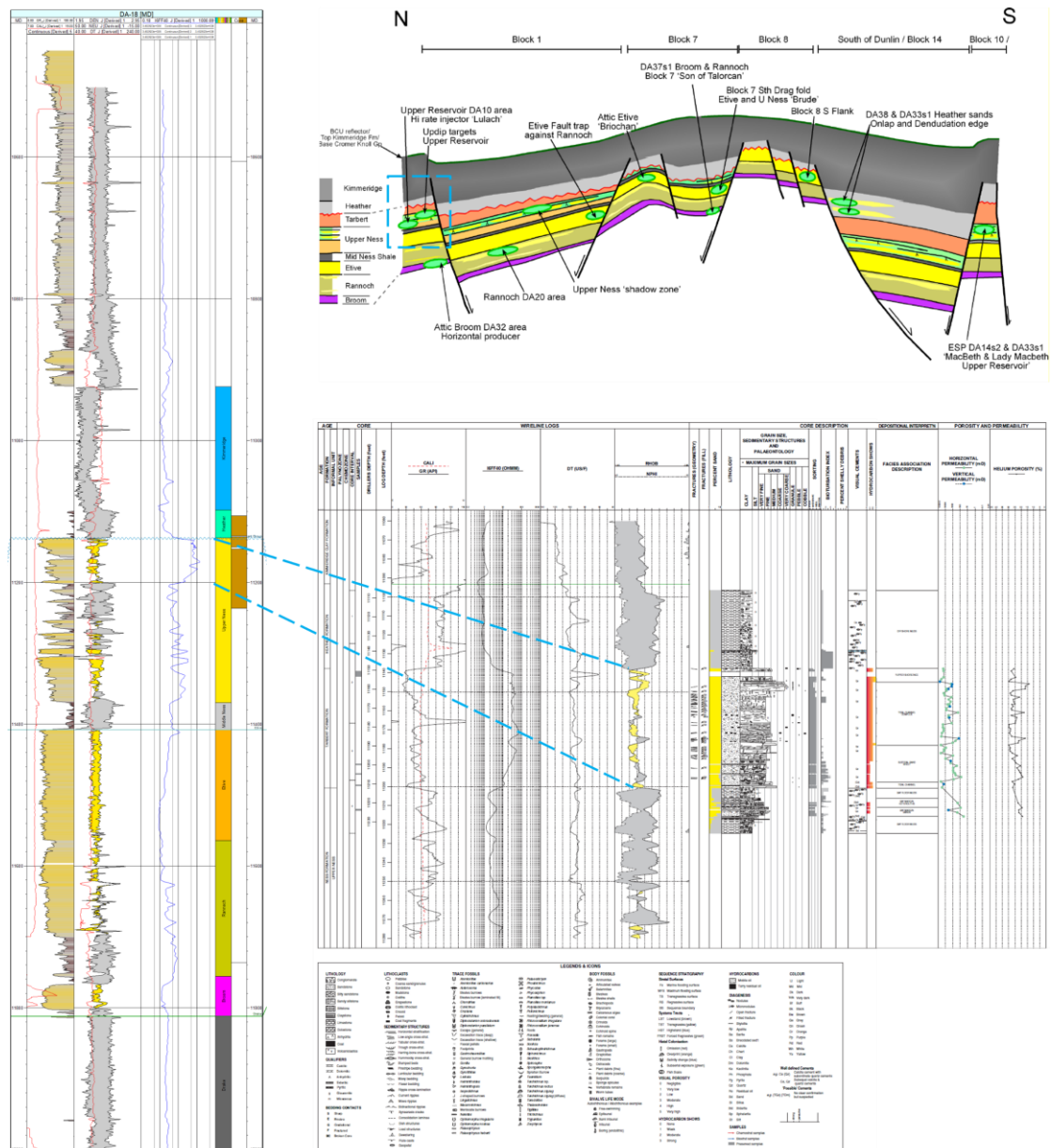


Figure 5.1 (continued) (b) The corresponding well-log is presented for the whole wellbore with the most prominent 'Brent group' formation ending on top with the 'Upper Ness Formation' covered by Heather and finally with the reservoir roof the 'Kimmeridge Shale' formation. A detailed well-log of the cored interval is shown on the right side giving further information on lithology, petrophysical parameters as permeability, helium porosity (well-logs and location maps provided by Fairfield Energy).

5.3.2 Experimental setup

5.3.2.1 Bio-grout experimental setup of high-pressure and temperature core-holder

A schematic of the experimental setup used for these experiments is shown in Fig 5.2. Rock cores were wrapped in RS PRO Black Polyolefin heat shrink tubing, then vacuum saturated with sterile tap water prior to being placed in the high-pressure high-temperature stainless steel Hassler-type core-holder. The heat shrink tubing was used to ensure a smooth contact with the inner rubber sleeve of the core-holder to prevent fluid bypass. The setup enabled recording of pressure at the inlet and outlet of the core. Continuous fluid injection was achieved with a constant pressure Cole Parmer Masterflex Ultra-High-Pressure dual-piston pump (max. flow rate 12 ml/min, max. operating pressure 124 MPa) and the core outlet pressure was regulated with a Swagelok Model 1000D backpressure regulator. The Hassler type core-holder used had a maximum pressure rating of 15 MPa and a maximum temperature rating of 90°C. Confining pressure was applied with an Enerpac P141, single-speed, hydraulic hand pump and was set for all experiments to $p = 13.8$ MPa (~ 2000 psi) based on the requirement to avoid by-pass flow for the highest injection pressure tested; $p = 11.18$ MPa at $T = 60^\circ\text{C}$.

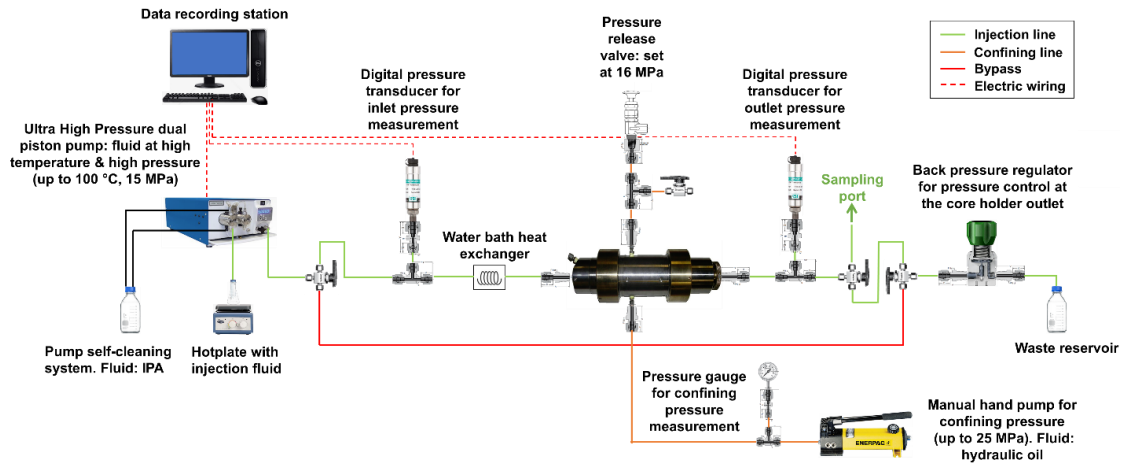


Figure 5.2. Schematic of the experimental setup of the bio-grouting experiment at *in-situ* wellbore pressure and temperature conditions. The fluid pathway is indicated by the green line starting at the high-pressure pump passing a water bath heat exchanger reaching the inlet of the Hassler type core-holder where the bio-grouting treatment occurs and leaves at the outlet via the backpressure regulator to a waste reservoir. The solid red line indicates a fluid bypass line to service the pump and backpressure regulator. The red dashed lines indicate the data logging system consisting of two pressure transducers at the inlet and outlet side of the core holder, as well as a data connection with the high-pressure pump for flow-rate monitoring and pressure setting connected to a data recording station.

The injection fluid passed from the high-pressure dual-piston pump through the high-pressure injection lines (stainless steel) with a diameter of 1/16", through a water bath heat exchanger that then connected to the high-pressure high-temperature stainless steel Hassler-type core-holder. Fluids exited the core via the backpressure regulator into a waste reservoir. A fluid line that bypasses the core-holder was also installed for servicing the pump at regular intervals during the experiment to prevent bio-clogging and piston damage due to bio-precipitation in the pump back chamber. This line was connected via two 3-way valves located at either side of the core-holder. The differential fluid pressure is calculated based on the absolute pressure measurements at the inlet and outlet of the core holder using ESI Technologies GS4200-USB digital pressure transducers. The high-pressure and high-temperature (HPHT) core holder and the injection lines are thermally insulated to reduce the heat loss from the injection fluid.

Operating in permeability measurement mode:

To measure initial and post-MICP treatment permeabilities, an additional sampling port was installed between the outlet of the core holder and the backpressure regulator to allow the outlet pressure to be bled to atmospheric pressure. Injection pressure was then ramped in a stepwise fashion to subject the core to a series of differential pressures and the corresponding flow rates were measured, in order to determine the core permeability. For all permeability measurements, the pore space was pre-flushed using multiple pore-volumes of sterile tap water prior to measuring the permeability in order to ensure that the fluid density can be assumed to be that of water. Initial core saturation is achieved by vacuum saturating the well-cores for a duration of 24 hr.

Setting the flow rate for bio-grouting:

To achieve a given flow rate of $Q = 0.5$ ml/min at the desired pressure and temperature conditions (Table 5.1), the following procedure was used: a) the backpressure regulator was closed, b) the downhole pressure on the injection pump was set, c) the injection fluid was heated according to the set wellbore depth via a hot-plate d) the heated fluid was injected through the water bath heat exchanger and the core-holder mounted with the rock sample and once the target pressure was reached, the pump stopped to maintain the target pressure, e) the target pressure was maintained stable for a duration of 20 min, then the backpressure regulator was opened and backpressure adjusted until the target flow rate was reached.

Selection of appropriate *in-situ* wellbore conditions:

The temperatures used in these experiments were selected based on the results in *Chapter 4*. For a given temperature, the likely wellbore depth was determined by using the apparent geothermal temperature gradient in the subsurface for the North Sea based on an average geothermal temperature gradient of $\sim 4.92^\circ\text{C}$ per 100 m depth and an average North Sea temperature of 5°C (personal communication, OGTC 2018) (Eq. 5.3):

$$\text{Depth} = \frac{(\text{Formation temperature} - \text{mean temperature of sea water})}{\text{Geothermal temperature gradient}} = [\text{m}] \quad (5.3)$$

Given the wellbore depth, the required fluid pressures (pore pressures) at the calculated injection depths were calculated assuming hydrostatic pressure (Eq. 5.4):

$$p_{\text{pore}} = \rho_w g h = [\text{Pa}] \quad (5.4)$$

Table 5.1 Simulated wellbore conditions for bio-grouting experiment

Simulated <i>in-situ</i> wellbore pressure and temperature conditions		
Wellbore depth as True vertical depth [m]	Pore pressure [MPa]	Temperature $^\circ\text{C}$
0	atm	20
914	9.14	50
1118	11.18	60

5.3.3 Bacterial strain and cultivation

A bacterial suspension of the ureolytic strain *S. pasteurii* (DSM, type 33:) was prepared. *S. pasteurii* were grown in Yeast Extract broth (13.0 g/l Yeast extract, 2.5 g/l NaCl, 1.0 g/l D-Glucose and 1.0 g/l K_2HPO_4) with 20 g/l of urea supplement under sterile conditions in an incubator shaker for 24 hrs at 30°C . Bacteria were then suspended with sterile tap water to a target optical density (OD_{600}) of 1.0. The OD_{600} was measured spectrophotometrically at a wavelength of $\lambda = 600$ nm, using 1 – cm path length cuvettes. This corresponds approximately to between 3.3×10^7 and 8.6×10^7 CFU/ml, calculated based on the linear relationship between *S. pasteurii* OD_{600} and CFU/ml at low optical densities (Ramachandran, S. K., Ramakrishnan, V., & Bang, 2001). The specific ureolytic activity k_{urea} (a measure of how much urea gets

hydrolysed per min per optical density) was calculated by measuring electrical conductivity over a five-minute interval, using the relationship in equation 4.8 (section 4.3.2, *Chapter 4*).

5.3.4 Cementation solution

For the cementation stage a calcium enrichment solution was used that contained a 1.0 M equimolar solution of CaCl₂/urea and was prepared fresh every day before injection by dissolving 22.2 g CaCl₂ and 12.0 g urea into 0.2 l of deionised water.

Injection protocol

Each well-core was conditioned overnight in a vacuum saturator to ensure 100% water saturation and no entrapped air. In all bio-grouting experiments a staged injection approach (Tobler, McLachlan and Phoenix, 2012; Minto *et al.*, 2016; Tobler *et al.*, 2018) was deployed with periods of static no-flow in-between (Table 5.2).

Table 5.2 Daily bio-grout injection schedule

Daily bio-grout injection schedule								
Day	Treatment cycle	Fluid	OD ₆₀₀	Bacterial Inj. (PV)	1.0 M CaCl ₂ /urea (PV)	Sterile tap water (PV)	Flow rate [ml/min]	Treatment duration [min]
1 - 9	AM	<i>S. pasteurii</i>	1.0	1			0.5	40
		Sterile tap water				0.5	0.5	20
		Static (No-flow)					0.0	60
		Cementing solution			2		0.5	80
		Sterile tap water				0.5	0.5	20
		Static (No-flow)					0.0	120
	PM	<i>S. pasteurii</i>	1.0	1			0.5	40
		Sterile tap water				0.5	0.5	20
		Static (No-flow)					0.0	60
		Cementing solution			2		0.5	80
		Sterile tap water				0.5	0.5	20
		Static (No-flow)					0.0	overnight

Each day consisted of two treatment cycles as summarised in Table 5.2: (1) A volume of 20 ml (1.0 PV) of bacterial suspension ($OD_{600} = 1.0$) was put into a water bath on a hot plate and left for 5 min to heat up to the required injection temperature and then injected at a flow rate of 0.5 ml/min for a duration of 40 min. (2) A pulse of water with a volume of 10 ml (0.5 PV) was injected in order to prevent mixing in the injection lines between the bacteria and cementing solution. (3) The pressure and temperature in the core were then maintained for a 1 hr static, no-flow period. This was to allow the microbes to settle from the aqueous phase and attach on the mineral surfaces of the sand grains (Schultze-Lam *et al.*, 1996). (4) 40 ml (2 PV) of the cementing solution with a concentration of 1.0 M equimolar $CaCl_2$ /urea was flushed through the core at a flow rate of 0.5 ml/min for 80 min followed by a pulse of tap water (0.5 PV) as spacer between the cementation fluid and the next injection cycle starting with bacterial suspension. (5) Between the AM and PM injection cycles, there was a 2 hr, static no-flow period, whereas between the PM and AM cycles the duration of the static period was overnight. (6) After day 5, every morning, two pore volumes of sterile tap water were flushed through the core prior to starting the first treatment cycle of the day, to further inhibit the formation of precipitate at the inlet to the core-holder.

This full treatment process was conducted over nine consecutive days, resulting in a total of 18 treatment cycles.

5.3.5 Permeability measurement

The bulk permeability was measured by injecting sterile tap water at three increasing flow rates (0.2, 0.35, 0.5 ml/min) into the core-holder. Once steady-state conditions were reached for one flow rate, the flow rate was increased. From this procedure, permeability was calculated based on Darcy's law:

$$k = \mu \cdot \frac{Q}{A} \cdot \frac{L}{\Delta P_{IN-OUT}} \quad (5.5)$$

where k is the permeability [m^2], μ is the dynamic viscosity of the fluid flowing through the core [$T = 20^\circ$: $\mu = 1.0016E-3$ Pas; $T = 50^\circ C$: $\mu = 0.5465E-3$ Pas; $T = 60^\circ C$: $\mu = 0.466E-3$ Pas] (Korson, Drost-Hansen and Millero, 1969), Q is the flow rate [$8.33E-9$ m^3/s], A and L are the cross-sectional area [$1.018E-3$ m^2] and length [0.077 m] of the core respectively, and ΔP_{IN-OUT} is the pressure differential across the core [Pa]. Permeability was derived by considering the average value of ΔP_{IN-OUT} when they reached a stable value during the test and using the

average flow rate recorded by the injection pump over this stable period. For the permeability measurements, the effect of temperature on the fluid viscosity was accounted for.

Table 5.3 Experimental conditions for each experiment with the corresponding core nr. and measurements, sampling and analyses conducted

Experimental conditions and analyses conducted										
		Wellbore conditions			Measurements/Sampling/Analyses conducted					
Exp.:	Core Nr.:	TVD [m]	P [MPa]	T [°C]	Permeability measurements [mD]	Effluent sampling for XRD	Micro xCT	FE-EPMA with EDX, WDX	W-SEM	Cryo-FIB/ FEG-SEM
Exp 1	211/23-3	0	atm	20	Initial and then from day 5 – day 9	x	x	x	x	-
Exp 2	211/23-4	914	9.14	50		x	x	-	x	-
Exp 3	211/23-5	1118	11.18	60		x	x	-	x	-
Exp 4	211/23-6	1118	11.18	60	Initial and every day	-	-	-	-	x

In addition to the three fully bio-grouted well-cores (Exps. 1-3, Table 5.3), a fourth (replicate) core (Exp 4, Table 5.3) was treated at the highest pressure and temperature conditions ($p = 11.18$ MPa, $T = 60^\circ$ C) in which permeability measurements were taken after every 2nd treatment cycle, in order to get a more detailed understanding of the relationship between permeability reduction during the earlier treatment cycles.

5.3.6 Effluent sampling and biomineral characterisation

Characterisation of the biominerals in the effluent was conducted to infer calcium carbonate polymorph formation under *in-situ* wellbore pressure and temperature conditions. Effluent samples were collected in 15 ml centrifuge tubes at the core-holder outlet (behind the back pressure regulator) each day during the PM cementation cycle. The collected biominerals precipitated from the effluent sample were separated first by centrifugation with an Eppendorfer 5804 R Centrifuge at 6000 RPM for 8 min. The supernatant was decanted, and the collected sediment pellet was resuspended in sterilised tap water, vortexed and re-centrifuged twice, to remove any remaining cell debris, urea and CaCl_2 . The precipitates were air-dried at 37°C in a Gallenkamp incubator for 24 hr before undergoing characterisation via X-ray Powder Diffraction Analysis (XRD).

The air-dried samples were analysed using an X-ray Diffractometer (Bruker D8 Advance) with a Cu K α shutter tube generating X-rays of a wavelength $\lambda = 1.5406 \text{ \AA}$ (0.15406 nm). Patterns were collected over the 2θ scan range $5\text{-}60^\circ$ using a step size of 0.02° , a scan speed of 0.6 s/step and a 0.6 mm X-ray slit size to reduce the divergence of the X-ray beam. Initial data evaluation was performed with DiFFRAC EVA, and quantitative information on the ratios of the calcium carbonate polymorph calcite and vaterite was obtained through a Rietveld refinement with TOPAS using the crystallographic information files for calcite (Markgraf and Reeder, 1985) and vaterite (Kamhi, 1963).

5.3.7 Micro xCT analysis of CaCO $_3$ distribution in the sandstone cores

The well-cores were scanned before and after treatment using a micro-X-ray CT scanner (Nikon XT H 225 LC). This resulted in 3D computer tomographic images with a voxel size of $0.047 \times 0.047 \times 0.047 \text{ mm}$ (corresponds to $47 \text{ }\mu\text{m}$ resolution). Images were acquired at an X-ray energy of 120 kV and 125 mA at the power of 15 W. Each core was vacuum saturated and imaged under water-saturated conditions. After bio-grout treatment, each core face was wrapped with parafilm and immediately scanned under saturated conditions. Reconstruction was conducted with the Nikon CT PRO 3D metrology software suite. Pixel based image segmentation was achieved with Image J - 3D Trainable Weka Segmentation (TWS) (random forests) plugin (Arganda-Carreras *et al.*, 2017) (Appendix C, S1). Successful segmentations were achieved after the data sets were run through a python script on Google Co-Lab (Appendix C, S2) where the average porosity for each CT slice was estimated as a function of distance from the inlet of the core and compared with the gravimetrically measured porosity results. Thresholding was applied in Thermofisher Scientific Avizo, 2019 to produce 3D volume renderings of the pore-space throughout the core. The change in the average porosity of each slice in the CT scan was calculated by subtracting the initial slice-averaged porosity from the post-treatment slice-averaged porosity. This provides an estimate of the volume of CaCO $_3$ biominerals as a function of distance along the z-axis of the core.

5.3.8 Scanning Electron Microscopy on polished blocks

5.3.8.1 Sample preparation for Scanning Electron Microscopy (SEM) analyses

Cylindrical polished blocks were prepared by Petrolab Ltd. The well-cores were first vacuum impregnated with an epoxy resin and sectioned into five equally spaced cylindrical blocks along the z-axis of the core at $z = 1.8, 19.25, 38.5, 57.75, 75.7$ mm} (Fig. 5.3). The blocks were polished with a Buehler EcoMet350 Grinder Polisher with a $0.5\ \mu\text{m}$ finish and then carbon coated to reduce surface-charging during imaging.

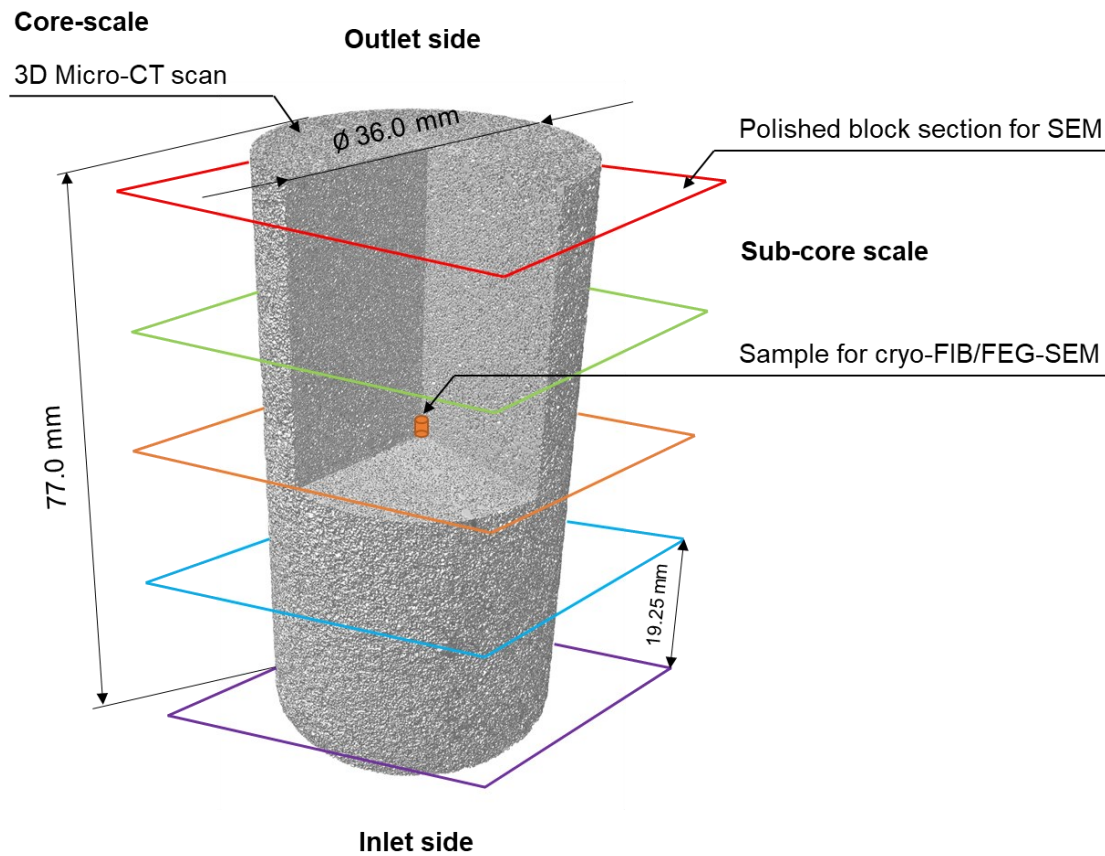


Figure 5.3. Schematic of a sandstone well-core. *Core-scale experiments and imaging techniques* are related to the bulk volume of the well-core. *Sub-core scale SEM imaging* is conducted on polished blocks with an equal spacing of 19.25 mm along the z-axis of the core. For the high resolution cryo-FIB/FEG-SEM imaging a sub-sample was generated under fully saturated conditions.

5.3.8.2 Tungsten Filament Scanning Electron Microscopy (W-SEM)

In pre-liminary investigations (Appendix C, S3) scanning electron microscopy was performed on highly polished blocks (carbon coated) of the well-core mounted in a Field Emission – Electron Microprobe Analyser (JEOL JXA-8530F Field Emission-EPMA). Based on these images (SI), Tungsten SEM images were collected. The polished blocks (Figure 5.3) from Exps. 1-3 (Table 5.3) were analysed in a Hitachi S3700 – tungsten filament Scanning Electron Microscope (W-SEM) with an Oxford Inca 350 with 80 mm X-Max detector. To compare the microstructure of the CaCO_3 biominerals at different temperatures, the polished blocks sectioned at $z = \{38.5 \text{ mm}\}$ (Fig. 5.3) were selected for all three pressure and temperature combinations ($p = \text{atm}$, $T = 20^\circ\text{C}$; $p = 9.14 \text{ MPa}$, $T = 50^\circ\text{C}$; $p = 11.18 \text{ MPa}$, $T = 60^\circ\text{C}$).

5.3.9 High resolution Scanning Electron Microscopy of biominerals

5.3.9.1 Cryo-Focused Ion Beam/Field Emission Gun-Scanning Electron Microscopy

High resolution cryo-FIB/FEG-SEM analysis was performed with support and access to a FEI Helios G4 CX Dual Beam - High-resolution monochromated FEG-SEM with precise Focused Ion Beam at the University of Leeds Electron Microscopy and Spectroscopy Centre (LEMAS). This was funded through the LENFF (EPSRC) access scheme. This was only conducted for one of the cores treated at $p = 11.18 \text{ MPa}$, $T = 60^\circ\text{C}$. This was carried out to provide high-resolution (HR) images of the final biominerals that formed under wellbore conditions. Such HR images enable extracting information on the mechanisms of biomineral formation.

5.3.9.2 Sample preparation for cryo-FIB/FEG-SEM

A sub-core of the MICP treated well-core from (Exp 4, Table 5.3) was cut at $z = \{38.5 \text{ mm}\}$ (Fig. 5.3) and kept saturated (submerged) with the effluent from the last treatment cycle in a beaker. From this cylinder, small sub-samples were cut off for cryo-Focused Ion Beam/Field Emission Gun - Scanning Electron Microscopy. The ideal sample size for the cryo-FIB/FEG-SEM stage is approximately 2 millimetres in diameter obeying a 1:2 diameter-to-length ratio

for the cryo-fracturing process. Sub-samples were prepared the day before analysis and kept stored (submerged) in the effluent of the last MICP treatment cycle in 2 ml Eppendorfer vials.

5.3.9.3 Sample preparation for cryo-fracturing

The above samples were glued onto a cryo-FIB/FEG-SEM stage and then lifted into a plunge freezer where liquid nitrogen was added to form a nitrogen slush. Plunge freezing down to a temperature of $T = -175^{\circ}\text{C}$ immediately preserved all the fluid phases present, including the microbes in their position in the porous media. Next, the stage was transferred to the fracturing chamber of the FIB/FEG-SEM. With a diamond knife, a fracture was induced in the middle of the sample where the top part broke off. As a last step, the fractured surface was Iridium coated to prevent the sample from surface charging during cryo-FIB/FEG-SEM analysis.

5.3.9.4 Cryo-FIB/FEG-SEM setup

For the analysis of the samples, a FEI Helios G4 CX Dual Beam - High-resolution monochromated FEG-SEM with precise Focused Ion Beam was used. The cooling system was provided by a Quorum Technologies PP3010T Cryo-SEM system. Elemental mapping was conducted with an Oxford Instruments Aztec EDS system with a 150 mm SDD detector. BSE and SE images and elemental mapping were acquired and processed using FEI and Oxford Instruments in-house software.

5.4 Experimental Results

Core-scale results

5.4.1 Permeability and porosity reduction under changing p, T conditions

In the baseline experiment at atmospheric conditions ($p = \text{atm}$, $T = 20^{\circ}\text{C}$) (Fig. 5.4) the well-core was treated without permeability measurements until day 5 to avoid any changes in pressure and temperature due to re-pressurisation of the experimental setup (as described in operating in permeability measurement mode, section 5.3.2.1)

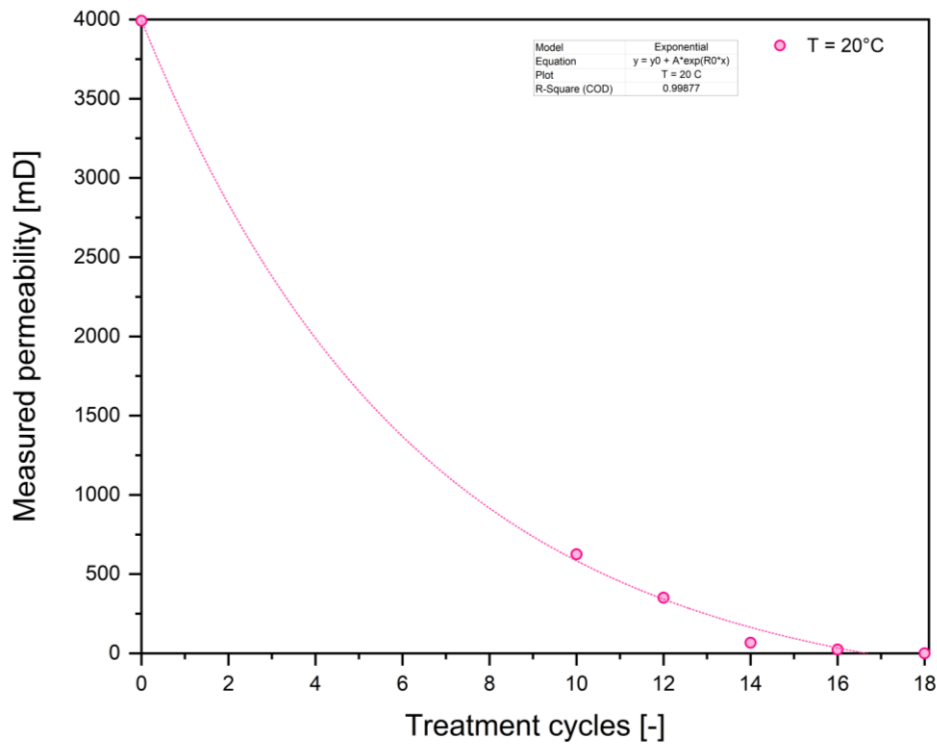


Figure 5.4. Results of the baseline bio-grouting experiment at atmospheric conditions ($p = \text{atm}$, $T = 20^\circ$) with the reduced permeabilities. Permeability measurements show the average permeability across the core and indicate an exponential decay behaviour with progressive bio-grout treatment cycles at atmospheric conditions. The dotted line is indicating the fitted curve for the individual datapoints.

At the injection stage of treatment cycle ten, a significant increase of the inlet pressure arose from 726 psi to 1757 psi (Fig. 5.5). This led to the conclusion that the injection port was very close to being plugged by biominerals. Since this occurred at the very end of the cementation cycle, the cycle was finished, and the core-holder opened the next morning prior to permeability measurements being taken to check for clogging. A small amount of biominerals had started to build up at the injection port. After cleaning the injection port, reassembly of the core-holder and re-pressurising the system, sterile tap water was injected to displace the pore fluids from the previous days treatment cycle in the core prior to measuring the permeability (i.e., first permeability measurement).

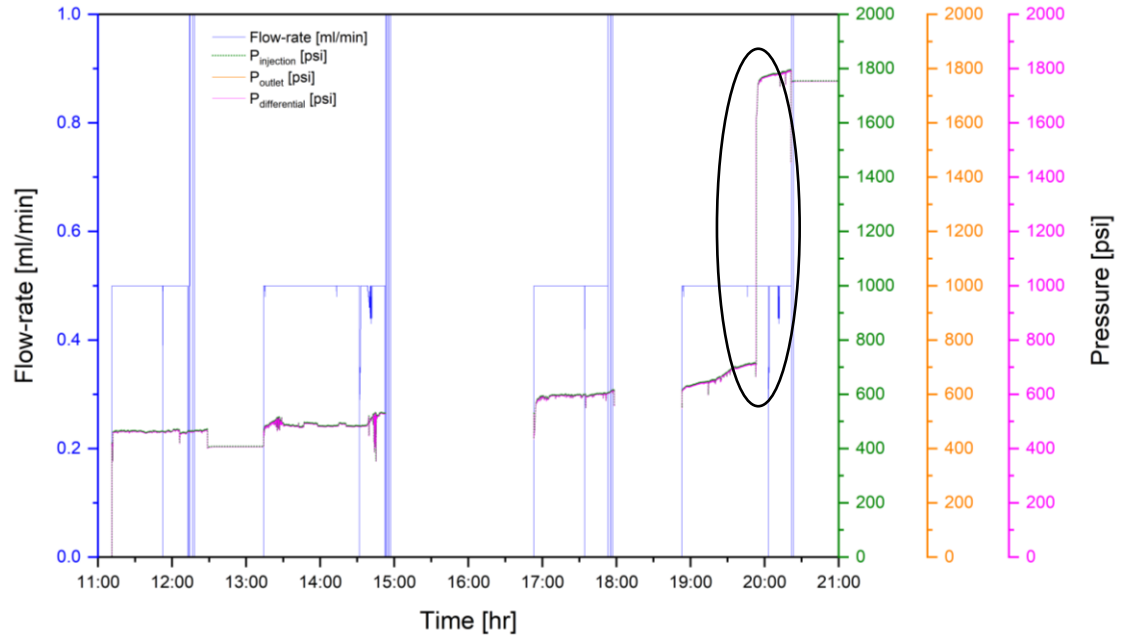


Figure 5.5. This figure shows the recorded flow rate on the y-axis; on the secondary y-axis: the recorded injection pressure (green), the outlet pressure (orange): $p_{\text{outlet}} = \text{atm}$ and the differential pressure (magenta): $p_{\text{differential}} = p_{\text{injection}} - p_{\text{outlet}}$ from the bio-grout treatment at ($p = \text{atm}$; $T = 20^{\circ}\text{C}$). The ellipse is indicating the sudden increase in pressure from 726 - 1757 psi, due to a buildup of biominerals at the injection port.

At atmospheric conditions ten treatment cycles of bio-grout led to a permeability reduction of 84% (Table 5.4). During the next eight treatment cycles, the pressure was continuously monitored to check for blockages and avoid equipment damage. In the following days, permeability reduced in smaller steps and after 18 treatment cycles, it was no longer possible to inject at 0.5 ml/min without injection pressures exceeding the operating limit of the core-holder.

Figures 5.6a and b show the reduction in permeability with increasing treatment cycles for all four experiments, conducted at 3 different pressure and temperature combinations (Exp 1-4, Table 5.3). In all experiments the permeability continues to reduce with increasing treatment cycles due to calcium carbonate crystal growth in the porous media. In all samples, the permeability is reduced by at least five orders of magnitude (Table 5.4). Figure 6b shows the same results normalised by the initial permeability of each core, to allow for direct comparison between samples.

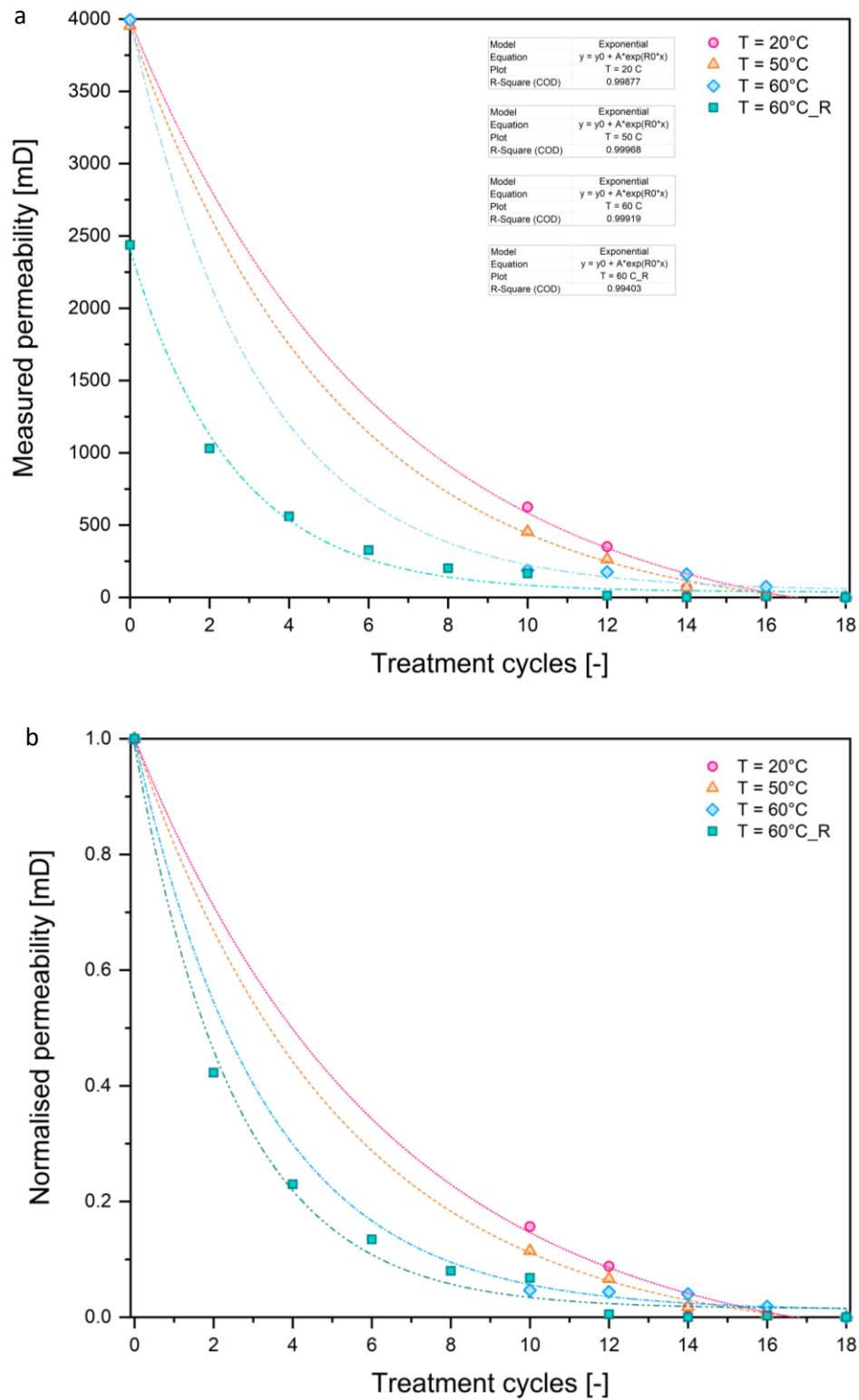


Figure 5.6. Results of the biogrout experiment at wellbore conditions with the reduced permeabilities. (a) Permeability measurements show the average permeability across the core. (b) Normalised permeability values for comparison indicate an exponential decay behaviour with progressing bio-grout treatment cycles at wellbore conditions. Dotted, dashed, dash-dot, dash dot-dot lines are indicating the fitted curves for the individual datapoints. Datapoints that are not visible are hidden by other datapoints.

Table 5.4 Permeability reduction after applying biogrout treatment under wellbore conditions

Permeability reduction results								
Bio-grout treatment:					Core Nr.:			
					211/23-3	211.23-4	211/23-5	211/23-6*
Day	Cumulative treatment cycles	OD ₆₀₀	Bacterial Inj. (PV)	1.0 M CaCl ₂ /urea (PV)	Permeability [mD] p = atm, T = 20°C	Permeability [mD] p = 9.14 MPa, T = 50°C	Permeability [mD] p = 11.18 MPa, T = 60°C	Permeability [mD] p = 11.18 MPa, T = 60°C R
0	Initial	-	-	-	3992	3954	3994	2437.68
1	2	1.0	1	2	-	-	-	1030.61
2	4	1.0	1	2	-	-	-	560.06
3	6	1.0	1	2	-	-	-	327.75
4	8	1.0	1	2	-	-	-	201.00
5	10	1.0	1	2	625.15	453.65	186.60	165.37
6	12	1.0	1	2	351.00	262.22	175.59	12.69
7	14	1.0	1	2	67.23	69.00	160.55	0.05
8	16	1.0	1	2	23.36	14.95	73.27	6.23
9	18	1.0	1	2	0.042	0.004	0.063	0.042

Comparing results for the differing pressure-temperature combinations, it is clear that as the pressure and temperature increase, the rate of permeability reduction in early cycles is increasing. Experiments 1 - 3 have very similar initial permeabilities between 3950 and 3994 mD. After 10 treatment cycles the permeabilities have dropped to values of 625, 453 and 186 mD for temperatures of 20, 50 and 60 °C respectively. The results in *Chapter 4* indicate pressure has limited/no influence on the ureolytic activity suggesting this is likely an effect of the increasing temperature on ureolytic activity; a measure of how fast the bacteria can convert urea into ammonium and carbonate ions.

5.4.2 Effluent biomineral characterisation

The results of the X-ray powder diffraction analyses of the biominerals collected from the effluent are visualised in Fig. 7 (a-c) and the results from the Rietveld refinements are summarised in Table 5.5. First it is important to note that the identification of calcium carbonate minerals in the effluent indicates that biominerals are transported through the sandstone core; this could be due to solution-based nucleation/formation of the calcium carbonate minerals.

Nucleation theory differentiates between solution nucleation (homogeneous) and surface nucleation processes (heterogeneous nucleation) (Cheong, Gaskell and Neville, 2013). Solution nucleation is the process where biominerals start to grow in solution and has been observed in other biomineral systems e.g., vaterite solution nucleation has been shown to occur through the nucleation of nano-crystallites that then grow in a spherulitic manner (Bots *et al.*, 2012) to form a mineral crystal. MICP, however, is generally associated with surface nucleation, in which the bacterial cell reduces the activation energy barrier required for precipitation and acts as a nucleation template for surface sorption (Ferris *et al.*, 2004; Gadd, 2010). The identification of calcium carbonate minerals in the effluent in these experiments (Table 5.3) implies that both solution nucleation and surface nucleation processes may be occurring.

Table 5.5 also shows a change in the mineralogy of the effluent over time. In abiotic systems, under standard conditions, the Ostwald step rule can be used to predict the crystallisation sequence, which after nucleation should transform from a non-crystalline to a crystalline calcium carbonate polymorph (Radha *et al.*, 2010). Starting at the lowest thermodynamically state, amorphous calcium carbonate (ACC) should transform to calcite, via vaterite intermediates at low temperatures ($<30^{\circ}\text{C}$), and to aragonite at temperatures above (60°C) (Ogino, Suzuki and Sawada, 1987; Radha *et al.*, 2010; Rodriguez-Blanco, Shaw and Benning, 2011; Bots *et al.*, 2012). In a biotic system, however, researchers suggest that during biomineralisation, the CaCO_3 polymorph selection can be strain specific (Saracho *et al.*, 2020).

In the baseline bio-grouting experiment at $p = \text{atm}$, $T = 20^{\circ}\text{C}$, the first detected biomineral phase on the diffractogram is vaterite, with low amounts of calcite (Fig. 5.7a, Table 5.5). Further treatment results in the first calcite peaks appearing in the effluent sampled from Day-2 of the experiment. From Day 1 onwards the amount of vaterite steadily decreases as the

amount of calcite present steadily increases (Table 5.5), until calcite dominates. These observations suggest that within the cores, during early treatment cycles calcium carbonate initially forms as vaterite, while in later cycles it is predominantly calcite. This indicates that the calcite observed was a result of transformation of vaterite following the Ostwald step rule (Radha *et al.*, 2010) and additional calcite biomineral formation during the successive treatment cycles also likely progressively more formed as calcite as a result of changing ureolytic activity or even possibly detachment of calcite which has formed on grain surfaces (during earlier treatment cycles) and later detached as a result of changing flow paths and changes in local fluid velocity.

At elevated pressure and temperature conditions, $p = 9.14$ MPa, $T = 50^{\circ}\text{C}$ (Fig. 5.7b, Table 5.5), hexagonal vaterite was the dominant phase until the 10th treatment cycle (Day 5). This is likely due to the high ureolytic activity of *S. pasteurii* leading to high supersaturation levels where metastable phases are more kinetically favored over the more thermodynamically stable mineral calcite. After the 10th treatment cycle, there is a very rapid drop in vaterite production with $> 90\%$ of calcite dominating the precipitates in the effluent, likely due to changing ureolytic activity resulting in lower supersaturation favouring more stable calcite to form (possibly on preformed calcite crystals).

At the highest pressure and temperature conditions ($p = 11.18$ MPa, $T = 60^{\circ}\text{C}$) (Fig. 5.7c, Table 5.5), hexagonal vaterite was found until the 12th treatment cycle, before calcite predominates. This may be due to the high hydrolysis rates of urea (under such conditions) (Xyla, Mikroyannidis and Koutsoukos, 1992; Warren *et al.*, 2001) led in this case to prolonged high sustained supersaturations leading to a prolonged production of metastable calcium carbonate crystals before transformation occurred.

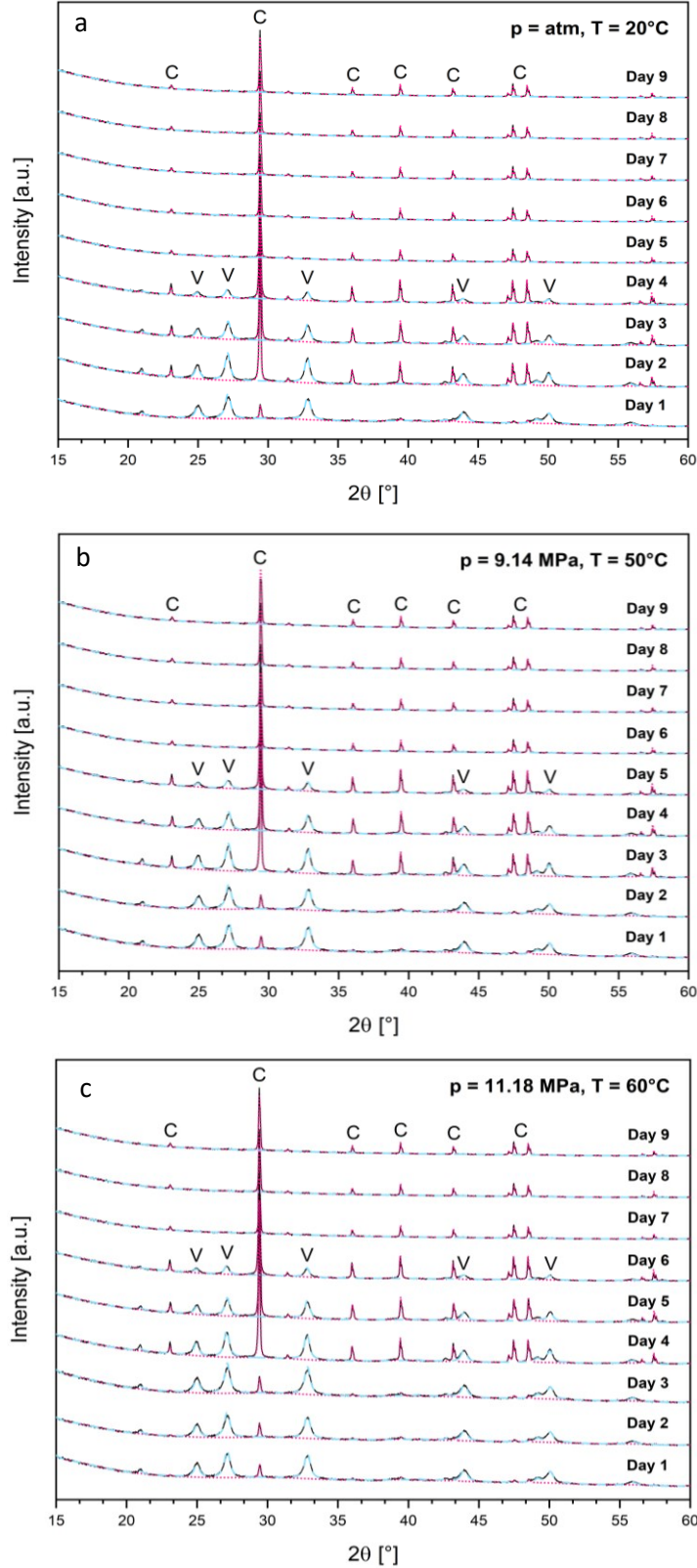


Figure 5.7. Biominerals analysed were retrieved from daily effluent sampling during the PM bio-grouting treatment cycle under *in-situ* wellbore pressure and temperature conditions. Corresponding XRD patterns and observations are shown in (a – c) from bottom to top (Day 1 – Day 9). (a) At atmospheric conditions ($p = \text{atm}$, $T = 20^\circ\text{C}$) calcium carbonate biominerals in the form of mixed hexagonal vaterite with small amounts of rhombohedral calcite formed. During the next treatment cycles calcite will become the more the dominant mineral phase (b) At $p = 9.14 \text{ MPa}$, $T = 50^\circ\text{C}$, hexagonal vaterite was the dominant phase until the 10th treatment cycle (Day 5), afterwards it could not be detected anymore. (c) At $p = 11.18 \text{ MPa}$, $T = 60^\circ\text{C}$, hexagonal vaterite was found over prolonged times until the 12th treatment (Day 6) cycle before mineral dissolution and recrystallisation to a more thermodynamically stable state into the mineral calcite occurred. Dotted and dashed lines are Rietveld refinement results for calcite (magenta) and vaterite (light blue).

Table 5.5 Results of the semi-quantitative Rietveld refinements with TOPAS, on the phase composition of the precipitate carbonate biominerals from the effluent, the values in between brackets are the uncertainties on the last digit of the weight percentage as determined by TOPAS

Rietveld refinements results from collected effluent samples										
	Exp.:	Exp 1			Exp 2			Exp 3		
	Core Nr.:	211/23-3			211/23-4			211/23-5		
Wellbore conditions	TVD [m]	0			914			1118		
	P [MPa]	atm			9.14			11.18		
	T [°C]	20			50			60		
Sample collection day		SUA [mM /min /OD]	Calcite [wt %]	Vaterite [wt %]	SUA [mM/ min/ OD]	Calcite [wt %]	Vaterite [wt %]	SUA [mM/ min/O D]	Calcite [wt %]	Vaterite [wt %]
Day 1		3.45	4.5 (3)	95.5 (3)	4.73	2.8 (5)	97.2 (5)	5.60	1.4 (7)	98.6 (7)
Day 2		3.43	25.5 (4)	74.5 (4)	4.85	4.3 (6)	95.7 (6)	5.45	5.1 (3)	94.9 (3)
Day 3		3.51	33.9 (5)	66.1 (5)	4.90	25.9 (4)	74.1 (4)	5.31	3.3 (7)	96.7 (7)
Day 4		3.42	50.3 (9)	49.7 (9)	4.86	32.1 (5)	67.9 (5)	5.49	27.2 (3)	72.8 (3)
Day 5		3.60	88.6 (7)	11.4 (7)	4.81	50.9 (9)	49.1 (9)	5.66	34.2 (5)	65.8 (5)
Day 6		3.55	88.9 (7)	11.1 (7)	4.92	90.2 (5)	9.8 (5)	5.72	52.1 (4)	47.9 (4)
Day 7		3.32	89.3 (4)	10.7 (4)	4.98	90.1 (9)	9.9 (9)	5.51	92.6 (3)	7.4 (3)
Day 8		3.50	89.5 (5)	10.5 (5)	4.84	91.3 (7)	8.7 (7)	5.33	94.2 (3)	5.8 (3)
Day 9		3.36	93.7 (6)	6.3 (6)	4.77	93.5 (8)	6.5 (8)	5.42	95.9 (4)	4.1 (4)

5.4.3 Micro xCT analyses of CaCO_3 distribution in sandstone cores

The distribution of bio-precipitation in the pore-space of each core was imaged using micro X-ray computed tomography. The left-hand column of Figure 5.8 shows the initial pore-space for each core in blue (whole core). For clarity of image, the middle and right-hand columns show data from a slice (marked A-B) that passes through the centre of the core. In the middle column of Figure 5.8, red indicates the distribution of CaCO_3 biominerals with a light blue background to make the biominerals more visible. The right-hand column in Figure 5.8, is a composition where blue indicates the remaining pore-space and red the precipitated CaCO_3 .

Figure 5.8 (a-c) shows the baseline experiment (Exp 1, Table 5.3) at atmospheric conditions ($p = \text{atm}$, $T = 20^\circ \text{C}$) before and after the bio-grout treatment. In the segmented initial pore space (Fig 5.8a, arrow) mm-thick diagonal layers are clearly visible crossing the well-core, these show the up-dip laminae. The post-grouting 3D slice reveals the location of the biominerals precipitated in the pore-space. Calcite is clearly precipitated close to the injection face. Away from the injection point, the precipitate is well distributed across the whole core, with a higher density of minerals being visible in the higher porosity laminae (arrows). This is likely due to a drop in the fluid velocity within the higher permeability layers.

Figure 5.8 (d-f) shows the results of the X-CT imaging at pressure and temperature conditions (Exp 2, Table 5.3) of $p = 9.14 \text{ MPa}$, $T = 50^\circ \text{C}$. Again, mm-thick diagonal layers (Fig. 5.8d, arrow) are visible, with more calcium carbonate precipitated within these higher permeability layers.

In Figure 5.8 (g-i) at a pressure and temperature of $p = 11.18 \text{ MPa}$, $T = 60^\circ \text{C}$ (Exp 3, Table 5.3), laminae are less distributed throughout the original core. Instead, there is a single very prominent thick lamina towards the outlet of the core. This is coincident with a thick accumulation of biominerals leading to the formation of an almost continuous layer of calcium carbonate blocking the pores (arrow).

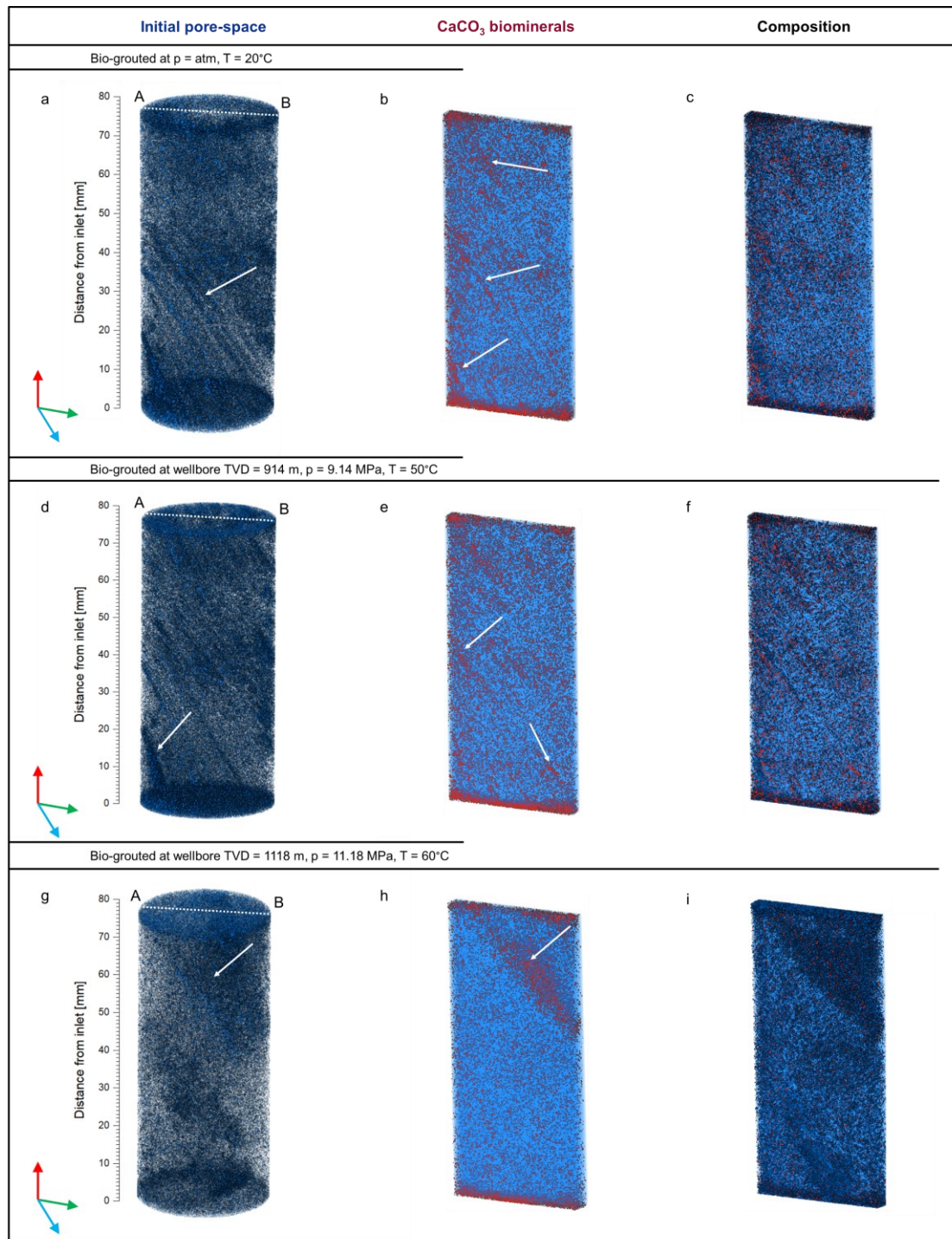


Figure 5.8. (a-i) 3D renderings are showing the segmented pore-space (blue) of the sandstone well-cores prior treating with bio-grout, the CaCO₃ bio-mineral distribution (red with light blue background to make the biominerals more visible) in the pore-space and a composition of the remaining pore-space with the biominerals. (a-c) shows the baseline experiment ($p = \text{atm}$, $T = 20^\circ\text{C}$). (b) The post-grouting 3D slice reveals the location of the biominerals precipitated in the pore-space. The biominerals are well distributed across the whole pore-space and in particular in the higher porous laminae (arrows) more bio-precipitates could be found potentially induced by a change in the flow hydraulics. (d-f) is showing the bio-grouting experiment at elevated pressure and temperature conditions ($p = 9.14 \text{ MPa}$, $T = 50^\circ\text{C}$). Again,

mm thick diagonal layers (d, arrow) with potentially higher porosities are crossing the well-core. (e) CaCO_3 biominerals are very well distributed across the core, again highlighting higher permeable areas (arrows) that allowed for more bio-precipitation. (g-i) at the highest pressure and temperature bio-grouting experiment ($p = 11.18 \text{ MPa}$, $T = 60^\circ\text{C}$) the overall distribution (without laminae) of the biominerals seems to be similar compared to $T = 20^\circ\text{C}$ and $T = 50^\circ\text{C}$. Fine, mm thick laminae are missing in the lower part of the core. Instead, there is a very prominent thick laminae in the upper right corner showing a larger accumulation of biominerals leading to the formation of an almost continuous layer of biominerals (h, arrow).

Using the segmented data from the X-ray CT images, the slice-average porosity was computed as a function of distance from the inlet. The initial porosity (Fig. 5.9a) of the cores determined from XCT ranged from 22.7 – 24.7 %, which is similar to that determined gravimetrically (24.8%). Post-treatment (after all treatment cycles were complete) slice-average porosities (Fig. 5.9b) were calculated using the same method and resulted in porosities as low as 6.00 – 8.05%. This corresponds to an average porosity reduction of $\sim 70\%$. Figure 5.9c compares the porosity reduction for the different temperature and pressure combinations. The greatest reduction was achieved at a temperature of 60°C , this reduces slightly at 50°C and is significantly less for the sample at 20°C .

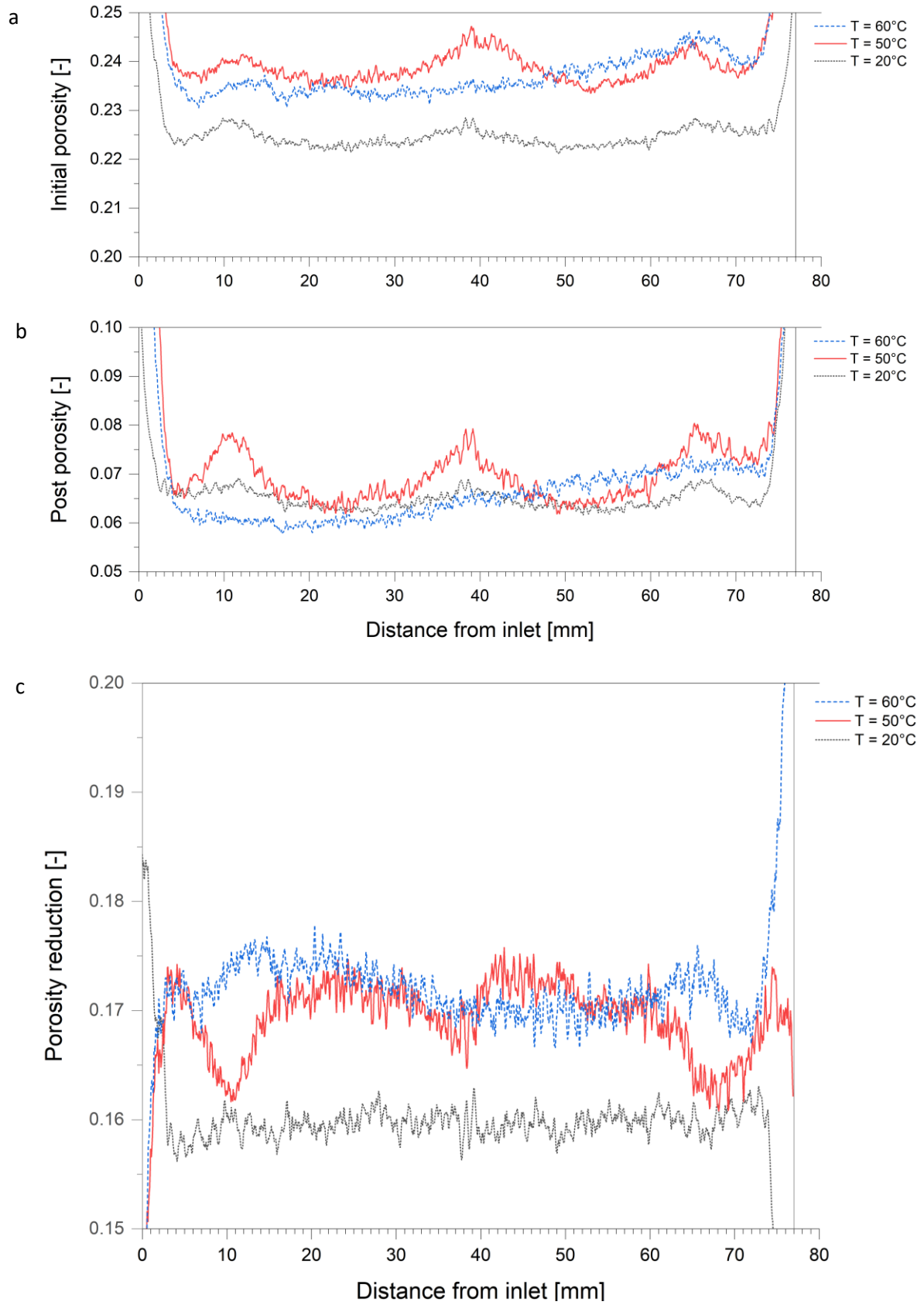


Figure 5.9. Porosity as a function of distance from the inlet computed from the individual CT image stacks. (a) indicates the initial porosity along the z-axis of the core in the untreated state. (b) shows the reduced pore-space after application of MICP over a period of 9 consecutive days. (c) indicates the absolute change in porosity for each CT slice over the length of the core.

Sub-core scale results

5.4.4 Tungsten Filament Scanning Electron Microscopy (W-SEM)

To investigate the spatial distribution and morphology of calcite crystals as a function of pressure and temperature, polished sections taken at a distance of $z = \{38.5 \text{ mm}\}$ from the inlet (Fig. 5.3) from Exps. 1-3 (Table 5.3) were analysed using Tungsten Filament Scanning Electron Microscopy. Representative back scattered electron images (BSE) are shown in (Fig. 5.10 a-i). At all grouting conditions, calcium carbonate phases could be identified as the relatively large anhedral and roughly circular regions/minerals with the lighter grey shading in the backscatter images (Fig. 5.10 a-i).

At atmospheric grouting conditions ($p = \text{atm}$, $T = 20^\circ\text{C}$) calcium carbonate biominerals can be observed to bridge the pore-space between sand grains (Qtz) (Fig. 5.10 a-b). In Fig. 5.10b a circular pattern can be identified (arrow), this indicates biomineral growth is promoted radially from a centre point. This morphology is indicative of spherulitic growth, a predominantly solution nucleation based growth mechanisms (or on microbes) (Bots *et al.*, 2012; Lin *et al.*, 2018). The progressively larger circles indicate the process is progressing with additional treatment cycles, likely via surface nucleation on the newly grown carbonate crystals. Spherulitic carbonate crystal growth is also observed at elevated wellbore conditions ($p = 9.14 \text{ MPa}$, $T = 50^\circ\text{C}$ and $p = 11.18 \text{ MPa}$, $T = 60^\circ\text{C}$) (Fig. 5.10 d-i). At 60°C , Fig. 5.10 g-i, the spherulite crystal is overgrown by faceted crystals, resulting in a rough crystal surface. This observation is common in the 60°C images and was not seen in the specimens treated at the lower temperatures.

Fig 5.11 shows details of the three crystals in Figures 5.10c, f and i, with additional annotation to show the observable layers within the crystal that relate to the individual treatment cycles. It is evident that the thickness of the biominerals varies between each treatment cycle. In Figure 5.11d, at $T = 50^\circ\text{C}$, the carbonate crystal shows two potential nucleation points; growth appears to occur near the surface of the sand grain and then merge with a second nucleation point (arrow). In Figure 5.11f, at $T = 60^\circ\text{C}$, it is evident that the outer edge of the biomineral becomes increasingly more faceted and increases in surface area as the number of treatment cycles increases. This indicates that in this case, the initial spherulite was overgrown by faceted

crystals (likely concurrent to a slower growth rate to achieve these faceted crystals), possibly indicating a decrease in the supersaturation during growth.

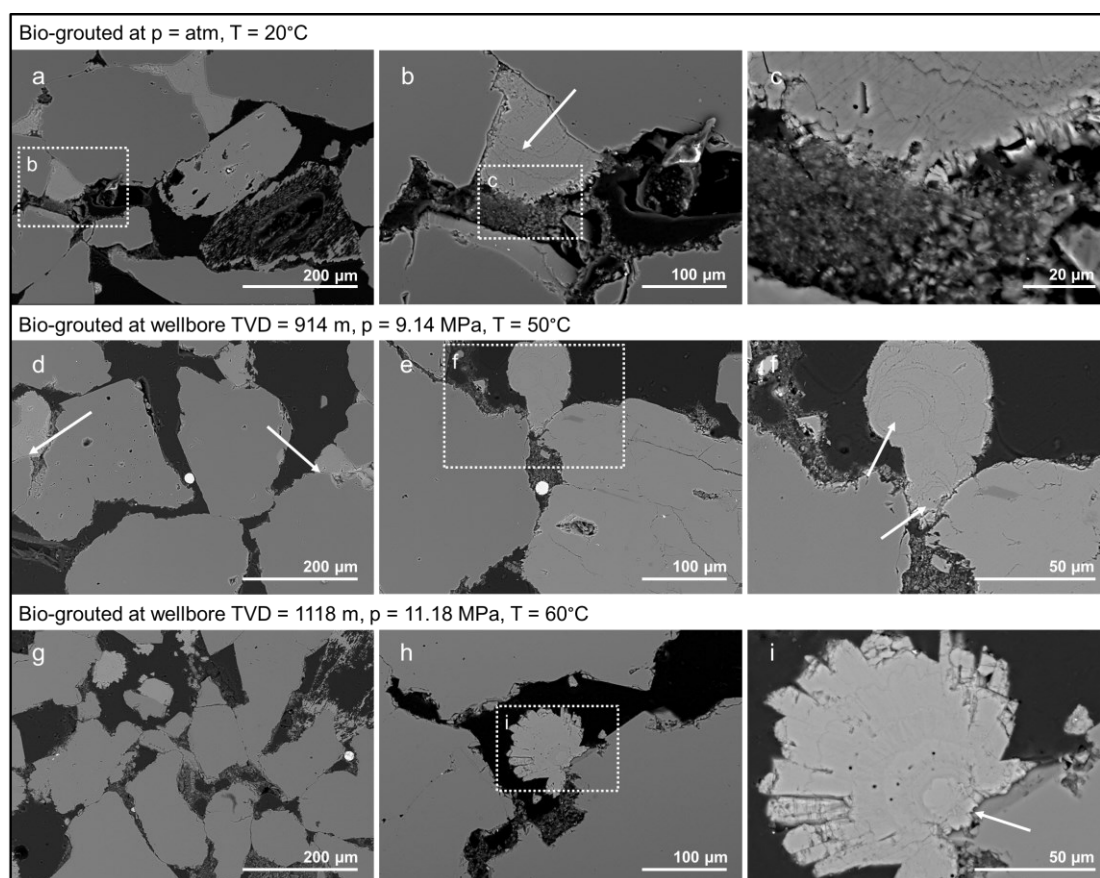


Figure 5.10. Highly polished blocks with resin impregnation were prepared from the bio-grouted well-cores under in-situ wellbore pressure and temperature conditions. For the analysis, a representative set of Back scattered electron images (BSE) with the corresponding observations are shown in (a – i). In (a – c) BSE images at atmospheric grouting conditions ($p = \text{atm}$, $T = 20^\circ\text{C}$) calcium carbonate biominerals bridge the pore-space in between the sand grains (Qtz). (b - c) Growth is promoted radially from a centre point and is progressing with additional treatment cycles via surface nucleation (small dots are indicating microbial activity) on the newly grown carbonate crystals. (d – f) At elevated wellbore conditions ($p = 9.14 \text{ MPa}$, $T = 50^\circ\text{C}$) spherulitic carbonate crystal growth dominates. (g – i) At the highest pressure and temperature conditions ($p = 11.18 \text{ MPa}$, $T = 60^\circ\text{C}$) spherulitic growth was very prominent with spherulites overgrown by faceted crystals (rough surfaces). In (i) the growth fronts of the carbonate crystal were extending radially with embedded microbes (black dots) and potential surface attachment (indicated by arrow).

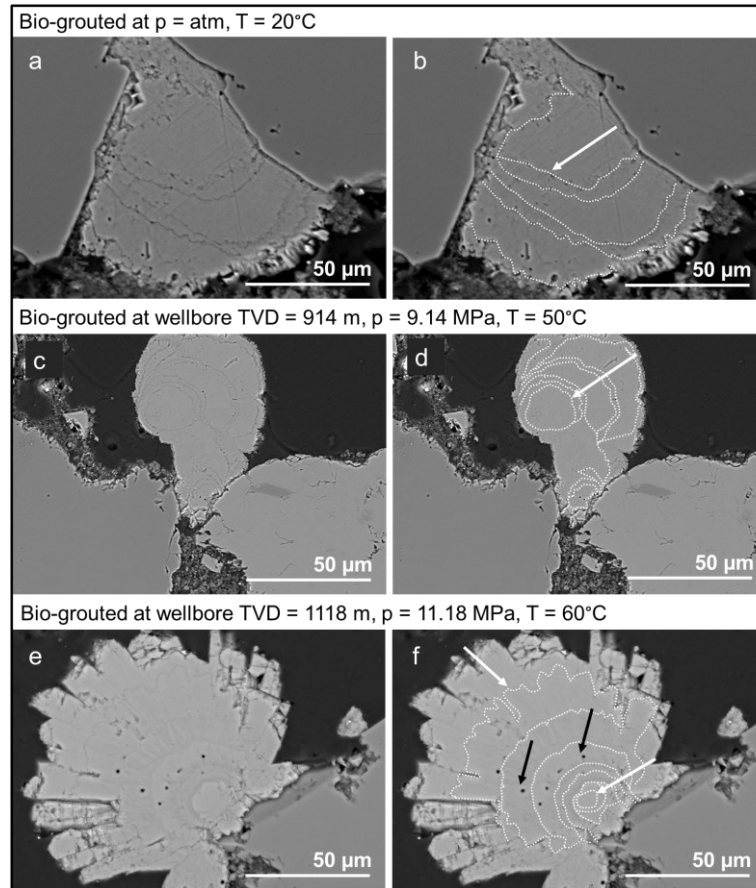


Figure 5.11. This figure is comparing the growth front behaviour of carbonate crystals under various pressure and temperature conditions (a – f). In (a – b) BSE images at atmospheric grouting conditions ($p = \text{atm}$, $T = 20^\circ\text{C}$) calcium carbonate biominerals bridge the pore-space in between the sand grains (Qtz) (small black dots are indicating microbial activity at the growth fronts. (b) the annotation is giving an idea how growth is promoted radially from a centre point and is progressing with additional treatment cycles via surface nucleation (c – d) At elevated wellbore conditions ($p = 9.14 \text{ MPa}$, $T = 50^\circ\text{C}$) spherulitic carbonate crystal growth dominates. The lower part of the carbonate crystal shows potential growth from the surface of the sand grain and might have merged with a solution nucleated spherulite. (d) the annotation indicates the spherulites with radially extending growth fronts. Microbial activity was found again at the crystal growth fronts (e – f) At the highest pressure and temperature conditions ($p = 11.18 \text{ MPa}$, $T = 60^\circ\text{C}$) spherulitic growth was very prominent with spherulites overgrown by faceted crystals. In the annotation (f) the growth fronts of the carbonate crystal were first extending radially as in (d) and the further the crystal extends the jagger this growth fronts become. It seems like these gaps were again filled up with new bio-precipitates and grew further. The black arrows indicate microbial activity not just at the growth front boundary, it is likely they are embedded in the crystal matrix.

5.4.5 Cryo-FIB/FEG-SEM

5.4.5.1 Surface and solution nucleation processes

Figures 5.12 and 5.13 are taken from a cryo-prepared sample (Exp 4, Table 5.3, Fig. 5.3) at the highest pressure and temperature conditions ($p = 11.18$ MPa, $T = 60^\circ\text{C}$) and show two different calcium carbonate crystal growth morphologies (Fig. 5.12e / Fig. 5.13 circle). In Figure 5.12, small random orientated CaCO_3 crystals/anhydral are shown, and in Fig. 5.13 radial (in circle) in Fig. 5.13a/spherical e.g., in Fig. 5.13 (c-e) crystals. This indicates the presence of two distinct calcium carbonate crystallisation mechanisms. In the first morphology, the crystals appear to be relatively randomly orientated, and near the surface of silicate minerals within the pores (Fig. 5.12). Additionally, these crystals do not fill the full pores of the sample. This suggests that during the first crystallization process, microbes accumulate around sand grain surfaces (Qtz). The negative surface charges of the bacterial cell walls then act as nucleation templates for calcium carbonate crystal growth (Schultze-Lam *et al.*, 1996; Stocks-Fischer, Galinat and Bang, 1999). Whereas in the second crystallization process, crystals appear to radiate outward from a central point, and form clusters of crystallites with a spherulitic morphology (Fig. 5.13). Additionally, in the areas with spherulitic morphologies, calcium carbonate tends to either fill the complete pore space in the sample, or block pores (Fig. 5.13), also observed in the W-SEM image (Fig. 5.10b, e, h). A high-resolution image of the morphology shows that these morphologies are composed of nanoparticles oriented spherically around a centre point (Fig. 5.13e). Such polycrystalline morphology suggests that the growth process is spherulitic growth (Bots *et al.*, 2012). Spherulitic growth is characterised by nucleation in solution, and growth through subsequent nucleation of calcium carbonate crystallites on the pre-existing carbonate surface (Fig. 5.13e). This (solution nucleation process) indicates that the observed crystals only became retained within the pores when their size minimizes migration through the pores.

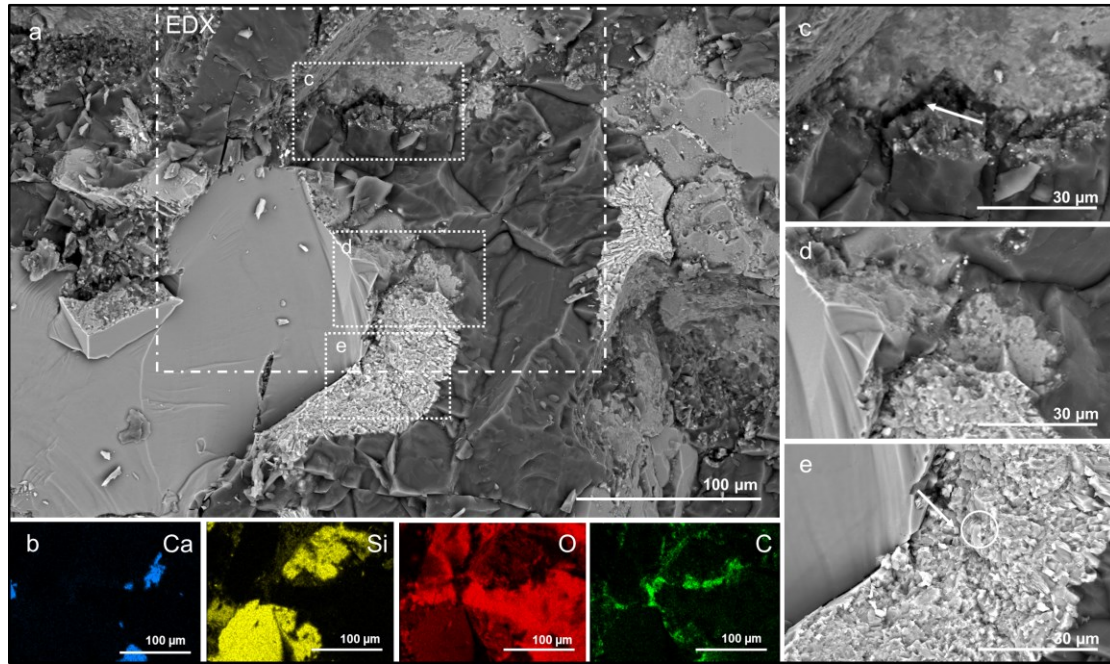


Figure 5.12. Small sub-samples were prepared, from the sectioned bio-grouted well-core under in-situ wellbore pressure and temperature conditions ($p = 11.18$ MPa, $T = 60^{\circ}\text{C}$), (39 mm from inlet). For the analysis, a representative set of Back scattered electron images (BSE) with Energy-dispersive X-ray maps (EDX) and corresponding observations are shown from the cryo-fractured sub-sample in (a – e). In BSE image (a) a surface nucleation process of CaCO_3 biominerals onto a sand grain (Qtz) is shown. (b) is an elemental map for the area of interest. (c) indicates microbial (arrow) activity with surface nucleation. (d) shows the interface between the sand grain (Qtz) and the bio-precipitates. (e) the interface between the sand grain (Qtz) and the morphology of the CaCO_3 biominerals.

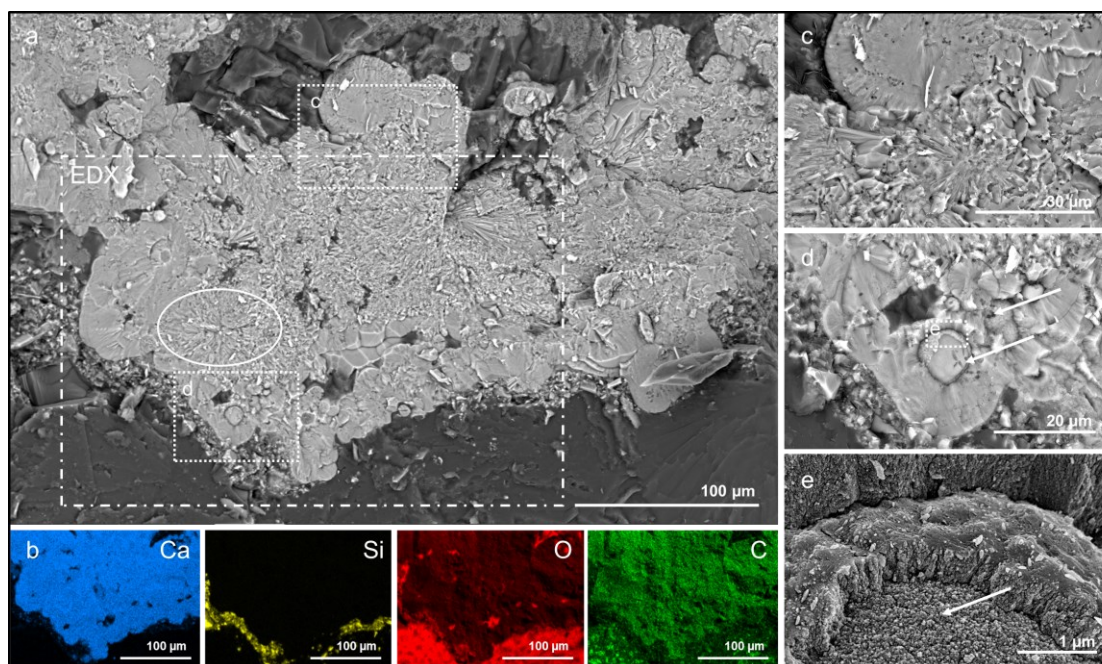


Figure 5.13. Small sub-samples were prepared, from the sectioned bio-grouted well-core under in-situ wellbore pressure and temperature conditions ($p = 11.18$ MPa, $T = 60^{\circ}\text{C}$), (39 mm from inlet). For the analysis, a representative set of Back scattered electron images (BSE) with Energy-dispersive X-ray maps (EDX) and corresponding observations are shown from the cryo-fractured sub-sample in (a – e). In BSE image (a) The circle indicates the radial growth of the carbonate crystals, likely spherulitic growth, which indicates a solution nucleation process, or on microbes suspended in solution of CaCO_3 biomineral formation (rather than nucleation on well-defined surfaces, e.g., minerals). Newly born CaCO_3 nano-crystallites were building up on each other extending in a typical spherulitic manner, which has been potentially identified as hexagonal vaterite. (b) is an elemental map for the area of interest. (c) shows typically radial extension of the carbonate crystals with a lot of microbial activity as seen as black dots. (d) presents a very well preserved spherulite with typical surface attachment of further nano crystallites. The arrows are indicating rod-shaped microbial imprints. (e) Secondary electron image (SE) of the surface of the spherulite showing the tiny nano-crystallites attaching

At the surfaces of the spherulites (Fig. 5.13c, d, e) microbial activity seems to be particularly abundant; microbial imprints of well-preserved rod-shaped bacteria cells are visible (Fig. 5.13d). The nanoparticulate/polycrystalline nature of the surface of a highlighted spherulite in (Fig. 5.13e) indicates that the bulb grows dominantly via surface nucleation.

Figure 5.14 shows calcium carbonate next to a sand grain, indicating that a pore filled with calcium carbonate (lighter grey shading in the backscatter image). In this area regions with spherical morphology (Fig. 5.14b and c) and with anhedral morphologies (Fig. 5.14d) are observed. This suggests that we were able to capture the growth of carbonate crystals in the pore-space between mineral interfaces (Qtz) as well as spherulitic growth (likely through solution nucleation or nucleation on microbes in solution). The whole pore-space in the image is filled in a chaotic manner with a mixture of both crystallization mechanisms visible. In the right image of Fig. 5.14c a crystal showing spherulitic growth is identified, with growth progressing radially from a centre point. The multiple layers have thicknesses up to 2 micrometres and are likely distinct mineral growth episodes associated with each treatment cycle. Microbial imprints are visible at the transition from one growth episode to the next due to the repeated injection of microbes with each treatment cycle (Fig. 5.14c, arrow).

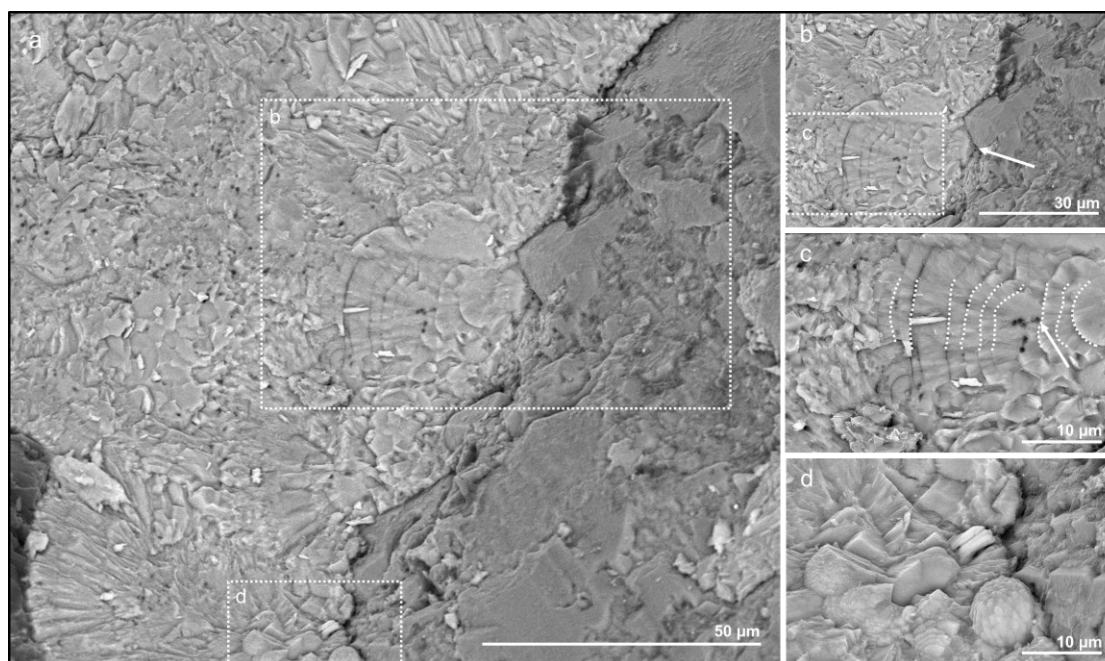


Figure 5.14. Calcium carbonate next to a sand grain, indicating that a pore filled with calcium carbonate. The filling of the pore occurred in a chaotic manner. (a) a combination of solution and surface nucleation processes led to the complete blockage of this pore. Spherulitic growth and also crystal formation with surface nucleation with microbes as negatively charged surface templates could be identified. (b) shows radial growth progression with lots of microbial activity involved; the microbes were also seen at the interfaces. With these radially extending growth rings the amount of treatment cycles could be counted (for 9 consecutive days). In (c) the BSE image is indicating the attachment of a further CaCO_3 crystal to another one with multiple layers of CaCO_3 extending radially. (d) is showing a typical spherulitic hexagonal vaterite crystal confirming the suggested nucleation mechanism.

To get a more in-depth view of the interface between the sand grain (Qtz) and the carbonate crystal, focused ion beam milling with a Gallium beam was conducted on the crystal shown in Figure 5.15a (location of milling is shown by the dotted line). Focused ion beam milling for a depth of 25 μm from the top of the surface was applied on the cryo-frozen sample (Fig 5.15a, dotted line). Fig. 5.15b revealed that there is surface attachment of microbes (indicated by arrows) occurring at the interface of the quartz and the carbonate biomineral. In the bulk precipitate, microbial activity was also abundant, and bacterial rods are visibly entombed in the matrix of the carbonate (Fig. 5.15b inset).

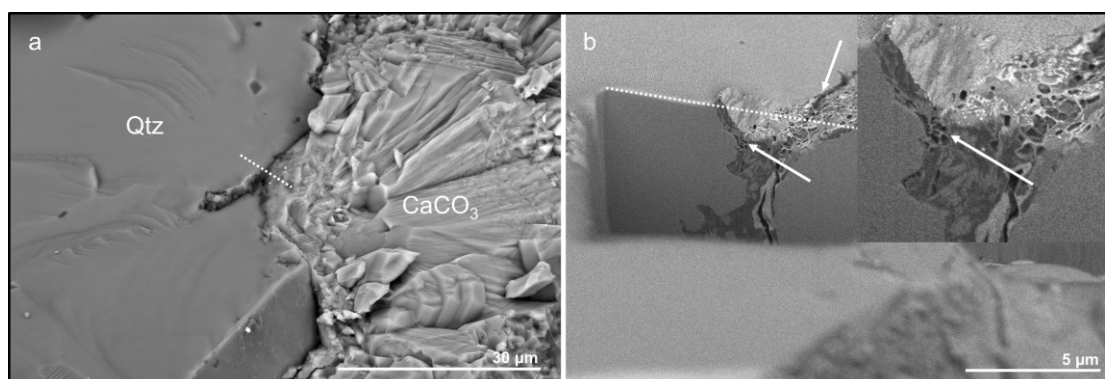


Figure 5.15. (a) The left panel is showing a sand grain (Qtz) - CaCO_3 biomineral crystal interface. The white dotted line is indicating the milling location across the two interfaces with the Focussed-Ion-Beam (FIB) – milling depth 25 μm . In the right panel (b) the white arrows indicated microbial activity at the interfaces that potentially led to surface nucleation and CaCO_3 crystal growth from this point. Furthermore, in the biominerals precipitated on the right a lot of entombed bacterial cells could be identified in the matrix of the carbonate (inset).

5.5 Discussion

5.5.1 Reduction of permeability

Minto *et al.* (2017) demonstrated a permeability reduction in Berea sandstone of over one order of magnitude in 6 MICP treatment cycles. Treatment results in greater porosity reduction close to the inlet (Minto *et al.*, 2017), with porosity close to the outlet remaining unaffected by MICP treatment. Tobler *et al.* (2018) reduced the permeability of a clean and homogenous Bentheimer sandstone via MICP treatment by 3 orders of magnitude (from 2400 – 2.7 mD) in 20 treatment cycles.

The results presented here demonstrate that MICP treatment at elevated and high pressure and temperature combinations can reduce permeability by at least 5 orders of magnitude after 18 treatment cycles (9 consecutive days). The most rapid drop in permeability was observed at $T = 60^{\circ}\text{C}$, with the first 10 treatment cycles producing a 96% permeability reduction from 3994 to 186.60 mD, compared to 89% (from 3954 to 453.65 mD) at $T = 50^{\circ}\text{C}$ and 84% (3992 to 625.15 mD) at $T = 20^{\circ}\text{C}$ (Fig. 5.6 and Table 5.4).

Based purely on ureolytic activity, as the enzyme has an optimum reaction rate between 45 to 65 °C (Krajewska, Van Eldik and Brindell, 2012), it might have been expected that a greater difference would be seen between 20 and 50°C than 50 to 60°C. However, this neglects the effect that temperature has on CaCO_3 saturation index and other abiotic factors. The combined effect of temperature on bacterial activity and on the saturation index potentially explains why 60°C resulted in a greater permeability reduction at fewer treatment cycles compared to the specimens treated at 50°C and 20°C. Meanwhile, the effect of increasing the pressure from 9 to 11 MPa is thought to have had minimal influence on permeability *Chapter 4* shows little effect of exposure to pressure on the rate of ureolysis.

5.5.2 Spatial distribution of calcium carbonate

MICP treatment resulted in fairly uniform calcite precipitation along the length of the cores (Fig 5.9). Although, initial higher porosity bands within the sandstone rocks (Fig. 5.8a, d, g) appeared to promote the growth of calcium carbonate crystals potentially due to changes in the flow hydraulics (Fig. 5.8b, e, h). In Fig. 5.9 it seems the higher porosity band in the 60°C treated core promoted a greater porosity reduction in that zone. This might be expected as the fluid velocity is likely to be lower in higher porosity zones. In comparison to the 50°C treated core, it seems that the location of the initially higher porosity bands experienced a lower porosity reduction. This may therefore indicate that it is not just dependent on the initial porosity but on the flow paths, local fluid velocities within each core and how these evolve over time with treatment. Bacterial attachment and mobilisation is dependent on fluid velocity and thus, lower velocities may promote attachment, and higher velocities restrict precipitation in a given zone.

5.5.3 Biomineral attachment and mineral growth

From the sub-core analyses conducted, it is evident that both, nucleation in solution or on microbes in solution (i.e., spherulitic growth), and heterogenous nucleation processes of calcium carbonate are present. That implies that carbonates are established due to multiple nucleation processes. In places where sufficient bacterial cells were present, the negatively charged surfaces of the microbes attract positively charged calcium ions from the cementing solution and act as nucleation templates to promote the growth of carbonate crystals (Fig. 5.14b) (Douglas and Beveridge, 1998). This is furthermore supported by the application of Focused Ion Beam milling, and Field Emission Gun - Scanning Electron Microscopy, which indicates that microbes have acted as nucleation templates that were indicated by radial growth from these surfaces (Fig. 5.15). Conversely, I suggest that in areas that were too far away from any microbial activity mainly solution nucleation appeared to dominate (Fig. 5.13). This led to the growth of bulb-shaped spherulites that may have grown big enough to clog pore throats, in addition to the heterogenous growth of minerals on surfaces (e.g., through attachment), and therefore both nucleation processes, divert fluid flow in the porous media, and reduce the permeability (Fig. 5.6 and Table 5.4).

5.5.4 Calcium carbonate crystal polymorphism/pseudomorphism

Rodriguez-Blanco, Shaw and Benning (2011) and Bots et al. (2012) have experimentally investigated calcium carbonate crystal polymorphism. According to the Ostwald step rule, in a first stage, very unstable amorphous calcium carbonate forms (<2 mins stable) before transformation into vaterite. Finally, depending on the environmental conditions, a more thermodynamically stable form is reached in the form of calcite (which in a pure system takes 5 h at 25°C; Rodriguez-Blanco, Shaw and Benning, 2011). The SEM images (Figures 5.11, 5.13, and 5.14) indicate that in the experiments presented here, spherulitic growth of calcium carbonate crystals dominate. It is well known that several carbonate polymorphs (aragonite and calcite) can form through spherulitic growth (Rodriguez-Blanco, Sand and Benning, 2017). Consequently, judgment based on the polymorphism alone is not straight forward. From the spherulitic appearance of the minerals in the images, this mostly resembles vaterite (Fig. 5.7). Though, during the final treatment cycles, dominantly calcite was detected in the effluent. Conversely, initially the effluent was dominated by vaterite (Table 5.5). These findings suggest

that whilst in early treatment stages, the main carbonate phase is metastable vaterite as spherulites (Fig. 5.7 and Table 5.5). This implies that calcium carbonate transformation into calcite should be occurring, and likely the spherulitic appearance in the images (Fig. 5.7) are ‘relics’ from spherulitically grown vaterite (pseudomorphism).

In the W-SEM (and cryo-SEM) images, faceted crystals (indicative of calcite) found only sporadically (e.g., Fig. 12e, arrow) with the majority of the phases being spherulitic calcium carbonate polymorphs. The dominance of calcite in the XRD analyses and the absence of rhombohedral calcite crystals in the images could be explained by one of two processes: (a) pseudomorphism (as described above), where the original morphology of the precursor phases is retained (in this case the vaterite phases), resulting in calcite crystals with spherulitic morphology. In nature, this is observed with ikaite ($\text{CaCO}_3 \cdot 6\text{H}_2\text{O}$) another metastable carbonate mineral that precipitates from alkaline waters with high phosphate concentrations (Sánchez-Pastor *et al.*, 2016). During the transformation into the anhydrous calcium carbonate polymorphs the original ikaite crystal shape is preserved as a pseudomorph (Sánchez-Pastor *et al.*, 2016); or (b) crystal growth in confinement (Schenk *et al.*, 2014), although similar spherulitic morphologies are observed in open pores (Fig. 5.10).

5.6 Conclusions

Bio-grout sealing experiments of sandstone well-cores from a North Sea offshore wellbore were treated under *in-situ* wellbore pressure and temperature conditions and permeability reductions of five to six orders of magnitude were achieved, reaching post-treatment permeabilities of $63\ \mu\text{D} - 4\ \mu\text{D}$ (Fig. 5.6 and Table 5.4). This was achieved in 18 treatment cycles conducted over 9 consecutive days of treatment (Table 5.4). This compares to the permeability of a conventional API G class cement with $\sim 10\ \mu\text{D}$ (personal communication, OGTC, 2018; Aguilar *et al.* (2016)) to be 2.5 times less.

Permeability reduction occurred more rapidly (in fewer treatment cycles compared to the lower pressure and temperature combinations with 84% at $T = 20^\circ\text{C}$ and 89% at $T = 50^\circ\text{C}$. Porosity reduction was higher in both the 50°C and 60°C compared to the 20°C specimens. The sandstone cores all contained heterogeneities (i.e., higher permeable sections). Higher permeability bands were found to be closely correlated with higher amounts of calcium carbonate precipitation, most likely due to influencing bacterial immobilisation.

Effluent bio-mineral characterisation (Fig. 5.7, Table 5.5) indicates that during the bio-grout treatment, during initial treatment cycles these phases form as vaterite, and over multiple progressive cycles transform into a higher stable crystal polymorph in this case calcite. Though, the change to the typical rhombohedral crystal structure in the BSE images was found sporadically (in Fig. 5.11e). The results were suggesting that one of the two processes known as pseudomorphism, where the original morphology of the precursor phase is retained or crystal growth in confinement (narrow pore throats) occurred.

Our results from sub-core scale imaging analysis present evidence for two nucleation processes: (i) biomineral crystal growth at surfaces and (ii) in solution was promoted. Spherulitic CaCO_3 growth with progressing addition of carbonate layers was observed (Fig. 5.14c).

These findings have important implications for applying this biogROUT mineral sealing technology successfully in the subsurface and for preventing uncontrolled fluid flow up to the surface from former oil and gas production zones.

Chapter 6

Conclusions and Recommendations

6.1 Conclusions

In a first step of a bigger research campaign investigating the potential application of Microbially induced carbonate precipitation, this PhD research has focussed on gaining new knowledge in the application of MICP as a subsurface mineral sealing technology under *in-situ* pressure and temperature conditions to potentially seal off leakage migration pathways of hydrocarbon wellbores.

The specific research objectives of this thesis were to:

1. Optimise the growth performance of *S. pasteurii* with a new low-cost/low-carbon nutrient media and assess performance based on biomass and enzyme kinetics.
2. Investigate the influence of temperature and pressure on the ureolytic activity of *S. pasteurii* and hence determine the depth range over which MICP might be applicable for wellbore sealing.
3. Investigate the sealing potential of MICP over a range of pressure and temperature combinations consistent with subsurface conditions in the North Sea.
4. Investigate the influence of temperature and pressure on the mineralogy and morphology of carbonate crystals produced via MICP and determine how these may influence the success of the biogrout as a future sealing technology.

5. Outline potential application of microbially induced carbonate precipitation for establishing environmental barriers in subsurface applications based on the results from MICP sealing ability under simulated depth conditions.

In this thesis the findings (organised as paper) were presented as conclusions in each *Chapter*. As a consequence, they are only presented in brief here. This final *Chapter* aims to recap the main conclusions of each paper according to the objectives listed above, and then to discuss the implications for the oil industry.

Each section within the conclusions (Section 6.1) consists of i) the key findings, ii) the contribution of new gained knowledge to existing knowledge. This is then followed by implications for the oil industry and recommendations for future work (Section 6.2 / 6.3).

6.1.1 Optimisation of the growth performance of *S. pasteurii* with a new low-cost/low-carbon nutrient media

A preliminary investigation was conducted to optimise the growth of *S. pasteurii* with various microbiology and non-microbiology grade nutrient sources and compared with the microbiology “gold standard” Brain Heart Infusion broth.

The results in *Chapter 3* showed that the average growth of *S. pasteurii* in a 24 hr time interval for various nutrient media ranged from 0.99 – 1.24 OD₆₀₀ (Tryptone the least efficient nutrient source to Brain Heart Infusion the most growth efficient nutrient source). Other nutrients i.e., LMG-139, Yeast Extract, Lab-Lemco Beef Extract and Marmite could achieve similar growth in a 24 hr time frame.

Based on the aforementioned results (OD₆₀₀ over time) the efficiency yield per media (OD₆₀₀/costs) was calculated and it was noted that the non-microbiology grade nutrient Marmite had the highest efficiency yield, this was followed by Brain Heart Infusion as the most common and widely used nutrient in Microbiology.

Further study of the enzyme kinetics revealed that Marmite, which is a waste-stream product from brewery yeast, is a more sustainable and cost-effective growth media than the commonly used Brain Heart Infusion broth. Finally, biomineral precipitation experiments showed that

similar quantities of biominerals are precipitated regardless of the growth media. Cost analysis based on the treatment of 1 m³ of porous media with the alternative nutrient media Marmite concluded that costs could be reduced by ~90%.

6.1.2 Contributions to existing knowledge

Research in this thesis on the Marmite as growth media for the bacteria makes a significant contribution to the potential for commercial viability of MICP at an industrial scale. Not only can yeast-based growth media reduce the cost, but it will also contribute to a future low-carbon circular economy: yeast has a much lower embedded carbon than beef, and if it can be sourced from the food processing industry, then the waste products from one industry are becoming the feedstock for another.

6.1.3 Investigation of the effect of temperature and pressure on whole *S. pasteurii* cells and their implications on metabolic activity

This section of the thesis investigated for the first time an independent study of temperature and pressure, and a combination of both, on whole *S. pasteurii* cells.

In a first stage, constant temperature batch exposure experiments were conducted on a bacterial suspension of *S. pasteurii* and pure urease enzyme from jack bean meal within a temperature range of $T = 20 - 90^{\circ}\text{C}$. The results in *Chapter 4* showed that temperature, as opposed to pressure, had the largest impact on ureolytic activity.

Experiments showed that the bacteria produced the most urease enzyme at $T = 60^{\circ}\text{C}$, which was found to be the optimum temperature for sustained ureolytic activity over extended periods of time. Higher temperatures of $T = 80^{\circ}\text{C}$ and $T = 90^{\circ}\text{C}$ indicated a short spike in high ureolytic activity for $t = 30$ min at $T = 80^{\circ}\text{C}$ and $t = 15$ min at $T = 90^{\circ}\text{C}$. After which the ureolytic activity reduced by approximately 2/3, with activity at the highest temperature ultimately dropping to zero.

For pure enzyme derived from jack bean urease, the ureolytic activity was similar to that of the bacteria at $T = 20^{\circ}\text{C}$. However, at a temperature of $T = 80^{\circ}\text{C}$ the enzyme was only active for 15 min before dropping to zero ureolytic activity, and at $T = 90^{\circ}\text{C}$ no activity was found.

Experiments were conducted to investigate the effect of gradual temperature ramping, as would be experienced when pumping to depth in a wellbore. Using *S. pasteurii*, temperature was gradually increased from ambient laboratory conditions to $T = 60, 70, 80$ and 90°C over a period of $t = 30$ min and then remained constant for a further $t = 30$ min. The experiments showed that the rate of temperature increase did not affect the ureolytic activity, which was the same as that found with the constant temperature experiments.

S. pasteurii were exposed to a range of constant pressures from $p = 1.0 - 15.0$ MPa achieved in a Hassler-type core holder for a duration up to $t = 2$ hr. The results indicated that pressure had no influence on the ureolytic activity. By increasing the pressure from 1.0 MPa, through 5.0 MPa and 10.0 MPa to 15 MPa, the ureolytic activity slightly decreased but the effect was not significant.

The effect of gradually increasing the pressure from atmospheric to $p = 15.0$ MPa was also investigated. Again, results showed a small drop in ureolytic activity after ramping, the magnitude of decrease was similar to that achieved when the pressure was fixed at 15 MPa. Hence, the rate of pressure change did not appear to affect the activity of the bacteria.

In a final set of experiments, temperature and pressure combinations for simulated depths of 1000 and 1500 m with their corresponding temperature according to a geothermal gradient and hydrostatic pressure were investigated. Results revealed very high ureolytic activity at $T = 54^{\circ}\text{C}$ and $p = 10.0$ MPa fluid pressure, whereas at a temperature of $T = 79^{\circ}\text{C}$ and $p = 15.0$ MPa fluid pressure, the ureolytic activity diminished significantly.

As an alternative technique, thermal induced carbonate precipitation (TICP) was explored as a pilot approach for temperatures found to be beyond bacterial and enzyme survival. Results suggested that thermal urea hydrolysis begins to occur at a temperature between $T = 120^{\circ}\text{C}$ and 140°C . At $T = 140^{\circ}\text{C}$ urea could be decomposed in less than 24 hr whereas at increased temperatures of $T = 160^{\circ}\text{C}$ and $T = 180^{\circ}\text{C}$ urea was thermally hydrolysed in less than < 12 hr and < 8 hr respectively.

6.1.4 Contributions to existing knowledge

The results gained from this study with batch experiments on bacterial suspension of *S. pasteurii* exposed to a range of temperature and pressure conditions and combinations of it

show the first time the applicability of MICP in a deep subsurface environment. Findings suggest that temperature has the biggest impact on bacterial urease production by *S. pasteurii*, whereas pressure has almost no effect. Experiments suggest that MICP is a viable technology up to a temperature of $\sim 60^{\circ}\text{C}$. For temperatures above 120°C , experiments showed that thermally induced carbonate precipitation could provide a viable alternative.

6.1.5 Mechanistic insights into the formation of biominerals *under in-situ* wellbore pressure and temperature conditions

This section of the thesis investigated the sealing capabilities of microbially induced carbonate precipitation under *in-situ* wellbore pressure and temperature conditions on well-cores retrieved from the Dunlin-Field in the North Sea. The sandstone cores were mainly from the Brent sequence and contained structural unconformities and laminae with alternating grain sizes.

Three temperature and pressure combinations were used in the experiments, based on the results of *Chapter 4*. These were: a baseline experiment at $T = 20^{\circ}\text{C}$, $p = \text{atm}$; *in-situ* wellbore temperature and pressure conditions of $T = 50^{\circ}\text{C}$ and $p = 9.14 \text{ MPa}$; and a temperature and pressure combination of $T = 60^{\circ}\text{C}$ and $p = 11.18 \text{ MPa}$, representing conditions at a depth of 914 and 1118 m (bgl) respectively.

Each core was treated with two cycles of MICP per day and the drop in permeability was determined as a function of the increasing number of treatment cycles. The permeabilities of the individual well-cores were reduced by 5 to 6 orders of magnitude (into the micro-Darcy range), after 18 consecutive treatment cycles. Experiments showed that permeability reduction occurred more quickly at higher temperatures.

Micro X-ray computed tomography was conducted for each core prior to, and post, treatment. Results showed that for all temperature and pressure combinations biominerals were well distributed throughout the pore-space and that, where higher porosity laminae were present, a larger amount of calcite had precipitated. Porosity reduction was found to be $\sim 70\%$ for all three bio-grouted cores, despite the core-scale observation that permeability reduction occurred more rapidly at $T = 60^{\circ}\text{C}$, no significant differences in calcite distribution were observed in the X-CT data.

Sub-core scale image analysis, in combination with effluent biomineral analysis, revealed new mechanistic insights into the formation of calcium carbonate biominerals under *in-situ* wellbore pressure and temperature conditions. Effluent bio-mineral characterisation indicated that during the bio-grout treatment, biominerals initially form as vaterite, and then transform into the higher stable crystal polymorph, calcite. Results from the sub-core scale image analysis (with Tungsten filament scanning Electron Microscopy (W-SEM) and cryo-Focused Ion Beam/Field Emission Gun Scanning Electron Microscopy (cryo-FIB/FEG-SEM) suggest that two distinct nucleation processes occur; biomineral crystal growth from nucleation on a grain surface and biomineral growth where nucleation occurs in solution. Spherulitic CaCO_3 growth occurred with progressive addition of carbonate layers associated with each treatment cycle. At the highest pressure and temperature combination (only) ($p = 11.18 \text{ MPa}$, $T = 60^\circ\text{C}$) spherulites with overgrown faceted crystals were identified. This type of crystal development, which may occur due to variations in the saturation index, could explain the more rapid permeability drop that occurred after 10 treatment cycles at $T = 60^\circ\text{C}$ (by 96%) when compared to the lower pressure and temperature combinations (89% at $T = 50^\circ\text{C}$ and 84% at $T = 20^\circ\text{C}$).

6.1.6 Contributions to existing knowledge

This study gives new insights into the formation of calcium carbonate biominerals formed during *in-situ* wellbore pressure and temperature conditions. So far, research in the literature has been mainly conducted on sand columns and or within very homogenous rocks in ambient conditions i.e., Berea or Bentheimer sandstone cores. This investigation targeted, in particular, the potential for microbially induced carbonate precipitation as a sealing technology at depths appropriate to the hydrocarbon industry. Furthermore, it is first study to investigate the mechanistics of the formation of biominerals at these high pressure and temperature conditions.

6.2 Implications for the oil industry

In this section the major implications of MICP for the oil and gas industry are discussed. Deployment depths and new fields of application in subsurface environments are outlined, including discussion of where MICP could bring advantages over traditional cementitious grouting technology.

Deployment depths for MICP will vary depending on the local geothermal gradient but are illustrated for the North Sea in Fig. 6.1. In the North Sea region, MICP would be deployable up to a depth of TVD = 1118 m which corresponds to a bottomhole temperature of $T = 60^{\circ}\text{C}$ and a depth of $\sim 1118\text{m}$ (*Chapter 5*). A pumping time of the biogrout of around 30 – 45 min to 1 km depth is calculated, which is sufficiently short for the bacteria to survive. The results in *Chapters 4* and *5* imply that biogrouting at this temperature and pressure combination would be possible and that it can be used to significantly reduce permeability. Once the temperature is in the range $T = 70^{\circ}\text{C}$ to $T = 140^{\circ}\text{C}$, no form of biogrouting is possible (*Chapter 4*); enzymatic precipitation of CaCO_3 breaks down and the temperature is not sufficiently enough for efficient thermal hydrolysis to occur. Once 140°C is exceeded (at a depth of 2744 m in the North Sea, Fig. 6.1) thermal hydrolysis of the urea (TICP) will occur. Future research should consider the feasibility of TICP at these temperatures, and specifically, the likelihood of precipitation occurring in the borehole. For example, it takes ~ 133 min (avg. pumping time/km depth ~ 37.5 min, personal communication, OGTC 2018) for fluids to reach a target depth of TVD = 3556 m. If it is assumed that the urea and the calcium source are pumped downhole as a mixed solution, with a bottomhole temperature of $T = 180^{\circ}\text{C}$, 50% of the urea could be hydrolysed by the time the solutions have reached the target depth and CaCO_3 precipitation might be induced in the wellbore. As a consequence, beyond a critical depth (to be determined by future research) it may be preferable to inject the urea and the calcium source as separate solutions.

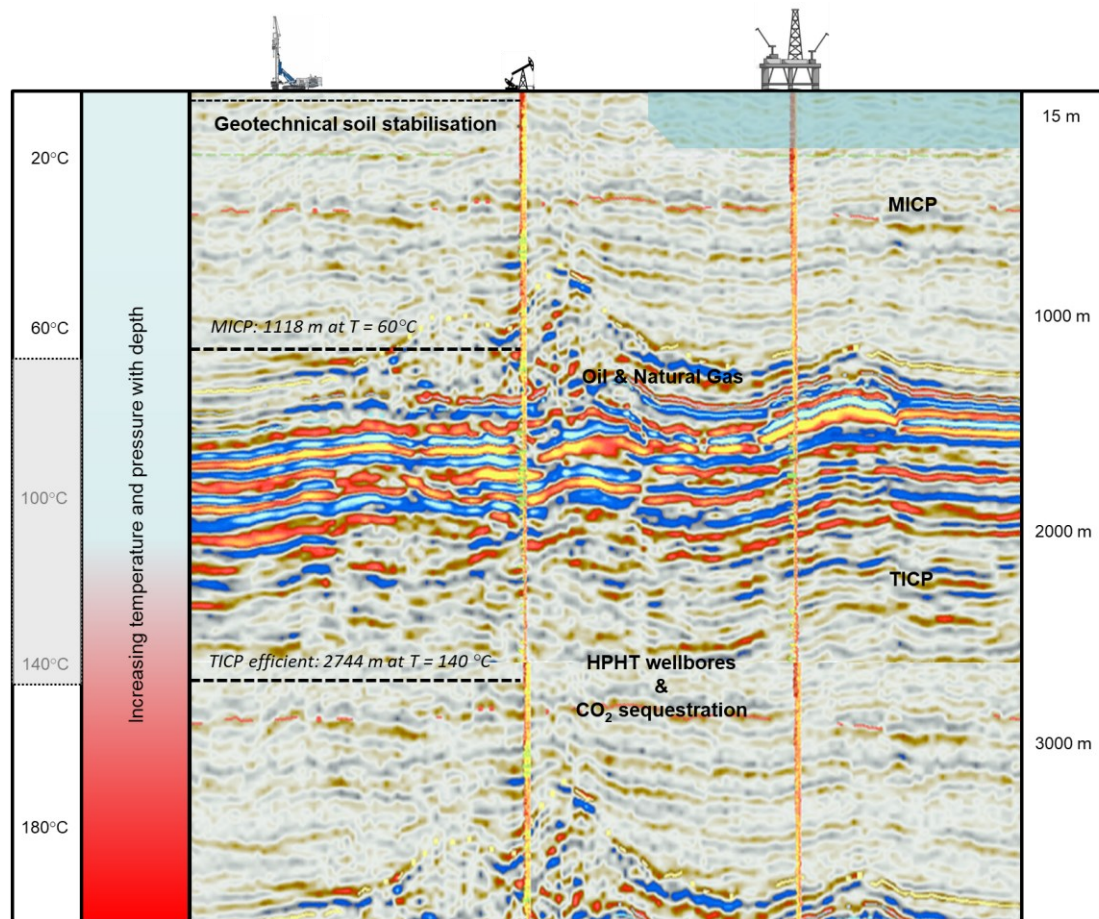


Figure 6.1: Deployment depths of MICP and TICP based on an average geothermal gradient of $\sim 4.9^{\circ}\text{C}/100\text{m}$ for the North Sea region. MICP indicates a maximum deployment depth of TVD = 1118 m. This could be for example a shallow reservoir or caprock formation. Whereas for TICP the corresponding depth would be above $> \text{TVD} = 140^{\circ}\text{C}$ for efficient thermal hydrolysis of urea. In between $T = 70^{\circ}\text{C}$ and $T = 140^{\circ}\text{C}$ as indicated in grey on the left-hand side of Fig. 6.1 is a grey zone. TICP would be theoretically possible within this zone, but due to the low temperature thermal hydrolysis of urea would take > 1 day and fluids may disperse meaning that calcium carbonate does not precipitate where required.

Microbially induced carbonate precipitation as a novel grout material, has two properties that can be advantageous over traditional cement grouts, depending on the desired application: (1) it has a small particle size $\sim 2 \mu\text{m}$; (2) it has a viscosity similar to water. The most common cementitious grout used in the Civil Engineering sector is Ordinary Portland Cement (OPC). The main disadvantage of OPCs is the average particle size of $70 \mu\text{m}$ in diameter (ASTM C150; ASTM, 2012). This has major implications for grout rheology and penetrability. Cement grouts are far more viscous and less penetrable than water, which is a problem when injecting cementitious grouts into fine apertures. Crawford and Groskopf, (1984) pointed out that the

minimum aperture size for a fracture or fissure needs to be roughly three times the particle size of the cement. That implies that cement grouts are limited in their application by their own particle size. A further limitation of OPCs is volumetric shrinkage that occurs during the setting of the cement, which may leave shrinkage cracks through which fluid leakage can occur. To overcome the particle size limitation, in the last decade cement research has advanced and ultrafine ($d_{95} < 15 \mu\text{m}$) and microfine cements ($d_{95} < 30 \mu\text{m}$) are the new standard in rock fracture grouting to penetrate fine aperture fractures (Tolppanen and Syrjaenen, 2003). However, Tolppanen and Syrjaenen (2003) note that the injection pressures required are enormous and for near-surface applications can be a problem. Even at greater depths, the requirement for high injection pressures will consume more energy during grouting. In comparison, bio-grouts with a viscosity similar to water of $\mu = 1.1 \text{ mPas}$ and bacterial cells in the range of $0.5 - 2.0 \mu\text{m}$ have the potential to penetrate very small aperture fractures, as well as pore-throats in porous sediments. This opens up a new world of potential applications.

Oil industry wellbores penetrate deep geological layers in the earth's crust and are subject to dynamic geo-mechanical stresses imposed by hydromechanical changes during production and stimulation, as well as due to tectonics. In addition, barrier materials can be subject to aggressive chemical degradation from natural and man-made fluids. For example, in a carbon capture and storage well, wellbore fluids will be acidic and could attack the wellbore cement leading to corrosion problems in the upper well construction (Carey *et al.*, 2007, 2010; Kutchko *et al.*, 2008, 2009; Huerta *et al.*, 2016). Corrosive fluids can attack the steel casing and tubulars or the cement sheath which bonds the steel casing to the rock formation and provides zonal isolation, thus preventing vertical fluid migration (Dusseault, Gray and Nawrocki, 2000) between production and non-production zones. As a result of such chemical and mechanical degradation mechanisms, there is a requirement within the oil industry for well repair solutions (Oil & Gas UK, 2020). The simplest and most traditional cement remediation technology is 'squeeze cementing' (Rike and Rike, 1982) where the cement grout is squeezed under high pressure in the annular gap between the steel casing strings to fix large cement defects. The most sophisticated approach in current use detects the compromised section of the well cement sheath with a cement bond log (CBL), seals the area with packers, then perforates the casing, washes and then cement (or sometimes resin) (PWC) is squeezed into the section to repair the wellbore (Delabroy *et al.*, 2017). A major disadvantage of using squeeze jobs is the high pump pressure needed to push the grout into the defective cement. For small defect apertures, pump

pressure may risk further fracturing, rather than sealing the defect. As a consequence, MICP, as a very low viscosity technology may have a significant advantage over existing techniques.

Figure 6.2 describes potential areas of application in which biogrout could be advantageous over cementitious grouts. The first area, when drilling into a potential hydrocarbon bearing formation, might be in sealing a caprock (an impermeable layer, often shale) that provides a seal against the upward movement of formation fluids (e.g., hydrocarbons) (Fig. 6.2a). Drilling-induced fractures caused by vibrations during the drilling process and geo-mechanical stress could result in a potential weak point in the later lifecycle of the wellbore (e.g., when well gets plugged and abandoned, the top seal of the well is compromised). In this case, biogrout could be used to seal fractures in the cap rock surrounding the wellbore. Another common issue is damage to the cement casing and the casing-formation interface (Fig. 6.2b). Here, low viscosity biogrout injections could potentially penetrate fine aperture fluid migration pathways. Ureolytic bacteria cells are in the range of 0.5 - 2.0 μm and can penetrate into fine cracks and deposit CaCO_3 to form a durable seal. Deeper in the wellbore, high porosity horizons, once used for hydrocarbon production or as injection zones for enhanced oil recovery (e.g., water flooding), could be sealed using biogrout (Fig. 6.2c). The fluids should be delivered to the existing perforations in the steel casing and injected into the formation of interest. Multiple injection cycles, as demonstrated in *Chapter 5*, have the capability to seal highly permeable sedimentary rocks such as sandstone. A further advantage of MICP over cementitious grouts is that CaCO_3 is more durable than cement; cement has a design life of decades, whereas natural geological analogues show that CaCO_3 veins are preserved over geological timescales (millions of years).

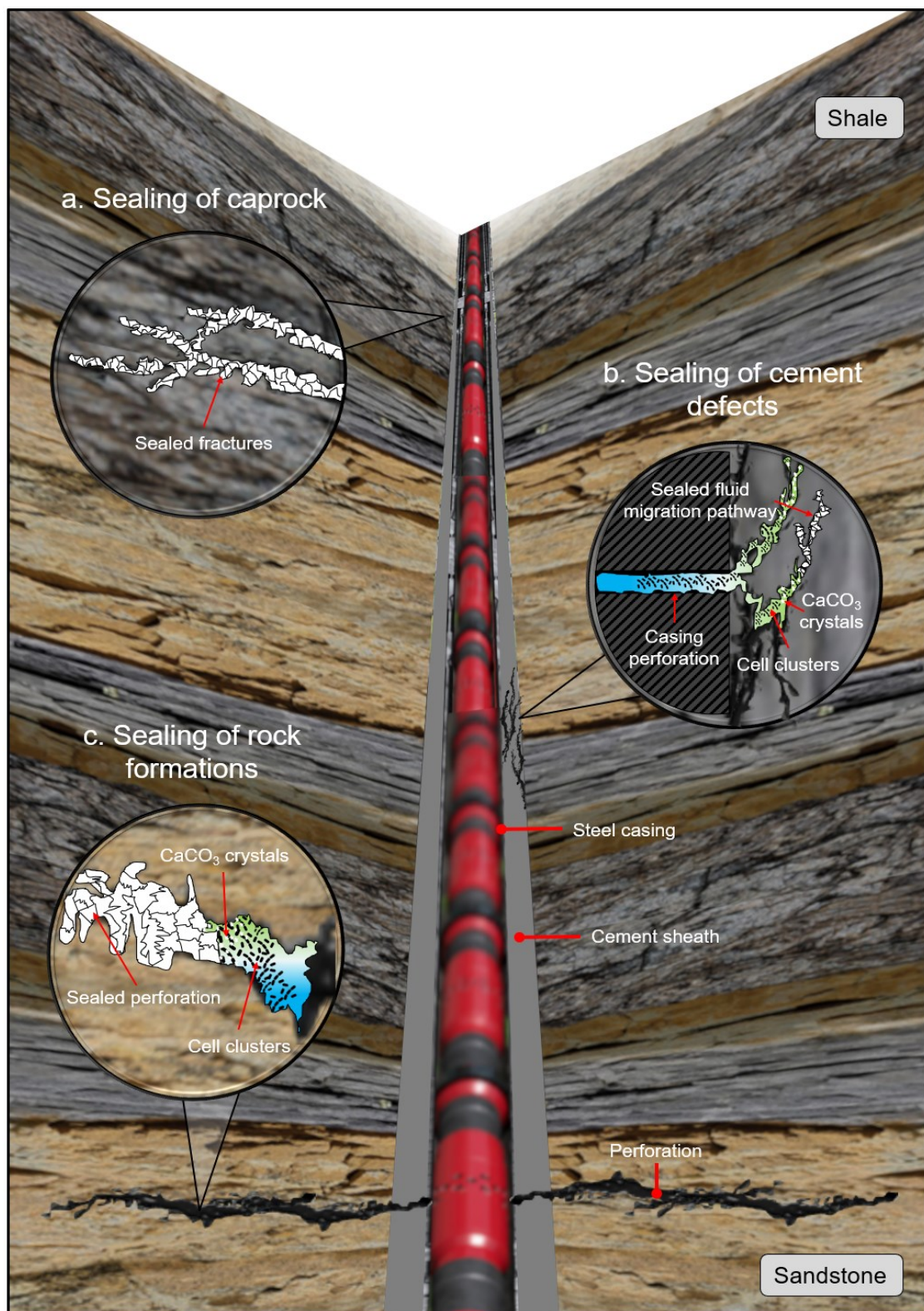


Figure 6.2: The figure shows a cut through of a conventional hydrocarbon wellbore with a reservoir section (bottom) – intermediate geological formations and on top a sealing layer as indicated as caprock. (a) is showing that potential caprock formations with shales could be compromised during drilling operations and cause major problems in the lifecycle of the wellbore and compromising fluid and pressure containment of the wellbore. (b) Sealing well cement

defects is another big opportunity of MICP to reach thin cracks, fissures and upward extending fluid migration pathways that are almost impossible to reach with conventional cementitious grouts. (c) Old and mature hydrocarbon wellbores reach at some point in their lifecycle an end and need to be plugged and abandoned in a safe and reliable way. MICP could be potentially injected through the reservoir's perforations (indicated as black veins) and establish a CaCO_3 environmental seal.

In summary, in the North Sea region MICP could be successfully deployed at depths up to ~ 1120 m ($p = 11.18$ MPa and $T = 60^\circ\text{C}$ – depending on the local geothermal gradient) as a new remediation technology for well cement sheaths, fractured caprocks and as a wellbore barrier element (WBE) to seal production and stimulation horizons prior to plugging and abandonment.

Thermally induced carbonate precipitation (TICP) could open up a new field of application for well temperature greater than $T = 140^\circ\text{C}$. More research is required though to demonstrate that CaCO_3 precipitation occurs and that it nucleates onto cement or rock surfaces, and hence could be a valuable new high temperature well sealing technology.

6.3 Recommendations for future work

The biominerals precipitated in the presence of the alternative nutrient media Marmite showed a higher percentage of vaterite precipitated compared to the Brain Heart Infusion from which similar biominerals were precipitated. This is attributed to the presence of a large variety of organic and inorganic compounds that effect both the kinetics of recrystallisation and the transformation sequence of ACC to calcite. Marmite is a commercial food product, and as such, has additives that are not required for MICP, as well as being sold at a premium. Future research should investigate direct use of the yeast-based waste streams from which Marmite is derived.

A major limitation when using alternative secondary waste stream products from the chemical and food processing industry is the lack of quality control. This is attributed as previously mentioned, to the fact that industry does not apply quality checks to waste steam products. Prior to use a company adopting this approach would need to be clear about the quality of product required. Additional quality checks, and if required subsequent media adjustments, might be needed to guarantee the nutrient media's performance on the large scale.

The biggest limitation on successful application of microbially induced carbonate precipitation in a wellbore environment is the temperature, which will be governed by the local geothermal gradient and will determine the depth to which the biogrout can be deployed. The temperature threshold lies between $T = 60^{\circ}\text{C}$ and $T = 70^{\circ}\text{C}$. Future research should investigate in more detail, the exact temperature at which ureolytic activity declines and inactivation of the enzyme occurs.

To determine the range of situations in which the biogrout might be deployable it is also necessary to investigate the effects of wellbore and reservoir fluids on crystal nucleation and growth. Future experiments should test the sealing capacity of MICP in oil-filled cores and in the presence of other commonly used oil industry fluids such as surfactants and scale-inhibitors.

In comparison to microbially induced carbonate precipitation, thermally induced carbonate precipitation (TICP) could potentially be deployed at depths of between ~ 2000 m to ~ 3500 m, depending on geothermal gradient. Further research on TICP should investigate whether carbonate precipitation from TICP can be controlled. For example, do the carbonate crystals produced using TICP nucleate on grain surfaces, thus reducing permeability, or do they nucleate in solution?

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List of Tables

Table 3.1	Composition of the six nutrient media studied	32
Table 3.2	Broth preparation for Experiment 3.....	34
Table 3.3	Results of the enzyme kinetics of BHI and MM medium. All the experiments on kinetics were conducted in duplicates $n = 2$	39
Table 3.4	Results of the mass difference of the precipitates from BHI and MM medium non-centrifuged and centrifuged. All the precipitation experiments were conducted in triplicates $n = 3$	40
Table 3.5	Results of the semi-quantitative Rietveld refinements with TOPAS, on the phase composition of the precipitated carbonate bio-minerals, the values between brackets are the uncertainties on the last digit of the weight percentage as determined by TOPAS	43
Table 3.6	Assumptions of MICP and cement jet grout treatment volumes for field-scale applications.....	45
Table 3.7	Cost breakdown for upscaling to field-scale application and comparison	45
Table 3.8	Greenhouse gas emission estimates/comparison per m^3 biogrout and traditional cement jet grout.....	46
Table 4.1	Simulated pressure and temperature combinations for the bacterial suspension at well depth	65
Table 5.1	Simulated wellbore conditions for bio-grouting experiment	84
Table 5.2	Daily bio-grout injection schedule.....	85
Table 5.3	Experimental conditions for each experiment with the corresponding core nr. and measurements, sampling and analyses conducted.....	87
Table 5.4	Permeability reduction after applying biogrout treatment under wellbore conditions	95
Table 5.5	Results of the semi-quantitative Rietveld refinements with TOPAS, on the phase composition of the precipitate carbonate bio-minerals from the effluent, the values in between brackets are the uncertainties on the last digit of the weight percentage as determined by TOPAS	99
Table S1.1	Shows the results of an Elemental Analysis conducted on triplicates ($n=3$) of biological reagents where the molecular structure was unknown in form of Total Carbon (TC) and Total Nitrogen (TN).....	154

Table S1.2 This table comprises of current unit costs of biological and chemical reagents needed for MICP. Costs per kilogram of the reagent retrieved on 26.05.2020. 156

List of Figures

Figure 1.1: Multiple routes for fluid migration from a cemented wellbore. Starting at the top with (a) between the cement – casing interface; (b) the cement plug and casing or production tubing; (c) in the cement matrix of the well plug; (d) through casing, tubing corrosion; (e) compromised or inadequate cement sheath; and (f) at the cement – geological formation interface. Figure reprinted from Celia *et al.* (2005).....3

Figure 2.2: This graph describes the Michaelis-Menten kinetics model. Where r_u is defined as the ureolysis rate in $[\text{mol l}^{-1} \text{ h}^{-1}]$ and the righthand side of the Michaelis-Menten model is described by two important parameters, (1) the maximum rate constant v_{max} , which is the maximum reaction rate an enzymatic reaction can have, (2) the Michaelis Menten constant k_m (half saturation constant) as the concentration where the enzymatic reaction is at half of the maximum rate.....14

Figure 2.3: Schematic of homogenous (from solution) crystallisation pathways for CaCO_3 . (a) the upper part shows the classic nucleation theory, and the middle and bottom paths show non-classical nucleation behaviour i.e., pre-cluster nuclei aggregation and stepwise phase transition. (b, c) is showing homogenous and heterogenous nucleation based on the classical nucleation theory. (d) the change of free energy during homogenous and heterogenous nucleation. (e, f) non-classical nucleation including pre-cluster nuclei aggregation and stepwise phase transition illustrated by Zhao *et al.* (2018).17

Figure 3.1. Summary of the growth media screening experiments, (a) OD_{600} and (b) the efficiency yield over a 24-hour period. The error bars represent the average $\pm$ SD of $n = 6$ replicates.....37

Figure 3.2. Growth curves of *S. pasteurii*, (a) in BHI and Marmite broth; b) linear relationship between CFU/ml vs OD_{600} for BHI and Marmite broth. The error bars represent the average $\pm$ SD of (a) $n = 6$ and (b) $n = 3$ replicates.....38

Figure 3.3. Summary of the enzyme kinetics experiment, (a) the hyperbolic Michaelis-Menten Fits and (b) the linearised version of the Michaelis-Menten curve is represented as the Lineweaver-Burk plot. In the case of Marmite and Brain Heart Infusion media, the maximum reaction rates are very similar. Dotted and dashed lines indicate the curve fits.....39

Figure 3.4. Comparison of the dry weight CaCO_3 precipitated from centrifuged/non-centrifuged *S. pasteurii* bacterial suspension (grey) compared to the theoretical possible weight gain (black). The error bars represent the average $\pm$ SD of $n = 3$ replicates.41

Figure 3.5. XRD patterns of calcium carbonate bio-minerals and crystallisation products from top to bottom: (a) rhombohedral calcite; (b) mixed hexagonal vaterite with rhombohedral calcite; (c) rhombohedral calcite; (d) hexagonal vaterite without distinctive calcite peak at $2\theta = 29^\circ$. Dotted and dashed lines are Rietveld refinement results for calcite (magenta) and vaterite (light blue).....43

Figure 4.1. Ramping profiles of the water bath temperature. For every individual temperature exposure experiment the heating rate was adjusted to reach the target temperature ($T = 60^{\circ}\text{C}$, 70°C , 80°C and 90°C) in $t = 30$ min with an additional 30 min of a hold period to simulate transport from surface to depth and injection.....	55
Figure 4.2. Schematic of the experimental setup of the pressure and combined pressure and temperature exposure experiments. The fluid pathway is indicated by the green line starting at the high-pressure pump passing a water bath heat exchanger reaching the inlet of the Hassler type core-holder with the PTFE liner and leaves at the outlet via the sampling port or backpressure regulator to a waste reservoir. The solid red line indicates a fluid bypass line to service the pump and backpressure regulator. The red dashed lines indicate the data logging system consisting of two pressure transducers at the inlet and outlet side of the core holder, as well as a data connection with the high-pressure pump for flow-rate monitoring and pressure setting connected to a data recording station.....	57
Figure 4.3. Constant temperature exposure results: Specific ureolytic activity for bacterial suspension exposed to different temperatures, from top to bottom: $T = 20 - 90^{\circ}\text{C}$ and for different durations: $t = 15 - 120$ min. All experiments were conducted in triplicate. Where error bars cannot be detected they might be hidden by the datapoint.....	60
Figure 4.4. Constant temperature exposure results: Specific ureolytic activity for Jack-bean urease suspension exposed to different temperatures, from top to bottom: $T = 20 - 90^{\circ}\text{C}$ and for different durations: $t = 15 - 120$ min. All experiments were conducted in triplicate. Where error bars cannot be detected they might be hidden by the datapoint.....	61
Figure 4.5. Temperature ramping and hold experimental results: Specific ureolytic activity after ramping to different temperatures in 30 min and additional hold of the temperature for 30 min. All experiments were conducted in triplicate. Where error bars cannot be detected they might be hidden by the datapoint.....	62
Figure 4.6. Constant elevated pressure results: Specific ureolytic activity for bacterial suspension exposed to different pressures and for different durations. The red datapoints are presenting the results of the baseline temperature exposure experiment ($T = 20^{\circ}\text{C}$ and $p = \text{atm}$). All experiments were conducted in triplicate. Where error bars cannot be detected they might be hidden by the datapoint.	63
Figure 4.7. Specific ureolytic activity after ramping fluid pressure to 15.0 MPa in 30 min plus holding the pressure constant for further 30 min. All experiments were conducted in triplicate. Where error bars cannot be detected they might be hidden by the datapoint.	64
Figure 4.8. Specific ureolytic activity after combined pressure and temperature exposure. All experiments were conducted in triplicate. Where error bars cannot be detected they might be hidden by the datapoint.	65
Figure 4.9. Schematic of the high-pressure, high-temperature vessel (PARR 4750). The vessel was modified with a PTFE liner with lid to avoid contact of the pressure and temperature exposed fluid within the stainless-steel vessel.....	68

Figure 4.10. This figure presents HPLC chromatograms and the corresponding calibration curve. All heat-treated urea samples were run in triplicate with a blank sample in between. (a-b) shows the results from thermally decomposed urea at 120°C and 140°C run over a Cogent Bidentate C18 column with 100% UPW as mobile phase ($RT_{urea} = 3.60$ min). At 140° a substantial drop in peak area of urea was observed indicating that urea is getting decomposed. (c) is the corresponding calibration curve.71

Figure 4.11. This graph is presenting the results of the thermal decomposition of a 1.0 M urea solution measured with a Cogent Bidentate C18 HPLC column (best peak shape and retention time). The remaining urea concentrations in solution are calculated based on the detected peak area (urea peak) from the chromatograms that are related to concentrations via the established calibration curves (Fig. 4.10c). All the samples were run in triplicate and where error bars cannot be detected they might be hidden by the datapoint.72

Figure 4.12. This graph is showing a comparison between the measured solution pH over time of a 1000 mM thermally hydrolysed urea (at 120, 140, 160 and 180°C) and a 1000 mM urea solution hydrolysed by a 1.0 OD₆₀₀ *S. pasteurii* bacterial suspension (black datapoints).73

Figure 5.1 (a) Location of the Dunlin Field in the north-northeast North Sea of the UKCS (Block 211/23a) with the selected wellbore DA-18 (211/23-A10) in Block 1 (blue frame).....80

Figure 5.1 (continued) (b) The corresponding well-log is presented for the whole wellbore with the most prominent ‘Brent group’ formation ending on top with the ‘Upper Ness Formation’ covered by Heather and finally with the reservoir roof the ‘Kimmeridge Shale’ formation. A detailed well-log of the cored interval is shown on the right side giving further information on lithology, petrophysical parameters as permeability, helium porosity (well-logs and location maps provided by Fairfield Energy).81

Figure 5.2. Schematic of the experimental setup of the bio-grouting experiment at *in-situ* wellbore pressure and temperature conditions. The fluid pathway is indicated by the green line starting at the high-pressure pump passing a water bath heat exchanger reaching the inlet of the Hassler type core-holder where the bio-grouting treatment occurs and leaves at the outlet via the backpressure regulator to a waste reservoir. The solid red line indicates a fluid bypass line to service the pump and backpressure regulator. The red dashed lines indicate the data logging system consisting of two pressure transducers at the inlet and outlet side of the core holder, as well as a data connection with the high-pressure pump for flow-rate monitoring and pressure setting connected to a data recording station.82

Figure 5.3. Schematic of a sandstone well-core. *Core-scale experiments* and *imaging techniques* are related to the bulk volume of the well-core. *Sub-core scale SEM imaging* is conducted on polished blocks with an equal spacing of 19.25 mm along the z-axis of the core. For the high resolution cryo-FIB/FEG-SEM imaging a sub-sample was generated under fully saturated conditions.89

Figure 5.4. Results of the baseline bio-grouting experiment at atmospheric conditions ($p = atm$, $T = 20^\circ$) with the reduced permeabilities. Permeability measurements show the average permeability across the core and indicate an exponential decay behaviour with progressive bio-grout treatment cycles at atmospheric conditions. The dotted line is indicating the fitted curve for the individual datapoints.92

Figure 5.5. This figure shows the recorded flow rate on the y-axis; on the secondary y-axis: the recorded injection pressure (green), the outlet pressure (orange): $p_{\text{outlet}} = \text{atm}$ and the differential pressure (magenta): $p_{\text{differential}} = p_{\text{injection}} - p_{\text{outlet}}$ from the bio-grout treatment at ($p = \text{atm}$; $T = 20^{\circ}\text{C}$). The ellipse is indicating the sudden increase in pressure from 726 - 1757 psi, due to a buildup of biominerals at the injection port.....93

Figure 5.6. Results of the biogrouting experiment at wellbore conditions with the reduced permeabilities. (a) Permeability measurements show the average permeability across the core. (b) Normalised permeability values for comparison indicate an exponential decay behaviour with progressing bio-grout treatment cycles at wellbore conditions. Dotted, dashed, dash-dot, dash dot-dot lines are indicating the fitted curves for the individual datapoints. Datapoints that are not visible are hidden by other datapoints.....94

Figure 5.7. Biominerals analysed were retrieved from daily effluent sampling during the PM bio-grouting treatment cycle under in-situ wellbore pressure and temperature conditions. Corresponding XRD patterns and observations are shown in (a – c) from bottom to top (Day 1 – Day 9). (a) At atmospheric conditions ($p = \text{atm}$, $T = 20^{\circ}\text{C}$) calcium carbonate biominerals in the form of mixed hexagonal vaterite with small amounts of rhombohedral calcite formed. During the next treatment cycles calcite will become the more the dominant mineral phase (b) At $p = 9.14 \text{ MPa}$, $T = 50^{\circ}\text{C}$, hexagonal vaterite was the dominant phase until the 10th treatment cycle (Day 5), afterwards it could not be detected anymore. (c) At $p = 11.18 \text{ MPa}$, $T = 60^{\circ}\text{C}$, hexagonal vaterite was found over prolonged times until the 12th treatment (Day 6) cycle before mineral dissolution and recrystallisation to a more thermodynamically stable state into the mineral calcite occurred. Dotted and dashed lines are Rietveld refinement results for calcite (magenta) and vaterite (light blue).98

Figure 5.8. (a-i) 3D renderings are showing the segmented pore-space (blue) of the sandstone well-cores prior treating with bio-grout, the CaCO_3 bio-mineral distribution (red with light blue background to make the biominerals more visible) in the pore-space and a composition of the remaining pore-space with the biominerals. (a-c) shows the baseline experiment ($p = \text{atm}$, $T = 20^{\circ}\text{C}$). (b) The post-grouting 3D slice reveals the location of the biominerals precipitated in the pore-space. The biominerals are well distributed across the whole pore-space and in particular in the higher porous laminae (arrows) more bio-precipitates could be found potentially induced by a change in the flow hydraulics. (d-f) is showing the bio-grouting experiment at elevated pressure and temperature conditions ($p = 9.14 \text{ MPa}$, $T = 50^{\circ}\text{C}$). Again, mm thick diagonal layers (d, arrow) with potentially higher porosities are crossing the well-core. (e) CaCO_3 biominerals are very well distributed across the core, again highlighting higher permeable areas (arrows) that allowed for more bio-precipitation. (g-i) at the highest pressure and temperature bio-grouting experiment ($p = 11.18 \text{ MPa}$, $T = 60^{\circ}\text{C}$) the overall distribution (without laminae) of the biominerals seems to be similar compared to $T = 20^{\circ}\text{C}$ and $T = 50^{\circ}\text{C}$. Fine, mm thick laminae are missing in the lower part of the core. Instead, there is a very prominent thick laminae in the upper right corner showing a larger accumulation of biominerals leading to the formation of an almost continuous layer of biominerals (h, arrow).101

Figure 5.9. Porosity as a function of distance from the inlet computed from the individual CT image stacks. (a) indicates the initial porosity along the z-axis of the core in the untreated state. (b) shows the reduced pore-space after application of MICP over a period of 9 consecutive days. (c) indicates the absolute change in porosity for each CT slice over the length of the core. .103

Figure 5.10. Highly polished blocks with resin impregnation were prepared from the bio-grouted well-cores under in-situ wellbore pressure and temperature conditions. For the analysis a representative set of Back scattered electron images (BSE) with the corresponding observations are shown in (a – i). In (a – c) BSE images at atmospheric grouting conditions ($p = \text{atm}$, $T = 20^\circ\text{C}$) calcium carbonate bio-minerals bridge the pore-space in between the sand grains (Qtz). (b – c) Growth is promoted radially from a centre point and is progressing with additional treatment cycles via surface nucleation (small dots are indicating microbial activity) on the newly grown carbonate crystals. (d – f) At elevated wellbore conditions ($p = 9.14 \text{ MPa}$, $T = 50^\circ\text{C}$) spherulitic carbonate crystal growth dominates. (g – i) At the highest pressure and temperature conditions ($p = 11.18 \text{ MPa}$, $T = 60^\circ\text{C}$) spherulitic growth was very prominent with spherulites overgrown by faceted crystals (rough surfaces). In (i) the growth fronts of the carbonate crystal were extending radially with embedded microbes (black dots) and potential surface attachment (indicated by arrow).....105

Figure 5.11. This figure is comparing the growth front behaviour of carbonate crystals under various pressure and temperature conditions (a – f). In (a – b) BSE images at atmospheric grouting conditions ($p = \text{atm}$, $T = 20^\circ\text{C}$) calcium carbonate bio-minerals bridge the pore-space in between the sand grains (Qtz) (small black dots are indicating microbial activity at the growth fronts. (b) the annotation is giving an idea how growth is promoted radially from a centre point and is progressing with additional treatment cycles via surface nucleation (c – d) At elevated wellbore conditions ($p = 9.14 \text{ MPa}$, $T = 50^\circ\text{C}$) spherulitic carbonate crystal growth dominates. The lower part of the carbonate crystal shows potential growth from the surface of the sand grain and might have merged with a solution nucleated spherulite. (d) the annotation indicates the spherulites with radially extending growth fronts. Microbial activity was found again at the crystal growth fronts (e – f) At the highest pressure and temperature conditions ($p = 11.18 \text{ MPa}$, $T = 60^\circ\text{C}$) spherulitic growth was very prominent with spherulites overgrown by faceted crystals. In the annotation (f) the growth fronts of the carbonate crystal were first extending radially as in (d) and the further the crystal extends the jaggier this growth fronts become. It seems like these gaps were again filled up with new bio-precipitates and grew further. The black arrows indicate microbial activity not just at the growth front boundary, it is likely they are embedded in the crystal matrix.....106

Figure 5.12. Small sub-samples were prepared, from the sectioned bio-grouted well-core under in-situ wellbore pressure and temperature conditions ($p = 11.18 \text{ MPa}$, $T = 60^\circ\text{C}$), (39 mm from inlet). For the analysis, a representative set of Back scattered electron images (BSE) with Energy-dispersive X-ray maps (EDX) and corresponding observations are shown from the cryo-fractured sub-sample in (a – e). In BSE image (a) a surface nucleation process of CaCO_3 biominerals onto a sand grain (Qtz) is shown. (b) is an elemental map for the area of interest. (c) indicates microbial (arrow) activity with surface nucleation. (d) shows the interface between the sand grain (Qtz) and the bio-precipitates. (e) the interface between the sand grain (Qtz) and the morphology of the CaCO_3 biominerals.108

Figure 5.13. Small sub-samples were prepared, from the sectioned bio-grouted well-core under in-situ wellbore pressure and temperature conditions ($p = 11.18 \text{ MPa}$, $T = 60^\circ\text{C}$), (39 mm from inlet). For the analysis, a representative set of Back scattered electron images (BSE) with Energy-dispersive X-ray maps (EDX) and corresponding observations are shown from the cryo-fractured sub-sample in (a – e). In BSE image (a) The circle indicates the radial growth of the carbonate crystals, likely spherulitic growth, which indicates a solution nucleation process, or on microbes suspended in solution of CaCO_3 biomineral formation (rather than nucleation on

well-defined surfaces, e.g. minerals). Newly born CaCO_3 nano-crystallites were building up on each other extending in a typical spherulitic manner, which has been potentially identified as hexagonal vaterite. (b) is an elemental map for the area of interest. (c) shows typically radial extension of the carbonate crystals with a lot of microbial activity as seen as black dots. (d) presents a very well preserved spherulite with typical surface attachment of further nano crystallites. The arrows are indicating rod-shaped microbial imprints. (e) Secondary electron image (SE) of the surface of the spherulite showing the tiny nano-crystallites attaching109

Figure 5.14. Calcium carbonate next to a sand grain, indicating that a pore filled with calcium carbonate. The filling of the pore occurred in a chaotic manner. (a) a combination of solution and surface nucleation processes led to the complete blockage of this pore. Spherulitic growth and also crystal formation with surface nucleation with microbes as negatively charged surface templates could be identified. (b) shows radial growth progression with lots of microbial activity involved; the microbes were also seen at the interfaces. With these radially extending growth rings the amount of treatment cycles could be counted (for 9 consecutive days). In (c) the BSE image is indicating the attachment of a further CaCO_3 crystal to another one with multiple layers of CaCO_3 extending radially. (d) is showing a typical spherulitic hexagonal vaterite crystal confirming the suggested nucleation mechanism. 110

Figure 5.15. (a) The left panel is showing a sand grain (Qtz) - CaCO_3 biomineral crystal interface. The white dotted line is indicating the milling location across the two interfaces with the Focussed-Ion-Beam (FIB) – milling depth 25 μm . In the right panel (b) the white arrows indicated microbial activity at the interfaces that potentially led to surface nucleation and CaCO_3 crystal growth from this point. Furthermore, in the biominerals precipitated on the right a lot of entombed bacterial cells could be identified in the matrix of the carbonate (inset)..111

Figure 6.1: Deployment depths of MICP and TICP based on an average geothermal gradient of $\sim 4.9^\circ\text{C}/100\text{m}$ for the North Sea region. MICP indicates a maximum deployment depth of $\text{TVD} = 1118\text{ m}$. This could be for example a shallow reservoir or caprock formation. Whereas for TICP the corresponding depth would be above $> \text{TVD} = 140^\circ\text{C}$ for efficient thermal hydrolysis of urea. In between $T = 70^\circ\text{C}$ and $T = 140^\circ\text{C}$ as indicated in grey on the left-hand side of Fig. 6.1 is a grey zone. TICP would be theoretically possible within this zone, but due to the low temperature thermal hydrolysis of urea would take > 1 day and fluids may disperse meaning that calcium carbonate does not precipitate where required. 123

Figure 6.2: The figure shows a cut through of a conventional hydrocarbon wellbore with a reservoir section (bottom) – intermediate geological formations and on top a sealing layer as indicated as caprock. (a) is showing that potential caprock formations with shales could be compromised during drilling operations and cause major problems in the lifecycle of the wellbore and compromising fluid and pressure containment of the wellbore. (b) Sealing well cement defects is another big opportunity of MICP to reach thin cracks, fissures and upward extending fluid migration pathways that are almost impossible to reach with conventional cementitious grouts. (c) Old and mature hydrocarbon wellbores reach at some point in their lifecycle an end and need to be plugged and abandoned in a safe and reliable way. MICP could be potentially injected through the reservoir's perforations (indicated as black veins) and establish a CaCO_3 environmental seal. 126

Figure S2.1 (a) Gram-staining of the Marmite growth media is showing gram-positive bacteria in the solution since they have a very thick cell wall made out of peptidoglycan, which retains the crystal violet dye. (b) In comparison, this image shows *S. pasteurii* stained from BHI media and confirms that this particular strain is growing in the new medium 155

Figure S1.1. High pressure exposure experiment. (a) is indicating healthy *S. pasteurii* cells tested at $p = \text{atm}$, and 1.0 MPa (arrow), (b) is showing partially intact cells at the lower left corner and on the upper right cell debris of the *S. pasteurii* cells is visible (arrows). 157

Figure S1.1. Image describes the segmentation workflow starting with the input image stacks, further selecting image features and label them according to a class system. Next, train the classifier and then apply the trained classifier onto the whole data set (Arganda-Carreras et al., 2017) 158

Figure S3.1. COMPO and SE map are giving insight about the sub-core scale location of the CaCO_3 biominerals. Ca and Si are WDX maps, whereas O and P maps are from standard EDX mapping. 164

Figure S3.2. COMPO and SE map are giving insight about the sub-core scale location of the CaCO_3 biominerals. Ca and Si are WDX maps, whereas O and P maps are from standard EDX mapping. 164

Figure S3.3. COMPO and SE map are giving insight about the sub-core scale location of the CaCO_3 biominerals. Ca and Si are WDX maps, whereas O and P maps are from standard EDX mapping. 165

Figure S3.4. COMPO and SE map are giving insight about the sub-core scale location of the CaCO_3 biominerals. Ca and Si are WDX maps, whereas O and P maps are from standard EDX mapping. 165

Figure S3.5. COMPO and SE map are giving insight about the sub-core scale location of the CaCO_3 biominerals. Ca and Si are WDX maps, whereas O and P maps are from standard EDX mapping. 166

Appendix A

Supplementary information to Chapter 3

SUPPLEMENTARY INFORMATION 1:

Results from elemental analysis

Table S1.1 Shows the results of an Elemental Analysis conducted on triplicates (n=3) of biological reagents where the molecular structure was unknown in form of Total Carbon (TC) and Total Nitrogen (TN)

Sample Nr.	Amount of samples in triplicates	Media	Constituen ts	Elemental analysis	
				TC (%) ± SD	TN (%) ± SD
1	3	BHI	Brain Heart Infusion	37.2(1)	10.9(1)
2	3	LMG 139	Peptone	43.0(2)	14.7
3	3	LLB	Lab Lemco Beef Extract	41.5(4)	13.7(1)
4	3	YE	Yeast Extract	39.9(1)	11.6(3)
5	3	TT	Tryptone	37.2(2)	10.7(4)
6	3	MM	Marmite	28.9(8)	7.4(3)

SUPPLEMENTARY INFORMATION 2:

Gram-staining technique to identify *S. pasteurii* in new alternative nutrient media

BHI and Marmite were inoculated by *S. pasteurii*, procedure as mentioned in section enzyme kinetics with a defined nutrient media and incubated for 20 hr. Standard microbiology gram-staining technique was applied to confirm that there is just a gram-positive strain growing in the growth solutions based on its cell wall structure. This technique was sufficient since the new growth media Marmite has a very high sodium chloride content (11g NaCl in 100 g serve = 11%) similar to BHI which means just halo-tolerant microorganisms of the domain Archaea, Bacteria and Eukarya (Margesin and Schinner, 2001) can survive in this media. Optical microscopy with Bright-Field mode highlights the presence of the same gram-positive bacteria (i.e., *S. pasteurii*) in both the Marmite medium and in the standard commercial Brain Heart Infusion broth.

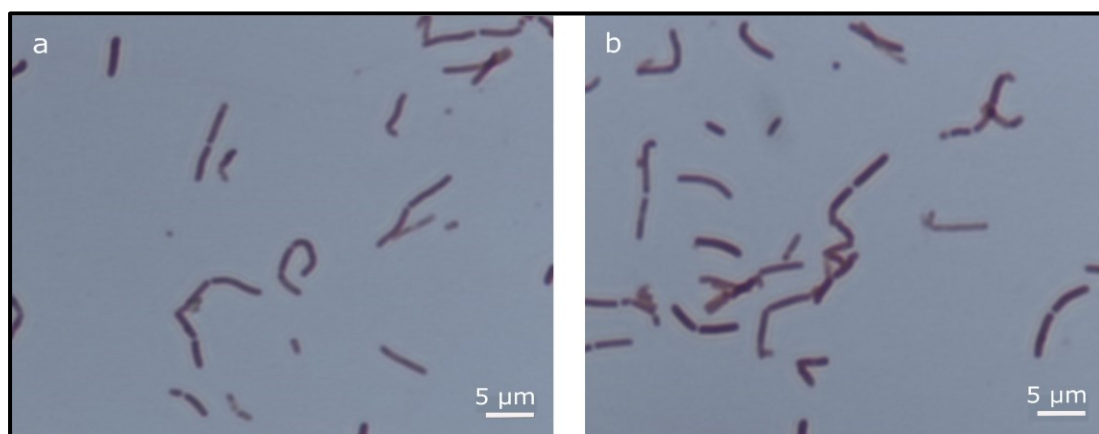


Figure S2.1 (a) Gram-staining of the Marmite growth media is showing gram-positive bacteria in the solution since they have a very thick cell wall made out of peptidoglycan, which retains the crystal violet dye. (b) In comparison, this image shows *S. pasteurii* stained from BHI media and confirms that this particular strain is growing in the new medium

SUPPLEMENTARY INFORMATION 3:

Unit costs of growth media constituents

Table S1.2 This table comprises of current unit costs of biological and chemical reagents needed for MICP. Costs per kilogram of the reagent retrieved on 26.05.2020.

Unit costs of biological and chemical reagents		
Reagents	Costs (£/kg)	Supplier
<i>Biological</i>		
Brain Heart Infusion	97.20	Sigma-Aldrich, UK
Marmite	7.36	Unilever, UK
Peptone	377.00	Sigma-Aldrich, UK
Lab Lemco Beef Extract	362.00	Thermo Scientific, UK
Yeast Extract	182.00	Sigma-Aldrich, UK
Tryptone	191.00	Sigma-Aldrich, UK
<i>Chemical</i>		
lab-grade urea	45.60	Sigma-Aldrich, UK
non lab-grade urea	0.14	Origin Fertilisers, UK
lab-grade CaCl ₂	5.80	Sigma-Aldrich, UK
non lab-grade CaCl ₂	0.09	Dow Ludington, USA

Appendix B

Supplementary information to Chapter 4

SUPPLEMENTARY INFORMATION 1:

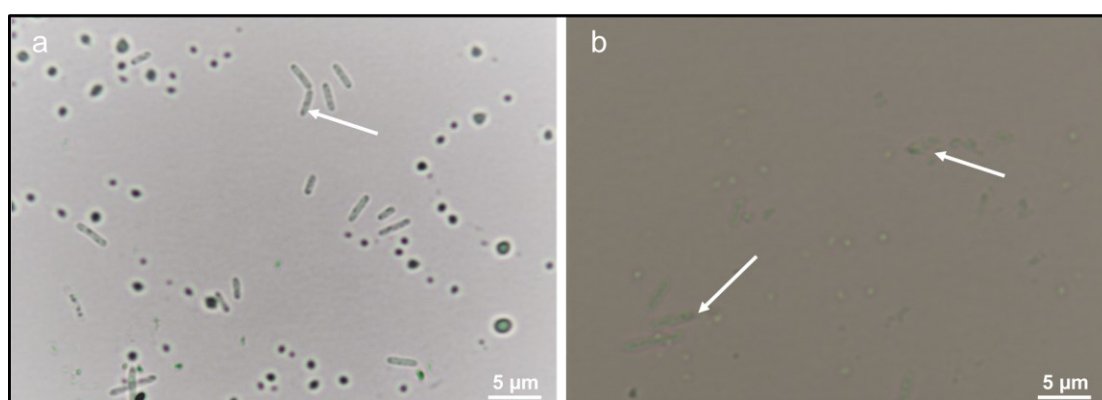


Figure S1.1. High pressure exposure experiment. (a) is indicating healthy *S. pasteurii* cells tested at $p = \text{atm}$, and 1.0 MPa (arrow), (b) is showing partially intact cells at the lower left corner and on the upper right cell debris of the *S. pasteurii* cells is visible (arrows).

Appendix C

Supplementary information to Chapter 5

SUPPLEMENTARY INFORMATION 1:

Image Segmentation (Machine learning based algorithm – 3D Weka Segmentation)

The Trainable Weka Segmentation (TWS) is a state-of-the-art image segmentation plugin in Fiji that uses a collection of machine-learning algorithms with a selection of image features to generate pixel-based segmentations.

The workflow used to segment the pore space from the xCT slices is described below:

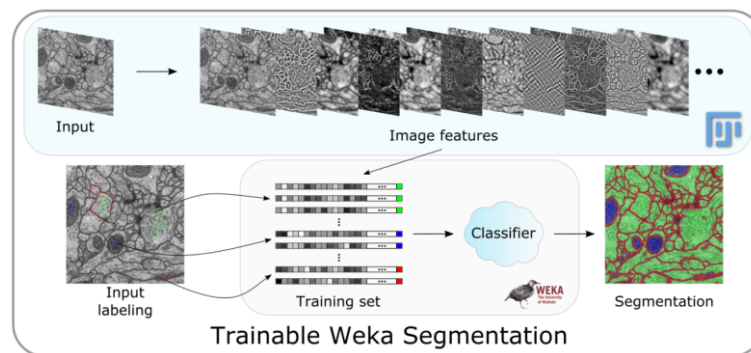


Figure S1.1. Image describes the segmentation workflow starting with the input image stacks, further selecting image features and label them according to a class system. Next, train the classifier and then apply the trained classifier onto the whole data set (Figure reprinted from Arganda-Carreras *et al.*, 2017)

In a first step, a training set (stack of images) of 400 XCT slices with dimensions of 400^3 pixel was established to train the classifier. The classifier itself was defined by two classes either pore space or bulk grain space also known as input labelling. In the GUI of the Weka Plugin in Fiji, the features were labelled pixel by pixel, pore space as class 1 and bulk grain space as class 2. Afterwards, the training of the classifier was initiated, and results were overlain with the corresponding class colours and can be toggled on or off to check if the segmentation result was successful. When the training of the classifier was successful, the trained classifier was applied to the whole data set (image stack of XCT slices). The size of the image stack (number of slices) needs to be wisely chosen, since the Weka segmentation process is very RAM intensive and the hardware of the workstation is the main limitation. The used datasets for training the classifier were in a range of 700-800 MB and the whole dataset with a size of 5.6 GB worked out on a Dell, Intel X-Celeron, 128 GB DDR4 RAM workstation.

SUPPLEMENTARY INFORMATION 2:

Porosity as a function of distance from inlet towards the outlet

The porosity calculation longitudinal of the axis of the well core was calculated on each slice from inlet to outlet based on the results of the 3D Weka segmentation. The following code below is describing the procedure being used by using Python 3.6 script in Google Co-lab:

#Installing some libraries and mounting g-drive

```
!pip install tifffile

from google.colab import drive
drive.mount('/content/drive')
```

#Importing and reading XCT image stack as tiff-file:

```
%pylab inline
import tifffile

img = tifffile.imread('/content/drive/MyDrive/fabian/Segmented core_01.06.2020_5
01 slices.tif')

img_slice = img[500]
fig, ax = plt.subplots(1, 1, figsize=(12, 12))
ax.imshow(img_slice)
plt.show()
```

#Define circular mask centre and area where calculation is going to be based on:

```
def create_circular_mask(h, w, center=None, radius=None):

    if center is None: # use the middle of the image
        center = (int(w/2), int(h/2))
```

```

if radius is None: # use the smallest distance between the center and image walls
    radius = min(center[0], center[1], w-center[0], h-center[1])

Y, X = np.ogrid[:h, :w]
dist_from_center = np.sqrt((X - center[0])**2 + (Y-center[1])**2)

mask = dist_from_center <= radius
return mask

h, w = img_slice.shape
mask = create_circular_mask(h, w, radius=350)
print(mask.shape)
masked_img = img_slice.copy()
masked_img[~mask] = 0

fig, ax = plt.subplots(1, 1, figsize=(12, 12))
ax.imshow(masked_img[300:500, 0:100], cmap="gray")
ax.imshow(mask[300:500, 0:100], cmap="inferno", alpha=0.5)
plt.show()

```

Computing the number of pixels in the mask

```

sum_slice = np.sum(mask)
sum_slice_grain = np.sum(img_slice.astype(bool))
print("Sum of pixels in mask ", sum_slice, "sum slice in image slice ", sum_slice__
grain, "porosity ", 1-sum_slice_grain/sum_slice)

```

Function to compute porosities and compute porosities on every slice

```

def create_porosity_calculator_function(sum_slice):
    def compute_porosity(img):
        sum_grain = np.sum(img.astype(bool))
        return 1.0-sum_grain/sum_slice

```

```

return compute_porosity

func = create_porosity_calculator_function(sum_slice)

porosities = np.array([func(slice_img) for slice_img in img])

# Using matplotlib to plot the porosities along the z-axis of core

fig, ax = plt.subplots(1, 1, figsize=(12, 12))
ax.plot(porosities)
ax.set_xlim(0, img.shape[0])
ax.set_ylim(0, 0.25)
ax.set_ylabel("porosity")
ax.set_xlabel("Distance from Inlet")

#Save the computed porosities in a text file

np.savetxt('/content/drive/MyDrive/fabian/porosities_intial', porosities)

```

SUPPLEMENTARY INFORMATION 3:

Electron Microprobe Analysis with EDX and WDX spectroscopy

Scanning electron microscopy was performed on highly polished blocks (carbon coated) of the well-core mounted in a Field Emission – Electron Probe Microanalyser (JEOL JXA-8530F field-emission EPMA). Backscattered COMPO images (BSE) and secondary electron images (SE) were showing the microstructure, while energy dispersive (EDX) and wavelength dispersive (WDX) X-ray spectroscopy were giving insights about the elemental composition and distribution at the selected area of interest. Five areas of interest were chosen along the z-axis of the core equally spaced (19.25 mm), and the microstructure analysed and presented as BSE images with four additional elemental maps = {Si, Ca, O, P} starting at the bottom $z = \{1.8 \text{ mm}\}$ to the top of the core $z = \{75.7 \text{ mm}\}$. The elemental maps were selected based on the known mineralogy of the sandstone well-cores consisting mainly out of Quartz grains (SiO_2), the carbonate biominerals (CaCO_3) and the *S. pasteurii* cell walls containing peptidoglycan a phosphor compound (P).

Results on the microscale reveal mineral plugging of voids and pore throats in between silica grains. On some BSE and SE images, it could already be identified that there are heterogeneities in the carbonate crystal formation due to microbial activity. The phosphorus elemental map shows nicely, where the microbes are located. This is due to phosphor compounds known as peptidoglycan in the cell wall of the bacterial cell. The calcium and silicon maps indicate the presence of calcium carbonates either blocking voids and pore throats or cementing the bigger quartz grains very well.

$z = 1.8 \text{ mm}$

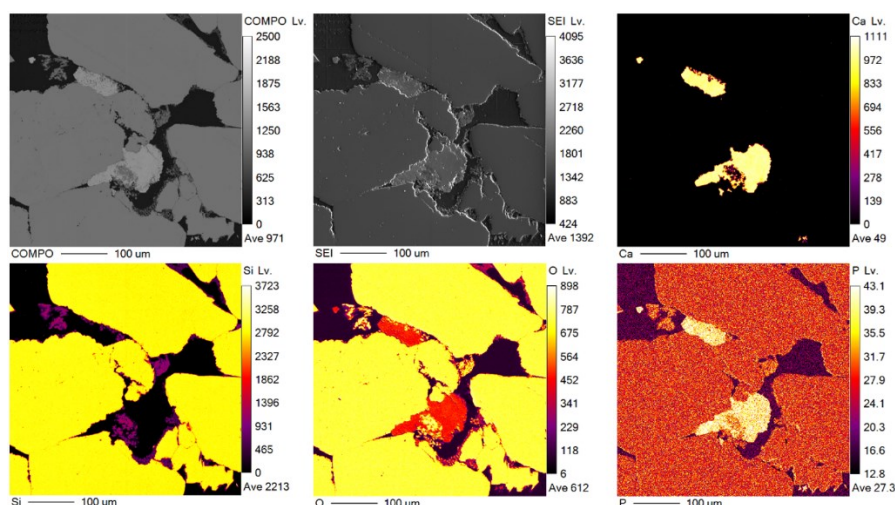


Figure S3.1. COMPO and SE map are giving insight about the sub-core scale location of the CaCO_3 biominerals. Ca and Si are WDX maps, whereas O and P maps are from standard EDX mapping.

$z = 19.25 \text{ mm}$

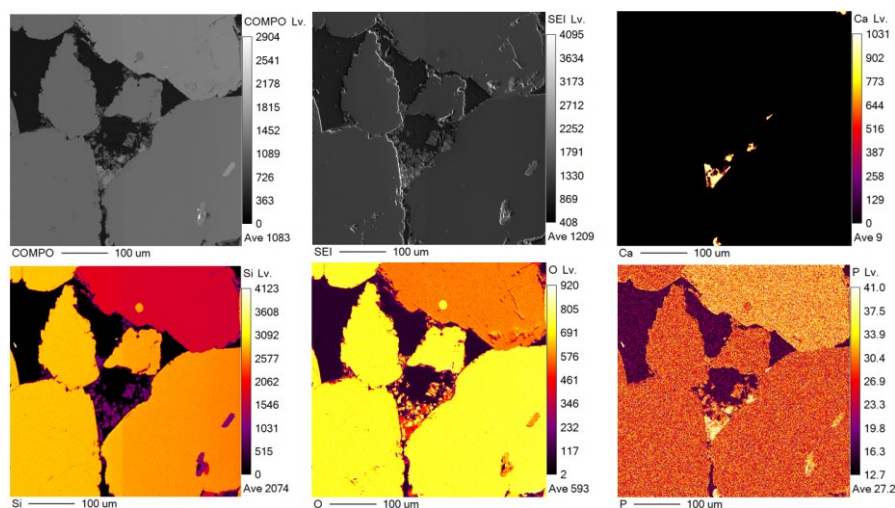


Figure S3.2. COMPO and SE map are giving insight about the sub-core scale location of the CaCO_3 biominerals. Ca and Si are WDX maps, whereas O and P maps are from standard EDX mapping.

$z = 38.50 \text{ mm}$

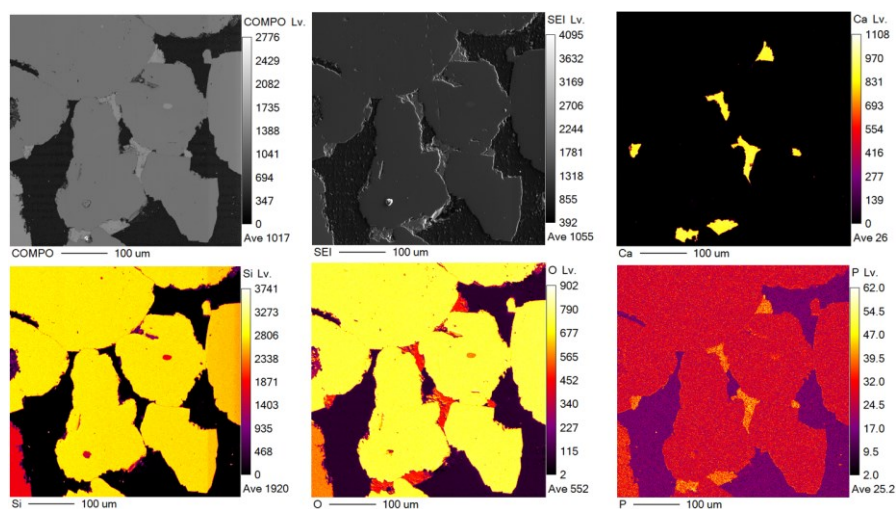


Figure S3.3. COMPO and SE map are giving insight about the sub-core scale location of the CaCO_3 biominerals. Ca and Si are WDX maps, whereas O and P maps are from standard EDX mapping.

$z = 57.75\text{mm}$

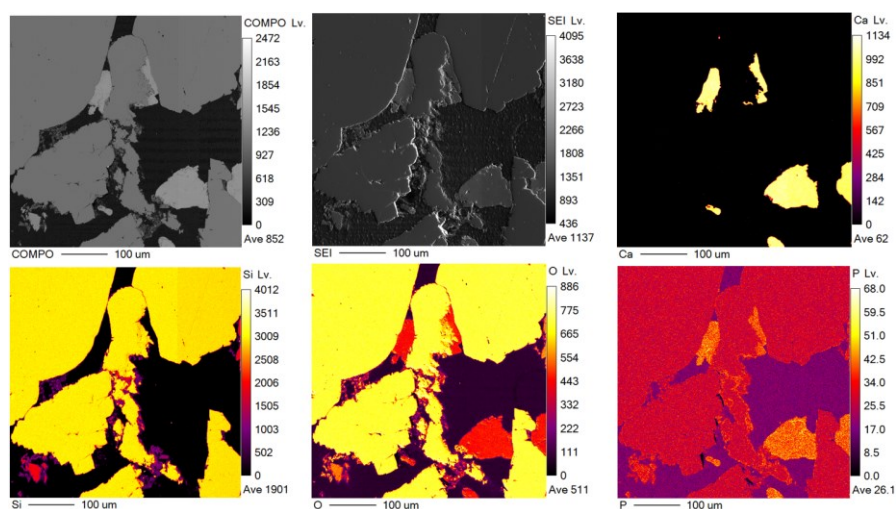


Figure S3.4. COMPO and SE map are giving insight about the sub-core scale location of the CaCO_3 biominerals. Ca and Si are WDX maps, whereas O and P maps are from standard EDX mapping.

$z = 75.2 \text{ mm}$

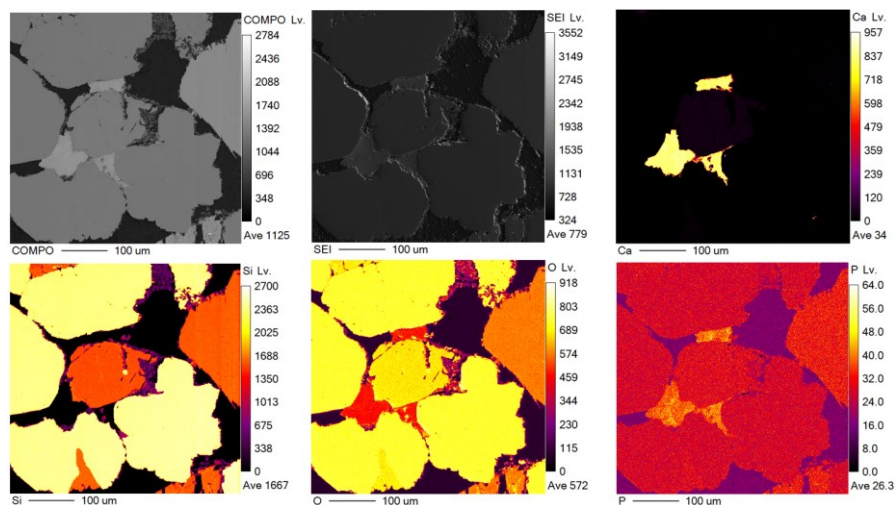


Figure S3.5. COMPO and SE map are giving insight about the sub-core scale location of the CaCO_3 biominerals. Ca and Si are WDX maps, whereas O and P maps are from standard EDX mapping.