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# Microbial stabilisation and kinetic enhancement of marine methane hydrates in both deionised- and sea-water

Mohammad Reza Ghaani [a], Jonathan M. Young [b, 2], Prithwish K. Nandi [a, 1], Shamsudeen Dandare [b], Christopher C.R. Allen [b, **], Niall J. English [a, *]

[a] School of Chemical and Bioprocess Engineering, University College Dublin, Belfield, Dublin 4, Ireland
[b] School of Biological Sciences, Queen's University Belfast, University Road, Belfast, BT7 1NN, Northern Ireland, UK

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ABSTRACT

The large quantity of marine methane hydrates has driven substantial interest in methane-gas-fuel potential [4-5], especially with the qualified success of Shensu (2017) and Nankai-Trough (2014 & 17) production trials via depressurisation (blighted ultimately by sanding out) [4], building on an earlier Malik-2008 trial for permafrost-bound hydrate. In particular, obviating deep-water-drilling approaches, such as the MeBO production rig (without such a drill bit) [5], together with blowout preventers [4], constitutes a tantalising cost-saving measure. Tailored means of addressing sand production by customised gravel packs, wellbore screens and slotted liners with from-seafloor drilling will be expected to lead to future production-trial success [4]. However, despite these exciting engineering advances and a few marine-mimicking laboratory studies of methane-hydrate kinetics and stabilisation from microbial perspectives [6,7], relatively little is known about the thermogenic or microbial origin of marine hydrates (Lanoil et al., 2001 Nov) [8], nor their possible formation kinetics or potential stabilisation by microbial sources as an exponent of Gaia's hypothesis, or within the context of "Gaia's breath" as regards global methane 'exhalations' [2]. Here, for the first time, we elucidate the methylotrophic-microbial basis for kinetic enhancement and stabilisation of marine-hydrate formation in both deionised- and sea-water, identifying the key protein at play, which has some similarity to porins in other methylotrophic communities. In so doing, we suggest such phenomena in marine hydrates as evidential of Gaia's hypothesis.



## 1. Introduction

Clathrate hydrates are non-stoichiometric crystalline inclusion compounds in which a water host lattice encages small guest atoms or molecules in cavities [1]. Methane hydrates are the most widespread clathrate in Nature in the permafrost and relatively shallow continental-shelf ocean regions, and constitute a significant energy resource [2–4]. The large quantity of marine methane hydrates has driven substantial interest in methane-gas-fuel potential [4,5], especially with the qualified success of Shensu (2017) and Nankai-Trough (2014 & 17) production trials via depressurisation (blighted ultimately by sanding out) [4], building on earlier Malik 2008 trial for permafrost-bound hydrate. In particular, obviating deep-water-drilling approaches, such as the MeBO production rig (without such a drill bit) [5], together with blowout preventers [4], constitutes a tantalising cost-saving measure. Tailored means of addressing sand production by customised gravel packs, wellbore screens and slotted liners with from-seafloor drilling will be expected to lead to future production-trial success [4]. However, despite these exciting engineering advances and a few marine-mimicking laboratory studies of methane-hydrate kinetics and stabilisation from

* Corresponding author.
** Corresponding author.
*E-mail addresses:* c.allen@qub.ac.uk (C.C.R. Allen), niall.english@ucd.ie (N.J. English).

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[1] Present address: Irish Centre for High-End Computing, Trinity Enterprise Tower, Pearse St., Dublin 2, Ireland.
[2] Present address: Almac Group, Seagoe Industrial Estate, Craigavon, BT63 5QD, Northern Ireland.

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microbial perspectives [6,7], relatively little is known about the thermogenic or microbial origin of marine hydrates [8], nor their possible formation kinetics or potential stabilisation by microbial sources as an exponent of Gaia's hypothesis, or within the context of "Gaia's breath" as regards global methane 'exhalations' [2].

In various further laboratory experiments on marine-(mimicking)-hydrate formation, beyond refs. 6 & 7, a largely muddled picture on kinetics has emerged, from bacterial inhibition [9], to promotion via biosurfactants and related compounds (*e.g.*, surfactin) [10], although very few studies relate to sea water or temperatures/pressures relating to seabed conditions, with marine sand/sediment. However, independently of the hydrate field, there is a deal of literature concerning the release of surfactant-like molecules (including surfactin) by marine bacteria and even by marine methylotrophs, and this raises the question of whether cultures of marine methylotrophs can enhance hydrate formation under conditions truly reflective of continental-shelf seafloor milieux, and, if so, the underlying mechanism(s) thereof [10].

Bearing this tantalising open question in mind, in ref. 10, we used deionised water to grow a methylotrophic culture, and isolate and clone a gene encoding a protein, which was then expressed and the protein the purified. In its purified form, we found that it caused a kinetic enhancement of methane hydrates – which we dubbed as 'GHP1'. We also used metagenomics analysis to characterise the pure protein, as well as its early-stage formation kinetics [10]. However, despite finding convincing experimental evidence of kinetic enhancement of methane-hydrate formation, it is not yet clear for such a protein - whether isolated or in its biological milieux, or, indeed, whether cultivated in sea- or deionised-water environments – has some degree of thermodynamic enhancement, and what the role of seawater *per se* might be in this process. Given these intriguing open questions, the present work embraces the prospect of applying metagenome analysis to shed light on the full gamut of mixed-culture system-behaviour and complexities, including the putative role of auxiliary molecules like ectoine, which may serve to enhance the effects of surfactants as they interact with water molecules, and unravelling the mechanistic contribution of proteins and peptides (possibly with rhamnolipid- or surfactin-like compounds) towards facilitating and regulating hydrate formation – and, crucially, stabilisation, cultivated with natural seawater. Indeed, disentangling the cocktail of underlying bio-mechanisms remain elusive; here, tackling these open questions serves as our potent motivation.

## 2. Methodology

### 2.1. Culture preparation

We grew a methylotroph mixed culture, to ascertain if (and how) its extracellular elements affects methane-hydrate formation and stabilisation at seafloor conditions. We used fresh seawater with Growth-A medium [11], to establish six 300 ml cultures, three of which had no additional carbon source (codified as 'C' 1, 2 & 3), and three of which had 0.3% v/v methanol added after Janiver et al. (dubbed 'T' 1, 2 & 3) [12]; incubation time was 18 days at 22 °C, and further details of preparation are directly below. T1 was visibly much cloudier than the T2 and T3, meaning higher levels of microbial concentration.

We used fresh seawater (82.5% v/v inoculum), obtained the evening before from Belfast Lough (Hollywood, Co Down, Northern Ireland) and stored overnight at 4 °C added to a medium comprising (g/L):

NaCl: 2.4, (NH4)2SO4: 1.0, MgSo4.7H2: 1.0, CaCl2: 0.2, FeSO4: 0.002, Na2MoO4.2H2O: 0.002, KH2PO4: 0.36, K2HPO4: 2.34, with 1 ml of Vishniacs' trace-elements solution.

The final added phosphate concentration was approximately $2.34/174 = 13$ mM. There was noted to be considerable phosphate precipitation after the final medium was made up. Seawater contains circa 1 M salt, so this does not affect osmolarity significantly. The phosphate was autoclaved separately.

Methanol has a density of 7.92 g/10 ml and a molecular weight of 32 g/mol; thus, this corresponds to a molarity of $(0.9 \times 1000/300 \times 0.792)/32 = 74$ mM.

The pH was measured as 6.9. Incidentally, Neufield et al. used an incubation temperature of 19 °C [13], whilst Janvier et al. used 30 °C [12].

### 2.2. Hydrate formation and mass-balance-based determination of hydrate-conversion yield

These cultures and seawater were then placed in a temperature-controlled hydrate-formation pressure vessel at 3.5 °C, and allowed to form methane hydrate (featuring chemically- and heat-treated marine sand, to neutralise any background effects), exposed to methane gas at 120 bar; further details are in 'Supplementary Information'.

The experimental apparatus for hydrate-formation and dissociation kinetics (as well as estimation of dissociation temperature) employed a pressure vessel fabricated using 316 stainless steel with internal volume of approximately 340 $cm^3$ (cf. Figs. S1 and S2). The vessel was agitated using a tilting shaker. A pressure transducer with an uncertainty of 0.2 MPa, was used to measure pressure, whilst a thermocouple with an accuracy of ±0.1 K was inserted into the cell to measure the inner temperature, with temperature/pressure readings every 2 s. Prior to each run, any possible remaining bacteria in the vessel was sterilised by washing with ethanol solution (25 wt%). Each hydrate-kinetics experiment began with charging the equilibrium cell with approximately 20 $cm^3$ of seawater, deionised water, or cultured bacterial solution. The main system was cooled to the desired temperature of 3.5 °C (to mimic seafloor temperatures), via the temperature-control system (cf. Supp. Info.). Once the system the desired temperature, the cell was evacuated for 3 min by vacuum pump to remove any residual air, and then pressurised to the desired pressure of 120 bar (again, reflecting continental-shelf seafloor conditions) using pure methane. Due to inevitable Joule-Thomson thermal contraction, the cell pressure was decreased slightly by decreasing the temperature; however, within less than 20 min, the temperature stabilised and remained so until the end of the chose hydrate-formation period. Then, a continuous slow pressure decline was observed during the hydrate crystal-growth stage (always under the constant-volume conditions), after nucleation. In practice, however, there is some small temperature fluctuation during hydrate formation (*i.e.*, the thermal trace of hydrate formation) due to its exothermic nature, but this is countered continually by the temperature-control system. Here, the average temperature of the production régime (*i.e.*, the temperature plateau) is considered as the starting temperature of the reaction. The system was kept at (or very near) the desired temperature of 3.5 °C for 24 h to gauge the yield towards hydrate over this period, and then the temperature was increased with continuous 0.5 K $h^{-1}$ rate up to 295 K to study hydrate-dissociation kinetics, and determine the dissociation temperature accurately at the ~100 bar level (as well as a second independent estimate of the yield by judging methane release). The reader is referred to Supp. Info. for further discussion of the apparatus.

The 'yield' for conversion to hydrate during formation (or, conversely, inferred when measuring dissociation) is calculated based on the number of absorbed moles of gas into the liquid/solid phase (*i.e.*, by monitoring gas-phase pressure drop continuously on a mass-balance basis), whilst the number of released moles of gas

may be measured directly during hydrate dissociation from the rise in system pressure. Naturally, the first step in this number-of-gas-phase-moles-from-pressure determination lies in defining accurate the *de facto* compressibility factor of the methane in the system, measured on our own system (tailored specifically by its slight temperature gradients and thermal inertia, in terms of the readily measurable temperature and pressure data), although we do of course make use of 'reference' literature values as a function of temperature and pressure. For this purpose, in five separate runs, the chamber was loaded with methane gas (and nothing else, after thorough cleaning of the type described above) at ~80–130 bar (spanning the entire gamut, and more, of recorded gas-phase pressure during hydrate formation/dissociation cycles) for up to five different pressure values. While all the inlet/outlet valve were closed, the reactor was heated up slowly from 3 to 5 °C whilst pressure and temperature were recording by computer, and these data for the five points were selected to calculate the compressibility-factor value, $z$. Due to no completely and utterly definitive measurements of the number of loaded moles into the system, the $z$-value of one $P/T$ pair was taken from Ref. [15] as the correct one: the exact number of gas moles in the chamber can be extracted using this hypothesis, via $PV = znRT$. Given that this number of moles, $n$, is the same for all the other $P/T$ points, the $z$ value of those other four points can be therefore inferred via the same equation over the five independent runs, and the mean and variance taken. These sets of lowest-variance points with three coordinates (temperature, pressure, and $z$-value) have been plotted as a 3D plot while the equation of a fitted surface to these points was obtained using a rational Taylor model [16], as

$$z = \frac{z_0 + A_{01}x + B_{01}y + B_{02}y^2 + C_{02}xy}{1 + A_1x + B_1y + A_2x^2 + B_2y^2 + C_2xy}$$

- (cf. Fig. S3 and Supp. Info.). This surface-fit equation was employed later to calculate the compressibility factor of methane at various pressures and temperatures in the hydrate-formation and dissociation runs from mass balances on thus-inferred gas-phase-number-of-moles data (from the gas-phase pressure), taking into account the temperature-variation of methane absorption in liquid with literature data for Henry's Law constants for methane [17]. Knowing the number of absorbed/released moles of methane, in addition to typical methane-hydrate cage-occupancy levels of 90% in the present P/T range [1], allows for the percentage yield to methane hydrate to be calculated.

### 2.3. Genomic analysis

Preliminary analysis was performed by aligning the sequences against the Refseq (complete) bacterial-, archaeal- and viral-genome database [10]. Statistically-significant differences in taxon abundances between sample groups at the class- and genus-taxonomic ranks were evident (cf. Fig. 2 and Fig. S8). This suggests that the methanol-enriched samples have a much higher proportion of Gammaproteobacteria - specifically Methylophaga (or something relatively close to it); indeed, this is not particularly surprising, given its methylotrophic nature [10]. About 30% of the treatment group's and about 50% of the control group's DNA sequences could not be classified using this method, but substantial improvement would result when aligned against a larger database, so as to elucidate functional-gene abundances. See Fig. 3 for the novel protein's sequence, which is discussed further in ref. 10, including mass spectrometry, cloning and purification.

## 3. Results and discussion

18 runs were performed under a variety of conditions to assess systematically various putative effects (cf. Table S1), primarily; (i) reproducibility and (ii) ageing, with a further suite of a half-dozen runs to gauge (iii) the effect of methanol levels, given its known effect as a thermodynamic inhibitor [1]. Reproducibility was in general good, performed over three independent runs (cf. Table S4 for deionised-water results, as an example). Examining the age of the culture on kinetics of yield (cf. Table 1 and Fig. 1), the most obvious effect is a strong promotion in yield for T1 and acceleration of hydrate-formation conditions relative to both seawater conditions and the reference-nutrient sample C1 (over double the seawater effect). However, it becomes clear that there is an important age effect: for T1, an "expiration effect" starts within a couple of weeks (akin to an "incubation" for declining promotion): the yield has decreased significantly after 23 + 12 days. Comparing the aged sampled with deionised water (DI), T1 ageing acts as powerful inhibitor, where it reduces the yield of hydrate formation even lower than pure DI. Naturally, deionised water lacks the salt and biological entities that serve to promote homogeneous nucleation even further in marine-sandy milieux. Indeed, this is consistent with the working hypothesis of protein origin of T1's dramatic, time-dependent 'bifurcated' effect (*vide infra*). For C1, on the right, ageing by 16 days aging also reduces the effect of the nutrition sample on hydrate formation, *i.e.*, it acts as an inhibitor. However, the dramatic promotion seen for T1 hydrate conversion vis-à-vis seawater is not as dramatic for C1, nor, by contrast, is the subsequent inhibition for aged samples. In the case of seawater, there is a weak ageing effect, with just a 4% decline in yield over ~3 weeks, and still at a higher level than deionised water. Similar very mild ageing effects were seen with further runs in the case of Dublin Bay seawater.

Turning to dissociation temperature, as a proxy for thermodynamic stabilisation, to examine potential methylotroph-culture effect thereon, Fig. S7 evinces an apparent trend of a slight increase (up to perhaps ~1 °C) at ~11–12 °C when less-aged T1 is

**Table 1**
Age-of-sample-effect. Yield estimate of hydrate produced during formation, along with dissociation-based estimate. 20 $cm^3$ of water was used, with 1 g of chemically-/heat-treated sea sand. The hydrate yield is estimated for both formation and dissociation. Intermediate storage of all samples was at 4 °C in a refrigerator. 'BSW' refers to 'Belfast Lough Seawater', whilst 'DI' denotes deionised water. The dissociation-based yield is typically higher, due potentially to release of some methane bubbles from solution upon heating (which developed during hydrate formation; cf. Supp. Info., and especially Fig. S6).

| Run no. | Process | Age (day) | Water type | Additive | | | |
|---|---|---|---|---|---|---|---|
| | | | | | Temp (°C) | Initial Pressure (bar) | Yield (%) |
| M22 | Form. | 1 | BSW | T1 | 2.8 | 119 | 26% |
| M22 | Dissoc. | 1 | BSW | T1 | 3.0 | 117.5 | 37% |
| M05 | Form. | 1 + 12 | BSW | T1 | 3.3 | 108 | 38% |
| M05 | Dissoc. | 1 + 12 | BSW | T1 | 3.4 | 104 | 42% |
| M12 | Form. | 23 + 12 | BSW | T1 | 3 | 104.7 | 8% |
| BSW | Dissoc. | 3 + 12 | BSW | – | 3.3 | 114.4 | 18% |
| DI | Form. | – | DI | – | 3.2 | 105.4 | 11% |
| Di | Dissoc. | – | DI | – | 3.3 | 104.5 | 12% |

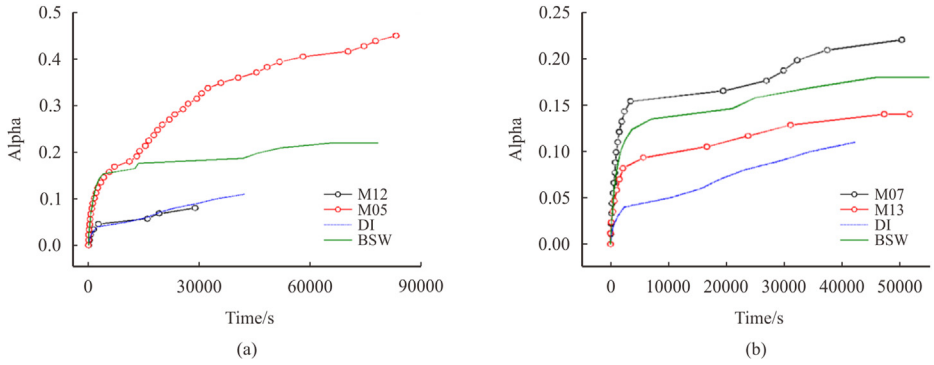


**Fig. 1.** Hydrate-formation yield versus time, showing age-of-sample effect for T1 on the left, and for C1-based conversion on the right. It is evident that, for T1 on the left, an "expiration reaction" starts after couple of weeks (akin to an incubation time), but the yield has decreased significantly after 23 + 12 days. Comparing the aged sampled with deionised water, T1 ageing acts as powerful inhibitor, where it reduces the yield of hydrate formation even lower than pure deionised water. This is consistent with a protein origin of this effect (see main text). For C1, on the right, ageing by 16 days aging also reduces the effect of the nutrition sample on hydrate formation, *i.e.*, it acts as an inhibitor. However, the dramatic promotion seen for T1 hydrate conversion vis-à-vis seawater is not as dramatic for C1, nor, by contrast, is the subsequent inhibition for aged samples.

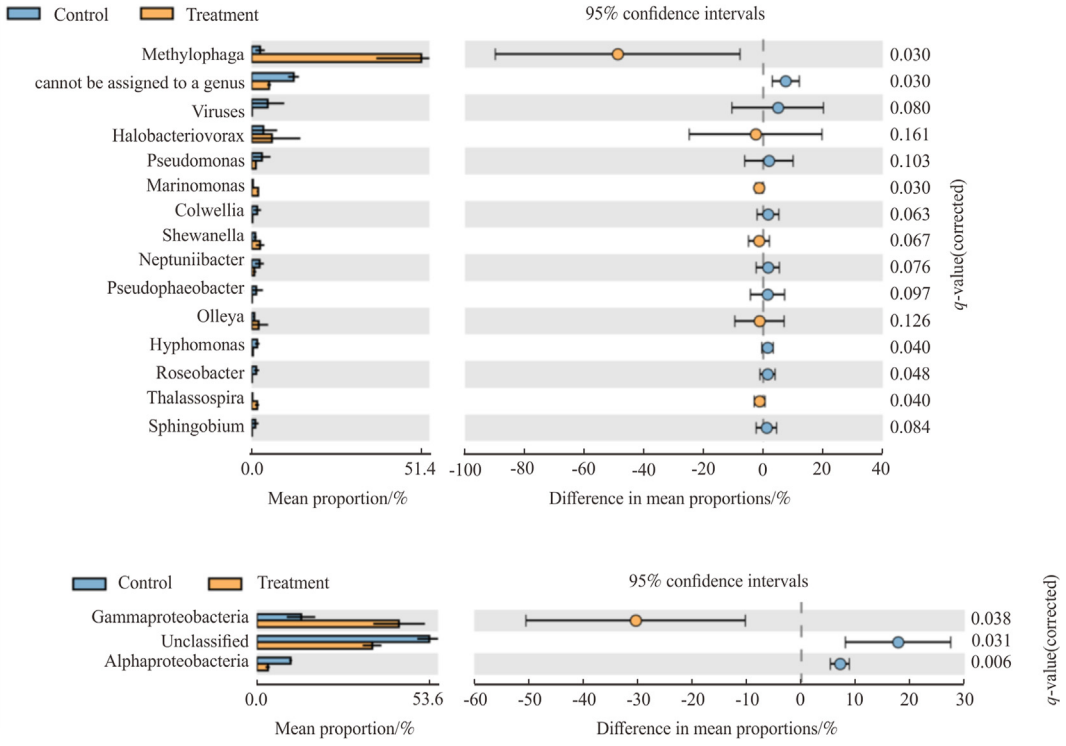


**Fig. 2.** Synopsis of statistically-significant differences in taxon abundances between sample groups of genus- (top) and class- (bottom) level taxonomic ranks. See Fig. S8 for further details.

1.MKLLKKSTLA TLVGAAALTA AGAANATIVV GGENGYEFSV DGNINQFFIA
51.SDQDSASASA NRDQDNQQVA NGLLPTFFGF NVAMPEINGL KVAARVSISP
101.STNNGSYVNS DAAMEQREAF ATVDGSFGQI MLGKGLGLYS ANNILLDQTL
151.YGVGATGLTA AGDQNTGQTS LGRIGYGYEY ANWRSQIRYT TPDMNGFKAA
201.VAVMDSDDFL TAKVVNSSTT SNQFEKDARY EASLSYATAF DGGSMKLWLD
251.GMTQDVRYSN ATGSEKSDAY TVGGQLVFGG IEAVAAYYDS KGQGVNGLGT
301.GAFTADNKAR EGDGYYAQLG YRFGGQTFVA ASYGKSTLDR DSANTVAGGV
351.ADLDTNSMTT IGIYHDVTAN LKLVAEYSKI ETEYHIQSSD DEVDVLSLGG
401.FISW

**Fig. 3.** The sequence of the novel protein underpinning the marked effects of the T1 culture. Protein-sequence coverage of 60% to methylophaga aminisulphidivorans, with matched peptides rendered in bold. Further details are provided in Supp. Info. and ref. 10.

used. This is in stark contrast with more aged T1 or deionised water, with DI water showing no age effect. This suggests that the T1 microbial culture confers greater thermal and thermodynamic stabilisation of marine-like methane hydrates vis-à-vis those produced synthetically in environments bereft of methylotrophs.

Despite measurements of essentially zero methanol (or, at least undetectable above the 1 mM level in the 'T' cultures or the 'C' nutrient case) prior to hydrate formation, we performed some tests on assessing the ultimate hydrate yield with DI water with the same treated marine sand (in the identical sand-to-water ratio). At 40 and 80 mM (roughly half and similar to the 74 mM used to set up the 'T' microbial culture, respectively, cf. 'Methodology'), the yield at 40 mM did not alter from ~6 ± 2% for pure-DI water, whilst that at 80 mM increased modestly to ~7.5%, although given the estimated yield's uncertainty of ~2% from multiple measurements (cf. Supp. Info.), the 80 mM-versus zero-methanol difference does not pass a 90% two-tailed Student's t-test for $H_1$, meaning that it is less probable that methanol concentration has a decisive effect on hydrate-conversion yield in the range studied. In any event, the lack of measured methanol means that this potential variable can be eliminated in rationalising both age-dependent and dramatic effects of the T1 culture at accelerating hydrate-formation kinetics, conversion yield and stabilising hydrate-dissociation condition to higher temperature: whilst young (less than a few weeks), hydrate promotion with respect to DI is found, with a decline in hydrate-formation propensity and stabilisation once more aged (acting as a *de facto* inhibitor).

Naturally, T1's intriguing time-dependent promotion/inhibition 'dichotomy' is suggestive of potential peptide-based activity underpinning this clear and substantial microbial effect. To elucidate the precise biological origin, we performed Refseq against standard bacterial-, archaeal- and viral-genome database [10]. Statistically-significant differences in taxon abundances between sample groups at the class- and genus-taxonomic ranks were evident (cf. Fig. 2). This suggests that the methanol-enriched samples have a much higher proportion of Gammaproteobacteria - specifically generally redolent of Methylophaga; indeed, this is not particularly surprising, given its methylotroph nature. In any event, we sequenced this earlier in ref. 10, and it is shown in Fig. 3, with 60% overlap to Methylophaga aminisulphidivorans, not unlike the general porin class.

## 4. Conclusions

We have established *prima facie* evidence of microbial enhancement and stabilisation of marine hydrates, mediated by a peptide not unlike methylotrophic porins [10]; ref. 10 did not discuss seawater and non-purified proteins, nor comment to any extent on measurements suggesting the possibility of thermodynamic stabilisation. Not only does this advance more direct evidence of Gaia's hypothesis [10,18], but, more speculatively, could signal the rôle of similar peptides in some of the earliest biotic processes in Earth's primordial history [19,20], as pre-biotic peptides formed at deep-sea vents were sequestered by early methylotrophs [14].

## Declaration of competing interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.petlm.2021.10.010.

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