Identification of Microorganisms in Hydrocarbon-Contaminated Aquifer Samples by Fluorescence In Situ Hybridization (CARD-FISH)

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Abstract

High loads of petroleum hydrocarbons in contaminated soils and sediments make these ecosystems difficult to study with molecular techniques. Among these sites, aquifers – environments with low turnover rates and, hence, slow-growing microbial communities–pose a great challenge for microbial ecologists.

Fluorescence produced by petroleum hydrocarbons coating sediment particles can be so strong that microscopic techniques are made impossible. Low microbial cell numbers pose further limitations for molecular analyses such as fluorescence in situ hybridization (FISH).

Here, we present a protocol for the separation of microbial cells from sediment samples of highly petroleum-contaminated aquifers. By excluding the strongly autofluorescing sediment particles, by concentrating microbial cells on membrane filters, and by using signal amplification in combination with FISH (CARD-FISH), we were able to quantify various microbial populations in this intriguing ecosystem.

Keywords: Aquifer, CARD-FISH, Cell quantification, Hydrocarbon contamination, Microbial community

1 Introduction

Contamination of aquatic and terrestrial ecosystems with hydrocarbons occurs worldwide and represents a major threat to the environment and human health. Alongside cost- and labor-intensive technological approaches, natural attenuation strategies exploiting microorganisms have become an alternative way to clean up those contaminated sites [1, 2]. Yet our understanding of the physiology and ecology of the natural microbial communities found at polluted sites is limited as the sites themselves often pose a challenge even to common analysis techniques.

Aquifers, like most nutrient-poor water bodies, are characterized by a low number of small-sized planktonic cells, with more than 90% of the microorganisms attached to sediment particles [3, 4]. Hence, studying microbes indigenous to an aqua-terrestrial

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ecosystem necessitates the enrichment of the planktonic fraction by filtering large amounts of water [5–7] or by directly analyzing sediment samples [8–10]. Most studies include both approaches [11–13]. Since particle-attached communities are often more active than their planktonic counterpart [14–17], sediment samples are likely to be more relevant when focusing on microbial processes, such as biodegradation [18]. However, background fluorescence and autofluorescence caused by sediment particles (i.e., clay) and especially hydrocarbons present a major challenge to any kind of microbial visualization technique in this environment. One of the techniques thus affected is fluorescence in situ hybridization (FISH) of rRNA – at large, a widely used cultivation-independent method to investigate population dynamics and interspecies relationships at the single-cell level [19–21]. This fact is also reflected in the very low number of published studies using FISH in contaminated aquifer sediments (six in total: [22–27]).

The FISH procedure generally consists of four parts:

1. *Fixation* of the sample containing the target cells.

   Fixation stabilizes macromolecules and cytoskeletal structures thus preventing lysis of the cells during hybridization. At the same time fixation permeabilizes the cell walls for the fluorescently labeled oligonucleotide probe molecules.

2. *Hybridization* of target cells with specific oligonucleotide probes.

   The fixed cells are incubated (hybridized) in a buffer containing the labeled probe at a specified temperature that favors the specific binding of the probe to the target. Ideally, only those probe/rRNA pairs will form which have no mismatches in the hybrid. Consequently, only target cells that contain the full signature sequence on their rRNA will be stained.

3. *Washing* to remove unbound probe.

   The subsequent washing step will remove all unbound probe molecules.

4. *Enumeration/quantification* of stained target cells.

   Finally, the hybridized cells are counted by epifluorescence microscopy.

Further developments of the FISH assay introduced additional steps to the common protocol, for example, signal amplification by catalyzed reporter deposition with horseradish peroxidase (HRP)-labeled oligonucleotides (CARD-FISH, [28]). A study on marine planktonic and benthic microbial assemblages showed that the quantification efficiency of FISH can be significantly enhanced by using the more sensitive CARD-FISH assay [29].

Alternative approaches for the quantification of certain target organisms in an environment are of course available (though without cell visualization) and used in many areas of microbial diversity research. Some of these methods require prior DNA extraction, and regardless of the chosen protocol, one has to keep in mind that this step will already have an influence on the outcome of the microbial composition study [30]. Some more bias is likely to be introduced when applying polymerase chain reaction (PCR)-based methods (e.g., quantitative real-time PCR [31–33]). In addition, setting up PCR protocols for samples from petroleum-contaminated samples can be rather challenging, as not only the hydrocarbons but humic substances are known to at least partly inhibit the PCR [34]. One technique for cell quantification without prior DNA extraction and amplification steps is flow cytometry. This approach works well for cell cultures [35], single-cell sorting [36], and also in combination with FISH [37]. However, hydrocarbons and particles in the sediments that remain in the sample even after physical/chemical cell separation techniques cause strong autofluorescence, leading to erroneous cell counts with the flow cytometer. Another issue with cytometry is the potential clogging and contamination of the fine tubings by such fine particles and hydrocarbons.

The challenge to visualize microbes in contaminated aquifers with (CARD)-FISH is to minimize both background fluorescence and the number of false-positive (CARD)-FISH signals caused, for example, by particle-bound probes. One way to reduce background fluorescence and false-positive signals is by detaching cells from sediment through physical and/or chemical means and density gradient centrifugation. However, the disadvantage with this procedure is that spatial distribution patterns of microbes are disrupted and steric partnerships are disintegrated. Thus, this method is commonly more suited to analyze the presence/absence and abundance of target organisms.

The CARD-FISH protocol we present here has been developed and optimized for hydrocarbon-contaminated aquifers, in particular heavily polluted aquifers containing large plumes of methyl tertiary butyl ether (MTBE) and benzene, toluene, ethylbenzene, and xylenes (BTEX) [38]. We obtained considerable improved permeabilization and hybridization efficiency (2- to 20-fold) by applying a laboratory microwave. In fact, permeabilization with Tris-EDTA buffer (1× TE) in the conventional oven resulted in CARD-FISH signals below the detection limit, whereas a short treatment with the histological microwave resulted in CARD-FISH signals with well-preserved cell morphologies. Additionally, using a histological microwave decreased hybridization time when compared to hybridization in a conventional oven. The latter usually requires two to twelve hours for the hybridization reaction, while hybridization using controlled microwave irradiation needs only 20 min to 2 h.

The detection efficiency of FISH in contaminated aquifers lies reportedly between 23 and 72% for *Bacteria* and *Archaea* together [24, 25, 27]. Depending on the degree of hydrocarbon contamination, we achieved an efficiency ranging from 100% in aquifer samples with lower hydrocarbon concentrations to 15% closest to the center of the contaminant plume being the least biologically active part [39, 40].

2 Materials

2.1 Sample Fixation

Formaldehyde (37%): best stored dark, stable for several months at room temperature.
Phosphate buffered saline (1× PBS): 137 mM NaCl, 2.7 mM KCl, 4 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.6.
Ethanol 96%.

2.2 Cell Separation

Tris-EDTA buffer (1× TE): 10 mM TrisHCl, 5 mM EDTA, pH 9.0.
Natrium pyrophosphate: 1 M.
Tween 80 (Reagent purchased from SERVA (http://www.serva.de)).
Sonication device: ultrasonic liquid processor.
Phosphate buffered saline (1× PBS) (*see* Sect. 2.1).
Nycodenz solution: 1.3 g ml^{-1}; 60% (w/v) in Milli-Q water; if autoclaved and stored at 4°C, stable for several weeks (Axis-Shield PoC, http://www.axis-shield.com).
Cellulose nitrate support filters: pore size 0.45 μm, diameter 47 mm (The filters were purchased from Sartorius (http://www.sartorius.com)).
Polycarbonate filters type GTTP: pore size 0.2 μm, diameter 47 mm. (The filters were purchased from Millipore (http://www.millipore.com)).

2.3 Cell Permeabilization

Low gelling point agarose: 0.1% [w/v], gel strength should be approx. 1,000 g cm^{-2} (Reagent purchased from Biozym (http://www.biozym.com)).
Ethanol 50%.
Tris-EDTA buffer (1× TE) (*see* Sect. 2.2).
Formaldehyde (37%) (*see* Sect. 2.1).
Hydrogen peroxide (H_2O_2): 0.1%, store at 4°C.

2.4 CARD-FISH Procedure and Counterstaining

Horseradish peroxidase (HRP)-labeled oligonucleotide probes: working solutions are prepared at a concentration of 50 ng μl^{-1} and stored in small portions (50–100 μl) in the dark at –20°C. Once thawed, HRP-labeled probes should be stored at 4°C where they are stable for up to 6 months (all probes were purchased from biomers.net (https://www.biomers.de) (*see* **Note 1**).

Hybridization buffer: 0.9 M NaCl, 20 mM TrisHCl (pH 8), 10% dextran sulfate, 0.02% sodium dodecyl sulfate (SDS), 1% blocking reagent, × ml formamide and × ml Milli-Q water; stable for 12 months if stored at −20°C. (Blocking reagent was purchased from Roche (https://www.roche-applied-science.com)) (*see* **Note 2**).

Phosphate buffered saline (1× PBS) (*see* Sect. 2.1).

Amplification buffer: 1× PBS (pH 7.6), 2 M NaCl, 10% dextran sulfate, 0.1% blocking reagent; stable for 12 months if stored at −20°C, at 4°C stable for 4 weeks (Blocking reagent was purchased from Roche (https://www.roche-applied-science.com) (*see* **Note 3**).

Hydrogen peroxide (H_2O_2): 0.0015% make fresh as required.

Fluorescein-labeled tyramide (Fluorochromes purchased from Invitrogen (www.invitrogen.com); custom labeled, see [28]; light sensitive, store at −20°C (*see* **Note 4**).

Ethanol series: 50%, 70%, and 96%.

Mounting medium containing a general DNA stain (i.e., 4′,6-diamidino-2-phenylindole, DAPI), light sensitive, store at 4°C.

Microscope glass slides and cover slips.

3 Methods

3.1 Sampling Procedure and Fixation

1. Take sediment sample and fix with formaldehyde (2% volume/volume [v/v] final concentration) at 4°C for 12–24 h (*see* **Note 5**).
2. Wash samples twice with a 1:1 mix of 1× PBS and 96% ethanol by pelleting at 15,000×*g* for 5 min and resuspend. For centrifugation we use 5810R centrifuge with swing-out rotor A-4-62 (Eppendorf (http://ww.eppendorf.com)).
3. Store washed samples in 96% ethanol at −80°C.

3.2 Cell Separation

1. Mix 200 μl of sediment sample with 700 μl 1× TE buffer and 100 μl of 1 M Na-pyrophosphate in a 1.5 ml tube.
2. Place tube into a water bath and heat it to 55°C for 5 min at 200 W in a laboratory microwave. For microwaving we use the laboratory microwave BP-111-RS (Microwave Research and Applications, Inc. (http://www.microwaveresearch.com)).
3. Cool sample down to room temperature.
4. Add 1 μl Tween 80 and vortex for 15 min at RT.
5. Sonicate on ice. For sonication we use Sonifier Model 250 (Branson (http://www.emersonindustrial.com)).
6. To separate dislodged cells from sediment particles, transfer sample to 50 ml tube and mix thoroughly with 22.5 ml 1× PBS and 2.5 ml 0.1 M Na-pyrophosphate.

7. Place 2 ml of Nycodenz solution at the bottom of the 50 ml tube using a syringe with a long needle.
8. Centrifuge at 4,000 rpm for 15–17 h at 4°C. For centrifugation we use 5810R centrifuge with swing-out rotor A-4-62 (Eppendorf (http://www.eppendorf.com)).
9. Transfer supernatant and Nycodenz layer to a clean 50 ml tube and mix sample.
10. Filter sample onto white polycarbonate filter. For filtration we use filter type GTTP, size 47 mm, pore size 0.2 μm (Millipore (http://www.millipore.com) together with cellulose nitrate support filter, size 47 mm, pore size 0.45 μm (Sartorius (http://www.sartorius.com)).
11. Wash filter twice with autoclaved Milli-Q water, air-dry, and store at −20°C. It is possible to store filters at −20°C for several months. Labeling of filters should be done using a lead pencil only.

3.3 Cell Permeabilization

1. To prevent cell loss during permeabilization, place filters facing down into 200 μl low gelling point agarose (0.1%) onto a Parafilm covered, even surface (i.e., glass plate) and dry filters in an oven at 35°C (*see* **Note 6**).
2. Remove filters from Parafilm by wetting with 50% ethanol and gently peel filters off, air-dry filters.
3. Section filters into pieces (and label sections with a lead pencil if necessary).
4. Place filter sections into a 1.5 ml tube containing 1 ml of 1× TE.
5. Permeabilize by microwaving in a preheated water bath at 65°C for 8 min at 1,000 W (100% power output) (*see* **Note 7**).
6. Cool tubes for 5 min at RT.
7. To stabilize cells for subsequent hybridization, postfix cells in 900 μl of 1× TE and 120 μl formaldehyde (37%) for 5 min at RT.
8. Wash filter sections with 1× TE.
9. Inactivate endogenous peroxidases with 0.1% H_2O_2 in 1× TE for 2 min at RT (*see* **Note 8**).
10. Wash filter sections twice with 1× TE.

3.4 Hybridization of Filter Sections (CARD-FISH) and Counterstaining

1. Mix 1,000 μl hybridization buffer (% formamide depending on probe used) and 3.3 μl HRP-probe working solution in a 1.5 ml tube (*see* **Note 9**).
2. Transfer filter sections to the hybridization mixture.

3. For hybridization place tube into a pre-warmed water bath and microwave at 46°C for 40 min at 500 W in laboratory microwave (*see* **Note 10**).
4. To equilibrate the probe-delivered HRP, transfer filter sections to 50 ml of 1× PBS and wash for 15 min at RT (*see* **Note 11**).
5. Mix 1,000 μl amplification buffer with freshly amended 0.0015% H_2O_2 and 1 μl fluorescein-labeled tyramide in a 1.5 ml tube (*see* **Note 12**).
6. Transfer filter sections to the amplification mixture (*see* **Note 13**) and place tube for 15 min at 46°C in a conventional hybridization oven in the dark (*see* **Note 14**).
7. Wash filter sections five times with Milli-Q water, dehydrate in increasing ethanol concentrations (50%, 70%, and 96%) and let air-dry in the dark.
8. It is possible to store filter sections at −20°C or continue with counterstaining filter sections with a DNA stain (i.e., DAPI-amended mountant solution) (*see* **Note 15**).
9. Put filter sections on glass slide for microscopic enumeration of cells (*see* **Note 16**).

4 Notes

1. Repeated freeze-thawing of probe working solutions will damage the peroxidase and might cause the appearance of numerous brightly fluorescent particles (precipitation of the probe) that do not show any signal in UV (DAPI) excitation. In addition, hybridization signals become dim and background is high.
2. The specific formamide concentration of the hybridization buffer is linked to the probe used. A database of probes and their specific formamide concentrations is available at probeBase (http://www.microbial-ecology.net/probebase). For the exact volume of formamide and Milli-Q water added to the hybridization buffer, refer to Table 1 in [28].
3. Amplification buffer is stored best in small aliquots of 1–2 ml at −20°C.
4. Different fluorochromes are available for CARD-FISH, for example, various Alexa Fluor dyes and coumarin-, fluorescein-, tetramethylrhodamine-, cyanine 3-, and cyanine 5-labeled tyramides. Because these succinimidyl esters can hydrolyze rapidly, all reagents have to be water-free, and the active dye stock as well as the tyramine HCl stock must be prepared a few minutes before use.

5. Due to the size of the HRP molecule, accessibility of probes to the cells may be discriminating. This is, e.g., reflected in the preference for ethanol fixation rather than fixing with the cross-linking agents paraformaldehyde or formaldehyde. The probability that not all organisms can be detected under the same conditions increases with the phylogenetic diversity of the target group. So it is recommended to use the signal amplification method only for probes with a restricted target group for which fixation and hybridization conditions can be readily achieved.
6. Before embedding the filters let the freshly heated agarose cool down to 35–40°C. The temperature for drying the agarose embedded filters is not crucial and can range from 20 to 50°C.
7. For alternatives to permeabilization with 1× TE and a laboratory microwave, see [28].
8. Alternatively incubate filter sections in 50 ml of 0.01 M HCl for 10 min at RT in order to inactivate endogenous peroxidases. Some microorganisms, e.g., from anoxic sediments, may contain peroxidases or enzymes with pseudoperoxidase activity. This can be tested by incubating a filter section in amplification buffer containing H_2O_2 and fluorescently labeled tyramides. Cells with peroxidase activities will show bright fluorescence. These enzymes have to be inactivated, for example, by treatment with hydrochloric acid.
9. The ratio of hybridization buffer to probe working solution (50 ng μl^{-1}) used in FISH is generally 300:1.
10. Alternatively to a laboratory microwave, you might also use a conventional hybridization oven. Length of hybridization time has to be adjusted accordingly (at least double the time as with a laboratory microwave).
11. Gently shaking the tube with 1× PBS during washing for 15 min at RT assists with the removal of unbound probe from the filter sections.
12. The volume of labeled tyramide added strongly depends on the nature of the sample. A ratio of 1:1,000 of fluorochrome to amplification buffer is generally sufficient to get bright signals. If hybridization signals are not sufficient (*see* **Note 16**), increase/decrease the ratio of added tyramide.
13. After washing in 1× PBS, you can remove excess liquid by dabbing filter sections on blotting paper, but do not let filter sections run dry before transferring into the amplification mixture.
14. After transfer of filter sections into amplification mixture, keep filters always protected from direct light due to presence of light-sensitive fluorochrome.

15. On white polycarbonate filters, background fluorescence after DAPI staining is always somewhat worse than on black membrane filters. Black filters, however, show high levels of background fluorescence at green excitation. Use shorter DAPI staining time and/or longer ethanol washing to improve background. Make sure that hybridized filters have been thoroughly rinsed in distilled water before DAPI staining.
16. Enumeration of cells might be hindered by:
    (a) High background fluorescence due to:
    - Too high tyramide concentration. Either decrease the tyramide concentration or increase the blocking reagent concentration.
    - Too high probe concentration. If the background is covered with tiny fluorescent dots, check the probe concentration; 0.2 ng μl^{-1} is plenty.
    - Too short washing after CARD. Prolonged washing in deionized water and/or several changes with freshwater may help.

    (b) Low signal intensity due to:
    - Low ribosome content of the target cells. Increase the tyramide concentration or the temperature during the tyramide signal amplification. A prolonged hybridization time (up to 15 h) may also help.
    - Too low tyramide concentration. Increase tyramide concentration.
    - The probe-delivered HRP has too low or no activity. Check the probe for age; the probe should be thawed only once and should not be stored in the fridge for more than 6 months. Also the pH of the PBS should be around 7.6. Check the H_2O_2 concentration and its age and the reactivity of the tyramide.
    - The HRP is badly coupled to the probe. The amount of unlabeled oligonucleotide can be estimated spectrophotometrically.
    - The HRP-labeled probe cannot penetrate the cell wall. Try different permeabilization protocols.

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