Microscopy and Fluorescence In-Situ Hybridization mini-study of four contaminant-degrading enrichment cultures

and

Biofilm formation study of the KB-1 and T3L cultures using confocal microscopy

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Abstract

Four environmentally relevant and contaminant-degrading enrichment cultures (KB-1, ACT 3, T3L, and ORCH4) from the Edward’s Laboratory at the University of Toronto were studied via traditional microscopy, laser scanning confocal microscopy, and scanning electron microscopy. Also, general bacteria and archaea probes were tested for all these cultures to determine their suitability for continuous monitoring by Fluorescence In-situ Hybridization (FISH). For the culture ACT 3, a Dehalobacter specific probe (Dhb_4) was designed in-silico and experimentally tested in order to determine the morphology of the Dehalobacter species in this culture as well as in the sub-culture WL 1,2 DCA. Based on the preliminary results obtained, the relative abundance of Dehalobacter in ACT 3 and WL 1,2 DCA was 27 and 39%, respectively. Biofilm formation for the cultures KB-1 and T3L was also studied via laser scanning confocal microscopy. Biofilm was identified in both cultures; however, KB-1 produced larger and thicker biofilms in the time period studied.

1. Introduction

Groundwater represents about 30% of the world’s freshwater supply. Past industrial activities involving improper disposal of wastes, spills, and others have caused aquifer contamination with a variety of chemical compounds. Thus, soil and groundwater contamination is a major concern. Biodegradation processes can help restore contaminated environments when adequate biological principles and engineering techniques are applied. Many soil and groundwater pollutants can be biodegraded under anaerobic conditions which are frequently present below the ground surface.

At the Edward’s Laboratory at the University of Toronto, we want to understand the microbial interactions and the diversity of microorganisms that can help to anaerobically biodegrade various contaminants such as: trichlorethene (TCE), trichloroethane (TCA), toluene, benzene, chloroform, polychlorinated biphenyls (PCBs), and a variety of pesticides, among others. Several cultures have been enriched through time to anaerobically biodegrade some of these compounds. The culture KB-1, composed mainly of organo-halide respiring bacteria (OHRB) from the genus Dehalococcoides Mccartyi, acetogenic bacteria, and methanogenic archaea, reductively dechlorinates TCE to ethene [1]. KB-1 is fed methanol and TCE. The culture ACT 3, composed primarily of Dehalobacter, Desulfovibrio,
and *Methanosarcinales* [2], is fed methanol, ethanol, and lactate as electron donors, and 1,1,1 trichloroethane (TCA) as electron acceptor. The culture T3L is a methanogenic consortium, containing organisms from the order *Methanosaeta*, *Methanospirillum*, and others, that uses toluene as an electron donor and CO₂ as electron acceptor [3]. The fourth culture, ORCH₄, utilizes benzene as an electron donor and CO₂ as an electron acceptor. This culture has organisms from the subphylums Deltaproteobacteria and Firmicutes, as well as methanogenic archaea [4]. TCE, TCA, benzene, and toluene are all currently included in the list of toxic pollutants of the Environmental Protection Agency (EPA) in the U.S.

Recently, the Edward’s laboratory has developed an interest in exploring PCBs anaerobic biodegradation. PCBs are toxic, generally very hydrophobic, compounds with a wide variety of adverse health effects [5, 6]. There are few bacterial strains and/or phylotypes that have been shown to have PCB dechlorinating activity; these include the isolates ‘*Dehalobium chlorocoercia*’ DF-1 (marine specie), *Dehalococoides mccartyi* CBDB1, and *Dehalococoides mccartyi* 195, as well as strain o-17 (co-culture), and the Chloroflexi phylotypes DEH-10 and SF-1 [7]. Some researchers have studied the biofilm formation of *Dehalococoides* containing microbial consortia in membrane biofilm bioreactors [8-10]. Although these studies have shown successful *Dehalococoides* growth, they are intended to treat groundwater contamination ex-situ. Nonetheless, in-situ bioremediation strategies can be more beneficial and cost-effective. In order to biodegrade PCBs in-situ, I hypothesize that a PCB-degrading microbial consortium should be present at and/or delivered to the soil and sediment interface, ideally forming biofilms (around the mineral grains) that can progressively dechlorinate these compounds.

For the Microbial Diversity 2013 course’s mini-project, I performed a mini-study of the four enrichment cultures mentioned above using microscopy and Fluorescence In-situ Hybridization (FISH). The objective of this mini-study was to understand the diversity and roles of the microorganisms forming these cultures as well as to learn an array of techniques to monitor enrichment cultures such as microscopy, gas and liquid chromatography, and isolation. I also performed a biofilm formation study on the KB-1 and T3L cultures using confocal microscopy.

## 2. Materials and Methods

### 2.1 Microscopy

Samples were taken from all cultures and observed with phase contrast microscopy and Differential Interference contrast microscopy (DIC). One or two drops of sample were placed on glass slides for direct observation. The samples were excited with light at 420 nm to observe the methanogens. Methanogens contain variable amounts of coenzyme F₄₂₀ which fluoresces when excited at 420 nm [11]. Scanning electron microscopy was used to visualize *Dehalococoides* in KB-1. Sample preparation involved fixing 50 µl of culture in 10 ml of 3% glutaraldehyde in 1X PBS overnight, filtration through a 0.2 µm polycarbonate membrane filter, sequential filter drying in 50% (10 min), 70% (10 min), 85% (10 min), 95% (15 min), 100% ethanol (15 min), critical point drying (performed with assistance from the staff of the Central Microscopy Facility at the MBL), platinum coating, and posterior imaging.

### 2.2 Enrichment for methanogens and acetogens in the KB-1 culture

For the culture KB-1, methanogens and acetogens enrichments were performed. Serum bottles with anaerobic medium were prepared as detailed in Chapter 8 of the Microbial Diversity manual. I performed a 20% V/V transfer into fresh medium from the KB-1 culture to a final volume of 60 ml.
Bottles were prepared by triplicate, 3 bottles enriching for methanogens with 62 mM of methanol and 3 bottles enriching for acetogens with 5 mM of acetate. To enrich for methanogens and acetogens, rifampicin and bromoethanesulfonic acid (BES) was added, respectively. Methane and acetate production was monitored by gas and liquid chromatography, respectively (please refer to Microbial Diversity manual for sample preparation details).

### 2.3 Fluorescence in Situ Hybridization (FISH)

A preliminary FISH assessment was performed for the following cultures: KB-1, ACT-3, T3L, and ORCH4. For these cultures, general FISH archaea (Archaea 195) and bacteria (Eub I – III) probes were tested. We wanted to determine if FISH studies are potentially suitable for theses cultures. For the culture ACT 3, two specific probes, Dhb_210 and Dhb_442, were designed for *Dehalobacter* using ARB and following some guidelines outlined by Behren et al. 2003, Kumar et al., 2005 and Kumar et al., 2006 [12-14]. The probes were purchased from Biomers (biomers.net). After the preliminary tests, a subculture derived from ACT 3, WL 1,2 DCA, was also tested to verify probe hybridization. This culture also contains *Dehalobacter*; it is fed 1,2 dichloroethane and hydrogen as electron donor.

**Fixation of culture samples**

For cell fixation, aliquots of each culture were placed in 10 ml of a 1% paraformaldehyde solution in 1X PBS. For KB-1, ACT 3, and T3L, 0.1 ml of culture were used. For ORCH4, 1 ml of culture was fixed. The fixation was performed for 2 hours at room temperature. The fixed solution was filtered into 0.2 μm (25 mm) polycarbonate membrane filters mounted on a vacuum filtration system with 0.45 μm cellulose nitrate support filters. 30 ml of Mili-Q water were applied after each sample filtration to rinse the fixation tubes and the filtration tower. Then, the filters were air-dried and store in petri dishes covered with parafilm in the -20°C freezer.

**Hybridization of fixed cells [15, 16]**

For the hybridization step, filter sections (1/8 of 25 mm filter) were cut and placed in parafilm covered glass slides. A preliminary test was performed with filters from the ACT-3 culture using probes Dhb_210 and Dhb_442 and 10% formamide hybridization buffer. The probes were originally diluted to 100 pmol/μl and aliquots were taken to obtain working solutions of 10 pmol/μl. For the hybridization step, 18 μl of hybridization buffer were mixed with 2 μl of probe working solution and applied directly to each filter section. The hybridization buffer consisted of 5M NaCl (900 mM), 1 M Tris/HCl buffer (20 mM), formamide (at corresponding percent volume concentration, e.g. 10%), 1 μl of 20% SDS (0.01%) and Mili-Q water to a final volume of 2 ml. The filter sections (on the surface of the glass slide) were placed in 50 ml falcon tubes. Each falcon tube had a piece of paper tissue soaked in the remainder hybridization buffer to keep the falcon tube’s atmosphere saturated with the buffer solution. The hybridization step was performed for 90 min at 46°C. The filters were removed and washed for 15 min at 46°C in pre-warmed washing buffer solutions (20mM Tris/HCl, 5 mM EDTA, 0.01% SDS, 0.45 mol NaCl for 10 % formamide hybridization buffer). The protocol followed is described in more detail in the 2013 Microbial Diversity course manual as well as in Glockner et al., 1996. The washing buffer was poured into petri dishes and the filters were retrieved and washed with sterile Mili-Q water for 10 seconds. After air-drying, the filters were mounted on glass slides with a 4:1 mix of Citifluor and Vectashield containing 1 μg/ml of DAPI. The filters were observed on a Zeiss Imager A.2 microscope equipped with a camera under a 100X magnification objective with a 1.4 numerical aperture.
### 2.4 Biofilm formation on glass cover slips

Glass cover slips were placed in 50 ml falcon tubes containing 15 ml of KB-1 or T3L enrichment cultures. The falcon tubes were kept anaerobic in the anaerobic glove box. The cover slips were removed from individual falcon tubes at \( t = 4, 9, \) and 15 days. The cover slips were placed in 10 ml of 4% paraformaldehyde solution overnight at 4°C. Then, the cover slips were washed in a solution of 1X PBS followed by sterile Mili-Q water. Once dried, one side of the cover slip was carefully wiped with ethanol to remove biofilm formed on one side. The cover slip was mounted on a glass slide (biofilm side down) with 3 µl of a 4:1 mix of Citifluor and Vectashield with DAPI at a concentration of 1 µg/ml. After, the glass slides were imaged using confocal microscopy with a Zeiss Laser Scanning Microscope (LSM) model LSM-700.

### 3. Results and discussion

#### 3.1 Microscopic observations of the enrichment cultures

Figures 3.1 to 3.4 show some of the images obtained for each culture. KB-1’s and ACT 3’s methanogens fluoresced intensely. KB-1’s morphology is dominated by rod-shaped cells. ACT 3 contains cocci and rods. Methanosarcina was observed in samples from ACT 3. Methanosaeta was observed in the T3L culture. The culture ORCH4 was difficult to observe under phase contrast; DAPI staining showed that small rods and cocci are present.

![Image](image.png)

Figure 1. KB-1 culture. Left: Methanogens that fluoresce when excited at a wavelength of 420 nm. Right: transmitted light image of the same frame area as the image on the left.
Figure 2. ACT 3 enrichment culture. Left and right: 100X phase contrast images.

Figure 3. T3L enrichment culture. Left: 100X phase contrast image. Right: DIC 63X image.

Figure 4. ORCH4 enrichment culture. Left: 100X phase contrast image. Right: DAPI stained sample on filter piece.
3.2 KB-1’s enrichments

As expected, methane production was observed in the bottles enriched for methanogens as detailed in figure 5. Acetate concentrations increased in the bottles enriched for acetogens and decreased slightly in the bottles enriched for methanogens as shown in figure 6. Figures 7 and 8 show microscope observations of KB-1’s enrichment. Methanosarcina was observed to bloom under the enrichment conditions probably due to the high concentration of methanol added. The acetogens had a rod-shape morphology and were best observed under the DIC channel.

Figure 5. Methane production by KB-1’s methanogens. Error bars represent the standard deviation of the mean.

Figure 6. Acetate production and consumption by KB-1’s acetogens and methanogens, respectively. Error bars represent the standard deviation of the mean.
Figure 7. Images obtained from a sample from KB-1’s methanogenic enrichment. Top left: methanogens under 100X phase contrast. Top right: Methanosarcina. Bottom left: Methanosarcina imaged with Laser scanning confocal microscopy, 63X. Bottom right: 3D model of Methanosarcina aggregate, maximum thickness observed = 6 µm.

Figure 8. DIC 63X images of the acetogens’ enrichments.
Given the importance of *Dehalococcoides Mccartyii* sp. In KB-1, I decided to perform Scanning Electron Microscopy (SEM) on samples from the KB-1 culture. A screen for *Dehalococcoides* was performed based on known published information revealing its morphological features [17]. Through SEM, I could confirm that *Dehalococcoides* in KB-1 also possesses the characteristic bi-concaved disc shape morphology and it is about 0.4 µm in diameter. Figure 9 shows some of the SEM images obtained.

![SEM images of *Dehalococcoides* in the K-B1 culture (disc-shaped cells).](image)

### 3.3 FISH results

FISH using the probe EUB I-III and archaea was successful for all the cultures, see appendix 1 for sample images of each culture. I performed cell counts on one piece of ACT 3’s filter for the Eub I – III and archaea probes. For the EUB I – III probes, I found 6.67 E 7 DAPI stained cells/ml of culture and 3.04 E 7 hybridized cells/ml of culture for a relative abundance of 46%. For the Archaea 915 probe, I found 5.37 E 7 DAPI stained cells/ml of culture and 2.3 E 7 hybridized cells/ml of culture for a relative abundance of 43%. The preliminary test performed with probes Dhb_210 and Dhb_442 revealed that probe Dhb_442 produced successful hybridizations as observed in figure 10. Dhb_210 did not.

![FISH images showing hybridizations](image)
I performed further testing of probe Dhb_442, according to the experimental matrix shown in table 1.

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Table 1. Experiments performed with probe Dhb_446.

The quality of the signal, indicating successful and easily recognizable hybridization, was best with 10% formamide hybridization buffer for ACT 3 and 30% formamide concentration + helper oligos for WL 1,2 DCA. Figure 11 shows the visual results of these hybridizations. For ACT 3, 6.73 E 7 DAPI stained cells/ml of culture and 1.82 E 7 probe Dhb_442 hybridized cells were observed. For WL 1,2 DCA, 2.2 E 7 DAPI stained cells/ml of culture and 8.55 E 6 probe Dhb_442 hybridized cells were observed. The previous results are equivalent to Dehalobacter relative abundances of 27 and 39% for ACT 3 and WL 1,2 DCA, respectively. In ACT 3, Dehalobacter was observed to have a rod-shaped morphology, forming occasional short filaments of two to three cells. In WL 1,2 DCA, it was observed as thin rods mostly forming long filaments often forming closed loops. Further tests with probe Dhb_442 and through analysis of 454 pyrosequencing data should confirm if ACT 3 and WL 1,2 DCA contain identical strains of Dehalobacter. The fact that the culture ACT 3 responds better to a 10% formamide concentration could be indicative of mismatch hybridization, i.e. as the formamide hybridization concentration is increased higher specificity (mismatch absence) to the probe sequence is expected. The negative control experiment showed that probe Dhb_442 doesn’t bind to other bacteria. However, hybridization “artifacts” or “impurities” were detected, i.e. bright dots are often observed which could be mistaken for bacterial cells. Hence, in all tests conducted, the hybridized cells were confirmed as “true cells” by observing them DAPI stained as well.
3.4 Biofilm formation for the cultures KB-1 and T3L

KB-1’s biofilm was first observed at $t = 4$ days. For T3L, biofilm was first observed at $t = 9$ days. As time progressed, the thickness of the biofilm increased. Nonetheless, greater biofilm coverage was observed for KB-1. The biofilm develop on different areas in the cover slips. Figures 12 and 13 show the development of the biofilm for these cultures. However, it is important to note that even though biofilm was clearly observed, it was not uniform or abundant throughout the cover slip. In principle, we could show that biofilm growth indeed occurred but further testing is necessary to understand how this biofilm develops, what factors may enhance it, how does it correlate with culture growth and product biodegradation, among others. It would be very interesting to perform an extensive search of biofilm related genes in the metagenome of these cultures. A quick survey of KB-1’s metagenome in MG-RAST showed presence of genes involved in exopolysacharide formation.
Figure 12. KB-1 biofilm at $t = 4$ days. Top right and left show different areas of the cover slip, the scale bar represents 5 µm. The bottom images show another of the cover slip, DAPI stained (left) and with transmitted light (right), scale bars are 5 and 10 µm, respectively.
Figure 12. KB-1 biofilm at $t = 9$ days. Top right and left show different areas of the cover slip, each frame is $100 \times 100 \, \mu m$, the scale bar represents $10 \, \mu m$. The bottom image shows a z-stack 3D model of the biofilm in the top right hand side image, the observed maximum thickness was about $4 \, \mu m$. 
Figure 13. KB-1 biofilm (top right) and T3L biofilm (top left) at $t = 15$ days. Each frame is 100 x 100 µm, the scale bar represents 10 µm. The bottom image shows a z-stack 3D model of KB-1’s biofilm, the observed maximum thickness was about 6 µm.

Conclusions

The mini-study performed on the four enrichment cultures from the Edwards’ Laboratory allowed me to better understand the nature of the syntrophic microbial interactions occurring in these cultures. I was also able to learn different culturing, enrichment, monitoring, and isolation techniques available. Due to the time frame of the mini-project, no isolation was completed. We were able to design a *Dehalobacter*
specific probe, probe Dhb_442, which showed good results for the cultures tested. Both cultures showed that the probe targets the *Dehalobacter* organisms. Biofilm development was indeed observed for the cultures KB-1 and T3L and biofilm thickness was successfully monitored by z-stack laser scanning and 3D model construction. I highly recommend this technique for biofilm monitoring studies. Biofilm development on glass slides or cover slips can also be monitored with FISH or CARD-FISH to gain knowledge on morphology, microbial associations, as well as their evolution in time and space.

**Acknowledgements**

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- Special thanks to Sara Kleindienst for teaching me FISH and helping me all through-out the project
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- Family and friends in Canada and Colombia, especially my parents: Oscar and Luz Amparo, and my husband, Kristian Medri.

The Microbial Diversity course was a wonderful, unforgettable experience, and I hope to contribute in the future, both with achievements in scientific research and financial aid to future students if life permits me to do so.

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Figure 8. DIC 63X images of the acetogens’ enrichments.

Figure 9. SEM images of *Dehalococcoides* in the K-B1 culture (disc-shaped cells).

Figure 10. FISH images for probe Dhb_210 (top) and probe Dhb_442 (bottom). From left to right: DAPI, probe, combined channel image. Scale bar represents 10 µm.

Figure 11. FISH images for probe Dhb_442. Top: culture ACT 3 hybridized with a 10% buffer; middle: culture WL 1,2 DCA(bottom) hybridized with a 30% buffer; bottom: negative control with a 10% buffer (KB-1). Scale bar represents 10 µm.

Figure 12. KB-1 biofilm at t = 4 days. Top right and left show different areas of the cover slip, the scale bar represents 5 µm. The bottom images show another of the cover slip, DAPI stained (left) and with transmitted light (right), scale bars are 5 and 10 µm, respectively.

Figure 12. KB-1 biofilm at t = 9 days. Top right and left show different areas of the cover slip, each frame is 100 x 100 µm, the scale bar represents 10 µm. The bottom image shows a z-stack 3D model of the biofilm in the top right hand side image, the observed maximum thickness was about 4 µm.

Figure 13. KB-1 biofilm (top right) and T3L biofilm (top left) at t = 15 days. Each frame is 100 x 100 µm, the scale bar represents 10 µm. The bottom image shows a z-stack 3D model of KB-1’s biofilm, the observed maximum thickness was about 6 µm.
Appendix 1. Mini-project presentation with additional microscopy images