# Cable bacteria generating self-potentials in hydrocarbon-contaminated soil? – A pilot study

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# Abstract

Det er beskrevet at trådformede kabelbakterier genererer elektriske spændingsforskelle over den suboxiske zone i sedimenter igennem langdistance elektron overførelse (LDET). Elektriske spændingsforskelle er gennemgående blevet målt i og omkring carbonhydrid-forurenet jord, hvilket er blevet hypotetiseret til at skyldes LDET i jordsystemet. I samtid med dette er kabelbakterier blevet fundet i kapillarzonen af carbonhydrid-forurenet jord i Tyskland. Dette ledte til teorien om at LDET fra kabelbakterier danner de målte spændingsforskelle i carbonhydrid-forurenet jord, hvilket i dette studie blev undersøgt ved at lede efter korrelation mellem måling af spændingsforskelle samt tilstedeværelsen af kabelbakterier i carbonhydrid-forurenet jord. Hertil blev jordprøver samlet ved at foretage en boring på en carbonhydrid-forurenet grund og elektriske spændingsforskelle blev målt ned igennem borehullet med elektroder. De målte elektriske spændingsforskelle stemte overens med tidligere publicerede målinger i lignende jordsystemer, genereret af LDET. 16S rRNA sekvensering skulle anvendes til at detektere tilstedeværelsen af kabelbakterier, men da ingen målbar mængde RNA blev ekstraheret fra jordprøverne, blev der ikke foretaget sekventering, og kabelbakterier blev ikke fundet i jordprøverne. 16S rRNA sekventering blev foretaget af en fortyndingsserie med en kendt abundans af kabelbakterier, hvilket viste en detektionsgrænse på $10^3$ kabelbakterieceller pr. $cm^3$ jord. Dette antyder, at hvis kabelbakterier var tilstede i jordprøverne, var det med en lavere abundans end detektionsgrænsen eller, at kabelbakterier blev udeladt af jordprøverne grundet problemer med prøvetagningen.

Filamentous cable bacteria have been discovered to generate electric potentials (EP), performing long-distance electron transfer (LDET) across the suboxic zone in sediment systems. Since EP, hypothesized to be generated by LDET, have extensively been measured in and around hydrocarbon-contaminated soil, and cable bacteria have been found in the capillary fringe of hydrocarbon-contaminated soil at a site in Germany, the theory, that cable bacteria performing LDET creates the EP witnessed in hydrocarbon-contaminated soil, is proposed. The objective of this study was to investigate the correlation between the presence of cable bacteria and EP in hydrocarbon-contaminated soil. Soil samples were collected from drilling at a hydrocarbon-contaminated area, and EP was measured down the drill hole. The EP distribution in the drill hole, corresponded to previous published EP measurements from similar test-sites, suggesting LDET. The

presence of cable bacteria was to be investigated through 16S rRNA sequencing of the soil samples, but no detectable amount of RNA was extracted and therefore no cable bacteria found. 16S rRNA sequencing of a dilution series with a known abundance of cable bacteria showed a detection limit of $10^3$ cable bacteria cells per $cm^3$ soil, suggesting that if cable bacteria were present in the hydrocarbon-contaminated soil, the abundance were below the detection limit, or cable bacteria containing soil was left unsampled due to sampling obstacles.

# Introduction

Electric potentials (EP) have extensively been witnessed in and around hydrocarbon-contaminated soil, and often the signal from the generated electric fields are strong enough to be measured from the soil surface (Giampaolo, Rizzo et al. 2014). These electric gradients are known as self-potentials (SP), where electrons are transferred from the fringe of the contamination into the surrounding soil, through long distance electron transfer (LDET). The occurrence of SP has been highly debated and is theorized to be facilitated either by bacteria interconnected by electric conducting pili, or by ore bodies, conducting bacterially released electrons away from the contamination, with the electrons ultimately being removed by other bacteria through respiration (Revil, Mendonça et al. 2010). Other components than biological activity have also been documented to contribute to the generation of SP, such as the flow of water itself (Thony, Morat et al. 1997), as well as the activity of chemical species, such as potassium ions in the groundwater, interacting with soil minerals (Maineult, Jouniaux et al. 2006).

The unusual large amount of biologically available carbon in a soil system due to hydrocarbon-contamination, will lead to a steep redox-gradient promptly depleting the groundwater from dissolved electron acceptors, such as oxygen, nitrate and sulfate, at the fringe of the contamination (Christensen, Bjerg et al. 2000, Anneser, Einsiedl et al. 2008). This redox-gradient with rapid depletion of electron acceptors will restrict the bulk of the microbial degradation of hydrocarbon, to the fringe of the contaminant plume (Anneser, Einsiedl et al. 2008). Furthermore the rapid reduction of electron acceptors around the contaminant plume, will create a veil of electron acceptor-depleted aquifer downstream the plume (Christensen, Bjerg et al. 2000).

Cable bacteria are a few cm long filamentous bacteria of the deltaproteobacterial family Desulfobulbaceae (Trojan, Schreiber et al. 2016), which span the suboxic zone in some aquatic sediment systems, where they are proposed to oxidize sulfide using oxygen as electron acceptor in

spatially separated but electrically coupled reactions (Pfeffer, Larsen et al. 2012). The two redox half-reactions from oxidation of sulfide with oxygen as terminal electron acceptor, is divided as following: $2O_2 + 8H^+ + 8e^- \rightarrow 4H_2O$ in the oxic zone and $H_2S + 4H_2O \rightarrow SO_4^{2-} + 10H^+ + 8e^-$ in the anoxic zone, leading to the balanced stoichiometry $2O_2 + H_2S \rightarrow SO_4^{2-} + 2H^+$. The spatial uncoupling of the reduction and oxidation is made possible through LDET, where electrons are moved from the anoxic part of the sediment to the oxic part of the sediment, through the multicellular bacterial filaments, spanning the suboxic zone (Pfeffer, Larsen et al. 2012). This spatial separation will leave a net negative charge in the oxic zone, and a net positive charge in the anoxic zone, which will create a measurable electric gradient through the suboxic zone (Risgaard-Petersen, Damgaard et al. 2014).

Cable bacteria of the Desulfobulbaceae family have been found in the capillary fringe of hydrocarbon-contaminated aquifer at a site in Germany (Müller, Bosch et al. 2016). The capillary fringe is the layer above the groundwater, in which water sieves up through pores between soil particles. The capillary fringe then constitutes a matrix of water-filled space and gas-filled space between soil particles, creating good conditions for gas exchange, such as oxygen, between the atmosphere and the groundwater (Jost, Haberer et al. 2015). Müller´s study suggests that cable bacteria span the suboxic zone, between the capillary fringe containing atmospheric oxygen and the contaminant plume, where they live in syntrophy with sulfate reducers, oxidizing hydrocarbon in the anoxic part of the contaminant plume, leaving sulfide as a bi-product. This resulting sulfide, generated in the anoxic part of the contamination, is then oxidized by coupling to oxygen reduction in the oxic part of the aquifer by LDET. The model proposed by Müller would entail a unique role for the cable bacteria in the degradation of the hydrocarbon contaminant, since the spatial uncoupling of sulfide oxidation and reduction of oxygen, will result in spatial coupling of sulfide oxidation and sulfate reduction in the anoxic part of the contaminant. The oxidation of hydrocarbon contaminant coupled to reduction of sulfate will then be independent of the flux of sulfate from the oxic part of the capillary fringe to the anoxic part of the contaminant plume, and the flux of sulfide from the anoxic part of the contaminant to the oxic part of the capillary fringe. This flux-independency, and spatially coupling of the internal sulfur cycle should ultimately speed up the degradation of the hydrocarbon contaminant significantly, through higher rates of oxidation of hydrocarbon through sulfate reduction.

If cable bacteria in fact perform long distance electron transfer in the capillary fringe of contaminated aquifer, a more heterogenous and 3-dimensional distribution of cable bacteria could be envisioned compared to the distribution known from sediment systems (Schauer, Risgaard-Petersen et al. 2014), due to the more heterogenous distribution of oxygen in the capillary fringe. This heterogeneity could then alter the EP distribution in a more unpredictable manner, relative to the EP distribution witnessed in sediment containing cable bacteria (Risgaard-Petersen, Damgaard et al. 2014).

The findings of cable bacteria in hydrocarbon-contaminated soil (Müller, Bosch et al. 2016) and the frequent occurrence of EP in, and around contaminated soil systems (Giampaolo, Rizzo et al. 2014), combined with the knowledge that cable bacteria conduct electricity through LDET (Pfeffer, Larsen et al. 2012), propose that the measured electric fields around hydrocarbon-contaminated aquifers is derived by LDET performed by cable bacteria in the fringe of the contaminant plumes. Based on this theory, the goal of this study is to test for correlations between the presence of cable bacteria and electric signals in a hydrocarbon-contaminated aquifer. The presence of electric signals will be investigated by measuring the EP with electrodes and the presence of cable bacteria will be investigated microscopically and by 16S rRNA sequencing.

An important part of this study is to establish the detection limit of cable bacteria cells needed in soil, in order to succesfully extract, amplify and sequence cable bacteria 16S rRNA from a sample. This will be investigated through a dilution series of sediment with a known abundance of cable bacteria, where amplification of 16S rRNA will be performed with bacteria-specific primers as well as Desulfobulbaceae-specific primers.

Due to complications with RNA-extraction from the soil collected at the hydrocarbon-contaminated site, no 16S rRNA sequencing results were obtained, leaving this study's main objective of finding correlation between electric signals and the presence of cable bacteria uninvestigated.

# Materials and methods

## Site description and sampling

Soil sampling took place at a former gasification plant in Horsens, Denmark (55°51´37.9”N 9°51´39.4”E). The soil at this area is highly contaminated due to previous dumping of spare products from coal burning, and is now a museum as well as a national test site for investigation and treatment of hydrocarbon-contamination in soil. Due to seasonal fluctuations, groundwater table level at this site have been recorded to vary up to 50 cm, with the groundwater table being highest in the winter season. A specific site, in the middle of the contaminated area and in the middle of a contaminant plume, at the old gas plant was chosen for sampling (Figure 1). Hydrocarbon-contaminated soil was collected, in January 2017, using a helical drill bit with approximately 12.5 cm between each turn. Soil was collected by drilling, 1 meter at a time, to a total depth of 7 meters. The soil at this site was constituted predominantly of fine sand, interrupted by occasional layers of clay. Hydrocarbon-contaminated soil was struck at approximately 55 cm below the soil surface, with the ground water table located at about 88 cm below the soil surface. Soil was sampled from each turn of the helical drill bit, and out of the 12.5 cm between each turn, about 8 cm of soil was accessible for sampling, leaving a depth of approximately 4.5 cm of soil unsampled at each sampling. Sampling was performed by scraping off the outer layer of soil on the drill bit with a sterilized spatula, followed by the sampling and homogenization of sample, by thoroughly mixing it with another sterilized spatula. At each sampling, 5-6 g of homogenized soil was collected, quick frozen in liquid nitrogen and then kept on dry ice until ultimately

**Figure 1. Map of the former gasification plant in Horsens. The red and green dots represent previous drillings, where red dots represent closed and unavailable drill holes and green dots represents drill holes conserved, and available for further studying. The yellow dot shows the location of the drill hole B404, in which samples was collected. The blue arrow indicates the direction of the groundwater flow.**

stored at -80°C for later use for 16S rRNA sequencing. For later Fluorescence In Situ Hybridization (FISH)-analysis, approximately 0.5 g of homogenized sample was added to a 2 mL tube preloaded with 1 mL 4% PFA, vortexed and placed on ice to fix the bacteria in the samples. Later, the fixed samples were washed twice in 1 mL 1xphosphate-buffered saline (PBS), resuspended in 1x PBS and 96% ethanol, at a ratio of 1:1, and stored at -18°C. Ultimately 56 samples were collected throughout the 7 meters. Upon drilling, a hollow plastic tube, with filters allowing groundwater to flow through, was fitted down the drill hole, and the opening of the hole was sealed, to conserve the drill hole for later use. This drilling was named B404.

## Electric potential measurement

In February 2017, the EP was measured from the groundwater table, down to the bottom of the B404 drill hole. An electrode, coupled to a voltmeter, was lowered down the drill hole and EP measurements were recorded every 10 cm, measuring both from the top of the hole to the bottom and vice versa. A puddle surrounding the drill hole at depth 0 was used as reference, for the EP measurements.

## Preparation for limit of detection

### Sampling

Incubated fresh water sediment (provided by Tobias Sandfeld) containing cable bacteria was sampled by collecting top 2 cm sediment with a cut-off syringe, followed by homogenization with a spatula. Some of the sediment was stored at -80°C, and some sediment was fixed in the same manner as with the soil samples and stored at -18°C.

### Identification and filament length estimate through FISH

The fixed sample was used to identify and count the cable bacteria cells present in the sediment by FISH. The sample was homogenized by ultrasonic treatment for 3x20 seconds at 30% power. To dilute, and immobilize the bacteria in the sample, 1 part sample was diluted in 8.8 part 0.1% sodium pyrophosphate and 0.2 part 1% agarose. 10 µL diluted sample was then transferred to a 6-well glass slide, coated with gelatin solution containing 0.075% gelatin and 0.01% $CrK(SO_4)_2$. Upon drying, the sample was dehydrated by dipping the slides for 3 minutes in 50%, 80% and 96% ethanol respectively. Each well was then stained by addition of 1 µL of probe DSB706 (Loy, Lehner et al. 2002), specific for Desulfobulbaceae bacteria, and labeled with the red fluorescent dye CY3, 1 µL of probe EUB338 mix(I,II,III), targeting all bacteria (Daims, Brühl et al. 1999), labeled with the green

fluorescent dye 6-FAM, and 8 µL hybridization buffer (0.9M NaCl, 20 mM Tris HCL, 0.01% SDS and 35% formamide). Hybridization occurred at 46°C for 90 minutes, inside a closed tube containing tissue paper soaked in hybridization buffer. Counterstaining was performed by applying 2 µL, 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI) to each well. An AxioVert 200M epifluorescence microscope with a 100x lens was used to detect stained cells using specific filters for CY3 and 6-FAM. Pictures were gathered and handled through the program AxioVision. The total length of cable bacteria filaments in the samples was estimated using the line intercept method (Newman EI 1966).

### Dilution series

Hydrocarbon-contaminated soil, gathered between the depth of 400-429 cm (sample 12) from drilling B404, was used to dilute the cable bacteria containing sediment. Sample 12 was used to dilute the sediment sample, since no 16S rRNA was succesfully extracted from this soil. Dilution was performed by homogenizing 1.5 g sediment and 1.5 g soil, taking out 1.5 g of the mixture as a sample, and diluting the rest further with more soil. 1.5 g was taken out for each dilution creating a dilution series consisting 6 different dilutions, called sample A-F. The dilutions consisted of 100%, 50%, 25%, 10%, 1% and 0.1% sediment respectively, where sample A was undiluted sediment and sample F was the most dilutet.

## Molecular methods for 16S rRNA extraction and sequencing

RNA was extracted from field samples as well as from dilution series samples, using the RNA PowerSoil® Total RNA Isolation Kit (Qiagen), according to the manufacturer´s protocol, which include both mechanical- (bead beating) and chemical (proprietary surfactant) lysis of cells. A few alterations from the protocol was conducted. Field samples were pooled in triplets, with a total use of 3 g of field soil per extraction. Furthermore, 2 parallel extractions were performed per triplet, and pooled together upon running the samples through the RNA Capture Column. For the dilution series samples, 1.5 g of sample was used per extraction. For both field samples as well as dilution series samples, resuspension of the isolated RNA pellet was performed using 50 µL instead of 100 µL nuclease free water.

Upon RNA extraction, DNA was removed from both field samples and dilution series samples using the Turbo DNA-*free*™ kit (Ambion), according to the manufacturer´s protocol, assuming ≤200 µg nucleic acid per mL sample.

Revers transcription was performed using the Reverse Transcription kit (Omniscript), altering the manufactorer´s protocol by using random hexamer nucleotides as primer, at a final concentration of 1 µM. 8 µL sample was used per reaction.

A preliminary round of PCR-amplification was performed, where two primersets were applied to both the field samples and dilution series samples, namely the bacteria-specific primerset Bac 341F (Muyzer, De Waal et al. 1993)/Bac 805R (Caporaso, Lauber et al. 2011) as well as the Desulfobulbaceae-specific primerset DSB 706F/DSBB+1297R(Kjeldsen, Loy et al. 2007). Each reaction was prepared with following: 12.5 µL 2xKAPA HiFi Hotstart mix, 0.5 µL 10 pmol/µL forward primer, 0.5 µL 10 pmol/µL reverse primer, 0.5 µL 10 mg/mL Bovine Serum Albumin (BSA), 3 µL template and 8 µL $dH_2O$. PCR was performed according to Table 1, where all the field samples was treated with 25 cycles of the denaturation-annealing-elongation step, dilution series samples with bacteria specific primers was treated with 20 cycles, and dilution series samples with Desulfobulbaceae specific primers was treated with 30 cycles. Only small amounts of PCR product were traceable from any of the field samples following the first round of PCR and the small amount detectable, could not be ruled out as being DNA-contamination. All field samples were then discarded and not processed further, except sample 12, which was used as a control in the dilution series.

Miseq sequence preparation was performed using the 16S Metagenomic Sequencing Library Preparation protocol from Illumina, with some alterations. The same two primersets as in the preliminary PCR, with overhang adaptors attached, was applied in the amplicon PCR and 2 µL of template was used. PCR was performed according to Table 1, where dilution series samples with bacteria specific primers was treated with 10 cycles of the denaturation-annealing-elongation step, and dilution series samples with Desulfobulbaceae specific primers was treated with 30 cycles. The Index PCR was performed with half the volume of all the substrates compared to the suggested reaction volume in the protocol. The samples were then diluted to 4 ng nucleic acid per µL, pooled together, and run on a Miseq (Illumina).

| Step | Temperature | Time |
|---|---|---|
| Initial denaturation | 95°C | 3 min |
| Denaturation<br>Annealing<br>Elongation | 95°C<br>55°C<br>72°C | 0.5 min<br>0.5 min<br>0.5 min |
| Final elongation | 72°C | 5 min |
| Cooling | 4°C | ∞ |

**Table 1. PCR-program used throughout the different PCR steps of 16S rRNA amplification. Only the amount of cycles in the denaturation-annealing-elongation step varied.**

## Sequence analysis

The output sequences from the Miseq sequencing were purified and analyzed through the programs Mothur (Schloss, Westcott et al. 2009) and R (version 3.4.0). Sequences with a relative abundance below 0.01%, in the different dilution series samples were disregarded. All sequences identified as *Desulfobulbus*-affiliated, which is the genus most closely related to cable bacteria, were aligned using the SINA online tool (Pruesse, Peplies et al. 2012) and then added to a phylogenetic tree, specific for cable bacteria (Trojan, Schreiber et al. 2016), using ARB (Ludwig, Strunk et al. 2004), to investigate which 16S rRNA sequences clustered with known cable bacteria sequences.

## Total Cell Count

Total cell count was performed on fixed field samples from depth 25-50, 400-429 and 665-694, corresponding to triplet sample 2, 12 and 18 respectively. Total cell count was also applied on the sediment used in the dilution series, as a means of comparison. The fixed sample was mixed 1:1 with 0.1% sodium pyrophosphate. The solution was homogenized by ultrasonic treatment for 3x 20 s at 30% power, and further diluted 1:10 in 0.1% sodium pyrophosphate. 60 µL of the diluted sample, were transferred to 10 mL 1xPBS and added 1 µL SybrGold. The sample was then filtered through polycarbonate filters (pore size, 0.22 µm) and counterstained with DAPI. The filtrate was investigated with an AxioVert 200M microscope with a 100x lens, and cells on the filter were counted in 30 random positioned grids, with a grid-area of $1.56*10^{-4}$ $cm^2$. The abundance of bacteria cells per grid were calculated and scaled up to the total area of the filtrate. The 60 µL filtrated

sample, was diluted 1:40 in total, corresponding to filtration of 1.5 µL undiluted sample. This was used to calculate the abundance of bacteria cells per $cm^3$ soil or sediment.

# Results

## Electric potential measurement

EP measurements, showed a difference in EP throughout the depth of the drill hole (Figure 2). A relatively steep reduction in EP is witnessed from the reference measurement at depth 0 to the groundwater table at 88 cm.

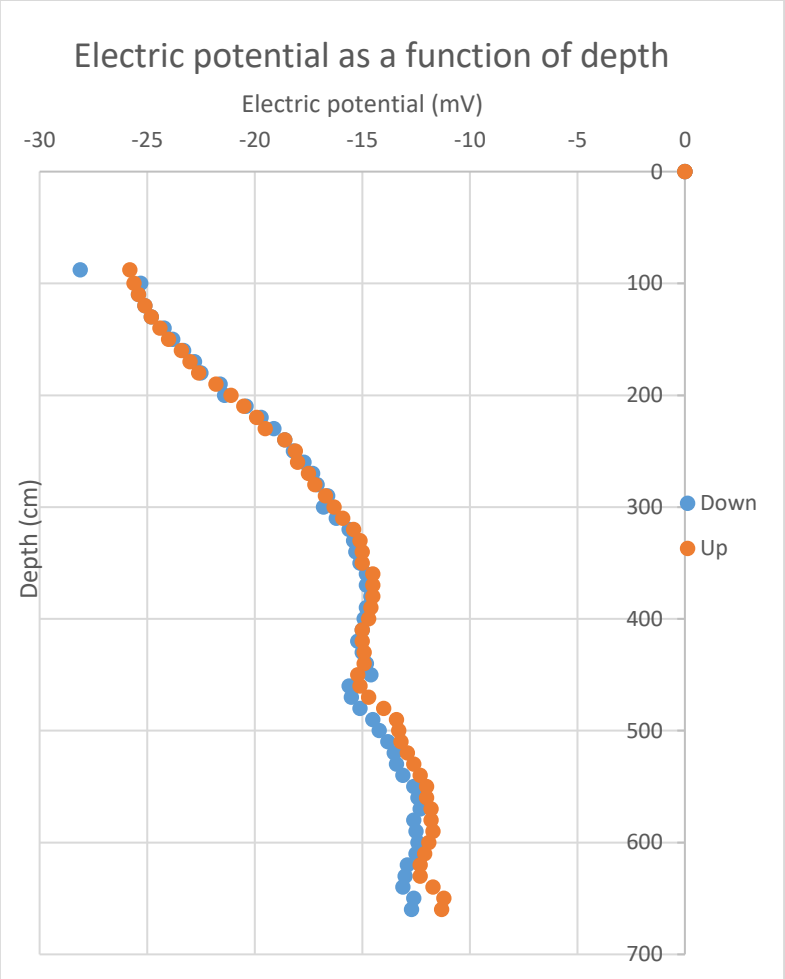


**Figure 2. EP measurements every 10 cm down drill hole B404. Blue dots represent measurements from lowering the electrode down the drill hole, and orange dots represent measurements from raising the electrode up the drill hole.**

## Limit of detection

### FISH analysis

Cable bacteria were identified in the sediment samples by double hybridization with probes EUB338 mix(I,II,III) and DSB706 (Figure 3). Total filament length estimate from the line intercept method, resulted in an estimate of $4.32*10^6$ µm cable bacteria filaments per $cm^3$. With a cable bacteria cell length estimate of 5 µm, inferred from Figure 3, and an estimated sediment density of 1.3 g per $cm^3$, leave an estimate of around 665,000 cable bacteria cells per g sediment. Since 1.5 g of diluted sediment was used for RNA extraction, the amount of cable bacteria cells potentially being available for 16S rRNA sequencing in the different dilution series samples should be approximately as presented in Table 2.

| Dilution sample | Potential number of cable bacteria cells available for 16S rRNA extraction |
|---|---|
| 12 | 0 |
| A | $10^6$ |
| B | $5.0 * 10^5$ |
| C | $2.5 * 10^5$ |
| D | $10^5$ |
| E | $10^4$ |
| F | $10^3$ |

**Table 2. The estimated amount of cable bacteria cells available for extraction of 16S rRNA in the different dilution series samples.**

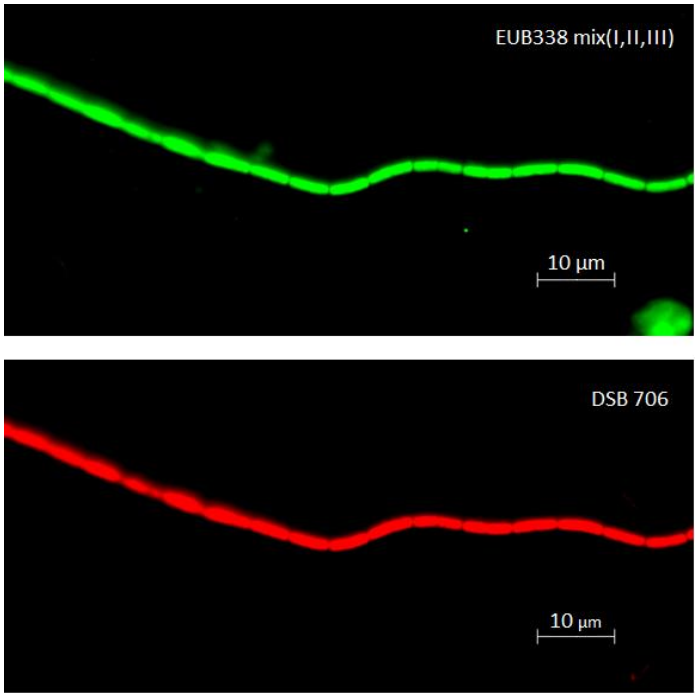


**Figure 3. Fluorescence microscopy pictures of cable bacteria from the FISH analysis. The top picture is stained with the bacteria-specific EUB338 mix(I,II,III) probe and the lower picture is stained with the Desulfobulbaceae specific DSB 706 probe. From the scalebar, a cell length of 5 µm was estimated.**

## Sequence analysis

The sequences obtained from the use of Desulfobulbaceae specific primers, did not align within the primers target sequence. It seems that the primers amplified 16S rRNA unspecifically, which ultimately led to the results being discarded and not processed further. From the amplification with bacteria specific primers, 12 different sequences affiliated with *Desulfobulbus* were obtained, in which 4 sequences clustered within a known group of cable bacteria, where all clustered in the proposed freshwater cable bacteria genus *Electronema*, with OTU00006 and OTU03328 clustering with the proposed species *palustris*, and OTU00014 and OTU00976 clustering with the proposed species *nielsenii* (Trojan, Schreiber et al. 2016) (Figure 4). Only these 4 sequences where accepted as cable bacteria, which led to a distribution of cable bacteria reads compared to the total amount of reads as presented in Table 3, and a relative abundance of cable bacteria in the different dilutions, is presented in Figure 5. Sample F shows only a small abundance of

| Sample | Total reads | Cable reads | Total reads per cable read |
|---|---|---|---|
| A | 37513 | 746 | 50.3 |
| B | 63292 | 1452 | 43.6 |
| C | 60346 | 1808 | 33.4 |
| D | 60018 | 1610 | 37.3 |
| E | 6443 | 96 | 67.1 |
| F | 1371 | 4 | 342.8 |

**Table 3. The total amount of reads, total amount of cable bacteria reads and the total amount of reads per cable bacteria reads, in the different dilution series samples.**

cable bacteria sequences with 4 reads, but cable bacteria 16S rRNA is in fact detectable in this dilution. No cable bacteria were found in either sample 12 or the negative control. The relative abundancy of cable bacteria sequences is evenly distributed among the different dilutions, as seen from Figure 5.

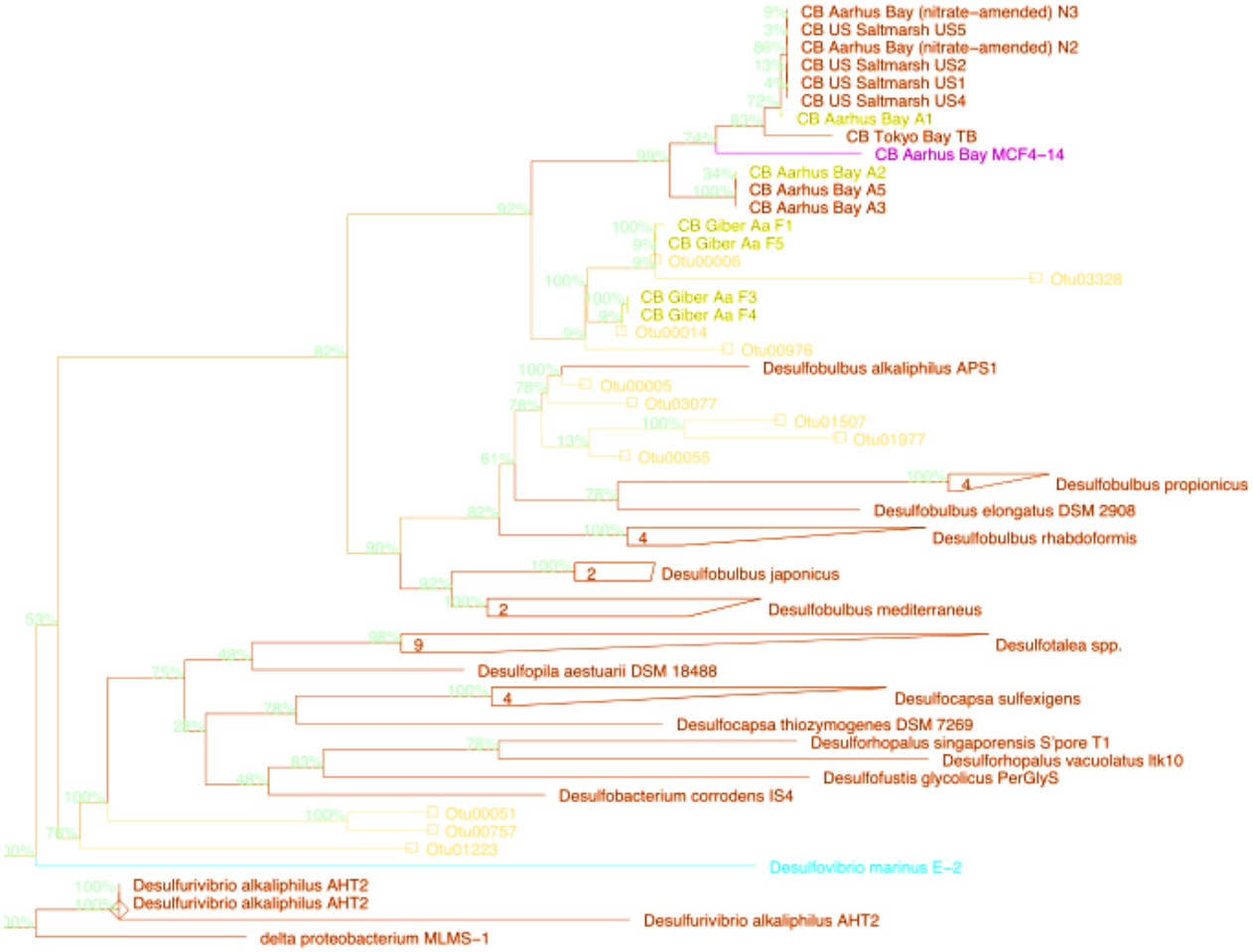


**Figure 4. The phylogenetic distribution of the 12 different sequences obtained, whereas 4 sequences aligns within a cable bacteria cluster. CB stands for cable bacteria and the taxa starting with "Otu" represents the sequences obtained from 16S rRNA sequencing.**

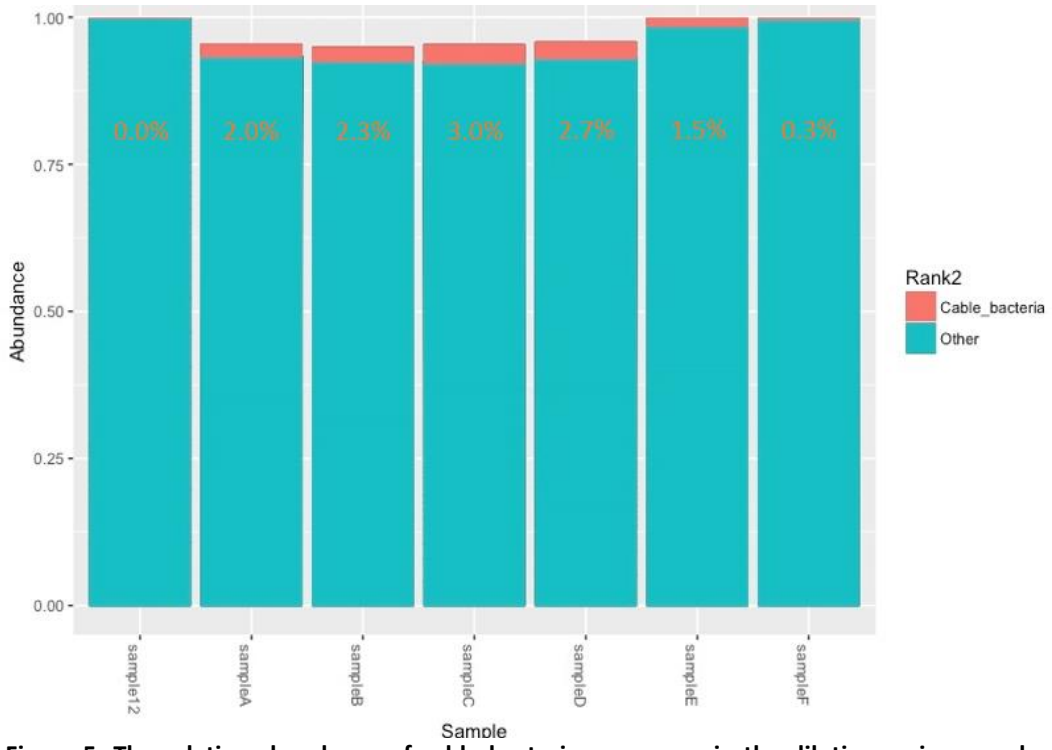


**Figure 5. The relative abundance of cable bacteria sequences in the dilution series samples, compared to all bacterial sequences gathered.**

## Total Cell Count

As shown in Table 4, the sediment used in the dilution series showed the highest abundance of bacteria cells with approximately 5 times more bacterial cells than counted in field sample 2, which was the field sample with the highest cell count. Although a few cells were seen from microscopy on sample 12, no cells encountered the grid, which resulted in a cell count of 0. This result should be viewed with skepticism as a relatively thick, brown layer covered the filter upon filtration, potentially hiding bacteria cells.

| Sample | Number of bacterial cells per $cm^3$ soil or sediment |
|---|---|
| Dilution series sediment | $2.6 * 10^7$ |
| 2 | $5.6 * 10^6$ |
| 12 | 0 |
| 18 | $1.5 * 10^6$ |

**Table 4. The abundancy of bacteria cells in different samples, estimated from Total cell count. The result from sample 12 is most likely not representative.**

# Discussion

Since no RNA was successfully extracted from the field soil, the objective of this study, to investigate correlation between the presence of cable bacteria and EP in contaminated aquifer, was left uninvestigated, although EP was measured.

The EP measurement showed a difference in EP throughout the investigated drill hole. The relatively negative potential at the depth of 88 cm (groundwater table), may represent cathodic activity. Below the depth of 88 cm, the electrical potential slowly rises again, which may represent anodic activity. The shape of the EP distribution corresponds to the EP model of LDET in the capillary fringe presented by (Revil, Mendonça et al. 2010), where the EP declines from the soil surface, down towards the contamination above the groundwater table, after which the EP starts rising again. This suggests that LDET is occurring in the capillary fringe of the contaminant plume investigated. The EP distribution does not follow the distribution seen in sediment with cable bacteria, where the EP is at its minimum at the cathodic part of the cable bacteria, and constant above this zone (Risgaard-Petersen, Damgaard et al. 2014). If the electric signals are created by cable bacteria performing LDET in the capillary fringe, the discrepancy in EP distribution could be due to a more heterogenous and 3-dimensional distribution of cable bacteria in the capillary fringe, compared to the sediment systems, making the EP distribution more unpredictable. Further, other factors besides LDET could impact the difference in EP, such as the flow of groundwater itself (Thony, Morat et al. 1997).

The sequence analysis from the limit of detection investigation showed that cable bacteria could be detected all the way down to a dilution of 0.1% cable bacteria containing sediment. This corresponds to $10^3$ cable bacteria cells per $cm^3$ being a sufficient abundancy of cable bacteria in soil system, to successfully detect the presence through 16S rRNA extraction and sequencing. Even though $10^3$ cable bacteria cells per $cm^3$ was the lowest abundance used in the dilution series, and proved sufficient to successfully detect cable bacteria in soil, the low abundance of 4 reads detected, suggests that cable bacteria could be left undetected, if present at an even lower abundance. Further, since dilution F showed 342.8 total reads per cable bacteria read, at least 342.8 reads will, in theory, be needed obtained from the lowest detectable abundance of cable bacteria, in order to detect their presence in a soil system. The absence of any detectable amount of RNA from the field samples suggests that if cable bacteria is present in the soil, the abundance of cable bacteria are either below the limit of detection of $10^3$ cells per $cm^3$, or the activity of the cable bacteria is lower

in the soil system compared to the sediment used in the dilution series, resulting in less rRNA in each cell. 3d modelling of cable bacteria distribution in the capillary fringe of the contaminant plume, knowledge of electric resistance in the soil system and electron transfer measurements from cable bacteria, could be combined and used as a tool to calculate the abundance of cable bacteria needed to generate the EP distribution measured down the drill hole. If the needed abundance is below the detection limit, cable bacteria could be present in the soil without being detected and still create a measurable EP.

At least two different species of freshwater cable bacteria were found in the dilution series sediment, where all 4 cable bacteria OTUs clustered in the proposed genus *Electronema* (Trojan, Schreiber et al. 2016). OTU00976 could potentially represent a new species, only aligning 94.1% with the proposed species *nielsenii*, in which the OTU clustered.

If combining the results of the estimated amount of cable bacteria from FISH-analysis, which corresponded to $4.32 * 10^6$ cable bacteria cells per $cm^3$ sediment, with the estimated amount of bacterial cells gathered from total cell count, corresponding to $2.60 * 10^7$ cells per $cm^3$ sediment, an estimated abundance of cable bacteria in the sediment is around 17%. Total cell count showed about 5 times as many bacterial cells in the dilution series sediment, compared to the field sample containing the highest abundance of bacteria cells (sample 2). Since 4 times as much field sample soil was used for RNA extraction compared to dilution series sediment, it would be expected that if the limiting factor of 16S rRNA extraction and amplification was bacteria cell abundance, a traceable amount of 16S rRNA would have been amplified from at least sample 2. This suggests that other factors are involved in the struggle to successfully extract and amplify 16S rRNA from the field samples. The most likely alternative factor would be that the cells from the field sample is less active and therefor produce and contain less rRNA, than the bacteria from the dilution series sediment. A massive upscaling in the amount of soil used for RNA-extraction could then potentially overcome the difficulties of extracting and amplifying 16S rRNA, and is recommended for further studies.

There are at least two possible mechanisms for oxygen-availability for cable bacteria in the contaminant plume, namely oxygen available from the capillary fringe, or dissolved oxygen from the groundwater. If the theory that cable bacteria perform LDET over the distance of a few cm, oxidizing sulfide with oxygen as the terminal electron acceptor in a spatially separated reaction (Pfeffer, Larsen et al. 2012), is accepted, and the model that cable bacteria are performing LDET in the fringe

of hydrocarbon-contaminated soil, with the use of dissolved oxygen from the groundwater as electron acceptor, is accepted as well, methods used in this study such as selection of test-site, sampling strategy and drilling technique, contains certain issues needed to be addressed. One of the issues is the site selection of drilling and sampling. The problem here is that the site of sampling is, with regards to the groundwater flow, located downstream highly contaminated soil. The high rates of microbial activity in and around the hydrocarbon-contaminated area (Anneser, Einsiedl et al. 2008), will most likely result in depletion of oxygen and other electron acceptors from the groundwater (Christensen, Bjerg et al. 2000), before the groundwater flows through the site at which the samples were collected. Furthermore, the site chosen for sampling is located in the middle of a contaminant plume and not at the edge of the contaminant plume, making the top and the bottom of the plume, the only fringes available for cable bacteria. This is a potential problem since the bottom and top of the plume are not covered by the groundwater flow, removing the possibility of groundwater delivering electron acceptors to the fringe of the contaminated plume. This means that the groundwater flow does not meet the fringe of the contaminant plume at any point in the drill hole that was sampled. These observations are most likely devastating for this model, since groundwater containing oxygen and other electron acceptors, will not meet the fringe of the contaminant plume. Since the sampling was performed in the middle of the winter, where the ground water table should be at its highest, the seasonal fluctuations of the groundwater table should not have an impact on criticism presented on this model, since the top of the contamination still won´t be covered by groundwater at any point throughout the year. The other suggested model, that cable bacteria creates EP through LDET in the capillary fringe of hydrocarbon-contaminated soil by spatially uncoupled oxidation of sulfide with oxygen as terminal electron acceptor (Müller, Bosch et al. 2016), doesn't contain the same issues concerning the availability of oxygen. In this model, oxygen is available from the hydrocarbon-contaminated capillary fringe, where LDET could occur. The seasonal fluctuations of the groundwater table could be a potential problem for this model, even though the capillary fringe will be surrounded by hydrocarbon-contamination throughout the whole year. The potential problem could be that cable bacteria would need to follow the seasonal fluctuations of the groundwater table to have access to both electron donor and electron acceptor throughout the whole year. The cable bacteria then need to be able to adapt rapidly to the

fluctuations of the ground water table, to keep performing LDET. The ability of cable bacteria to do so is currently not known, and needs to be investigated further.

The way in which the drilling was performed as well as the way in which samples were collected throughout the depth of the drilling could underlie certain issues. The helical structure of the drill bit used for collecting soil, made it difficult to represent the total, continuous depth when sampling. This led to the sampling of approximately 8 continuous cm per helical turn on the drill bit which had a size of about 12,5 cm. This left approximately 4,5 cm unsampled soil at each sampled depth, which corresponds to about 1/3 of the total depth being unsampled. This could potentially pose a problem since cable bacteria are up to a few cm long (Pfeffer, Larsen et al. 2012), and for that reason might as well be present in the unsampled part of the soil, creating the chance that cable bacteria that are present in the soil, is left unsampled. This problem can be overcome by using hollow-stem auger drilling, which is drilling with a drill bit containing a hollow center, collecting soil in a continuous matter. The use of a drill bit with a hollow center will also result in a way larger sample size being collectable for each depth, which will be useful for an upscaling of RNA extraction. Further, if only the model with cable bacteria performing LDET in the capillary fringe of hydrocarbon-contaminated soil is accepted, there is no reason to drill and sample much lower than the groundwater table, since the cable bacteria will be present in the capillary fringe, which will save a lot of work on sampling, laboratory work and analyzing of results.

## Conclusion

Though no cable bacteria 16S rRNA was successfully extracted from the field samples, EP measurements suggest LDET in the capillary fringe of the hydrocarbon-contaminated soil. The limit of detection of cable bacteria from 16S rRNA sequencing was found to be $10^3$ cells per $cm^3$ suggesting that if cable bacteria were present in the capillary fringe of the contaminant soil, the abundance were below the detection limit, or wasn't sampled due to obstacles from using a helical drill bit. For similar studies, it is suggested to focus the sampling around the capillary fringe, to use a hollow stem auger drill for continuous sampling, and to scale up the amount of soil used for extraction of RNA, to hopefully gather more than 342.8 reads from 16S rRNA sequencing, which is the theoretic amount of reads needed to detect cable bacteria at the detection limit.

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