Preliminary investigation into the influence of bacteria in marine sediments

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Introduction:

Recent investigations of offshore oil and gas sites off the coast of West Africa, with water depths ranging from 500m to greater than 2000m, have encountered clays with anomalously high undrained shear strengths at shallow sediment depths, as shown in Figure 1. These 'high strength crusts' are of great interest to the designers of deep-sea oil pipelines because they influence the depth of self-weight embedment that occurs when pipelines are laid on the seafloor. Of equal interest, however, is the sharp decline of shear strength with depth, returning to strengths generally associated with normally consolidated sediments by about 1 metre below sea floor (mbsf). This rapid reduction of strength may suggest a level of vulnerability of the crust, though to what extent is uncertain.

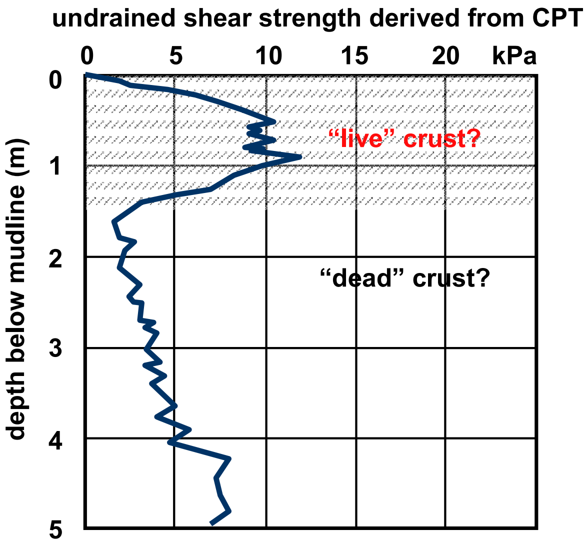
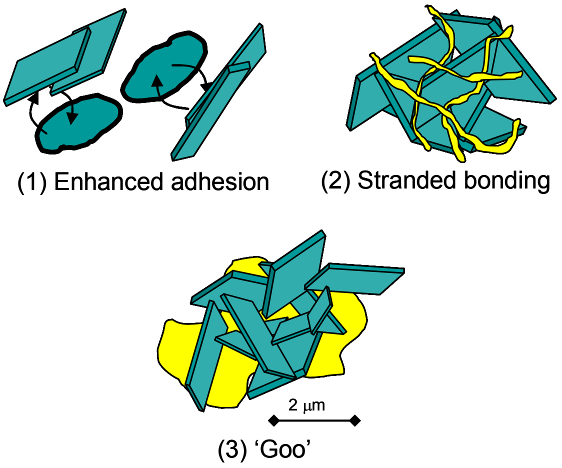


Fig. 1: *Undrained shear strength profile determined from CPT showing a 'crust' between 0.5 and 1mbsf, (modified after Ehlers et al. [1]).*

The origins of these crusts, and why they apparently exist only within the top few metres of sediment, is currently unknown. This phenomenon is, however, not restricted to the West African deep sea clays (Hooper [2]). Similar crusts have been observed world-wide, so an understanding of their origins and their possible alteration by pipeline installation and operation is important for deep sea pipeline designers.

This research hypothesises that bacteria and/or burrowing invertebrates (see Figure 3b) may be involved in the formation of the crusts observed in these sediments. However, only the former is discussed here. Mechanisms proposed for bacterial enhancement of sediment strength are shown in Figure 2, and described as follows: 1) enhanced adhesion by ion exchange between bacteria and clay platelets, 2) stranded bonding of clay platelets; and 3) reinforcement with 'goo' consisting of bacterial polysaccharides.



(1) Enhanced adhesion

(2) Stranded bonding

(3) 'Goo'

Fig. 2: *Proposed mechanisms for bacterially-enhanced soil*

The direct influence of bacteria on soil properties is currently little understood, and their presence has largely been ignored in the geotechnical testing of sediments for pipeline design. Pipelines are laid on and within the upper few decimetres of the seabed and, therefore, into sediment potentially containing high numbers of bacteria. The sea bed is initially at a temperature of 4°C at these extreme water depths. Oil may emerge from wells into the pipelines at over 160°C, however. We therefore propose to investigate the possible influence of bacteria on such sediments, and the possible short and long-term effects of a hot pipeline placed in contact with them.

Literature Review:

It has been shown that bacterial activity in marine sediments rivals that in terrestrial environments. Parkes et al. [3] review existing data on bacteria in marine sediments and show that the highest populations of bacteria occur within the top metre of sediment. The excretion of extracellular polymeric substances (EPS) by bacteria has been considered by Bhaskar and Bhosle [4] who suggest that EPS may be found in the following forms: biofilms, microbial mats (slime-like), discrete particles (capsular) and free dissolved matter. The presence of EPS has been associated with 'assisted sediment binding' in shallow marine and tidal environments.

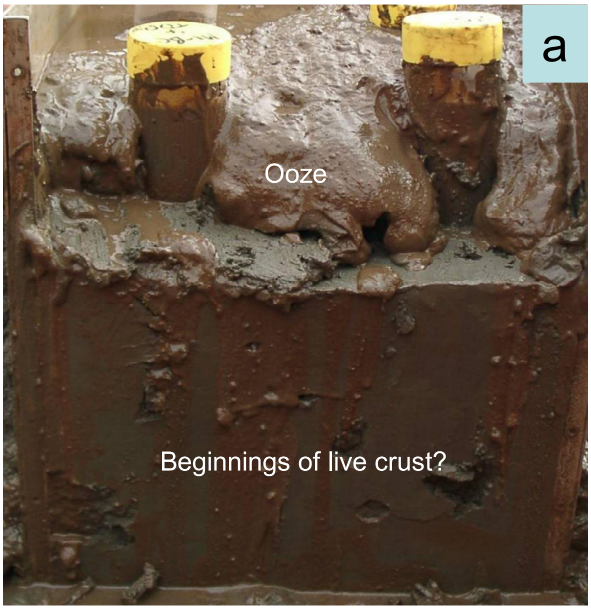
Bennett et al. [5] hypothesised the possible aggregation of clay through polymer bridges. Turley et al. [6] later showed that polymer bridges form between bacteria and clay platelets during their fall through the water column, thereby possibly enhance aggregation of clay at the seabed.

Deflaun and Mayer [7] investigated the development of bacterial polysaccharides on and within intertidal sediments. They concluded that the process of clay platelet bonding was initiated with the growth of filaments followed by fibrous webbing and finally continuous films. Clay particles were trapped or embedded by these films.

Dade et al. [8] showed that the presence of bacteria (*A. atlantica*) can enhance the viscosity and yield stress of kaolin clay at very high water contents through exudation of polysaccharides. It was observed that the level of enhancement was smaller in sandy sediments, probably owing to the significantly larger surface area of clays per unit volume of polysaccharide.

Materials and Methods:

The current research focuses on soft clays from the coast of West Africa (WA clays) in water depths of about 1400m (see Figure 3).



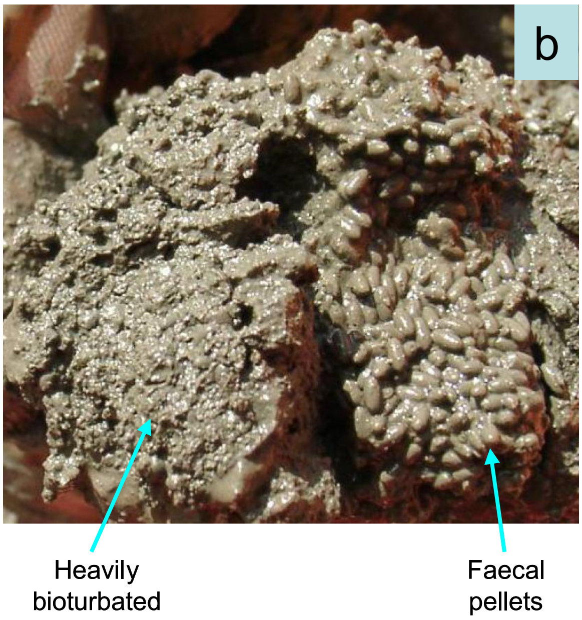


Fig. 3: (a) *Typical WA clay boxcore demonstrating ooze overlying firmer 'crust' material, and (b) evidence for burrowing invertebrate activity*[1]

The mechanical properties of these sediments are presented by several authors (Puech et al. [9], Thomas et al. [10] and [1]) and they are summarised here. The sediments encountered represent a wide range of depositional environments. Clay water contents generally range from 150% to 250% (see Figure 4), with very high plasticity, typically between 70% and 120%, but increasing to 150% near the seabed [9]. Carbonate contents in the form of crushed shells less than 30μm, generally range between 5% and 15% of the dry soil weight. [10] suggest that the organic content of these sediments may range between 2% and 6%.

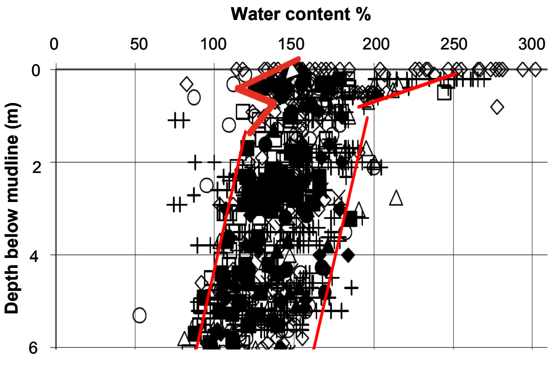


Fig. 4: *Water content plotted against depth with approximate upper and lower bound values (modified after Nauroy [11])*

To investigate the influence on mechanical soil properties of specific culturable bacteria present in WA clay, two commercially available DNA extraction kits were used; QIAamp Stool Mini Kit (QIAamp) and ZR Soil Microbe DNA Kit^{TM} (ZR).

Four samples of reconstituted WA clay were prepared following the procedures recommended in the kit. As only minimal amounts of DNA can be obtained using the extraction kits, the recovered DNA is 'amplified' using a polymerase chain reaction (PCR). The PCR process theoretically amplifies the existing DNA by a factor of over 10^9 (Howe [12]) using a simple, direct enzymatic process. The process involves heating and cooling cycles of a reaction mix containing the original DNA. This allows the melting and annealing of DNA strands to generate a complementary strand of each existing DNA strand. The process of repeated temperature cycling allows the PCR to be largely automated [12].

PCR reaction mixes were made up in aseptic conditions in a Perspex set-up box with a built-in UV irradiation light to suppress environmental contamination. DNA-extraction, environmental and water samples were included in the PCR amplification process to provide negative controls, and to check that the DNA extraction process and PCR set-up had captured the 'correct' DNA. Four general 16S RNA primers (two forward, two reverse) for prokaryote DNA were used in the PCR reaction mix. General 16S primers (see Table 1) were utilised to maximise the numbers of bacterial DNA amplified. A drawback of this, however, is the increased likelihood of also amplifying contaminants.

Table 1: *Primers used for targeting prokaryote DNA*

Name	Primer sequence
16S P1f	GTGCCAGCAGCCGCGGTAATAC
16S P2r	TCTACGCATTTCACCGCTACAC
16S P3f	CTTGTACACACCGCCCGTCACACCATC
16S P3r	TACCTTGTTACGACTTCACCCCA

PCR reaction mixes were analysed by gel electrophoresis to provide a visible check on any presence of contamination, and to check that the PCR had worked by the primers annealing to the DNA. The distance that a given PCR product travels through the gel is inversely proportional to its molecular weight. Inspection of the gels under UV light shows bands corresponding to each PCR product, which can then be compared with a given DNA ladder of known sizes to determine the length of the product (in base pairs). A typical result for WA clay after DNA extraction and the PCR process is shown in Figure 5.

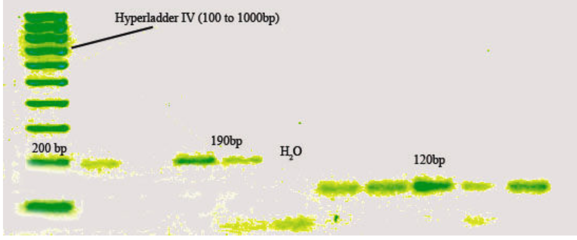


Fig. 5: *Contamination-free gel showing clear bands of 120 and 190 base pairs for P1+P2 and P3 and P4 primers, respectively*

To identify the amplified PCR products, DNA cloning was used to separate the different PCR products and obtain sequences from each. Cloning is required when general primers are used to differentiate between multiple amplified DNA with different sequences. If more specific primers are used, direct-sequencing may be used without the need for cloning.

Thirty four cloned DNA plasmids were recovered using a Qiagen Miniprep kit and sent to a commercial sequencing company. All but four returned positive results. Twenty samples returned an uncultured bacterial sequence. The inherent difficulty in amplifying and sequencing bacterial DNA from deep marine sediments is the likelihood of encountering new, uncultured species. These can not be purchased from a culture collection, and therefore can not be grown in the laboratory. Of the remaining ten sequences, three suggested the presence of *Marinobacter sp* (see Figure 6), which provided a viable option for laboratory experiments based on its growth characteristics and occurrence in marine waters.

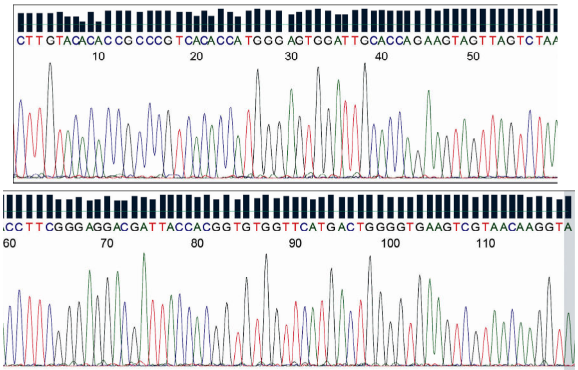


Fig. 6: *A section of the sequencing result for* M. aquaeolei

Marinobacter aquaeolei sp was purchased as a freeze-dried sample from the NCIMB and revived following the supplied procedures. *M. aquaeolei* is a moderately halophilic and mesophilic bacteria, and was isolated from the "head of an oil-producing well on the offshore oil/gas platform" (Huu et al. [13], p367) off the coast of Vietnam. The bacterium is rod-shaped, 0.4 to 0.5μm in width and 1.4 to 1.6μm in length. [13] observed growth between 13 and 50°C, and at pH between 5 and 10. Optimal growth occurred at 30°C and at a pH of 7.3.

Bio-Geotechnical Experiments:

To facilitate the testing of mechanical properties, including the undrained shear strength of sediment with and without the addition of *M. aquaeolei*, new laboratory testing equipment was designed. Experiments involving biological influences require adequate controls measure to ensure safe working environments and allow validation of test results.

The proposed experimental programme comprises a series of ball penetrometer tests in three sterile sub-samples of WA clay. Autoclaving is the most common method of soil sterilisation involving moist heat (Wolf and Skipper [14]). Samples are prepared in Perspex boxes, with dimensions of 300x150x250mm (LxWxD). Two sterile samples are inoculated with a liquid culture of *M. aquaeolei* previously grown at 30°C for 48 hours. The third sterile sample is not inoculated and serves as a control. All three sample boxes are placed in a specially designed 'clean-box' (Figure 7) with UV irradiation light, and allowed to consolidate under self-weight.

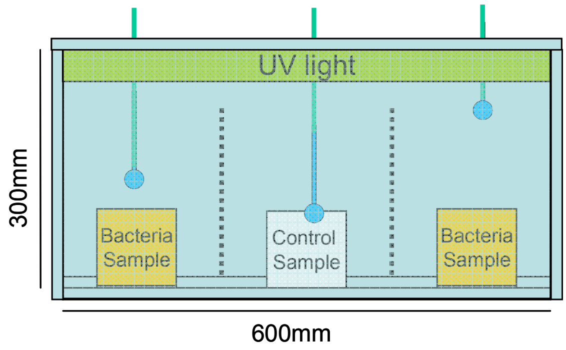


Fig. 7: *Proposed clean-box for testing of bacterial samples with control*

The clean-box allows the testing environment to be controlled whilst a ball penetrometer is used to measure the comparative increases in undrained shear strength (s_u) over time for both sterile and inoculated samples. Boundary effects will influence the test results; however, this series of experiments are concerned with comparative, rather than absolute, values of s_u.

The s_u values of the laboratory-prepared samples are expected to be between 1 and 10kPa, and sample sizes are relatively small. This therefore requires the design of a new mini-ball penetrometer capable of resolving very small undrained shear strengths. The new ball penetrometer is made of a hollow plastic ball with an outer diameter of 1" (25.4mm) attached to two hollow aluminium shafts (see Figure 8) and designed to penetrate clay with a shear strength up to s_u = 5kPa. This penetrometer has an area ratio of 15:1 following the definition given by Yafrate et al. [15] and therefore allows the measurement of very small penetration resistance in very soft clay. Following Stewart and Randolph [16], a ball factor of $N_t = 10$ has been used, thereby giving a maximum design load of Q = 15.7N.

The outer shaft (S2) connects directly to the ball's shell and serves as a 'tube seal' for the inner shaft (S1), preventing ingress of water and mud. S1 connects to an internally-mounted strain-gauged copper-beryllium diaphragm at the equator of the ball (see Figure 8). A third tube (S3) is placed outside S2 and protects the penetrometer from measuring the shaft friction on S2. The ball is pushed into the clay by applying load to S1. The protecting tube is pushed into the clay at the same rate and applied loads will also be directly measured using a 25N load-cell attached at the top of S1.

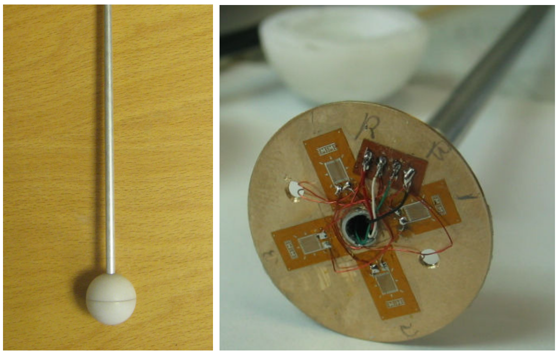


Fig. 8: *New 1" mini-ball penetrometer*

The influence of temperature on the growth of bacteria in WA clay, and hence, on s_u will also be undertaken. Heating mats producing temperatures of up to 65°C (in sample) will be placed around core samples and further ball penetrometer tests completed.

By comparing the measured s_u with (i) non-heated inoculated samples, and (ii) sterile controls, the possible adverse affects of hot oil pipeline installations on potentially vulnerable crusts will be investigated.

The effect of bacteria on the intrinsic properties including Atterberg limits and moisture content will be considered and the variability of s_u with liquidity index using the fall cone will also be investigated.

Discussion:

The intrinsic soil properties of reconstituted clays have been shown to differ from naturally sedimented clays (Burland [17]). [17] suggested that natural sedimentation lines lie above and parallel to the normal consolidation line for reconstituted samples when plotted on a graph of v versus log p′, and which consequently are a function of the initial water content. Advice on how best to reproduce deep sea sediment deposition history in the laboratory is limited. Sills [18] used very slow, self-weight consolidation of Ketelmeer mud in settling column experiments with pore pressure measurements.

No protocols have yet been developed to consider the inoculation of sedimenting laboratory samples with bacteria. Furthermore, the effect of liquid growth medium (in which bacterial cultures are prepared in the laboratory) on soil mechanical properties has not been investigated. Based on the current knowledge of the profile of in situ water content with depth (Figure 4), the current research will focus on a representative in situ water content (w) of about 200% and a liquid limit (LL) of 150%. WA clay will be liquefied at a water content (w) of 200% + 50% = 300% and then sterilised using an autoclave. The consistency of this slurry is similar to liquid growth medium broth and it will therefore be autoclaved using normal sterilisation cycles. The slurry will then be slowly added to cylinders containing: 1) sterile liquid growth medium, and 2) liquid growth medium containing bacteria at a known initial concentration.

Initial bacteria concentration within the prepared liquid growth medium (seawater agar yeast peptone) will be determined under aseptic conditions after incubation of the liquid growth medium for 48 hours at 30°C. 1:10 serial dilutions followed by plating out on solid agar growth medium (Zuberer [19]) with five replicate plates for each dilution level will be used to determine bacterial concentrations.

Similarly, protocols for determining Atterberg limits and moisture contents for samples inoculated with bacteria have not yet been developed. Therefore, the following procedure will be considered. All samples will be prepared by reducing the water content to close to the plastic limit through gentle heating and evaporation. Samples will then be sterilised twice using an autoclave with an extended sterilisation cycle of 1 hour, following [19]. A second 1 hour sterilisation cycle will be completed after three days of moist incubation to kill any remaining bacterial spores.

Sub-samples with a range of water contents are to be created by adding liquid medium with a known concentration of bacteria. Control samples without bacteria will also be prepared and tested with the fall cone apparatus.

Difficulty in achieving complete sterilisation of soil is to be expected due to the relative impermeability of clay close to its plastic limit. To check the effectiveness of the dual autoclaving process, clay samples will be streaked on agar plates and incubated. If no bacterial growth is observed, the clay will be considered sterile. An alternative method may be to autoclave the WA clay in liquidised form (as per the slow sedimentation set-up) prior to evaporation. This method, however, requires access to large aseptic areas for evaporation.

Conclusions:

This paper introduces the hypothesis that the presence of bacteria in deep-sea clays may be the cause of anomalous deep-sea clay crusts. Initial microbiological investigations of these materials have identified *M. aquaeolei* as a bacterium present in situ and culturable in the laboratory. A description of the proposed laboratory set-up to investigate the influence of this bacteria on the properties of WA clay is presented, including the development and construction of a new mini-ball penetrometer. This paper also provides discussion on foreseeable challenges in clay sterilisation, and the formulation of procedures and protocols necessary for testing both sterile and inoculated WA clay samples.

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