MICROBIAL INHIBITION OF METHANE

CLATHRATE HYDRATES

by

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ABSTRACT

Two microbial species were tested for inhibition of methane hydrates in a stirred crystallizer (subcooling of 2.34 K). The ice associating *Chryseobacterium* sp. *Strain C14*, grown in 0.5 wt% Tryptic Soy Broth (TSB) delayed hydrate nucleation, on average, by 30.3 hours compared to 37.9 hours for the PVP solutions. *Escherichia coli TG2* in 0.5 wt% TSB was used as a non ice associating bacteria control and surprisingly had the longest induction period of 118.1 hours, suggesting that it was 3 times more effective as a hydrate inhibitor than PVP. The 0.5 wt% aq. TSB solution without bacteria delayed hydrate nucleation an average of 6.7 hours, whilst bacteria without TSB also showed significant inhibition. However, for the bacteria and bacteria + TSB systems, nucleation times were far more sporadic and time dependant than the simple systems of pure water and PVP.

PVP decreased hydrate growth rate but increased gas consumption by nearly 4 fold. TSB without bacteria promoted gas consumption by over 2 fold but exhibited a slightly higher growth rate than the pure water solution. Reasons for the differences in growth profiles may be a result of the observed morphological differences in the hydrate phase. *Chryseobacterium* in 0.5 wt% aq. TSB had a distinct time dependency in growth characteristics and promoted growth rate almost 3 fold. *E. coli* in 0.5 wt% TSB showed a unique S-curve growth profile where the initial growth rate was very low. The differences in growth profiles of the two bacteria suggest different inhibition mechanisms. Ice-associating proteins likely play a significant role in hydrate formation, especially for *Chryseobacterium* which has shown inhibition of ice recrystallization. However, the interaction of other non-ice associating macromolecules may play a primary role in the observed inhibition and that biofilm formation may act as a barrier between the gas-liquid and/or heterogeneous nucleating solid-liquid interfaces which may help explain the significant inhibition observed by *E. coli*.

Considering that both species of bacteria yielded significant hydrate inhibition, albeit somewhat unpredictable, but since the procedure is simple, the potential of employing bacteria as ‘Microbial Hydrate Inhibitors’ looks promising. However, consistent inhibition will be a challenge to overcome so that these organisms could be used as other KHI solutions.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>$A_\lambda$</td>
<td>Optical density of solution of light at wavelength $\lambda$ (nm)</td>
</tr>
<tr>
<td>AA</td>
<td>Anti-agglomerants</td>
</tr>
<tr>
<td>AFP</td>
<td>Antifreeze protein</td>
</tr>
<tr>
<td>AFGP</td>
<td>Antifreeze glycoprotein</td>
</tr>
<tr>
<td>CR</td>
<td>Crystallizer</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>F</td>
<td>Fresh solution, no hydrate history</td>
</tr>
<tr>
<td>IBP</td>
<td>Ice-binding proteins</td>
</tr>
<tr>
<td>KHI</td>
<td>Kinetic hydrate inhibitor</td>
</tr>
<tr>
<td>LDHI</td>
<td>Low dosage hydrate inhibitor</td>
</tr>
<tr>
<td>M(No.)</td>
<td>Memory solution, hydrate history</td>
</tr>
<tr>
<td>MD</td>
<td>Molecular dynamics simulation</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>PVCap</td>
<td>Polyvinylcaprolactam</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SV</td>
<td>Supply vessel</td>
</tr>
<tr>
<td>TH</td>
<td>Thermal hysteresis</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
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</table>
## SYMBOLS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Unit</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta \mu$</td>
<td>J</td>
<td>Chemical potential driving force of hydrate formation</td>
</tr>
<tr>
<td>$\sigma_{ef}$</td>
<td>J.m$^{-2}$</td>
<td>Specific surface energy</td>
</tr>
<tr>
<td>$c$</td>
<td>-</td>
<td>Numerical shape factor</td>
</tr>
<tr>
<td>$G_{CH_4}$</td>
<td>mol</td>
<td>Moles of methane converted into hydrate phase</td>
</tr>
<tr>
<td>$n$</td>
<td>-</td>
<td>Number of hydrate forming units</td>
</tr>
<tr>
<td>$N_T$</td>
<td>mol</td>
<td>Total moles of methane converted to hydrate phase</td>
</tr>
<tr>
<td>$R_G$</td>
<td>mol.min$^{-1}$</td>
<td>Rate of hydrate formation</td>
</tr>
<tr>
<td>$t_i$</td>
<td>hours</td>
<td>Induction time</td>
</tr>
<tr>
<td>$T_L$</td>
<td>°C</td>
<td>Temperature of solution in crystallizer</td>
</tr>
<tr>
<td>$v_h$</td>
<td>m$^3$</td>
<td>Volume of a single hydrate repeating unit</td>
</tr>
<tr>
<td>$W(n)$</td>
<td>J.mol$^{-1}$</td>
<td>Work required to nucleate n hydrate forming units</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I have been extremely privileged to have been mentored by Peter Englezos and would, firstly, like to express how much of an honour it has been to work under his supervision. It has been a pleasure to work with someone so accomplished in the hydrate field and his academic support and guidance has been truly invaluable. On top of this, I would like to thank Peter Englezos, UBC and NSERC Strategic Grants for their financial support throughout this research project.

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Finally, I would like to thank all of my friends and family for their support and love. Specifically, for those, whom I miss very much, back home in the UK – such as my Mother, Father, Dave, David, Leah and Eifion. In Vancouver, I would like to thank Helen and Janice for all of their help, affection and inspiration, especially when I first moved here, and Jess for keeping me motivated and happy. All of whom I feel very lucky to have in my life.
Dedicated to my Mother
1 INTRODUCTION

1.1 Overview

Clathrate hydrates are ice like crystalline phases that can form in oil and gas pipelines reducing or plugging flow completely. Hydrates are favoured by high pressures and low temperatures and can form above zero degrees Celsius making hydrate formation typically a far greater problem than ice formation in oil and gas pipelines.

Oil and gas companies spend billions of dollars per year on some methods for controlling hydrate formation [1]. One of the main methods is to inject a chemical hydrate inhibitor, such as methanol, upstream and recover it in downstream processing. However, the industry is now moving towards using low dosage inhibitors that are less toxic and require less processing compared to the traditional thermodynamic hydrate inhibitors [2].

Recent studies have shown that antifreeze proteins (AFPs) found in some cold resistant organisms show hydrate inhibition that is more effective than that of a conventional low dosage hydrate inhibitor, polyvinylpyrrolidone (PVP), at the same concentrations [3]. However, at almost ten times the price compared to PVP, the cost is the limiting factor for commercial application of AFP [4].

AFPs are found in certain species of fish, insect, plants, fungi and bacteria [5], [6]. From a commercial view, bacteria have the advantage of being relatively easily mass produced whilst having the opportunity to be genetically engineered to contain or secrete other AFPs from different species.

The cold resistant bacteria, Chryseobacterium sp. strain C14, was isolated by Walker's group from freeze thaw cycling of a soil sample obtained from the Rocky Mountain range in Canada [7]. From ice crystal morphology experiments, this strain of Chryseobacterium is believed to use AFP as a method of cold survival [8], but the protein cannot be easily purified and thus its identity is not proven.

The primary objective of the work presented herein is to determine the potential of Chryseobacterium as a hydrate inhibitor and, secondly, to perform a critical study in an attempt to identify other microbial derived inhibitors and their potential.
1.2 Clathrate Hydrates

Clathrate hydrates are crystalline phases of water cavities that contain guest molecules that hold the structure together. The definition of the word “clathrate” is to have a lattice-like structure which is pierced, intermittently, with holes or gaps. This definition along with the word “hydrate”, meaning to contain water, resulted in the aptly coined term “clathrate hydrate”. However, the term clathrate hydrate is commonly shortened to simply “hydrates” as is the case in this thesis.

It is widely believed that the discovery of hydrates can be credited to Sir Humphrey Davy in 1811. Davy reported in a lecture to the Royal Society that he had observed crystallisation of “oxymuriatic gas”, now known to be chlorine, in the presence of water. However, Davy noted that, at the same conditions, pure chlorine did not crystallize [9]. This discovery eventually resulted in a great interest within the scientific community to find other hydrate forming species and fuelled a drive to understand the phenomena in general.

![Figure 1 Lω-H-V phase equilibrium curves for pure component hydrate formers. Data obtained from [10–14].](image_url)
Hydrates can form with many different chemical species, gas or liquid. The species that becomes enclathrated into the hydrate phase is termed the “guest” substance. Just like the gas, liquid or solid phases, hydrate equilibrium is given by a phase diagram governed by temperature and pressure conditions as shown in Figure 1.

The hydrate formation driving force is favoured by increasing pressure and decreasing temperature. The term driving force refers to the difference between the operating conditions and the hydrate equilibrium conditions – it can be defined in terms of temperature or pressure difference. The driving force determines the severity and rate of hydrate formation.

The differences between phase diagrams of the different guest species is a result of differences in molecular structure, size, shape and polarity of the guest species. Most molecular species larger than 10Å cannot form hydrates, whilst the smallest molecules, such as hydrogen, only form under a very large pressure. The guest species and sometimes the conditions (e.g. for cyclopentane hydrates) define the crystalline structure of the hydrates.

The three most common hydrate crystalline structures are type 1 (sI), type 2 (sII) and type H (sH). Type sI and sII can form with just a single guest species or with multiple guests whilst type sH hydrates require at least two guest species to form and are a relatively new discovery [15] compared to sI and sII [16], [17]. All of these structures can be modelled by a repeating unit consisting of a specific number of polyhedral cavities. The cavities are shown in Table 1 with the corresponding number per repeating unit given for the three most common types of hydrate structures. Hydrate cavities are composed of hydrogen bonded water molecules with the guest held in place by Van der Waals dispersion forces. The cavities are named after the convention of \( n_m \), where \( n \) is the number of edges and \( m \) is the number of \( n \) faces. For instance, the cavity, \( 5_{12} \), means that the cavity consists of 12 pentagonal faces.

Some guests, such as methane and hydrogen sulphide can form hydrates by both occupying the small cages and large cages of the hydrate crystal. These single component hydrate formers are referred to as simple hydrates. Other species, such as n-butane and benzene, are too large to occupy the small cage but are small enough to fit in the larger cages and will thus only form with the presence of a smaller guest such as methane or nitrogen. These smaller guests are referred to as help gases, in these cases.

Ideally, there should be one guest molecule per cavity, however, not all cavities are occupied. Gas hydrates are non-stoichiometric inclusion compounds and the guest to water ratio, termed the 'hydration number', varies depending on the species and pressure/temperature conditions. For instance, an ideal type sI hydrate should contain 8 guest molecules to 46 water molecules (see Table 1), which
corresponds to a hydration number of 5.75. However, an actual methane type sI hydrate has been proven through various studies, such as direct measurements, raman and nuclear magnetic resonance (NMR) spectroscopy to have a hydration number ranging from 5.8 to 6.2 [18–20]. New insights through molecular dynamics (MD) simulations suggest that even hydrate growth rate may be proportional to the hydration number [21].

Studies have shown that for small molecular species and at extreme conditions multiple guests can occupy a cavity, such as hydrogen at very high pressures which forms a type II hydrate with two guest molecules in the smaller cavity and four guest molecules in the larger cavity [22].

Table 1 Structure properties of the three most common types of clathrate hydrates (sI, sII, sH) with corresponding cavity type and number. Data and crystal structures obtained from [23]. With permission from Nature Publishing Group.

<table>
<thead>
<tr>
<th>Cavity type [average cavity radius, nm] {Coordination number}</th>
<th>Structure (formula)</th>
<th>Single hydrate repeating unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small (S) &lt;br&gt; 5^{12} &lt;br&gt; [0.395] {20}</td>
<td>Cubic sI &lt;br&gt; (2S.6L.46H₂O)</td>
<td></td>
</tr>
<tr>
<td>Medium (M) &lt;br&gt; -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large (L) &lt;br&gt; 5^{12}6² &lt;br&gt; [0.433] {24}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cubic sII &lt;br&gt; (16S.8L.136H₂O)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
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<td>Small (S) &lt;br&gt; 5^{12} &lt;br&gt; [0.391] {20}</td>
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<td>Medium (M) &lt;br&gt; 4^{5}6³ &lt;br&gt; [0.406] {20}</td>
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<td>Large (L) &lt;br&gt; 5^{12}6⁴ &lt;br&gt; [0.571] {36}</td>
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1.3 Hydrate Crystallization

Hydrate formation is a crystallization process. Thus, when the system is changed from a state below the hydrate equilibrium curve to a state above the equilibrium curve, hydrates form via a time dependant, two step, process of hydrate nucleation followed by hydrate growth. Understanding the way in which hydrates crystals form will help develop better industrial hydrate applications such as flow assurance methods in oil and gas pipelines.

Hydrate formation requires water and a guest, meaning that, the formation is heavily dependent on the availability of the two components. In most cases, water is in excess of the guest due to the low concentration of most guests in water (THF being a significant exception). Thus, solubility and saturation of the guest in the water phase is one of the most important parameters governing hydrate formation.

The induction time refers to the time taken for the first nascent hydrate crystal to form. After this point, hydrate crystals can grow spontaneously throughout the system governed by mass transfer and diffusion processes. The subject of crystallization has been extensively studied since the 1900's and a large knowledge base has been developed from this [24]. However, there are still numerous areas within the field that are not yet fully understood.

The term hydrate kinetic experiments was coined in the early 80's for experiments that involved the analysis of hydrate nucleation time and growth rate [25–27]. This is typically achieved by using a vessel of an aqueous solution in contact with a gas phase at conditions above the hydrate equilibrium curve. The resulting hydrate formation is then analysed by recording the pressure drop in the system (see Section 2). Many factors affect hydrate formation such as solubility of the guest, driving force, agitation rate, concentration of hydrate precursors and liquid-gas interfacial surface area. The consequence of this coupled with the inherent stochasticity of hydrate formation makes it difficult to reproduce results in different apparatus configurations, although observed trends are generally transferable [28].

Due to the time dependence of hydrate crystallization, modelling of formation kinetics has proved to be a challenging task and there are still many unknowns concerned with this area. However, hydrate nucleation remains substantially more difficult to predict and model compared to hydrate growth. A summary of the two steps is given in the following sections.
1.3.1 Nucleation

Nucleation refers to the process at which nuclei agglomerate to a critical size allowing for spontaneous nuclei growth throughout the system and marks the start of the hydrate growth phase.

As with any crystallization processes hydrate nucleation is either initiated in the presence of impurities, heterogeneously, or in a pure system, homogeneously. In homogeneous systems, a critical number of hydrate nuclei need to agglomerate through stochastic molecular interactions before the system can spontaneously nucleate. Increasing the supercooling, through cooling or increasing pressure, is believed to reduce the work and number of critical nuclei needed for spontaneous nucleation \[29\]. Pure water, for example, can exist as a liquid up until around -40 °C but ice nucleation frequently occurs at higher temperatures below 0 °C \[30\]. The nucleation time is thus not a single deterministic point but a probability distribution.

As far as the flow assurance engineer is concerned, hydrate nucleation is almost always triggered heterogeneously in a pipeline or process, meaning that as soon as the hydrate equilibrium phase has been crossed, hydrates will nucleate quicker than the ideal homogeneous system.

In order to be able to better predict the onset of hydrate nucleation, several researchers have tried to quantify the distribution of nucleation at which hydrates will form by supercooling the system at a constant rate and recording the percentage of samples crystallized at different temperatures \[31–33\]. However, the measured distributions are far from being universal.

One proposed model for both homogeneous and heterogeneous hydrate nucleation is derived from classical nucleation theory, and is defined in the form of Eq. (1) \[29\]. This equation is based on classical nucleation theory which has been shown to successfully predict other systems such as bubble nucleation temperatures at the immiscible liquid-liquid interface of superheated n-pentane and ethylene glycol, when compared to experimental results \[34\].

\[
W(n) = -n \Delta \mu + c v_h^{2/3} \sigma_{ef} n^{2/3} \quad \text{Eq. (1)}
\]

\[
\Delta \mu = \mu_{hs} - \mu_h
\]

W(n) (J.mol\(^{-1}\)) is the work needed to nucleate n hydrate forming units. \(\Delta \mu\) (J) is the degree of supersaturation in terms of chemical potential and defines the driving force of the system, that is to say, it is the difference between the chemical potential of the, pre-hydrate, water-guest solution, \(\mu_{hs}\), and the formed hydrates, \(\mu_h\). For hydrates to be able to nucleate, \(\Delta \mu\) needs to be greater than 0. The numerical
The specific surface energy can be represented in terms of a “bubble” or cluster of nucleating units as shown in Figure 3. The specific surface energy is governed by the contact angles of the cluster which is reduced in the case of the cluster adsorbed to a substrate surface and at the gas-liquid interface when compared to the spherical cluster in bulk solution. This is graphically shown in figure A of Figure 2. The work and nuclei units needed for nucleation is reduced when the contact angle is 90° and 60° compared to the spherical clusters at 180° for homogeneous nucleation. Figure B in Figure 2 also shows that increasing pressure of the system reduces the work requirement for nucleation.

In heterogeneous nucleation, foreign particles or bodies will give a certain value of the wetting angle depending on the substrate properties. It has been determined, using the methods described above, that hydrate nucleation of cap shaped clusters requires less work than the spherical clusters in the bulk solution, thus hydrates will more likely nucleate at the gas-substrate interface over the bulk [29]. The model also shows that for heterogeneous nucleation, certain additives can change the preference for nucleation at the gas-liquid interface. However, nucleation may have preference to occur at the gas-liquid interface simply due to the localised supersaturation of the guest gas at the interface film [35].

Figure 2 Diagrams depicting a nuclei cluster of n units forming (A) spherically in the bulk solution, as is the case for homogeneous nucleation, (B) cap-shaped on a substrate surface such as solution impurities or the vessel wall and (C) lens shaped between the liquid-gas interface (adapted from [29]). With permission from Elsevier.
Figure 3 Work, $W(n)$, and number of nuclei per cluster, $n$, required for cluster formation for (A) heterogeneous nucleation of cap shaped clusters of contact angle $60^\circ$ and $90^\circ$ and homogeneous nucleation of contact angle $180^\circ$ at 10 MPa and (B) homogeneous nucleation at 10, 20 and 40 MPa (data obtained from equations published in [29]). With permission from Elsevier.

Until the recent advances in computer processing power, simulating and experimentation on homogeneous nucleating systems proved to be extremely difficult. Today, molecular dynamics (MD) simulations have become a popular tool for modelling hydrate formation and dissociation [21], [35–38].

An MD simulation, used to model homogeneous hydrate nucleation has successfully shown the formation of the critical nucleus leading to spontaneous hydrate growth from a solution of dissolved guest within water [37]. The snapshot A through to C in Figure 4 shows the stochastic variation in the orientation of molecules and the formation and breaking of bonds. At point D, a single $\text{5}^{12}$ hydrate cavity can be seen to have formed around a methane guest molecule. Methane molecules are then shown to “adsorb” to the pentagonal faces of the cavity allowing further hydrogen bonding between
water molecules. By point H, several partially formed hydrate cavities can be seen to have formed. Another MD simulation, gives evidence that stochastic localized concentration of guest molecules triggers nucleation as opposed to the labile hydrate clusters theory [39].

The disadvantage with using MD simulations is that it requires a very large computational power. To keep the experiment run time manageable, the simulation usually has to be run at very high driving forces, in supersaturated solutions, that may not provide a realistic model at standard conditions. The experiments also have to be conducted for short time periods, usually less than a microsecond. This means that it is very difficult to perform an experiment that combines nucleation, growth and/or dissociation of hydrates. However, continuing advances in computing power should help broaden the boundaries of MD simulation and provide a greater understanding of hydrate nucleation. Understanding the mechanisms of nucleation could help develop future more powerful KHIIs. In fact, some MD simulations have already been used to identify previously unknown hydrate inhibitors [40].

Figure 4 Snapshots from a molecular dynamics (MD) simulation of a methane hydrate nucleating within 1μs (refer to reference for full simulation video). Gray lines: water molecules; red lines: hydrogen bonds; orange spheres: methane molecules that do not become enclathrated in this simulation; blue spheres: methane molecules that become enclathrated in this simulation; violet sphere: the methane molecule that initiated the first hydrate cavity. From (Walsh et al., 2009) [37]. Reprinted with permission from AAAS.
1.3.1.1 Memory Effect

Several workers have shown that reformation of hydrates in solutions that have had hydrates previously formed and dissociated can nucleate faster [25], [31], [32]. Practically, this is an important phenomenon to understand for flow assurance engineers who will need to recover accordingly after a hydrate plug has formed and dissociated in an oil and gas pipeline.

Generally speaking, there are two theories for why hydrate memory occurs. The first theory is that a solution retains a metastable ordered structure, analogous in some ways to anisotropic liquids. Anisotropic liquids are usually long chained molecules and/or aromatics that are ordered in a semi-crystalline liquid state that forms just after the melting point or just after the crystallization point depending on if the solution is being warmed or cooled, respectively. In the case of hydrates, the metastable region is much less pronounced and defined and even the belief that some labile order remains in the solution is still debated.

Evidence for the labile order theory include kinetic experiments where thawed ice has shown to have an induction time lower than tap water which in turn is lower than hot water [25]. However, this observation may have come from tiny ice nucleation seeds that didn't have time to melt. Residual structures of predominantly ice like and hydrate like precursors (albeit in small amounts) have been shown to be present after methane hydrate dissociation in an MD simulation 15-20 °C above the hydrate equilibrium point (290 K at 1.5 MPa) [41]. However, the researcher concluded that the concentration of hydrate clusters was too low for it to be the cause of the memory effect and states that the higher localized methane concentration is likely the main reason for hydrate memory. Another MD simulation showed that no residual structure remained around methane molecules in solution after hydrate melt, although the main focus of their study was on the subsequent hydrate growth from the localized supersaturation of dissociated hydrate solution [21].

Localised guest concentration is the second main theory for hydrate memory which arises from the belief that because the guest exists in a compressed form within the hydrate, subsequent dissociation results in local supersaturation of the guest. The higher saturation of guests would then provide a greater chance for an embryonic hydrate crystal to form. However, this would not completely explain why miscible liquid guests, such as THF, observe hydrate memory. But, molecular evidence from a neutron diffraction method has shown that the structure of water before hydrate formation does not differ from the structure of water after hydrate decomposition, suggesting that residual structure isn't responsible for the memory effect [42].
1.3.2 Growth

After spontaneous nucleation has occurred in the system, hydrates are free to grow and agglomerate throughout the bulk solution. Similarly to ice formation, hydrate formation is an exothermic process. Several factors affect hydrate growth with a major factor being heat and mass transfer of the guest and forming crystals. More specifically these are, the rate at which the guest can transfer to the hydrate growing region, the rate at which the particles are forming defined by the driving force (see Section 1.3.3), and the rate at which heat from crystal formation is transferred away. In a quiescent water-gas interface, hydrates will grow via film growth at the interface [43]. In agitated systems, the film is constantly being broken, increasing the overall rate of growth. Additives can also alter the film growth mechanism (see Section 1.4.2.1).

Much interest has been shown for hydrate growth in both micro and macro scale [21], [44–46]. Some pertinent points from the micro scale investigations, in terms of hydrate inhibition, come from morphology experiments in which the effect of KHIs can be observed through the distortion of forming crystals.

From a flow assurance point of view, the rate at which hydrates agglomerate essentially determines the scale of the problem, with the worst case being complete loss of flow through hydrate plugging. It is believed that the major factor effecting the rate of hydrate growth is the driving force which is defined as the degree of supercooling (i.e. the difference between the operating conditions and the hydrate equilibrium point of the solution) which is directly proportional to hydrate growth.

It has been shown using MD simulations that methane molecules in the liquid phase are attracted to forming cavities of a growing hydrate crystals as this is believed to be more energetically favourable than forming a new hydrate particle in the bulk liquid solution [21]. However, it should be noted that due to the current restrictions of MD simulations (see Section 1.3.1), these simulations were performed in supersaturated solutions that were artificially created from a dissociating hydrate crystal modified to contain 30-50% less methane than a fully occupied hydrate. This meant that the solution had almost 200-300 times the concentration of methane compared to experimental equilibrium solubilities which meant that the methane molecules were much less energetically favoured to stay in the liquid bulk solution compared to the growing hydrate crystal surface, resulting in increased growth rate compared to experimental observations. This was accommodated for by implementing an “active transport” force on the methane molecules which supposedly replicates the experimental environment.
Using crystallization theory coupled with mass transfer phenomena the kinetics of hydrate growth has been modelled and has shown to successfully fit experimental results for simple hydrate formers, albeit for a specific apparatus and conditions [27].

1.3.3 Driving Force

Often, the driving force is simply expressed in terms of subcooling or pressure difference which is an accurate representation for simple hydrate formers like methane in the presence of LDHIs that do not significantly change the phase diagram [47]. Operating conditions for the experiments performed in this thesis are plotted against the methane hydrate phase diagram shown in Figure 5 to illustrate the driving force.

![Figure 5 Methane hydrate phase diagram with operating conditions at a driving force of 730 kPa (subcooling of 2.34 K). Phase boundary calculated using CSMHYD [48].](image)
1.4 An Industrial Issue

1.4.1 Flow Assurance

Clathrate hydrates have been a scientific curiosity since their discovery over two centuries ago but they became a significant industrial issue at the turn of the 19\textsuperscript{th} century during the boom in growth of the oil and gas industry.

What was first believed to be ice formation in natural gas pipelines, causing blockages and reduced flow, was later found to be clathrate hydrates. This was first proven when hydrate crystals were formed in a laboratory flow loop above the melting point of ice \[49\]. Today, hydrates are a far more important issue now that many oil and gas producing reservoirs are located in extreme environments that have naturally low temperatures and/or very high pressures.

For example, hydrates blocked and hindered the progress of two deepwater drilling operations, significantly increasing the risk associated with the process \[50\]. But, hydrates gained notoriety in 2010 after the first effort to mitigate the leaking oil during the Deepwater Horizon oil spill failed after hydrate formed in the relief device. This resulted in the formation of a hydrate plug whilst simultaneously increasing the buoyancy of the device causing it to tip \[51\].

Flow Assurance Engineering is a relatively recent specialist field in petroleum engineering that focuses on providing optimum delivery of oil and gas through a pipeline network or a process. Hydrate prevention and control represents a significant proportion of flow assurance engineering and due to the variation in composition and conditions of pipelines each project represents a unique complex challenge.

Some methods used to inhibit ice formation have been applied to hydrate inhibition, such as the depression of the freezing point using colligative substances, termed thermodynamic hydrate inhibitors (THIs) such as alcohols, glycols and salts. However, numerous differences exist between ice and hydrates. Non-colligative hydrate inhibitors, termed kinetic hydrate inhibitors (KHIs), interact with hydrate crystallization to either delay the nucleation time, inhibit the growth rate or alter the crystal morphology but they may have little effect on ice crystallization.

Currently, the convention within the oil and gas market is believed to still be predominantly THI injection of methanol or glycols with concentrations of up to 60 wt\%. However, considering that the
cost associated with THI injection is usually in the millions of dollars per year per field [1], [2] there is a growth in the adoption of low dosage hydrate inhibitors (LDHIs) such as KHIs or anti-agglomerants (AAs) which require less than 2 wt% to be effective.

Although some believe that LDHI research is approaching the top of the 'S-curve', in terms of industrial application, there is also a market for developing so called 'green' hydrate inhibitors that are also cost effective [52].

![Figure 6 Methane hydrate phase equilibrium curves for pure solution, 5 wt% sodium chloride, 25 and 40 vol% methanol. Methane hydrates can form left of the corresponding curve. Phase equilibria data generated from CSMHYD [48].](image)

**1.4.1.1 Thermodynamic Hydrate Inhibitors**

All THIs inhibit hydrates in a colligative manner. That is to say that the inhibitor increases the amount of hydrogen bond competition, thus depressing the freezing point of water and the equilibrium point of clathrate hydrates. An easy way to visualize this is by looking at the effect of THIs on the hydrate phase diagram, which effectively shift the equilibrium to the left (as shown for methane hydrates in Figure 6) so that the operating conditions lie outside of the hydrate forming region.
Common THI’s include methanol, glycols (e.g. MEG) and salts (solubility limited). Salts are usually only applied if the driving force is low enough, otherwise the salt can precipitate out of the solution leading to the undesirable phenomena of salting-out [53], [54]. The miscible chemicals, methanol and glycols, are believed to be still the most common method of hydrate formation control in industry as they are very simple to apply and should significantly reduce the risk of hydrate formation if applied correctly. The major disadvantage of THIs is the cost associated with the high dose of these chemicals and the additional processes required to recover the THI downstream [1], [2]. Environmental considerations, especially with the associated toxicity of methanol and glycols are another significant disadvantage of THIs.

1.4.1.2 Kinetic Hydrate Inhibitors

Instead of shifting the hydrate phase boundary, which is the case for THIs, KHIs interact with the hydrate crystallization kinetics to delay the nucleation time and have negligible effect on the phase boundary. This means that, hydrates, given infinite time above the hydrate phase boundary will eventually form in an aqueous KHI solution. However, by accurately predicting the delay in nucleation time and knowing the retention time of the solution in a pipeline, hydrate formation can be effectively avoided. As discussed in Section 1.3.1, the hydrate nucleation time is defined as a probability distribution and it is thus essential that the worst case scenario be considered when applying a KHI to industry. Thus, KHIs are driving force limited with, typically, an effective maximum subcooling of less than 12 °C [52], [55]. This reliability issue and limitation is likely the biggest disadvantage of KHIs when compared to other hydrate control methods. However, the major advantage of KHIs is the potential savings compared to THIs, and as a consequence many fields (50 – 70 prior to 2006 [52]) have converted from THIs to LDHIs. For example, one North Sea natural gas production site, experienced great difficulties, such as salting-out, high maintenance costs and glycol water pollution, whilst using MEG as a THI and successfully switched to a KHI with the result that the cost of hydrate inhibition was reduced by 80% [55].

Traditionally, KHIs were based around water soluble polymers such as PVP and PVCap. Today, many companies have developed in-house proprietary chemicals that can delay nucleation time by several days, depending on the driving force. The history and state of the art of KHIs has been extensively reviewed elsewhere [52] and has been successfully applied in the field [55–58].
The selection and application of KHIs to the field depends on several considerations such as flow rate, gas/oil ratio, subcooling, pipeline size, well depth/size, retention time, water cut, salinity and other chemical injections (e.g. for corrosion, wax and asphaltene deposition). Thus, there is no 'rule of thumb' when it comes to applying a KHI in industry and each project requires customization. As an example, a 10% mixture of PVCap and N-methyl N-vinyl acetamide (VIMA) was first tested in a laboratory flow loop before being applied in an 800 ft subsea well producing 11-15 MMSCFD gas (>99 % methane) and very little condensate, with a water flowrate of 13 BWPD [59]. The KHI mixture was injected via a half inch umbilical line to the subsea manifold where it was subsequently injected continually throughout the year, into the 4 inch producing pipeline at a rate of 3 GPD. The pipeline was roughly operating in the hydrate forming zone (at 12 MPa and 10 – 12 °C) for a length of 6.4 miles. The KHI was designed to perform for planned shut in periods and because of the low dosage, no additional treatment downstream was required. In fact, the quality of the product was improved compared to the prior methanol inhibition process used in the field. Additionally, switching to the KHI saved the operator up to 40 % in operating costs whilst being far less of an environmental risk compared to methanol inhibition [59].

One mechanism for KHI nucleation time delay stems from classical nucleation theory, as described in Section 1.3.1. The KHI is believed to act as a substrate for nucleating clusters thus reducing the effective contact angle or wetting of the cap-shaped adsorbed cluster.

This could be one explanation for the mechanism of KHIs that would increase the effective contact angle and thus the work required for nucleation. In fact, many KHIs have a combination of hydrophobic and hydrophilic properties that could interact with the surface energy in this way.

Several MD simulations of inhibitor interactions with hydrate formation have been performed [35], [40], [60]. One study performed simulations on 4 different known inhibitors PEO, PVP, PVCap and VIMA and found that PEO and PVP did not shown any binding interaction whilst PVCap and VIMA did show adsorption to the hydrate surface [60].

**A Model KHI: Polyvinylpyrrolidone (PVP)**

PVP was first discovered to possess hydrate inhibition during the mid 90's and has since been used by many researchers as a model KHI due to its relatively simple structure, solubility, availability and cheap cost. It is considered a relatively moderate to weak strength KHI with the main appeal being economical.
As far as KHIs go, PVP is considered a reasonably green alternative, with the downside that it takes a long time to biodegrade. Also PVP monomer (n-vinyl pyrrolidone) is highly toxic to aquatic life [61], a pertinent environmental consideration in deep sea applications. Although the low dosage required for hydrate inhibition may render the environmental aspects of PVP insignificant, especially in relation to other inhibitors like methanol.

The discovery of PVP as a KHI may have come from its known interaction with other crystallization processes [62–65]. PVP is a known cryoprotective agent, which is applied to ice sensitive freeze drying of biological specimens [65]. PVP is a thickener and is used at much higher concentrations as a cryoprotective agent compared to the KHI application. This is to prevent the formation of deleterious ice and instead keep the solution in an amorphous glassy phase [65], an industrial process known as vitrification. PVP has been known to exhibit interesting binding interactions with co-solutes in aqueous solutions [62].

PVP is a, synthetic, non-colligative, hydrate inhibitor that delays hydrate nucleation but shows no thermal hysteresis (TH) with respect to ice [66]. This is in contrast to antifreeze proteins (AFPs) that are known to have TH and are believed to bind to ice [67] and, more recently, to THF hydrates [68], suggesting a different inhibition mechanism by the two KHIs. In fact, over 20 years ago, it was shown using DSC measurements and classic crystallization theory that PVP and AFP, in ice forming systems, use two entirely different growth inhibition mechanisms [69]; AFP binds to ice crystals whilst PVP does not bind significantly.

PVP appears to act as an effective hydrate nucleation inhibitor. However, some experimental studies have shown that after hydrates nucleate, catastrophic growth ensues that is even greater than in the absence of the inhibitor [1], [70–72]. One such observation was recorded in a temperature ramped flow loop that showed greater hydrate agglomeration but less overall hydrate growth in the presence of 0.5wt% PVP [71]. Another study used PVP to actually promote hydrate formation, in an agitated autoclave, for gas storage applications and stated that the increased growth was likely due to the lack of a hydrate cap formation in the presence of PVP caused by dendrite crystal growth and the slower growth rate [70]. Morphology experiments of methane/propane hydrates, in a quiescent system showed that catastrophic growth only occurred at 1.0 wt% PVP but not at lower concentrations [72].

The promoter effect of PVP has also been investigated in simulations. A recent publication used Monte Carlo simulations of PVPs interaction with hydrates that suggested that depending on the orientation of
the molecule in the simulation, hydrate growth could either be promoted or inhibited [73]. A MD simulation that tested PVP in a supersaturated methane-water film showed that PVP interacts with nucleating clusters below a critical nucleation size but does not interact with hydrates of two times the critical size (of ~400 water molecules), providing evidence that PVP acts as a nucleation inhibitor and a growth promoter [35]. Another interesting result from this MD simulation was that PVP did not show interaction with the cluster surfaces but rather changed the solution's surface energy [35], validating results from an older publication of a different MD simulation [60], that PVP does not inhibit nucleation by adsorbing to nascent hydrates.

1.4.1.3 Anti-Agglomerants

AAs are a type of surface active LDHI that affects hydrate growth by dispersing the hydrate phase into a slurry, thus preventing agglomeration and subsequent pipe blockage. AAs have been successfully applied in various pipelines and processes over the last 10 years [74–76]. They are believed to interact with hydrate crystals by binding to their surface making the crystals surface hydrophobic [77]. This results in the fine hydrate particles being forced out of the water phase and into the oil phase of the pipeline, reducing the amount of water available for the hydrates to agglomerate. Although this makes AAs very versatile at theoretically limitless driving forces, they cannot always be used in situations that have too little oil phase for the hydrates to be dispersed into. These limiting cases can occur for high gas or high water cut pipelines. Emulsion formation when using AAs could also be problematic in that it can severely reduce the water-cut quality, requiring additional treatment steps downstream.

1.4.2 Hydrates as a Storage Medium

When hydrates form the guest gas becomes, effectively, compressed into the crystalline phase. Some of the potential applications of this are carbon sequestration, hydrogen storage and natural gas transport [23], [70], [78].

1.4.2.1 Hydrate Promoters

To increase the maximum achievable hydrate growth for storage applications, chemical additives, termed promoters, can be applied to increase the kinetics of hydrate crystallization. They could be
considered as the opposite of KHIs. However, some promoters can delay nucleation time but increase hydrate growth, such as that observed by PVP (see Section 1.4.1.2).

Many promoters are surfactants, that alter the surface tension of the solution and exist as micelles in solution [79]. Sodium dodecyl sulfate (SDS) is a well known surfactant that has been used to promote both rate and final growth of hydrates as well as nucleation time. SDS is anionic and amphipathic.

Surfactants are believed to interact with the hydrates growing at the film, forcing them to the metal walls of a vessel around the liquid-gas interface, forming a porous hydrate layer around the walls but less in the centre [79]. The mechanism is believed to be due to the hydophilic head attracting water molecules whilst the hydrophobic tail is attracted to the gas, mediating the gas-water contact, acting as nucleation centres.

SDS forms as micelles in solution (water is not included in the micelles) and the promoter performance increases significantly once a critical micellar concentration (CMS) is reached [80].

One of the most powerful surfactants known can be attributed to surfactin, a biologically-derived surfactant obtained from the bacteria, *Bacillus subtilis* [79].
1.5 Biological Hydrate Inhibitors

There is a need to develop 'green' kinetic hydrate inhibitors as most of the common LDHIs, although far safer than methanol or glycol injection, are still toxic [52]. This has lead to a drive, in the past 5 to 10 years, into the research of biologically-derived KHIs [66], [68], [81–84].

Over 60 years ago Scholander discovered that blood plasma from fish caught below ice, in waters of -1.7 to -1.8 °C, on the east coast of Canada, existed in a supercooled state underwater but when exposed to the same temperature in a water tank above the surface the solution froze [85]. This difference occurred due to the presence of ice seeds that nucleated ice formation in the surface water tank but were not present in the supercooled water below the ice. Scholander showed that the fish could regulate salts in their blood to match that of the sea water and concluded that this was the mechanism for the observation. However, it wasn't until much later that proteins were identified as playing a significant role in preventing the fish plasma from freezing in some cold hardy species [86]. Since then, antifreeze proteins and antifreeze glycoproteins (AFGPs) have been extensively studied in ice-forming systems and have recently been studied in hydrate-forming systems. The following section will review the knowledge in these areas.

1.5.1 Ice-Binding Proteins

Some species of bacteria, fish, insects and plants, living in freezing environments, use proteins to control ice formation so as to have a better chance of survival. These are generally referred to as ice-binding proteins (IBPs). Ice-structuring proteins (ISPs) or ice-associating proteins (IAPs). The term IBPs will be used in this thesis.

The proteins found in different species vary depending on their required function. Certain proteins can inhibit ice formation, the AFPs and AFGPs, whilst others promote or control ice formation, termed ice nucleating proteins (INPs).

1.5.1.1 Anti Freeze Proteins

Recent publications have shown that AFPs are strong KHIs and this has led to an increase in interest within this area [66], [81–83]. However, AFPs have been studied far more for their interaction with ice rather than hydrates, and a significant knowledge base has developed [5], [67], [87], [88].
Knowledge from Ice Forming Systems

In terms of species survival against freezing environments, AFPs use the proteins’ TH and ice recrystallization inhibition (IRI) properties to control deleterious ice formation. The TH is defined as a difference between the melting and freezing points of ice in the presence of AFP, whilst IRI is used to protect against the formation of large ice crystals at high cooling rates or when the temperature is at a high sub-zero range.

The AFPs are believed to act by binding to ice crystal surfaces. Some AFPs have shown to have a higher hydrophobic region that is believed to bind to the ice surface by replicating the shape of ice surfaces [67]. The inhibition of growth on specific ice planes results in faceted crystal growth, causing the ice front to grow with local curvature, a thermodynamically undesirable result described by the Kelvin effect [89]. Others suggest that AFPs inhibit nucleation by binding to heterogeneous ice nucleators [90].

There are three main fish AFP structures, types I, II and III, which usually have TH values of less than 2 °C at concentrations found in the organism. There are also two extended classes of AFPs, termed hyperactive AFPS (due to the relatively high TH) and AFGPs, which are polypeptides with polysaccharide side chains. Most fish with AFPs live near cold water surfaces, where ice is nucleating, rather than in supercooled deep waters.

The AFPs with the highest TH are termed hyperactive and are found in some species of insects such as the mealworm beetle, Tenebrio molitor, (TmAFP [91]) and the spruce budworm moth, Choristoneura fumiferana, (CfAFP [92]). More recently they have been found in some species of bacteria and fish, such as the Antarctic bacterium, Marinomonas primoryensis, (MpAFP [92]) and the winter flounder, Pseudopleuronectes americanus, (wfAFP [92]).

The effect of environmental temperature was investigated on a species of Antarctic fish that showed a reduction in 60 % and 20 % of two different blood AFGP concentrations when exposed to 4 °C for 16 weeks [93].

Knowledge from Hydrate Forming Systems

Two AFPs obtained from the type I winter flounder (wfAFP) and CfAFP have shown to change the morphology of THF hydrate crystals, suggesting AFP binding to the crystal surface [83]. This study also showed significant inhibition, stronger than PVP, in crystallization experiments. This research group then validated that wfAFP is also an effective hydrate inhibitor in a propane hydrate system [94].
From both publications, the group suggested that the hydrate memory effect is eliminated by the presence of AFP.

A recent study showed that AFP type III HPLC12, obtained from the fish ocean pout, gave stronger nucleation delay of natural gas hydrates than a conventional KHI, PVCap, in a stirred cell [95]. Some researchers have suggested that AFP suppress both hydrate nucleation and growth [66], [82]. The effect of wfAFP was shown to depress initial methane hydrate growth curves, similarly to PVP and PVCap, and gave initial non-linear growth profiles compared to pure water solution [82]. However, the overall hydrate conversion was not investigated. Another publication confirmed the observation of initial non-linear growth in the presence of type III HPLC12 ocean pout and TmAFPs and concluded that AFP adsorption on hydrate crystals limited the initial crystal surface area for hydrate growth [66].

AFP binding to THF hydrates has been shown through fluorescence of crystals bound with green fluorescent protein (GFP) tagged AFPs [68], suggesting that this may be the method of hydrate inhibition of AFPs.

To the authors knowledge, hydrate thermal hysteresis (TH) and hydrate recrystallization inhibition, in the presence of AFPs, has yet to be investigated.

### 1.5.2 Other Proteins

Although AFPs, due to their association to ice, have become a popular potential KHIs to screen, very little investigation on the effect of other proteins (apart from those used as controls in the above studies, including cytochrome C) has been completed. Although crystal binding is one hydrate inhibition interaction, several other mechanisms have been proposed, such as interference with gas-liquid interface (see Section 1.4.1.2). Thus, potentially, some non ice associating proteins could act as a hydrate inhibitor.

Even for ice forming systems, it has been suggested that dissolved polymers, in general, could affect hydrate formation, albeit much less than ice-active proteins [96]. This is believed to be due to mass transfer limitations from slow diffusion rates of macromolecules away from the ice forming surface.

GFP, a protein that is not known for ice-association, was shown to inhibit hydrates formed in the presence of a methane-ethane-propane mixture, greater than the inhibition of PVP [81]. The GFP is likely not inhibiting due to surface binding as GFP did not bind to THF crystals [68].
A mutant wfAFP, A17L, that had no ice-associating properties, showed THF hydrate inhibition stronger than the corresponding ice association wfAFP [83]. This suggests that the mechanism for hydrate inhibition may be vastly different from ice inhibition.

1.5.3 Other Biological Inhibitors

Cationic starches, such as tapioca starch, have been shown to weakly inhibit hydrate nucleation at 0.5 wt% [97]. One recent publication has shown that, chitosan, a linear polysaccharide product obtained from deacetylation of chitin (a component of crustacean shells) gave weak nucleation inhibition of methane hydrates in a stirred autoclave [98]. However, this was not compared with a model KHI such as PVP.

Amino acids (glycine, L-alanine and L-valine) have very recently been investigated for their ability as a “green” THI which showed a similar shift in the hydrate phase boundary as methanol [99]. However, due to the solubility limitations, (< 4 mol% at pH of 7), they are unlikely to be an industrial alternative. But, KHI interactions of amino acids was not investigated.
1.6 Microbe Relationship with Hydrates

The niche research gap of microbial hydrate inhibitors forms the basis of the author's research objectives (see Section 1.7), but very little has been published on the subject. In fact, very little is known about the interaction of microbial species with hydrates in general, with the majority of research focused on organisms that abide within natural gas hydrate reserves [100–102].

For all forms of life on Earth, living organisms have a remarkable ability to adapt and thrive to extreme conditions. Perhaps, most impressive of all is the resilience of bacteria. To give one example, the *Cyanobacteria* species survived for 10 days exposed to low earth orbit from the International Space Station [103].

In consideration, it is no surprise that an abundant array of species survive in the coldest environments found on Earth, examples of which can be found in Table 2. However, the cold-resistant mechanisms used by the number of different cold hardy organisms are as varied and complex as the organisms themselves. Some species employ the use of AFPs or INPs to interact with forming ice crystals whereas others remain supercooled or can survive freezing.

Many KHIs, such as PVP, were discovered because of their known ice affinity. This meant that known ice associating chemicals, such as AFPs and INPs, were traditionally screened for potential hydrate inhibitor or promoter applications. Results of such experiments have shown various positive inhibition and promotion of hydrates using IBPs [82], [83], [104]. However, all of these experiments were conducted with purified proteins, which are discussed separately in Section 1.5.1.

Many microbial species interact with the formation of ice, yet, to the author's knowledge, only one publication has been released on microbial interaction with hydrates for the purpose of hydrate inhibition [84]; the results and method of their work will be reviewed in the following section.
Table 2 Examples of organisms that have cold tolerance using different survival mechanisms. Thermal hysteresis values are given at the concentrations found in the organism.

<table>
<thead>
<tr>
<th>Taxonomy</th>
<th>Isolated from</th>
<th>Known survival mechanism(s)</th>
<th>Notes [reference]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bolyphantes index</strong> Spider</td>
<td>Snow surface Trondheim, Norway</td>
<td>Anti freeze agent in haemolymph</td>
<td>Anti freeze agent could be a protein but has not been proven TH of 5.2°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[105]</td>
</tr>
<tr>
<td><strong>Chryseobacterium sp. strain C14</strong> Bacteria</td>
<td>Soil sample Rocky Mountains, North America</td>
<td>Believed to contain AFP</td>
<td>Shown ice morphology changes similar to AFP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[8]</td>
</tr>
<tr>
<td><strong>Gymnodraco acuticeps Fish</strong></td>
<td>Ross Sea Antarctica</td>
<td>Production of AFGP in blood serum and intestinal fluid</td>
<td>Blood TH of 1.55°C Intestinal TH of 2.15°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[106]</td>
</tr>
<tr>
<td><strong>Gymnodraco acuticeps Fish Larvae</strong></td>
<td>Ross Sea Antarctica</td>
<td>Physical prevention of ice nucleation through underdeveloped gills and other barriers</td>
<td>Has significantly less AFP compared to adult</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Production of AFGP in blood serum and intestinal fluid</td>
<td>Blood TH of 0.25°C Intestinal TH of 0.38°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[106]</td>
</tr>
<tr>
<td><strong>Moraxella sp. No. 82 Bacteria</strong></td>
<td>Sand sample Ross Island, Antarctica</td>
<td>Production of antifreeze lipoprotein (AFLP)</td>
<td>First AFP discovered in Antarctic bacteria</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TH of 0.104°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[107]</td>
</tr>
<tr>
<td><strong>Psuedomonas antarctica IN-74</strong> Bacteria</td>
<td>Ross Island, Antarctica</td>
<td>Secretion of INP into extracellular medium</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[108]</td>
</tr>
<tr>
<td><strong>Pytho deplanatus Beetle Larvae</strong></td>
<td>Fallen spruce bark Rocky Mountains, North America</td>
<td>Incapacl of producing ice nucleating molecules Glycerol and trehalose Accumulation</td>
<td>Supercooled down to -54°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Water and glycogen reduction</td>
<td>[109]</td>
</tr>
</tbody>
</table>
1.6.1 Microbial Hydrate Inhibitors

To the author's knowledge, this is the second publication to investigate microbial hydrate inhibition with the other publication coming from Walker's group at Queens University [84]. Their method was to test the inhibition of THF hydrates, in terms of percentage of samples crystallized after a given time at atmospheric pressure and 0 °C in the presence of the microbial species *Escherichia coli*, *Chryseobacterium* sp. Strain C14, *Pseudomonas putida*, *Pseudomonas syringae* and *Pseudomonas borealis*, all of which, apart from *E. coli*, are believed to contain various IBP.

Their results showed some promise with *Chryseobacterium* providing the strongest inhibition at almost 50% less mean samples crystallized after 5 hours compared to the water-THF control solution. All other bacteria tested negative with similar mean numbers of samples crystallized as the water-THF case.

It should be noted that THF is miscible in water and forms type sII hydrates below 4.4°C which means that although it may well provide an easy way of forming hydrates at atmospheric pressure, it may not be an accurate representation of hydrates that form in oil and gas pipelines mainly due to its miscibility with water. As this is opposite to most industrial cases in which hydrates form with immiscible guests at the water-guest interface in multiphase flow, giving rise to some dispute as to how effective THF is as a model hydrate former. However, some KHIs have shown both inhibition of THF hydrates and hydrocarbon guest hydrates, proving that, at least in some cases, THF is a suitable model hydrate.

Another important property of THF is that it is highly toxic to micro-organisms such as the microbial community found in activated sludge in a study to examine THF pollution in waste water treatment [110]. Walker's group encountered the most problems with the cells aggregating and showed no cell viability after each trial. However, in terms of hydrate inhibition, it would not necessarily matter if the bacteria were either lysed or viable as long as the nucleation time or hydrate growth were inhibited.
1.6.2 Freeze Stresses on Micro-organisms

The challenges faced by organisms in cold environments can be summarized into the main categories listed below and should prove helpful in understanding hydrate stresses on micro-organisms.

1. Exposure to lower temperatures (negating ice)
2. Ice formation around the organism (extracellular)
3. Ice formation inside of the cells of the organism (intracellular)
4. Osmotic changes in the surrounding environment

Organisms use two main strategies in freezing conditions, that is freeze tolerance or freeze avoidance. Those that are freeze tolerant can use small cryoprotective agents such as carbohydrates (e.g. trehalose), glycerol, amino acids and salts to depress the freezing point of the solution or make use of dissolved polymers that are believed to reduce ice formation by reducing the rate of mass transfer of the macromolecular molecules away from growing ice [96].

Several cold responses shared by many micro-organisms involve the production or degradation of chemicals through genetic and molecular mechanisms [96], [111]. Identification of these chemical changes in a solution is important to understand for this research so that any observed hydrate inhibition from the bacteria could, theoretically, be traced to a specific chemical or interaction.

The proceeding section summarizes the current understanding of cold and ice response mechanisms shared by many cells in general. For the purpose of this research, the cold tolerance of organisms that are most likely to have hydrate inhibition properties will only be discussed in detail with the focus on cold survival mechanisms of *Escherichia coli* as a model prokaryote due to the vast amount of publications on this particular species. Further reading on microbial cold response can be found elsewhere [111].

All cells make use of the lipid bilayer membrane as a barrier between the cell components and the solution. Because of the importance of the cell membrane in a freezing environment, most cells will be affected by the same factors when exposed to freezing temperatures. Vesicles of lipids that have been manipulated to encapsulate simple sugars, thus representing a model cell, have shown responses that do correspond to observed actual cell behaviour in a freezing environment [112].

The most damaging implications of an environment below the freezing point to the survival of micro-organisms is the formation of ice. The way in which ice damages cells is believed to be a combination
of many stresses that are not fully understood, but ice damage is known to be heavily dependant on the rate of cooling. Both very slow and very fast cooling are damaging to cells for different reasons with an optimal cooling rate giving the best cell survival percentage [113].

When freezing occurs, the solution around the cells will become more concentrated with solutes due to the uptake of water into the ice phase. This leads to two possible thermodynamic responses by the cell. Either ice forms inside the cell or water leaves the cell due to the newly formed osmotic gradient [113], [114]. The ratio of intracellular ice to water flux is heavily dependent on the rate of cooling. Rapidly cooled solutions will experience high intracellular ice whilst slow cooling will result in little or no intracellular ice.

Extracellular ice always forms before intracellular ice due to the apparent lack of ice nucleators within the cell [115] and the inability for ice nuclei to flow freely across the plasma membrane at temperatures above a critical point that depends on the cell type, which is typically in the range of -5°C to -10°C [116]. Most cells have the ability to remain supercooled, at temperatures above -5°C, as shown in Table 3. This table also shows that without external ice the cells do not freeze until much later, providing evidence that ice nucleators do pass through the membrane after a certain temperature initiating heterogeneous nucleation in the cell [116].

**Table 3 Degree of supercooling for different types of cells, organisms or tissues in the absence and presence of external ice.**

<table>
<thead>
<tr>
<th>Specimen (reference)</th>
<th>Degree of supercooling, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>External ice</td>
</tr>
<tr>
<td>Guinea pig testis [117]</td>
<td>-6 to -10</td>
</tr>
<tr>
<td>Vesicles of lemon [118]</td>
<td>&lt;-7</td>
</tr>
<tr>
<td>Insect larvae [119]</td>
<td>-10 to -15</td>
</tr>
<tr>
<td>Yeast [120]</td>
<td>-10</td>
</tr>
</tbody>
</table>
1.6.2.1 Intracellular Ice Formation

Intracellular ice formation prevents any cell activity but doesn't necessarily result in cell death, the most damaging effect comes from mechanical stresses on the membrane and cell organelles from expansion and contraction of freezing and thawing ice. Intracellular ice formation may lead to partial tears in the cell membrane which may lead to selective release of molecules of a certain size. The observed selectivity of membranes during freezing has been used to obtain crude recombinant protein purification through a simple freeze-thaw process [121].

Subsequent thawing of intracellular ice can lead to ice recrystallization in which large crystals appear leading to severe mechanical stress on the cell components [113].

Another result of intracellular ice damage comes from reactive oxygen species (ROS) that can damage the cell membrane, DNA and cellular proteins. The presence of ROS during freeze-thaw cycles is believed to come from the breakdown and subsequent release of electrons from the mitochondrial electron transport chain [122].

1.6.2.2 Extracellular Ice Formation

In the case of extracellular ice formation, the solution surrounding the cell will become more concentrated with solutes leading to an osmotic gradient causing water to flow from the cell. This will result in cell shrinkage which may lead to deleterious mechanical stress and eventual cell membrane collapse and lysing of the cell.

1.6.3 Ice Tolerance versus Hydrate Tolerance of Micro-organisms

In the case of the formation of ice, the water within the bacteria cell is able to freeze under certain conditions. However, in the case of hydrates, a guest would need to be present inside the cell for intracellular hydrate formation to be possible. The cell membrane of the bacteria would likely prevent the transfer of many guest species. If hydrates cannot form in the cell then on the formation of extracellular hydrates, water must leave the cell to balance the osmotic gradient formed from the effective solute concentration of the solution. This would mean that hydrate formation, regardless of formation rate, would always lead to water loss and eventual lysing of the cells through cell shrinkage. This is in contrast to ice formation, where rapid formation rates can lead to intracellular freezing with almost no cell volume change.
If we hypothesize that intracellular hydrate formation cannot occur then this would pose an intriguing phenomenon of being able to selectively crystallize the water in solution over the water present in the cell. Considering that freezing and thawing has been applied to microbiological processes such as a crude protein selection [121], hydrate formation may prove useful as another tool in these areas. For example, if one cell species in a mixture contains a hydrate guest whereas another cell species does not, then theoretically, either of the cells could be selectively separated or destroyed by controlling hydrate formation conditions.

Further experimentation and discussion on this effect is beyond the scope of this project, however, it would be an exciting hypothesis to prove as the implications would mean that, not only could potential new applications be discovered, but also the mechanisms of cell damage through ice formation could be validated by testing against a hydrate formation control.

1.6.4 Biofilms

Most natural occurring microbial communities are found attached to an interface, such as a rocks surface or on top of a liquid pool as part of community of various species intertwined in a network called a biofilm [123–126]. Biofilms were discovered in the early 1930's and gained focus in the 1980's due to numerous problematic occurrences in medicine and industry, such as biofilm formation on medical instruments, in human tissues, and associated with corrosion on marine vessels and pipelines. In the last decade, the focus has shifted to understanding biofilms on a molecular and genetic level. However, there are still many unknowns as to the specific molecular interaction in biofilm generation, especially as it is believed to be dependent on the microbial composition of the community in which there are infinite combinations [126].

Hydrate formation is greatly related to the amount of exposed liquid-(gas/liquid/substrate) interface, especially if the interface has a high concentration of the guest (See Section 1.3). Any mass transfer limiting barrier between these interfaces should, theoretically, delay nucleation time and inhibit growth. Biofilms consist of bacteria immobilized in a matrix of extracellular polymeric secretions (EPS) (50 – 80 % total biofilm mass), amongst other secreted matter, and, thus, could, theoretically, play a role as a hydrate inhibitor. The composition and structure of EPS varies depending on the microbial species, conditions and nutrient availability [127].

Although, the main component of a biofilm is water (up to 90 – 97 wt% water content [126], [127]), the mass transfer barrier could still be significant. For instance, it is believed that biofilms impact the mass
transfer of dissolved gas in solution, specifically the mass transfer of oxygen from the bulk liquid solution to the biofilm interface [128].

However, even if the biofilm could act as an interface barrier, in terms of industrial application, biofilms may not be feasible, not only because they have yet to be investigated for their hydrate inhibition but also because they have come as a hindrance to oil and gas pipelines rather than a solution. For example, to enhance recovery or pressure control, sea water is sometimes injected into reservoirs. Some anaerobic species in the sea water can produce corrosive by-products, such as hydrogen sulphide, and can survive as biofilms on the pipeline surface [129]. To prevent pitting and corrosion of the pipeline, nitrate ions (\(\text{NO}_3^-\)) are sometimes injected with the injected sea water to kill and prevent the formation of these biofilms [129].

Although the macroscopic study performed for this thesis is not a completely suitable method to determine the role of the biofilm on hydrates, it was not the initial focus of the investigation. Future work and recommendations have been given in Section 5.1.
1.7 Research Objectives

As discussed, the flow assurance industry is trending towards the use of the more economical LDHIs over the more conventional THIs (See Section 1.4.1). With the ever increasing demand of the oil and gas industry associated with an increase in subsea well heads, it now poses a great environmental responsibility for flow assurance engineers. This was highlighted by the recent BP Oil Spill disaster that could have been significantly mitigated if it weren't for the unscheduled formation of hydrates in a purge pipeline [51].

Microbial processes have been used for centuries in the production of essential food products and are used in many industries as a cheap and clean alternative. Some cold hardy microbial species use ice-associating proteins such as AFPs to control or prevent freeze-thaw damage. Some ice-associating agents, such as AFPs, have also shown hydrate-association characteristics. AFPs have hydrate inhibition strength comparable to PVP, a conventional KHI [66], [81–83], [104], however, to the authors knowledge, only one paper has been published on the matter of microbial hydrate inhibition [84].

The specific objective of this work is to determine the effect of Chryseobacterium sp. Strain C14, a known ice-associating bacteria, on hydrate formation, and compare this to control bacteria with no ice-associating properties. Whilst, the general objective of this thesis is to determine the potential of a new class of microbial hydrate inhibitors.

Microbial inhibition of THF hydrates has previously been studied in the presence of growth media, TSB [84]. Some of the results from previous work, especially from the bacteria, Chryseobacterium sp. strain C14, showed some promise, however, due to the toxicity and miscibility of THF in water, some questions over the suitability of the THF guest have been raised, as discussed in Section 1.6.1. Plus, the complex TSB mixture may have influenced hydrate formation and was not studied individually as a control. The goal of this work is to simplify the system, with the aim of specifically identifying a bacterial strain or certain molecular species from the bacteria that may interact with hydrate formation. To achieve this, a well understood technique for measuring hydrate formation was used with a simple guest hydrocarbon, pure methane, in a stirred autoclave (see Section 2). Methane was chosen as it
forms a simple type sI hydrate structure and is a good analogue to an industrial system, where the guest resides in a separate phase to water. A relatively low driving force of 750 kPa (subcooling of 2.34 K) was used (see Figure 5) so as to observe any weak inhibition of the solutions. Thus, any small inhibition could be explored and developed. The gas uptake technique allows both hydrate nucleation delay and growth inhibition to be observed.

Due to the time dependency of microbial solutions, it is of interest to measure the effect of hydrate formation on a microbial solution that has had hydrates formed and dissociated in prior experiments, these are labelled 'memory runs'. It should be noted that the memory effect in most other kinetic hydrate formation publications only concern the observation of faster hydrate nucleation compared to the fresh run due to either residual crystalline structures and/or localized guest concentration (see Section 1.3.1.1). However, because of the presence of bacteria in some of the solutions, the stage of the bacteria’s life cycle amongst other responses will likely play a key role in the hydrate formation of memory runs. How this effects hydrate formation is another important objective of this work, as reliability and consistency of hydrate inhibitors is, understandably, of great importance.

1.7.1 List of Experiments

The research goals of this work were primarily to determine the effect of a known ice-associating bacterial strain, on hydrate formation. To give the best chance of survival of the bacteria, and thus hydrate inhibition, *Chryseobacterium* sp. strain C14 (hereafter referred to as *Chryseobacterium*) was tested with growth media tryptic soy broth (TSB) at a chosen concentration of 0.5 wt%, which is also a typical KHI concentration. All other experiments could be considered as control to this experiment (ID 5.0) and a brief description of each set of runs is given below. Complete results of each run can be found in Appendix A and are discussed in Section 3.

Pure water experiments (ID 1.0 to 1.6) were required to validate the method and apparatus along with providing the benchmark to which every other experiment can be compared.

The growth media TSB at 0.5 wt% was tested without any bacteria (ID 2.0 to 2.2) to determine if this complex solution, of peptides, salts and carbohydrates, has an effect on hydrate formation.

*Chryseobacterium* was extracted from agar plates, as described in Section 2.1.3.4, to determine how the bacteria affects hydrate formation without growth media (ID 3.0 to 3.3).

*Escherichia coli* TG2, due to its role as a well researched, model prokaryote was tested to determine the effect of bacteria that aren't known for their ice associating proteins on hydrate formation. In
accordance with the *Chryseobacterium* experiments, *E. coli* was tested without and with growth media TSB at 0.5 wt% (ID 4.0 to 4.2 and ID 6.0, respectively).

Finally, in order to gauge the strength of any observed inhibition, a model KHI, PVP was used, firstly at equal concentration to the bacteria in TSB media solution of 0.5 wt% and secondly at 0.2 wt%. The lower concentration was chosen so as not to make the solution too viscous and it has been reported in other publications that an optimum PVP concentration for inhibition can be observed at lower concentrations than 0.5 wt%.
The inhibition apparatus is a semi-batch, stirred vessel used for forming gas hydrates, shown in Figure 7, and has been verified and used by other researchers in our laboratory [130], [131]. The hydrates form in the crystallizer (CR), shown in Figure 8, which is a 323 mL cylindrical vessel made of SS316 stainless steel with a liquid solution sample size of 140 mL. The crystallizer has two Plexiglas windows for viewing hydrate formation. A magnetic stirrer coupled to an external rotating magnetic shaft is used to agitate the solution and rotates at a set speed of 330 rpm with an uncertainty of 25 rpm. This stirring speed was chosen as it provided the most reliable mixing without the risk of decoupling from the stirrer shaft. A SS316 stainless steel detachable baffle is also used to reduce the solutions vortex and provide reliable mixing.

Figure 7 Flowsheet of the kinetic hydrate formation apparatus (adapted from [131]). With permission from Elsevier.
The reference vessel (RV) is maintained at a constant pressure of 2650 kPa and is used for the PID control system to measure the pressure difference between the crystallizer and the reference vessel. In this case the desired pressure in the CR is 3.5 MPa so the pressure difference (i.e. CR-RV) is set at 850 kPa (i.e. 3500-2650 = 850 kPa). When the pressure difference drops below 850 kPa the PID will open the control valve and allow gas from the supply vessel (SV) to enter the crystallizer thus maintaining the crystallizer at a constant set pressure of 3.5 MPa. The pressures are measured at an uncertainty of 25 kPa using Rosemount smart pressure transducers, model 3051 (Norpac controls, Vancouver, B.C.).

Ultra high purity (UHP) grade methane was obtained from Praxair Technology Inc. and used for all trials. Distilled and deionized water was used and autoclaved prior to use in the microbiological trials. PVP with an average molecular weight, of approximately 10 kDa was obtained from Sigma Aldrich.

Figure 8 (Left) Cross-section view of crystallizer showing the two Plexiglas windows on either side of the cylindrical vessel (Right) 3-pronged detachable baffle used to control mixing in the crystallizer. Reprinted with permission from (Ju Dong Lee et. al., 2005) [130]. Copyright (2005) American Chemical Society.
The crystallizer is submerged in a water bath that is temperature regulated by a chiller circulating cooled glycol. Due to environmental temperature fluctuations around the apparatus the temperature of the bath fluctuates by 0.1 °C, as shown in Figure 9 which shows the chiller being run for three days. The temperature of the liquid and gas phase in the crystallizer is measured with Omega Type T copper-constantan thermocouples Tolerance Class 1, with an error of 0.1 °C (Omega Engineering Inc.).

Figure 9 Temperature of a water solution in the crystallizer left for a period of 3 days showing natural fluctuations in the temperature of the environment and adjustments made by the chiller. The thermocouple used for this measurement is the same used for all the results shown in this thesis.
2.1 Gas Uptake

To analyse the strength of hydrate inhibitors, the delay in nucleation time and/or growth reduction needs to be measured. One method is to measure the consumption of hydrate forming substance (methane in this work) over time. This method has been widely used and verified in literature [1], [27], [82].

2.1.1 Calculating Moles Consumed

All experiments are run semi-batch at constant pressure (i.e. 3.5 MPa) with methane as the guest gas. The supply vessel provides the constant pressure in the crystallizer through the use of a PID control valve.

As hydrates form, gas is consumed into the crystalline phase and the supply vessel will thus undergo a pressure drop over time until the maximum amount of hydrates for the system has been reached. The supply vessel contains methane, a non-polar species, and is operating between 3.5 and 7.0 MPa at temperatures of 0 to 3 °C. At these conditions, the gas can be modelled with the equation of state below [132]:

\[ n = V_{SV} \left( \frac{P}{zRT} \right)_{SV,0} - V_{SV} \left( \frac{P}{zRT} \right)_{SV,t} - V_{CR} \left( \frac{P}{zRT} \right)_{CR,0} + V_{CR} \left( \frac{P}{zRT} \right)_{CR,t} \]

The pressure in the crystallizer remains constant in all of the experiments performed within this thesis, thus:

\[ n = V_{SV} \left( \frac{P}{zRT} \right)_{SV,0} - V_{SV} \left( \frac{P}{zRT} \right)_{SV,t} \]

The volume of the supply vessel, \( V_{SV} \), is 312cm³. The compressibility factor, \( Z \), can be calculated using the Pitzer correlation which assumes a linear relationship of the form:

\[ Z = Z^0 + \omega Z^1 \]

The acentric factor, \( \omega \), for methane is taken to be 0.011 [133] and \( Z^0 \) and \( Z^1 \), are given by:

\[ Z^0 = 1 + B^0 \frac{P_r}{T_r} \]

\[ Z^1 = B^1 \frac{P_r}{T_r} \]
The reduced conditions were simply calculated using the critical values for methane which are widely accepted and published as [133]:

\[ T_{c,C1} = 190.56 \text{ K} \text{ and } P_{c,C1} = 4590 \text{ KPa} \]

The reduced pressure was calculated using direct pressure readings from the supply vessel. Whereas the reduced temperature was assumed to remain constant at 273.8 K (±0.5 K).

The second virial coefficients are given as [132]:

\[ B_0 = 0.083 - \frac{0.422}{T_r^{1.6}} \text{ and } B_1 = 0.139 - \frac{0.172}{T_r^{4.2}} \]

These equations assume linearity of the compressibility factor, which is an accurate simplification at low to moderate pressures where the compressibility factor is almost linear. The equation for \( B_0 \) is used to calculate \( Z^0 \), (the largest component of \( Z \) ) and is given in Figure 11. The operating conditions between 3.5 MPa and 7.0 MPa at 267 K to 286 K has been shaded. At these conditions the correlation is within 2% of an accurate Benedict/Webb/Rubin (BWR) based equation of state correlation by Lee and Kesler [134].
Figure 10 Pitzer correlation curves predicting the second virial coefficient, $Z^0$, for reduced temperatures, $T_r$, 0.8 to 4.0 at low to moderate reduced pressures, $P_r$. The operating conditions highlighted include pressure ranging from 3.5 MPa to 7 MPa at temperatures ranging from 267 K to 286 K for pure methane gas.

2.1.2 Analysis

A typical gas uptake curve is shown in Figure 11. The moles of gas consumed, $G_{\text{CH}_4}$, is shown on the upper y-axis and the temperature of the solution, $T_L$, is shown on the lower y-axis against time in hours from when the conditions were set at time 0. Before hydrates nucleate, gas dissolves into the liquid phase as shown in region (a). Once hydrate nucleation occurs (at 69.8 hours in Figure 11), there is a sharp increase in temperature due to the exothermic nucleation process followed by a steady growth in the solution. Hydrate growth continues as long as the hydrate crystals are exposed to both free water and guest, the mixing in the vessel facilitates this. The highest concentration of guest and water is at the liquid-gas interface, once this has been blocked by a hydrate cap, the crystallization process becomes severely mass transfer limited until it eventually stops and plateaus as shown in part (c) of Figure 11.

The final hydrate growth, $N_T$, was calculated by taking the moles after the gas uptake curve had reached a plateau, including the moles of gas dissolved in solution before nucleation. This was
converted into a percentage of hydrate conversion by applying the stoichiometric ratio of 6 moles of water to 1 mole of methane, thus finding out how much water is needed for the specific moles of gas consumed and dividing it by the amount of moles of water available in the solution (~7.8 moles of water). The hydration number of 6 was chosen based on observed cage occupancies of methane hydrates [18–20].

To observe if any nucleation inhibitors affected the hydrate growth rate, $R_G$, the gradient of the gas uptake curve (after nucleation) was calculated by approximating a 1 hour section of the curve (20 minutes after the induction time) as linear. This section of the curve was chosen as it, generally, provided the most consistent and linear profile throughout each experiment set.

Figure 11 A typical gas uptake curve obtained from the kinetic hydrate inhibition apparatus (experiment ID 5.0M2, see Figure 30). Region (a) represents the amount of gas dissolution into the liquid phase, (b) consists of hydrate nucleation at 69.8 hours followed by steady hydrate growth until region (c) where mass transfer limitations from hydrate formation at the gas-liquid interfaces slows growth down until a plateau has been reached at 100 hours.
2.1.3  Experimental Method

2.1.3.1  General Sample Preparation
A 140 mL sample is prepared in a cylinder and flask. 2 mL of distilled water is added to the solution to accommodate for the volume of the liquid exit pipe from the CR. Initially the water bath is empty allowing the top of the CR vessel to be removed with a wrench. The baffle and magnetic stirrer is removed and cleaned initially with 70% aqueous ethanol using Kimwipe tissues. The CR is drained of any solution then sprayed and soaked with 70% ethanol for 15 to 30 minutes. 70% ethanol is used as it is a widely known disinfectant.

After ethanol disinfection, the CR is filled and drained numerous times with distilled water using a syringe to remove any excess ethanol and impurities. The baffle and magnetic stirrer are also rinsed several times with distilled water.

Finally, the CR, baffle and stirrer are cleaned and dried with a high pressure air hose. The sample is then poured into the CR with the stirrer and baffle reinstalled. The CR lid is then placed back on top of the vessel.

To ensure that the CR is free from gas impurities the CR is purged three times with methane at 2 to 3 MPa over a period of 30 minutes. Venting is done slowly enough to prevent temperature from dropping to below 0 °C, due to the Joules-Thomson effect, so as not to risk formation of ice nuclei.

After purging, the water bath is filled with cooled water and circulated with a rotating stirrer shaft. Insulation is then fastened around the entire water bath to minimize temperature fluctuations. The system is allowed to reach the equilibrium temperature which takes anywhere between 6 to 12 hours.

2.1.3.2  Experiment Start Up
The pressure of the reference vessel is checked and ensured to be at 2650 kPa. The supply vessel is then charged from a methane gas cylinder to a pressure between 5 MPa and 6 MPa.

When an experiment is ready to start, the CR is firstly charged with methane from the supply vessel using the manual gas feed needle valve (V5) to roughly 2.5 MPa (under hydrate phase boundary). The CR is given 15 minutes for the temperature to reach equilibrium. After which the CR is charged to 3.5 MPa and V5 is fully opened. Another 15 minutes is given to allow the pressure and temperature to settle in the CR. The experiment is then started by initiating the data logger and the magnetic stirrer at a speed of 330 rpm (±25 rpm).
The experiment is left to run until the gas consumption plateaus at which point the data logger and the stirrer are turned off and venting and decomposition of the hydrates can begin. The hydrate dissociation process usually takes over an hour of slow venting.

2.1.3.3 Memory Procedure

The hydrate “memory” effect is described in the introductory section of this thesis. It usually affects hydrate formation by reducing the nucleation time of the preceding runs. The phenomenon is still not fully understood but is heavily time dependent from when the last hydrate (or ice formed from Joules-Thomson cooling) completely dissociates. To ensure reproducibility, this time delay is kept constant at 4 hours from when the last hydrates and/or ice dissociates. This point is marked by temperature returning to equilibrium and observation through the Plexiglas window.

After the 4 hour waiting period, the experiment follows the same procedure as described in the experiment start up section above.

2.1.3.4 Microbial Specific

Microbial solutions were tested for inhibition with and without the growth media TSB. The bacteria without growth media are referred to as extracted bacteria.

The TSB growth media and the bacteria *Chryseobacterium sp Strain C14* and *Escherichia coli TG2* were kindly provided by Walker's group at Queen’s University. The *Chryseobacterium* was isolated previously through a freeze-thaw cycling of a soil obtained from the Rocky Mountains, Canada [7].

**Bacteria in Growth Media**

Solutions of 0.5 wt % TSB were made up in 200 mL sealed flasks of distilled water and autoclaved at 121 °C and 0.1 MPa for 20 minutes in a Sanyo MLS-3780 autoclave. The growth medium was then inoculated with a single colony forming unit from an agar plate under sterile conditions and within 3 days of the solution being autoclaved. The solution was then placed in an incubator at 27 °C for 5 to 7 days, after which the optical density was recorded, and the solution was transferred to a fridge at 4 °C to induce cold tolerance. The solution was left for 5 to 7 days before the optical density was recorded for a second time (the value given in the results) and then transferred into the CR (which had been sterilized with 70 % ethanol). The solution was left in the CR for 12 to 24 hours at the operating temperature of 273.8 K before the first run was started.
Extracted Bacteria

For each solution, three 10 mL autoclaved distilled water solutions were made up with single colony forming units to have a bacteria optical density from 0.5 to 1.5 at absorbance of 625 nm wavelength. These solutions were then mixed with 112 mL autoclaved distilled water and put into a fridge at 4 °C for 5 to 7 days. After which, the solution was transferred into the CR (which had been sterilized with 70 % ethanol). The solution was left in the CR for 12 to 24 hours at the operating temperature of 273.8 K before the first run was started.
3 RESULTS

3.1 Summary

All experiments were carried out in a stirred kinetic hydrate forming apparatus, using methane as the guest hydrate former at 3500 (±50) kPa at 273.8 (±0.2) K, as described in Section 2. The stirring rate was constant at 330 (±25) rpm, unless stated otherwise. To determine inhibition characteristics of the different solutions, both nucleation delay and growth profiles have been analysed. A summary of the average results obtained from each solution is given in Table 4. For results of each individual run, the reader is referred to Figures 22 through 32 and Table 7 in Appendix A. A unique ID is assigned to each solution, details of each solution including number and type of runs performed can be found in Table 6 of Appendix A.

Table 4 Kinetic hydrate formation results of all experiments in terms of average induction time, growth rate and total hydrates formed.

<table>
<thead>
<tr>
<th>ID</th>
<th>System</th>
<th>No. of Runs</th>
<th>Induction Time, t</th>
<th>Growth Rate, R&lt;br&gt;mol.min⁻¹</th>
<th>Total Hydrates Formed, N&lt;br&gt;mol</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 – 1.4</td>
<td>Water (High Agitation)</td>
<td>5</td>
<td>0.2</td>
<td>0.00060</td>
<td>0.173</td>
<td>13</td>
</tr>
<tr>
<td>1.5 – 1.6</td>
<td>Water</td>
<td>2</td>
<td>0.4</td>
<td>0.00027</td>
<td>0.119</td>
<td>9</td>
</tr>
<tr>
<td>2.0 – 2.2</td>
<td>0.5 wt% TSB aq.</td>
<td>3</td>
<td>7.1</td>
<td>0.00040</td>
<td>0.286</td>
<td>22</td>
</tr>
<tr>
<td>3.0 – 3.1</td>
<td>Extracted Chryseobac (Low)</td>
<td>2</td>
<td>0.5</td>
<td>0.00037</td>
<td>0.127</td>
<td>10</td>
</tr>
<tr>
<td>3.2 – 3.3</td>
<td>Extracted Chryseobac</td>
<td>2</td>
<td>3.1</td>
<td>0.00030</td>
<td>0.120</td>
<td>9</td>
</tr>
<tr>
<td>4.0</td>
<td>Extracted E. coli</td>
<td>1</td>
<td>2.3</td>
<td>0.00037</td>
<td>0.123</td>
<td>10</td>
</tr>
<tr>
<td>4.1</td>
<td>Extracted E. coli</td>
<td>1</td>
<td>9.3</td>
<td>0.00018</td>
<td>0.099</td>
<td>8</td>
</tr>
<tr>
<td>4.2</td>
<td>Extracted E. coli</td>
<td>1</td>
<td>3.8</td>
<td>0.00020</td>
<td>0.093</td>
<td>7</td>
</tr>
<tr>
<td>5.0</td>
<td>Chryseobac in 0.5 wt% TSB aq.</td>
<td>1</td>
<td>30.7</td>
<td>0.00072</td>
<td>0.274</td>
<td>21</td>
</tr>
<tr>
<td>6.0</td>
<td>E. coli in 0.5 wt% TSB aq.</td>
<td>1</td>
<td>118.5</td>
<td>0.00007</td>
<td>0.284</td>
<td>22</td>
</tr>
<tr>
<td>7.0 – 7.5</td>
<td>0.2/0.5 wt% PVP aq.</td>
<td>6</td>
<td>38.3</td>
<td>0.00015</td>
<td>0.407</td>
<td>32</td>
</tr>
</tbody>
</table>
Figure 12 Average induction times (columns) and individual runs (dots) of all experiment ID Sets: (1) Water; (2) TSB; (3) Extracted Chryseobacterium; (4) Extracted E. coli; (5) Chryseobacterium in 0.5 wt% TSB; (6) E. coli in 0.5 wt% TSB; (7) 0.2/0.5 wt% PVP (refer to Table 6 for details on each system). The sample size (n) of each set is shown and descriptive error bars are given as plus and minus one standard deviation from the mean.

The water systems (ID 1.0 – 1.6), as expected, observed the fastest nucleation time whilst the highest delay in nucleation, surprisingly, came from the control E. coli in 0.5 wt% TSB (ID 6.0) with an average induction time of 118.5 hours. This was almost 3 times longer than that observed by PVP at 0.2 and 0.5 wt% (ID 7.0 – 7.5) which showed an average induction time of 38.3 hours. All average induction times (fresh and memory runs) are ranked in Figure 12.

The average initial growth characteristics of all experiment sets are compared in Figure 13. The highest initial growth rate was observed by the Chryseobacterium in 0.5 wt% TSB solution (ID 5.0) with the lowest coming from the E. coli in 0.5 wt% TSB solution (ID 6.0). The highest hydrate conversion, shown in Figure 14, in term of moles of gas consumed, was observed by 0.2/0.5 wt% PVP (ID 7.0 –
7.5) whilst the pure water case (ID 1.5 – 1.6) shared the lowest observed final growth with the extracted bacteria experiments (ID 3.0 – 4.2).

Figure 13 Average initial growth rate (columns) and individual runs (dots) of all experiment ID Sets: (1) Water (high agitation is shaded); (2) TSB; (3) Extracted Chryseobacterium; (4) Extracted E. coli; (5) Chryseobacterium in 0.5wt% TSB; (6) E. coli in 0.5wt% TSB; (7) 0.2/0.5 wt% PVP (refer to Table 6 for details on each system). The sample size (n) of each set is shown and descriptive error bars are given as plus and minus one standard deviation from the mean.
Figure 14 Average final hydrate growth (columns) and individual runs (dots) of all experiment ID Sets: (1) Water (high agitation is shaded); (2) TSB; (3) Extracted Chryseobacterium; (4) Extracted E. coli; (5) Chryseobacterium in 0.5wt% TSB; (6) E. coli in 0.5 wt% TSB; (7) 0.2/0.5 wt% PVP (refer to Table 6 for details on each system). The sample size (n) of each set is shown and descriptive error bars are given as plus and minus one standard deviation from the mean.
3.2 Water and PVP Benchmarks

For a sample size of 15 runs, the clean water solutions without any inhibitors (ID 1.0 – 1.6) gave reliable and consistent induction times with averages of 0.2 and 0.4 hours for stirrer speeds of 380 and 330 rpm, respectively (Figure 22 and Figure 23). This observation confirms that higher agitated systems should nucleate faster than quiescent and less agitated systems. These results also validate the consistency of these simple systems which had small standard deviations of 0.04 and 0.05 hours, respectively, which were likely mostly effected by the stochasticity of hydrate nucleation and the errors of the measuring instruments. All of the pure water memory runs nucleated faster than their corresponding fresh run but the differences were very small at less than 5 minutes. Memory runs were started 4 hours after hydrate dissociation and, at this driving force, the memory affect is assumed negligible. Growth characteristics were also consistent with pure water at 330 rpm (ID 1.5 – 1.6) giving an average initial growth rate, $R_G$, of 0.00027 mol.min$^{-1}$ (standard deviation 0.00004 mol.min$^{-1}$) and a final hydrate conversion of 9% (standard deviation 1%).

As expected, the 0.2/0.5 wt% PVP sample (ID 7.0 – 7.5) also gave consistent induction times with an average induction time of 38.3 hours and a standard deviation of 8.83 hours. Note that due to the similarities in the induction times of PVP at 0.2 wt% compared to 0.5 wt%, the averages were taken over both concentrations. The PVP growth characteristics observed catastrophic growth, similar to other published experimental observations and simulations (see Section 1.4.1.2). Figure 16 shows the comparison of a 0.2 wt% PVP growth curve against a pure water curve and another growth promoter TSB (all other growth curves can be found in Appendix A and follow a similar trend).

The final amount of hydrate growth, in Figure 16, for PVP, is over 3 times more than the water case. It was observed, during the trials, that for the PVP solutions, hydrates remained in a slurry until the very end, whereas for the water case, hydrates would be in a slurry until a hydrate cap at the liquid-gas interface would form and grow slowly (see Figure 15). This observation coincides with the increase in hydrate growth for the PVP solutions as the crystallization growth doesn't become as mass transfer limited as for the case where a hydrate cap forms. This would also explain the larger linear section of the PVP growth curve and the slower plateau formation. This coincides with the mechanism proposed in a previous publication [70], where PVP is believed to change the morphology of hydrates, thus, lowering agglomeration rate and preventing hydrate cap growth.
Figure 15 Images of the crystallizer window after hydrates have formed and the growth curve has plateaued for (A) pure water case, producing hydrate cap, (B) 0.5 wt% TSB solution, producing hydrate cap with trapped bubble layer and (C) 0.2 wt% PVP, producing a thick hydrate slurry.

Figure 16 Hydrate growth curves comparing water, PVP and TSB cases. PVP and TSB are shown to undergo catastrophic growth after nucleation. Growth curves from experiments ID 1.6M3, 2.1F and 7.3M1 are used as examples, for all growth curves see Appendix A.
3.3 Growth Media TSB

The water and PVP samples could be considered the most reproducible systems out of all of the experiments due to the simplicity of the solution. However, if we now consider the more complex media solution of 0.5 wt% TSB without the presence of bacteria (ID 2.0 – 2.2), the induction times were far more scattered than the water and PVP cases, with an average of 7.1 hours at a standard deviation of 4.2 hours.

Looking at the typical composition of the growth media TSB in Table 5, the complexity is apparent with a composition of various peptides, salts and carbohydrates. From a flow assurance point of view, it is not surprising that the TSB mixture affects hydrate formation as many of these components, such as the peptides, will likely interact with water at a molecular level. For instance, both the peptide monomers, amino acids [99], and the longer peptide chains, proteins [83], have been shown to interact with hydrate formation.

The salt in TSB, that typically makes up 25% of the mixture, initially stands out as a potential source of the observed inhibition, as salts are a well known THI and could theoretically reduce the driving force and thus delay hydrate nucleation time. However, as only 0.5 wt% TSB solution was used, the effect of 0.125 wt% salt concentration will likely be too little for any THI interaction to be noticed. For instance, the effect of 0.5 wt% NaCl on methane hydrate equilibrium shifts the phase diagram by less than 50 kPa (Figure 17). Thus, it is unlikely that the salt, or any of the components, in the 0.5 wt% TSB solution is acting as a THI at this low concentration. Experiments at higher driving forces would help prove this.

Table 5 The major components of tryptic soy broth (TSB) growth media with the role of each component in terms of bacteria growth [135].

<table>
<thead>
<tr>
<th>Substance</th>
<th>Type (Role)</th>
<th>Weight Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic Digest of Casein</td>
<td>Peptides (Nitrogen source)</td>
<td>0.57</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>Salt (Osmotic balance)</td>
<td>0.17</td>
</tr>
<tr>
<td>Enzymatic Digest of Soy Meal</td>
<td>Peptides (Nitrogen source)</td>
<td>0.10</td>
</tr>
<tr>
<td>Dipotassium Phosphate</td>
<td>Salt (Buffer)</td>
<td>0.08</td>
</tr>
<tr>
<td>Dextrose (Glucose)</td>
<td>Carbohydrates (Energy source)</td>
<td>0.08</td>
</tr>
</tbody>
</table>
In consideration, if we assume that the delay in hydrate nucleation from the 0.5 wt% TSB solution occurs via KHI interaction, we can rule out the interaction of salt in the mixture, thus concluding that the various peptides in the solution from the enzymatic digest of casein and soy meal must be the major source of inhibition. Whilst the simple glucose sugar at a mere 0.04 wt% in the 0.5 wt% TSB solution may also play a small role in the KHI's strength.

The growth characteristics of the 0.5 wt% TSB solution also suggest that the inhibition acts kinetically, as the growth is increased compared to pure water cases, see Figure 16. If the TSB were a THI then the growth would be expected to be reduced due to the lower driving force. It was observed, that unlike the PVP, TSB did not remain in a slurry but formed a hydrate cap as was the case for the pure water experiments. However, for the hydrate cap formed in the TSB solution, what looked like trapped bubbles could be seen throughout the hydrate layer (Figure 15). This may have made the hydrate layer more porous allowing greater mass transfer of the guest to the solution compared to the pure water case. The reason for this observation is unknown to the author, however it may be related to the foaming properties of the TSB solution.

*Figure 17 Methane hydrate equilibrium curves with NaCl at 0.0, 0.5 and 5.0 wt% as calculated using CSMHYD [48]. Experimental operating temperature of 273.8 K has been highlighted for reference.*
3.4 Bacteria in TSB Growth Media Solutions

Addition of a single colony of *Chryseobacterium* or *E. coli* into the 0.5 wt% TSB solution and allowing to culture over a week in an incubator at 27 °C yields significantly stronger nucleation inhibition performance compared to just 0.5 wt% TSB, as shown in Figure 18. *Chryseobacterium* in 0.5 wt% gave an average induction time of 30.7 hours but varied significantly with a standard deviation of 29.1 hours. *E. coli* in 0.5 wt% TSB gave the longest observed induction time with an average of 118.5 hours with a large standard deviation of 59.4 hours.

Bacteria use the TSB growth media to convert the small molecules found in the solution, such as amino acids, peptides, carbohydrates and salts into large, complex, cell structures that contain predominantly macromolecular components, such as proteins, polysaccharides and lipids, all of which could affect hydrate formation. One possible reason for the increase in inhibition, when bacteria are cultured in TSB compared to pure TSB, is that the bacteria could be increasing the concentration of KHIs in the solution. However, the ability of *E. coli* to inhibit hydrates more than *Chryseobacterium* suggest AFPs are not the primary KHI source and that other molecular species are contributing a larger affect. Although identification of the KHI source is extremely difficult on a macroscopic scale, some possible sources of inhibition are discussed in Section 4. Overall hydrate growth of 0.274 and 0.284 mol for *Chryseobacterium* and *E. coli*, respectively, in 0.5 wt% TSB is very similar to the overall hydrate growth observed by that of clean 0.5 wt% TSB (0.286 mol). However, the growth profiles of the 3 different solutions were markedly different, as shown in Figure 19. For *E. coli* in 0.5 wt% TSB, there is a distinct 'S shaped' growth profile, where initially the rate is very low, at an average of 0.00007 mol.min⁻¹, nearly 4 times lower than the pure water case. It was observed that hydrate crystals grew, initially, above the interface and then later throughout the solution, which coincides with the gas uptake curve (morphology not shown).

The *Chryseobacterium* in 0.5 wt% TSB experiment shown in Figure 19 has a very high rate of growth (0.00080 mol) that quickly plateaus. However, this solution observed an interesting time dependence shown in Figure 20, where the gas uptake curve appears to get significantly more steep from the first fresh run (F) through to the last memory run (M5). The high rate of hydrate growth is also represented by the large temperature peaks shown in Figure 20, where the solution was raised by over 1 °C. No other solution gave noticeable solution temperature rises due to the relatively low driving force used. It
was observed, especially for the later memory runs, that hydrates grew non-uniformly and throughout the crystallizer.

Figure 18 Comparison of induction times for 0.5 wt% TSB (ID 2.0 – 2.2), Chryseobacterium in 0.5 wt% TSB, $A_{625\text{nm}} = 0.83$ (ID 5.0) and E. coli in 0.5 wt% TSB, $A_{625\text{nm}} = 0.31$ (ID 6.0). Individual runs are shown as green dots for fresh runs and as small black dots for memory runs, whilst the averages of each set are shown as shaded columns. Descriptive error bars are shown as plus and minus one standard deviation from the mean.
Figure 19 Hydrate growth curves comparing water, TSB, Chryseobacterium in TSB and E. coli in TSB cases. Growth curves from experiments ID 1.6M3, 2.1F, 5.0M2 and 6.0M2 are used as examples, for all growth curves see Appendix A.

Figure 20 Hydrate growth curves, including the solution temperature profile for Chryseobacterium in TSB cases (ID 6.0) for Fresh (F) and Memory runs (M1 – M5).
3.5 Extracted Bacteria Solutions

In an attempt to determine how much of an effect the TSB growth medium was having compared to the bacteria in the solution for experiments ID 5.0 and 6.0, colonies of bacteria were extracted from agar plates and made up to a 30 mL aqueous solution and mixed with 112 mL autoclaved distilled water (See Section 2.1.3.4 for details). It should be noted that, due to the difficulties of this method, concentrations as high as the bacteria in TSB growth media could not be met.

Induction times for the extracted Chryseobacterium and E. coli (experiments ID 3.2 – 4.2) are shown in Figure 21, and are seen to vary greatly. The relative concentration of each solution is in terms of optical density are given in Table 6 of Appendix A. Initially a lower concentration of 10 mL and 20 mL Chryseobacterium from agar plates in 132 mL and 122 mL distilled water, respectively, were tested and showed similar induction times to pure water cases and, for that reason, have not been included in the discussion but are shown in Figure 25 of Appendix A.

Extracted E. coli gave generally more consistent induction times over Chryseobacterium and showed the strongest inhibition with an average induction time of around 5 hours over the 3 solutions tested (See Figure 27 - 29 in Appendix A). Apart from experiment ID 3.3, all of the fresh and last memory runs gave low induction times, with a peak in induction time during the mid runs, suggesting an optimum inhibition (See Figure 21). None of the extracted bacteria experiments significantly altered the hydrate growth characteristics compared to clean water case (See Figure 13 and Figure 14). However, there is a weak trend that shows the extracted Chryseobacterium increases growth rate whilst extracted E. coli decreases growth rate. This is in conjunction with that observed by the two bacteria in growth media.
Figure 21 Comparison of induction times for extracted Chryseobacterium (ID 3.2 – 3.3) and extracted E. coli (ID 4.0 – 4.2). Each run of a solution is in order of length of time in the crystallizer, with the freshest case starting at the left (green dot) and running to the oldest case on the right (red dot) for each solution set. Individual runs are shown as black dots whilst the averages of each set are shown in light shade.
4 DISCUSSION

4.1 Microbial Response and Hydrate Inhibition

The bacteria in TSB (experiments ID 5.0 - 6.0) are exposed to a highly challenging environment. They are in a relatively low nutrient solution whilst being exposed to mechanical and osmotic stresses from hydrate formation. Thus when considering the extracted bacteria without growth media solutions (experiments ID 3.0 – 4.0), the bacteria are exposed to an even more harmful environment and will likely employ numerous survival responses to survive these conditions. The mechanisms which Chryseobacterium and E. coli use to adapt to these environments are highly complex, not fully understood and likely very different.

Based on previous morphology studies, the Chryseobacterium is expected to secrete an protein with IRI properties, possibly a AFP to survive ice stresses [8], and based on how the bacteria was isolated (via a freeze-thaw process) [7], it was expected that the bacteria would also have a relatively high tolerance to a hydrate forming system. Although it is not yet clear how bacteria tolerance is correlated with inhibition strength, a simplistic view would be to say that higher inhibition would be observed by those species that produce the highest concentration of AFPs, and although Chryseobacterium did mediate increased hydrate inhibition compared to TSB alone, the performance was not as impressive and less consistent than the inhibition seen in the E. coli experiments, suggesting that AFPs are not the primary source of inhibition in these solutions.

However, for the Chryseobacterium in 0.5 wt% TSB trial, the growth rate was greatly increased with large temperature peaks during hydration formation (Figure 20), a unique observation that wasn't seen in any other solution. Increased rate of crystal growth is characteristic of some adsorbing ice-associating proteins, albeit mainly ice-nucleating proteins. However, the Chryseobacterium is putatively believed to contain an AFP, and the actual molecular structure and properties of this ice-associating protein is unknown and may indeed have some promoting ability, as ISPs are generally not homologous between species [67]. Furthermore, the growth rate appeared to get progressively steeper from the initial to the final memory run (Figure 20). This could be due to the Chryseobacterium metabolising more ice-associating proteins after hydrates have formed and dissociated, in a response to the exposed stresses.
However, unlike the *Chryseobacterium* in growth media, the extracted *Chryseobacterium* solutions only weakly promoted growth rate (experiment ID set 3 in Figure 13). The reason for this is likely due to the low concentration of these extracted solutions compared to the *Chryseobacterium* in growth media case (absorbance of the solutions are given in Table 6). This theory would coincide with the weaker inhibition observed by the extracted *Chryseobacterium* shown in Figure 12. However, this observation may also be related to the absence of TSB and thus the inability of the bacteria to synthesize certain hydrate interfering molecules.

The extracted bacteria experiments in Figure 21 show large variations in induction times for each solution with some runs showing significant nucleation inhibition suggesting that the bacteria can inhibit hydrates without the presence of growth media. Considering the complex time dependant behaviour of bacterial solutions plus the unknown interaction of bacteria with hydrates, the observed variation in induction time of each solution is of little surprise. The dramatic variations suggest that there is significant change in the composition of the solution during each trial. These changes could come about, directly or indirectly, through changing concentrations of viable bacteria, providing evidence that hydrate inhibition is dependent on viable bacteria count. Further studies on a smaller scale could shed light on what aspects of the bacteria are giving the greatest hydrate inhibition.

Another interesting observation in these extracted bacteria cases is that there is a significant time dependency to the effectiveness of the microbial solution as an inhibitor, especially with the *E. coli* solutions which look as if they could fit a bell curve where a maximum inhibition would be met after a certain time, shown in Figure 21. If we assume that viable bacteria are correlated with inhibition strength then this observation may be due to some acclimatizing stage followed by a death phase of the bacteria. For *E. coli*, it has been shown that, in a starved state such as this, the bacteria can become even more resistant to environmental stresses such as heat shock and more pertinently to osmotic gradients and oxidative stresses [136], [137] that are apparent in freezing environments and which may also come from exposure to hydrate forming environments. After non-lethal exposure to such stresses, the bacteria can become even more resistant [138]. The response to these stresses can be the metabolism of various molecules, such as specific polypeptides [136] that may correlate with the observed hydrate induction times.

In an attempt to understand how bacteria, in general, could inhibit hydrates, some potential molecular sources of inhibition are summarized below.
4.1.1 Cell Membrane

One such component present in all cells is the phospholipid bilayer membrane. The membrane consists of a hydrophilic head/hydrophobic tail arrangement with a complex structure of specific functional proteins and other macromolecules embedded into the phospholipid wall. Due to the amphipathic nature of the lipids, the molecules are energetically favourable in the bilayer arrangement compared to individually in the bulk solution and form micelles. Mechanical stresses, such as ice formation, can tear the membrane. Any small tear will reform back into the favourable bilayer structure but large tears can result in the membrane collapsing and forming smaller lipid vesicles [111]. Tearing of the membrane can also selectively release certain molecules [121].

The interaction of the amphipathic membrane may be one of the reasons for the observed increase in hydrate inhibition of the bacteria solution compared to the growth media solution. However, the phospholipids are more in keeping with the hydrate promoting surfactants that are commonly micelle-forming agents [79]. Hydrate promotion was observed for bacteria in the presence of TSB, however, the final conversion was very similar to the pure TSB solution, making it difficult to determine if the bacteria showed additional growth promotion. If the cells were acting as a surfactant, then the extracted bacteria without growth media should also increase hydrate conversion; this was not observed (see Figure 14). It is unlikely that cell membranes promote hydrate growth since microorganisms form cells where water is included in the cell, which is the opposite to the SDS surfactant-mediated micelles where water is excluded. It is possible, however, that the concentration of extracted bacteria was too small so that the critical micellar concentration had not been reached. Another reason could be that the cells lysed very quickly in the absence of growth media.

Nevertheless, hydrate inhibition strength and/or hydrate promotion may be correlated with the number of intact cell membranes and thus related to the number of living cells and to the number of lysed cells (i.e. collapsed membranes). Recommendations for testing this hypothesis are given in Section 5.1.2.

4.1.2 Non-Ice Associating Polymers

One theory states that some macromolecules can delay the crystallization process by reducing the mass transfer rate of hydrate forming elements (i.e. guest molecules, water molecules and nascent cages) by simply getting in the way of hydrate forming sites [96]. Also, due to the differences in crystalline structure between ice and hydrates, non-ice association polymers may be hydrate-associating. There is
some published evidence that non-ice associating proteins can effect hydrate formation (see Section 1.5.2).

The growth media, TSB, delayed hydrate nucleation and enhanced hydrate growth more than predicted simply by the concentration of solutes, suggesting a KHI interaction. On analysis of the typical components of TSB, it is likely that peptides were acting as the KHI (as presented in Section 3.4).

Introducing a bacteria culture in this system will result in the metabolism of the TSB components, but the synthesis of longer chained proteins that likely have greater hydrate inhibition. The synthesis of other complex polysaccharides may also contribute to the hydrate inhibition, as it has been shown that starches and carbohydrates can also show some hydrate inhibition [97].

4.1.3 Biofilm Formation

Biofilms can form from a single species in a nutrient-rich environment at the gas-liquid interface. If the biofilm were effective at reducing the surface contact area between the guest and the water solution, then hydrate formation should be inhibited.

It was traditionally believed that, in an undernourished environment, biofilms do not form because survival of the bacteria requires them to detach and search for new nutrient sources, this is opposite to nutrient-rich environments where the bacteria are able to settle and form a biofilm [123], although certain E. coli strains show enhanced biofilm production in a relatively nutrient-poor environments representing both conditions used here. E. coli is a known single species biofilm former [125], [126]. If we hypothesize that the E. coli in the 0.5 wt% TSB solution is forming a biofilm then we would expect far greater inhibition than in the undernourished extracted bacteria solutions. Indeed, the E. coli in 0.5 wt% TSB showed the greatest inhibition, whilst being relatively consistent. The initial slow growth rate shown in the S curve growth profiles for the E. coli in 0.5 wt% solution could also be the reduction in mass transfer rate through the biofilm at the interface. Whereas, this growth profile was not observed in the extracted bacteria or pure TSB solutions. However, if the above were true, then the ability of the extracted E. coli to inhibit hydrate nucleation would mean that biofilms are not the only KHI source in the solution. Interestingly, there is no evidence of biofilm formation in Chryseobacterium (Wu and Walker, unpublished).
4.2 Effect of Conditions

After each run, hydrates are dissociated and the bacteria are exposed to perhaps their greatest stress of the experiment, through mechanical stress fluctuations, ice formation (from J-T effect of venting), osmotic fluctuations and recrystallization. Although much effort was put in to try and keep the rate of hydrate dissociation for each solution constant, the different additives and amount of hydrates meant that this was almost impossible. Thus, variations in rate, likely added to the inconsistency of the microbial solutions inhibition performance.

Another important factor to consider is the effect of pressure on the bacteria. But, because the pressure is relatively low at 3.5 MPa, the impact is likely negligible. For instance, high pressure processing (HPP) is used in the food industry to sterilize food products at pressures far higher than that used here [139–142]. For example, one HPP study showed that 200 MPa (at 20 °C) for a period of 90 days had no effect on an aqueous bacterial community found in sour Chinese cabbage [142]. It is also believed that proteins will not denaturing at pressures lower than 200 MPa [143], and thus, protein denaturation is unlikely to be an issue in these experiments.
5 CONCLUSIONS

It has been determined that the ice-associating bacteria, *Chryseobacterium sp. strain C14*, and a model, control, bacteria, *Escherichia coli TG2*, can both inhibit methane hydrate nucleation significantly in a stirred tank crystallizer, either with or without the presence of growth media. *E. coli* in 0.5 wt% TSB growth media showed the strongest inhibition with an average induction time of just over 3 times that observed by PVP, followed by *Chryseobacterium* in 0.5 wt% TSB with an average similar to PVP. However, all of the microbial systems gave less consistent hydrate inhibition compared to the simpler single component solutions. It is suggested that the inconsistencies come through changing concentrations of various KHIs in the solutions due to microbial response to the environment and concentration of viable bacteria. Thus, in the microbial systems, the inhibition strength of these solutions appeared as a complex time dependency of concentration of metabolised molecules by the bacteria. This was more apparent for the induction times observed by the extracted bacteria (without TSB), where, what appeared to be, an optimum inhibition was observed after a certain duration in the crystallizer (Figure 21). It is hypothesised that the reason for this is due to a microbial acclimatizing phase followed by a death phase.

Bacteria without the TSB growth medium also showed hydrate inhibition, albeit, far weaker than the bacteria with growth media (Figure 12). However, this deduction is made complicated by the fact that both TSB (without bacteria) and the bacteria (without TSB) both gave some hydrate inhibition. On top of this, the optical density of the extracted bacteria (without TSB) was different to the bacteria in growth media case, and because a relationship between bacteria concentration and hydrate inhibition is unknown, a concluding relationship cannot yet be made. However, the results are useful as it shows that bacteria can inhibit hydrates either with or without growth media.

The reason why the growth media TSB at 0.5 wt% (see Table 5 for composition) observed modest hydrate inhibition is unknown. However, due to the small amount of salt in the solution and the increase in overall hydrate conversion (Figure 16), it is suggested that the TSB medium as a whole is not acting as a THI, but that the complex peptide mixture is acting as a KHI.

The two microbial systems of *E. coli* and *Chryseobacterium* in TSB growth media gave markedly different hydrate growth profiles but gave similar overall hydrate conversion to that of TSB (without bacteria). *Chryseobacterium* in TSB increased the growth rate whilst *E. coli* in TSB initially retarded the growth rate before accelerating growth rate and plateauing, giving an S-shaped growth profile.
(Figure 19). The differences in growth profile may give indications as to how the two solutions are acting as hydrate inhibitors. Due to the steep growth profiles and large temperature rises observed for the Chryseobacterium in TSB solution (Figure 20), it is suggested that this, and the observed inhibition, may be the result of the putatively determined ice-associating molecules used by the organism that may be binding to hydrate nuclei surfaces. This hypothesis is based partially on the results of another publication that has shown that AFPs can bind to THF hydrates [68]. The growth profiles of the E. coli (in TSB) and Chryseobacterium (in TSB) solutions are significantly different, suggesting different molecular sources that are effecting hydrate formation.

However, the reasons for the observed hydrate inhibition by the, non-ice associating, bacteria E. coli is still a mystery. One possible explanation could be that the E. coli in growth media TSB formed a biofilm between the liquid-gas interface, reducing mass transfer of the guest to the bulk solution. The observed S-shaped growth profiles may be the result of the initial mass transfer limitation over the biofilm. Supporting evidence for this hypothesis is the observation that hydrates, in the E. coli in TSB solution, initially formed above the interface and later formed throughout the solution.

However, E. coli (without TSB) also inhibited hydrate nucleation and didn't give an S-shaped growth profile, suggesting that biofilm formation wasn't the source of inhibition in these experiments, especially as it is generally believed that biofilms do not form in extremely undernourished environments [123]. For these solutions it is suggested that non-ice associating macromolecules, such as that found in the TSB growth media, are responsible for the hydrate inhibition.

Thus, potential sources of inhibition for both species of bacteria are numerous and no real conclusions from these results can identify a single source, especially as the hydrate inhibition is likely due to the cumulative effect of multiple inhibitors. Potential sources of hydrate inhibition could be the cell membrane, secreted proteins or sugars such as trehalose. Further microscopic scale experiments would be needed to identify the actual source of inhibition. However, the major conclusion to be made is that two species of bacteria inhibited hydrates greater or in comparable strength to PVP. Considering the abundance of species of bacteria on Earth, it is remarkable that these two species, chosen for this research, gave such strong inhibition, and in all likelihood, a stronger hydrate inhibiting bacteria exists. Couple this with the relative ease of mass producing bacterial processes and the potential of a green technology, the future of microbial hydrate inhibitors looks promising. Further research will hopefully validate these results and identify sources of the microbial hydrate inhibition so that the solutions can be manipulated to improve the consistency of inhibition.
5.1 Future Work and Recommendations

5.1.1 Validation

This work has shown that both an ice associating and non-ice associating microbial species can inhibit the nucleation of hydrates. However, one of the biggest challenges with experiments on hydrates, is the replication between systems and thus, I believe, firstly, that validation of the presented results is the most important recommendation for future work.

Due to the far greater inhibition of the bacteria in growth media solutions, it is recommended to perform future experiments with the bacteria in a media solution, although extracting bacteria using a different method such as via centrifuge or dialysis may prove more effective than that employed in this thesis.

Examples of future validation work could be the variation of;

- **Conditions** (e.g. higher driving force)
- **Guests** forming hydrate structure sII or sH (e.g. ethane/propane/butane mixtures)
- **Apparatus** such as microscale analysis (e.g. morphology, DSC)
- **Bacteria** (e.g. *Pseudomonas putida*, *Pseudomonas borealis*, *Marinomonas primoryensi* [92])

5.1.2 Testing of Hypotheses

The reason for the observed hydrate inhibition in the microbial and, even in the growth media, TSB systems are unknown and the hypotheses given in the previous sections have yet to be tested. Being able to pinpoint the reason for the observed inhibition would allow several important questions regarding feasibility to be answered. For instance, the nucleation time of the microbial systems were inconsistent, a highly undesirable trait for a hydrate inhibitor. Identification of why this is happening could shed light on a solution to fix the problem. The recommended hypotheses and suggested methods to test are;

**Hypothesis 1:** Microbial hydrate inhibition is related to the cell membrane

- Test hydrate inhibition in systems that contain liposome vesicles that represent empty cells, as described in other cryobiological related research [112].
- Test hydrate inhibition of lysed cell membranes prepared via centrifuge.
Hypothesis 2: Microbial hydrate inhibition is related to viable cell count

- Test hydrate inhibition of stationary phase and compare to dead bacteria samples and/or lysed cells (e.g. use autoclave).

Hypothesis 3: Specific molecules secreted by *E. coli* and *Chryseobacterium* in response to environmental stresses are causing hydrate inhibition

- Expose bacteria to hydrates and/or ice for varying lengths of time and then observe hydrate inhibition.
- Use bacteria in stationary growth phase straight out of the incubator and test hydrate inhibition (i.e. test null hypothesis).
- Test a bacteria solution for hydrate inhibition using DSC, then remove sample and lyse bacteria. Separate major components using techniques such as centrifuge. Test separated component groups again in DSC, to see major contributors to hydrate inhibition. DSC is recommended as this test would need to be performed at the microscale level.

Hypothesis 4: Biofilms are contributing to hydrate inhibition

- Use bacteria that are well known for their biofilm formation and manipulate to form a biofilm. Test hydrate inhibition of solution with the bacteria before and after biofilm formation. A quiescent system may be necessary at first.

5.1.3 Alternative Work

5.1.3.1 The Effect of Hydrate Formation on Microbial Species

Most research relating hydrates to microbes are involved around relatively exotic species that abide within natural reserves of natural gas hydrates [144–146], such as *Bacillus subtilis*, which produces the powerful hydrate promoter surfactin [79]. Although there is proof that microorganisms can adapt and survive in hydrates, little is known about how microorganisms survive and adapt to hydrates crystallizing in their surrounding environment. However, there has been much interest in various organisms' survival and response to ice [147] and ice formation [111], [113], [115], [148–150].
Currently, it is believed that the rate of cooling below the freezing point of water determines whether the majority of ice forms inside of a cell or outside of a cell, both of which have unique consequences [111]. But, there are still many unknowns concerning this area.

Due to the similarities of hydrates to ice, one would generally expect a similar response from organisms exposed to hydrate forming systems and ice forming systems. However, the differences between the two crystal structures could yield some significant differences.

For instance, hydrates require a guest, and so whereas ice can form relatively easily inside the cell, hydrates would additionally require a guest within the cell. This would result in the absence of intracellular ice, regardless of cooling rate. The consequence of this would be, first most, that the current hypothesis of intracellular and extracellular ice formation could potentially be validated by observing changes in bacteria in the presence of forming ice, at different cooling rates, against a hydrate control. Furthermore, hydrate treatment on microorganisms could yield novel results that may have application in other industries such as cryopreservation or crude molecular purification, that are analogous to other ice forming systems [113], [121].
REFERENCES


APPENDIX A KINETIC HYDRATE EXPERIMENT RESULTS

Table 6 Summary of all 77 experiments in terms of experiment ID, solution and number of runs. The optical density is shown for microbial solutions.

<table>
<thead>
<tr>
<th>ID Set</th>
<th>Solution</th>
<th>No. of Runs</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fresh</td>
</tr>
<tr>
<td>1.0 – 1.4</td>
<td>142 mL water (high agitation: 380 ±25 rpm)</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>1.5 – 1.6</td>
<td>142 mL water</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>2.0 – 2.2</td>
<td>142 mL 0.5 wt% TSB aq.</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>3.0</td>
<td>132 mL water</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>10 mL extracted <em>Chryseobacterium</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$A_{625\text{nm}} = 0.71$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.1</td>
<td>122 mL water</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2x10 mL extracted <em>Chryseobacterium</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$A_{625\text{nm}} = 0.43, 0.87$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.2 – 3.3</td>
<td>112 mL water</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>3x10 mL extracted <em>Chryseobacterium</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(ID 3.2) $A_{625\text{nm}} = 0.92, 1.08, 1.09$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(ID 3.3) $A_{625\text{nm}} = 1.30, 1.27, 1.06$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.0 – 4.2</td>
<td>112 mL water</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>3x10 mL extracted <em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(ID 4.0) $A_{625\text{nm}} = 0.55, 1.08, 1.02$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(ID 4.1) $A_{625\text{nm}} = 1.16, 1.45, 0.78$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(ID 4.2) $A_{625\text{nm}} = 1.22, 0.74, 1.13$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>142 mL <em>Chryseobacterium</em> in 0.5 wt% TSB aq.</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>$A_{625\text{nm}} = 0.83$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>142 mL <em>E. coli</em> in 0.5 wt% TSB aq.</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>$A_{625\text{nm}} = 0.31$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.0 – 7.3</td>
<td>142 mL 0.2 wt% PVP aq.</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>7.4 – 7.5</td>
<td>142 mL 0.5 wt% PVP aq.</td>
<td>2</td>
<td>0</td>
</tr>
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</table>
A.1 ID: 1.0 – 1.4 Pure Water

Solution: 142 mL water
Conditions: 3500 kPa; 273.8 K; 380 rpm

Sample size = 9

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
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</tr>
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<tbody>
<tr>
<td>$t_i$</td>
<td>hrs</td>
<td>0.19</td>
</tr>
<tr>
<td>$R_G$</td>
<td>mol.min$^{-1}$</td>
<td>0.00060</td>
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<tr>
<td>$N_T$</td>
<td>mol</td>
<td>0.173</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>13</td>
</tr>
</tbody>
</table>

Figure 22 Kinetic hydrate formation results for pure water cases (ID 1.0 to 1.4). Showing the average, $\bar{x}$, and standard deviation, $s$, of the induction time, $t_i$, growth rate, $R_G$, and final hydrate growth, $N_T$ (top left), induction times of each run (top right) and hydrate growth profiles of each run (bottom).
A.2 ID: 1.5 – 1.6 Pure Water

Solution: 142 mL water
Conditions: 3500 kPa; 273.8 K; 330 rpm

Sample size = 6

<table>
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<tr>
<th></th>
<th>( \bar{x} )</th>
<th>( s )</th>
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<tbody>
<tr>
<td>( t_i ) hrs</td>
<td>0.36</td>
<td>0.05</td>
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<tr>
<td>( R_G ) mol.min(^{-1} )</td>
<td>0.00027</td>
<td>0.00004</td>
</tr>
<tr>
<td>( N_T ) mol</td>
<td>0.119</td>
<td>0.019</td>
</tr>
<tr>
<td>( % )</td>
<td>9</td>
<td>1</td>
</tr>
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</table>

Figure 23 Kinetic hydrate formation results for pure water cases (ID 1.5 to 1.6). Showing the average, \( \bar{x} \), and standard deviation, \( s \), of the induction time, \( t_i \), growth rate, \( R_G \), and final hydrate growth, \( N_T \) (top left), induction times of each run (top right) and hydrate growth profiles of each run (bottom).
A.3    ID: 2.0 – 2.2 Growth Media TSB

Solution: 142 mL 0.5 wt% TSB aq.
Conditions: 3500 kPa; 273.8 K; 330 rpm

<table>
<thead>
<tr>
<th></th>
<th>t_i</th>
<th>R_G</th>
<th>N_T</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hrs</td>
<td>mol.min(^{-1})</td>
<td>mol</td>
</tr>
<tr>
<td>x</td>
<td>7.1</td>
<td>0.00040</td>
<td>0.286 *</td>
</tr>
<tr>
<td>s</td>
<td>4.2</td>
<td>0.00006</td>
<td>0.010 *</td>
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</table>

* Sample size = 5, see Table 7

Figure 24 Kinetic hydrate formation results for growth media TSB cases (ID 2.0 to 2.2). Showing the average, \( \bar{x} \), and standard deviation, \( s \), of the induction time, \( t_i \), growth rate, \( R_G \), and final hydrate growth, \( N_T \) (top left), induction times of each run (top right) and hydrate growth profiles of each run (bottom).
A.4 ID: 3.0 – 3.1 Extracted Chryseobacterium in Water

Solution: 132 mL water, 10 mL extracted Chryseobacterium (ID 3.0)
122 mL water, 20 mL extracted Chryseobacterium (ID 3.1)

Conditions: 3500 kPa; 273.8 K; 330 rpm

<table>
<thead>
<tr>
<th>Sample size = 7</th>
<th>$\bar{x}$</th>
<th>$s$</th>
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<tbody>
<tr>
<td>$t_i$ hrs</td>
<td>0.5</td>
<td>0.4</td>
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<tr>
<td>$R_G$ mol.min$^{-1}$</td>
<td>0.00037</td>
<td>0.0002</td>
</tr>
<tr>
<td>$N_T$ mol</td>
<td>0.127*</td>
<td>0.009*</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>10*</td>
</tr>
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</table>

* Sample size = 6, see Table 7

Figure 25 Kinetic hydrate formation results for extracted Chryseobacterium in water cases (ID 3.0 to 3.1). Showing the average, $\bar{x}$, and standard deviation, $s$, of the induction time, $t_i$, growth rate, $R_G$, and final hydrate growth, $N_T$ (top left), induction times of each run (top right) and hydrate growth profiles of each run (bottom).
ID: 3.2 – 3.3 Extracted Chryseobacterium in Water

Solution: 112 mL water, 30 mL extracted Chryseobacterium (ID 3.2)
112 mL water, 30 mL extracted Chryseobacterium (ID 3.3)

Conditions: 3500 kPa; 273.8 K; 330 rpm

<table>
<thead>
<tr>
<th>Sample size = 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_i$ (hrs)</td>
</tr>
<tr>
<td>R_G (mol.min$^{-1}$)</td>
</tr>
<tr>
<td>N_T (mol)</td>
</tr>
<tr>
<td>%</td>
</tr>
</tbody>
</table>

Figure 26 Kinetic hydrate formation results for extracted Chryseobacterium in water cases (ID 3.2 to 3.3). Showing the average, $\bar{x}$, and standard deviation, $s$, of the induction time, $t_i$, growth rate, R_G, and final hydrate growth, N_T (top left), induction times of each run (top right) and hydrate growth profiles of each run (bottom).
A.6 ID: 4.0 Extracted E. coli in Water

Solution: 112 mL water, 30 mL extracted E. coli

Conditions: 3500 kPa; 273.8 K; 330 rpm

Sample size = 6

<p>| | | |</p>
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<thead>
<tr>
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<tbody>
<tr>
<td>$t_i$</td>
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</tr>
<tr>
<td>$R_G$</td>
<td>mol min$^{-1}$</td>
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</tr>
<tr>
<td>$N_T$</td>
<td>mol</td>
<td>0.123</td>
</tr>
<tr>
<td></td>
<td>%</td>
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Figure 27 Kinetic hydrate formation results for extracted E. coli in water cases (ID 4.0). Showing the average, $\bar{x}$, and standard deviation, $s$, of the induction time, $t_i$, growth rate, $R_G$, and final hydrate growth, $N_T$ (top left), induction times of each run (top right) and hydrate growth profiles of each run (bottom).
A.7  ID: 4.1 Extracted E. coli in Water

Solution: 112 mL water, 30 mL extracted E. coli

Conditions: 3500 kPa; 273.8 K; 330 rpm

Sample size = 10

<table>
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<tr>
<th></th>
<th>$\bar{x}$</th>
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<tbody>
<tr>
<td>$t_i$</td>
<td>hrs</td>
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<td>$R_G$</td>
<td>mol.min$^{-1}$</td>
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</tr>
<tr>
<td>$N_T$</td>
<td>mol</td>
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</tr>
<tr>
<td></td>
<td>%</td>
<td>8$^*$</td>
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$^*$Sample size = 7, see Table 7

Figure 28 Kinetic hydrate formation results for extracted E. coli in water cases (ID 4.1). Showing the average, $\bar{x}$, and standard deviation, $s$, of the induction time, $t_i$, growth rate, $R_G$, and final hydrate growth, $N_T$ (top left), induction times of each run (top right) and hydrate growth profiles of each run (bottom).
A.8  ID: 4.2 Extracted E. coli in Water

Solution: 112 mL water, 30 mL extracted E. coli

Conditions: 3500 kPa; 273.8 K; 330 rpm

Sample size = 7

<table>
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<th></th>
<th>x</th>
<th>s</th>
</tr>
</thead>
<tbody>
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<td>( t_i ) hrs</td>
<td>3.8</td>
<td>4.5</td>
</tr>
<tr>
<td>( R_G ) mol.min(^{-1} )</td>
<td>0.00020</td>
<td>0.00002</td>
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<tr>
<td>( N_T ) mol</td>
<td>0.093</td>
<td>0.009</td>
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<td>( N_T ) %</td>
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</tbody>
</table>

Figure 29 Kinetic hydrate formation results for extracted E. coli in water cases (ID 4.2). Showing the average, \( \bar{x} \), and standard deviation, \( s \), of the induction time, \( t_i \), growth rate, \( R_G \), and final hydrate growth, \( N_T \) (top left), induction times of each run (top right) and hydrate growth profiles of each run (bottom).
A.9    ID: 5.0 Chryseobacterium in Growth Media TSB

Solution: 142 mL Chryseobacterium in 0.5 wt% TSB aq.

Conditions: 3500 kPa; 273.8 K; 330 rpm

Sample size = 6

<table>
<thead>
<tr>
<th></th>
<th>$\bar{x}$</th>
<th>s</th>
</tr>
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<td>$t_i$</td>
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<tr>
<td>$R_G$</td>
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<tr>
<td>$N_T$</td>
<td>mol</td>
<td>0.274</td>
</tr>
<tr>
<td></td>
<td>%</td>
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</tr>
</tbody>
</table>

Figure 30 Kinetic hydrate formation results for Chryseobacterium in growth media TSB cases (ID 5.0).
Showing the average, $\bar{x}$, and standard deviation, $s$, of the induction time, $t_i$, growth rate, $R_G$, and final hydrate growth, $N_T$ (top left), induction times of each run (top right) and hydrate growth profiles of each run (bottom).
A.10  ID: 6.0 E. coli in Growth Media TSB

Solution: 142 mL E. coli in 0.5 wt% TSB aq.
Conditions: 3500 kPa; 273.8 K; 330 rpm

<table>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>$t_i$ hrs</td>
</tr>
<tr>
<td>$R_G$ mol.min$^{-1}$</td>
</tr>
<tr>
<td>$N_T$ mol</td>
</tr>
<tr>
<td>%</td>
</tr>
</tbody>
</table>

Figure 31 Kinetic hydrate formation results for E. coli in growth media TSB cases (ID 6.0). Showing the average, $\bar{x}$, and standard deviation, $s$, of the induction time, $t_i$, growth rate, $R_G$, and final hydrate growth, $N_T$ (top left), induction times of each run (top right) and hydrate growth profiles of each run (bottom).
A.11  ID: 7.0 – 7.5 Polyvinylpyrrolidone (PVP)

Solution: 142 mL 0.2 wt% PVP aq. (ID 7.0 – 7.3)
142 mL 0.5 wt% PVP aq. (ID 7.4 – 7.5)

Conditions: 3500 kPa; 273.8 K; 330 rpm

Sample size = 8

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<th>s</th>
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* Sample size = 4, see Table 7

Figure 32 Kinetic hydrate formation results for polyvinylpyrrolidone (PVP) cases (ID 7.0 to 7.5). Showing the average, $\bar{x}$, and standard deviation, s, of the induction time, $t_i$, growth rate, $R_G$, and final hydrate growth, $N_T$ (top left), induction times of each run (top right) and hydrate growth profiles of each run (bottom).
## A.12 Individual Run Results

*Table 7 Complete list of all 77 experiments showing corresponding solution state (fresh or memory), nucleation time, $t_i$, growth rate, $R_G$, and final hydrate growth, $N_t$.*

<table>
<thead>
<tr>
<th>I.D.</th>
<th>State</th>
<th>Date yymmdd</th>
<th>$t_i$ hours</th>
<th>$R_G$ mol.min$^{-1}$</th>
<th>$N_t$ mol %</th>
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<tbody>
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<td>1.0*</td>
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<td>0.00061</td>
<td>0.171</td>
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</table>

*Higher stirrer speed of 380 ±25 rpm.

†Supply vessel depleted before gas uptake curve reached plateau. The final amount of hydrate growth could not be determined.

‡Stirrer failed some time after hydrate nucleation. The final amount of hydrate growth could not be determined.
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<tr>
<th>I.D.</th>
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<th>(R_G) mol.min(^{-1})</th>
<th>(N_i) mol</th>
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</table>

*Stirrer failed some time after hydrate nucleation. The final amount of hydrate growth could not be determined.

†Stirrer failed at an unknown time during the experiment. The experiment was abandoned.
<table>
<thead>
<tr>
<th>I.D.</th>
<th>State</th>
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<th>( R_G ) mol.min(^{-1})</th>
<th>( N_i ) mol</th>
<th>%</th>
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</table>

Due to the large amount of growth in these experiments, the SV was depleted before the growth curves plateaued. Increasing the SV was not an option as the control valve could not hold higher pressure drops.