The Berries - revisited

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Introduction

The Purple Berries of Sippewissett are macroscopic aggregates of purple sulfur bacteria, found in the Great Sippewissett Salt Marsh (Falmouth, Cape Cod, MA). They occur only in certain tidal pools, that are both separated from the main marsh channels and have a depth of at least 30 cm. These pools are flooded only at very high tides, but presumably stay oxygenated. The berries have a characteristic purple color and vary in size between 1 and ~10 mm, most are found on top of the sediment.

Previous research in the Microbial Diversity class mainly focused on microscopy and physiology. The berries mostly consist of tight clusters of coccoid purple sulfur bacteria (PSB). This assumption is based on the purple pigments of the cell, the intracellular sulfur granules and the presence of bacteriochlorophyl a in the extract (Seitz et al., 1993; Sharp, 2001). In addition a number of rod-shaped bacteria, long filamentous cyanobacteria, diatoms and even nematodes can be found within the berries, although their presence is limited to the channels between the PSB clusters (Banin, 1997).

Molecular studies of the microbial community of the berries yielded somewhat contradictory results. Most 16S sequences belonged to Cytophaga and sulfate-reducing bacteria (SRB), while only one PSB clone could be found (Banin, 1997). Although these results allow proposing a microbial food-web, where sulfur is cycled between SRB and PSB, the question who is the main constituent of the berry still remains unanswered on a molecular level.

A straightforward explanation for the apparent lack of PSB sequences in previous libraries is the tight clustering of the PSB within the berry facilitated by an uncharacterized extracellular matrix. This matrix could prevent effective DNA extraction and therefore lead to the skewed 16S libraries reported previously.

The goal of this study was the development of a rigorous DNA extraction method, which would lead to representative 16S libraries and the characterization of the extracellular matrix.

Materials and Methods

1. Sampling Site

Great Sippewissett Salt marsh is located at 41◦35'N and 41◦40'W along the western shore of Cape Cod, Massachusetts, USA. Samples were exclusively collected from a single pool marked "X" in Figure 1. The pool is an oval of about 1.5 by 1 m, the water depth is about 30 cm. It is a slightly elevated compared to the surrounding marsh land.

The water is clear and oxygenated. The berries were found along the western side on top of the sediment and collected by hand. Most were about 3-10 mm in diameter. Batches of ~20 berries were stored in 50 ml Falcon tubes in pond water ~20 cm from a light source at room temperature. The berries did not change their shape or appearance during the three week period of the experiments.

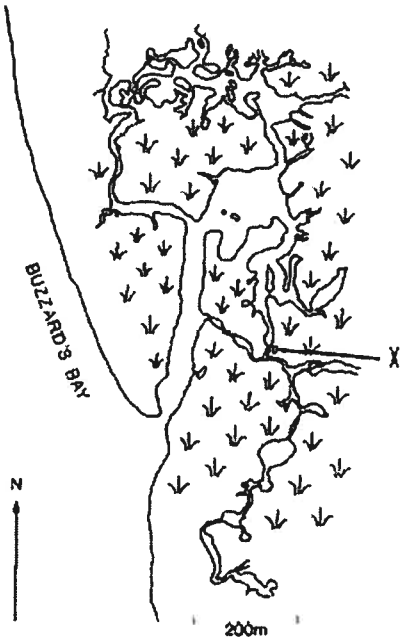

Figure 1: Great Sippewissett Salt Marsh. The berries were sampled from a pool marked X on a slightly elevated peninsula (after Seitz et al., 1993)

2. DNA extraction

"old school" Phenol-Chloroform extraction

One berry was ground to a fine powder with dry ice in a pre-cooled porcelain mortar. The powder was incubated in 3 ml Lysis buffer (50 mM Tris pH 8, 50 mM EDTA, 350 mM NaCl, 2 % Sarcosine, 8 M Urea) for 10 min at RT. The nucleic acids were isolated and cleaned by a standard Phenol-Chloroform-Isoamylalcohol 25:24:1 extraction sequentially incubating for 60, 30 and 15 min. From the final aequous layer the DNA was then precipitated by adding 1/10 vol 3M NaOAc and 2 vol EtOH and centrifuging at 10,000 g for 20 min. The pellet was washed twice with 70% EtOH and dried in the flow hood for 15 min. The DNA/RNA was resuspended in 1 ml TE-Buffer (10 mM Tris-Cl, 1mM EDTA pH 8) overnight.

MoBio soil kit, MoBio plant kit

The berry was homogenized in an Eppendorf tube with a fitting steril pestle (South Jersey Precision Tool and Mold Inc. 749521-1590) in 200 µl TE-buffer pH 8 for about 5 minutes until the largest remaining chunks were less than a millimeter. The DNA was extracted according to manufacturer's recommendation, except that the initial bead-beating step was prolonged to 5 minutes. The soil kit is intended to be used on humic

acid-rich samples, the plant kit is better in degrading waxy cuticles and the extensive polysaccharides encountered in plants.

3. PCR

The 16S gene was amplified from all three samples using general eubacterial 8F and 1492R primers. For tRFLP a FAM labeled 8F primer was used. The PCR was run with 60C annealing temperature and 1:30min extension times for 30 cycles.

The presence of PSB was checked by PCR with a PSB-specific 25F (AGA GTT TGA TCM TGG CTC) and the general 1492R primer using the same cycling conditions, but an annealing temperature of 55C.

4. tRFLP

The FAM-labeled PCR product was digested with Rsa I at 37C for 3 hours, cleaned on Micro-Con filters, eluted with 10 mM Tris-Cl, dried down in a speed vac and sent to Michigan State University for the capillary run.

5. Cloning and Sequencing

The PCR product was cloned into the TOPO-TA 2.1 vector (Invitrogen) according to manufacturer's recommendation and plated on LB-Amp-Xgal. White colonies were picked and grown up in 200 micl LB-Amp. The cells were spun down at 3000g for 1 hr, the pellet resuspended in 100 micl 10 mM Tris-Cl and boiled for 15 min. 1 µl of the lysed cells were cleaned up for sequencing using ExoSap-It (Amersham) and sent off to Michigan State University.

6. Berry dissociation

A single berry was homogenized as described under MoBio kit in 2. The solution used in the homogenization was the same as the one in the following incubation step. The solutions were buffered with 10 mM MES for pH 5 or 10 mM Hepes for pH 7 or pH 8. The berries were incubated overnight with cellulase, sulfatase, chitinase at pH 5 and 7, 1M Urea at pH 5 and 7 and 10 mM EGTA pH 8. For the aggregation experiment an equal amount of CaCl2 was added to the EGTA tube.

Dissociation of the berries was monitored microscopically and documented via digital photography.

Results and Discussion

1. DNA extraction

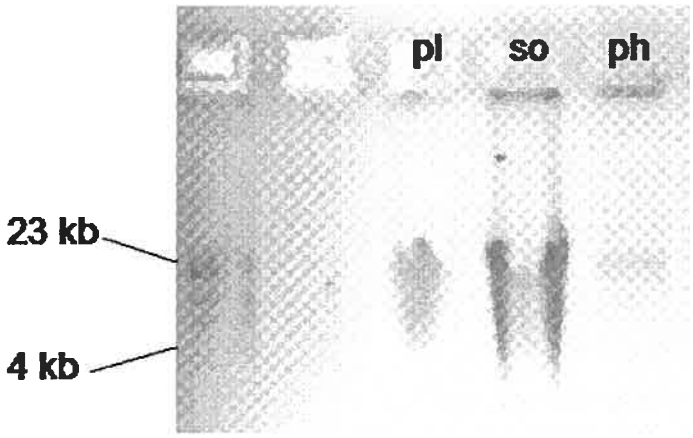

Figure 2: DNA preparations.
pl = MoBio plant kit
so = MoBio soil kit
ph = Phenol-Chloroform extraction

DNA was successfully isolated from the berries with all three techniques. The intensity of the Phenol-Chloroform band is lower because only 1/100 of the total DNA was loaded (1/10 in the other two lanes). The size of the DNA in the range of ~3 to >25kb is suitable for PCR amplification.

2. 16S PCR

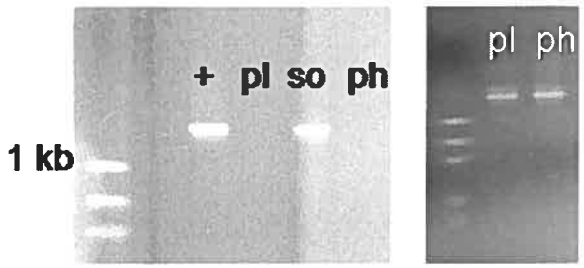

Figure 3: 16S PCR

The PCR amplification using eubacterial 8F and 1492R primers was successful. The right panel uses 1 micl of template per 50 micl reaction, the amount had to be adjusted to 0.1 micl for the plant kit and phenol-chloroform preparations. This is an indication for inhibitors still present in the DNA preparations.

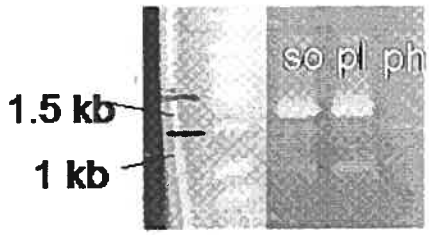

Figure 4: Purple Sulfur Bacteria-specific 16S PCR

The PSB primers give a product of the expected size in the MoBio kit preparations. There are minor bands, which could probably be removed by using a higher annealing temperature. This result was not followed upon due to time constraints.

3. tRFLP

A FAM-labeled 8F + the general 1492R and a Rsa I digest was used for tRFLP analyses of the three DNA preparations.

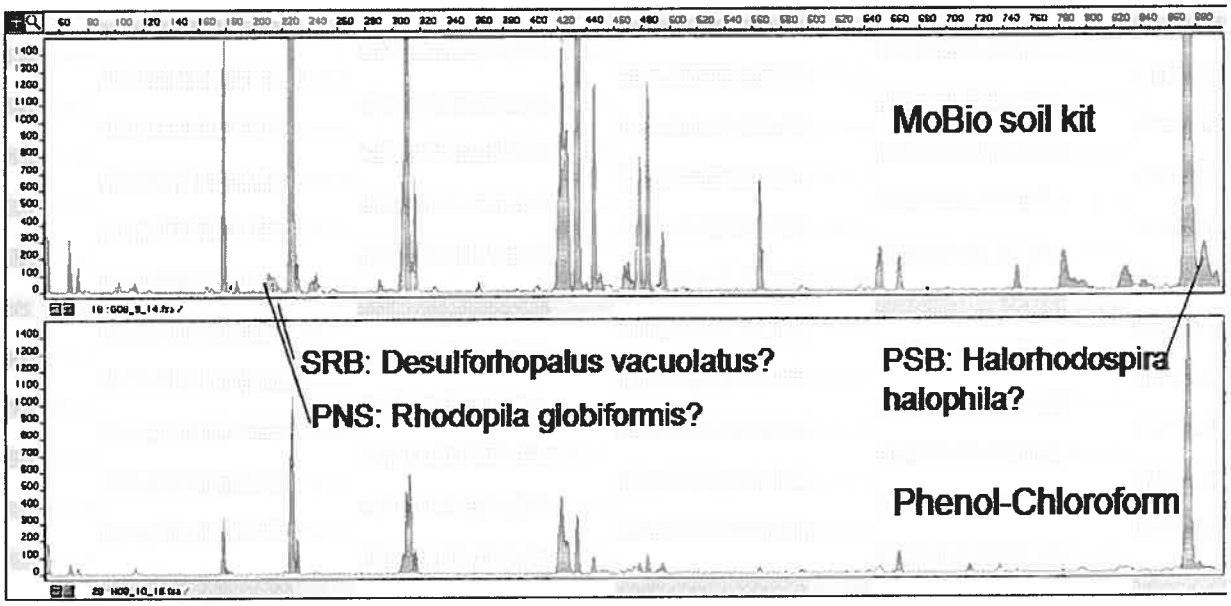

Figure 5: tRFLP

The tRFLP gives a total of 56 peaks, which corresponds to at least 56 species/strains present in the berry (there might be multiple organisms with the same Rsa I restriction pattern). The diversity is much more complex than expected. Previous DGGE experiments (Banin, 1997) had found, that ~10 species constituted the berry community. Not all of the 56+ species are necessarily an integral part of the berry. Despite of the intensive rinsing with dH20 from the outside, a considerable number of environmental organisms must be present within the porous berry structure. To address this question one would have to repeat this experiment several times and only count peaks, that are consistently present, as members of the berry community. In addition, running a tRFLP

on the surrounding pond water would allow subtraction of the resulting peaks as contamination. The TAP-tRFLP program from the RDP website (http://rdp.cme.msu.edu) can be used to assign possible species to the peaks. The designation is preliminary and has to be backed up by other experimental evidence. Putative PSB, SRB and PNS peaks can be observed.

There are less peaks in the Phenol-Chloroform preparation (lower panel), however since the overall peak height is lower (i.e. less DNA was loaded into the capillary), the smaller peaks simply did not show up. Therefore MoBio Soil kit and Phenol-Chloroform are assumed to perform similarly and quite satisfactory. The results from the plant kit, where one could expect more diversity due to its optimization for dealing with complex polysaccharides, did not arrive in time for this report.

4. Cloning and Sequencing of 16S genes

Due to time constraints, it was not possible to wait for the tRFLP results before deciding which library to use for 16S cloning and sequencing. The MoBio soil kit was picked randomly. Only one out of the 96 sequencing reactions gave a readable result. The sequence is 87% similar to Spirochaeta isovalerica. The identity (or even the presence) of purple sulfur bacteria in the berries remains mysterious.

5. Examining the berry matrix: Hydrolases and pH

Berries homogenized with a pestle in an Eppendorf tube still maintain dense clusters of purple cells (Fig 6). Seitz et al. showed that treatment with Triton X/Tween or SDS/Proteases had no effect on the integrity of the berry, arguing against hydrophobic molecules or proteins as the main constituents of the matrix, that keeps the cells in clumps. Polysaccharides are a possibility. To address this, mashed berries were incubated with crude preparations of Hydrolases (Chitinase, Sulfatase, Cellulase) at varying pH. Even high concentrations of an enzyme mix (100 u/ml cellulose, 1 mg/ml SIGMA-Sulfatase) seemed to have just a minor effect on the berries. Chitinase seemed to encourage the growth of contaminating heterotrophs, presumably because the enzyme preparation was very crude and consisted mainly of "tasty nutrients".

Incubating the berry fragments at pH 5 (buffered with 10 mM MES) overnight loosened up the cell clusters considerably (Fig 7). The fragments became more fluffy and increased in volume. The cells also lost some color and became orange, however most of them seemed to survive under these conditions (highly retractile, Fig 7 cells to the left). The dissociation was not quantitative, a lot of clumps, both of live and dead cells remained. Longer incubation allowed small highly motile rods to take over the culture (Fig 8).

6. Examining the berry matrix: EGTA

Contrary to the results from Seitz et al. incubation in 1M Urea overnight at pH 5 or 7 did not dissociate the cells. This incubation did turn all cells black, i.e. they were presumably dead.

Ethylene glycol-bis(2-aminoethyl)-N,N,N¢,N¢-tetraacetic acid (EGTA) is a chelating agent specific for Ca^{2+}. It is strong enough to remove Ca^{2+} from biogenic assemblages. Incubation of a thoroughly mashed berry with 10 mM EGTA pH 8 completely dissociated the berries and freed the cells (Fig 9).

The process is reversible, after addition of 10 mM CaCl2 to the dissociated cells, they started to form clusters of 100s of cells overnight (Fig 10). The clusters are not as dense as the original berry.

If India Ink is applied to the slide, the ink rapidly diffuses through the water channels between the cluster, but the ink particles cannot enter the space between the cells (Fig 11). This clearly indicates the presence of some material that fills the intercellular space in the loose clusters.

The drastic effect of EGTA and the possibility to encourage cell clustering suggests, that Ca^{2+} plays a major role in maintaining the integrity of the berry, presumably by forming ionic bridges between some kind of polymer (Fig 11). This is consistent with the reaction on acidic pH. The nature of this matrix-forming polymer is still elusive. A complex polysaccharide(mixture) is very suggestive.

The complete dissociation of the cells allows counting the supposed purple sulfur bacteria (the highly refractile coccoid cells). In a medium sized berry (~5 mm diameter) there are 4x10^6 cells. This number seems rather low, however one has to take into account, that a berry is not a massive colony of bacteria. There are a lot of water-filled channels, openings and chambers.

Summary and Conclusions

An extraction with standard methods gives a reasonable amount and quality of DNA. The key step is probably an initial thorough physical shearing of the berry. A special pestle with a fitting Eppendorf tube or grinding frozen berries with dry ice worked well. The diversity of the berry community is surprisingly high. 56 peaks were observed in tRFLP. Attempts of sequencing 16S genes and thereby identifying purple sulfur bacteria failed.

The matrix of the berries seemed at least partially based on ionic interactions. Removal of Ca^{2+} lead to complete dissociation of the berry. The process was reversible in the laboratory. This dissociation sets the stage for future culturing attempts. Known numbers of cells could be transferred directly into agar shakes selecting for PSB. If a significant percentage of the cells form colonies, these are prime candidates for the main berry-forming bacteria.

The matrix forming polymer could be a polysaccharide. A more thorough examination of the effects of different Hydrolases is needed. Another somewhat exotic, but not unprecedented possibility is DNA, which could be tested by Nuclease treatment.

References

Banin U, 1997 Microbial Diversity Mini-Project Report

Seitz A, Nielsen T and Overmann J, 1993. Physiology of purple sulfur bacteria forming macroscopic aggregates in Great Sippewissett Salt Marsh, Massachusetts. FEMS Microbial Ecology 12: 225-236

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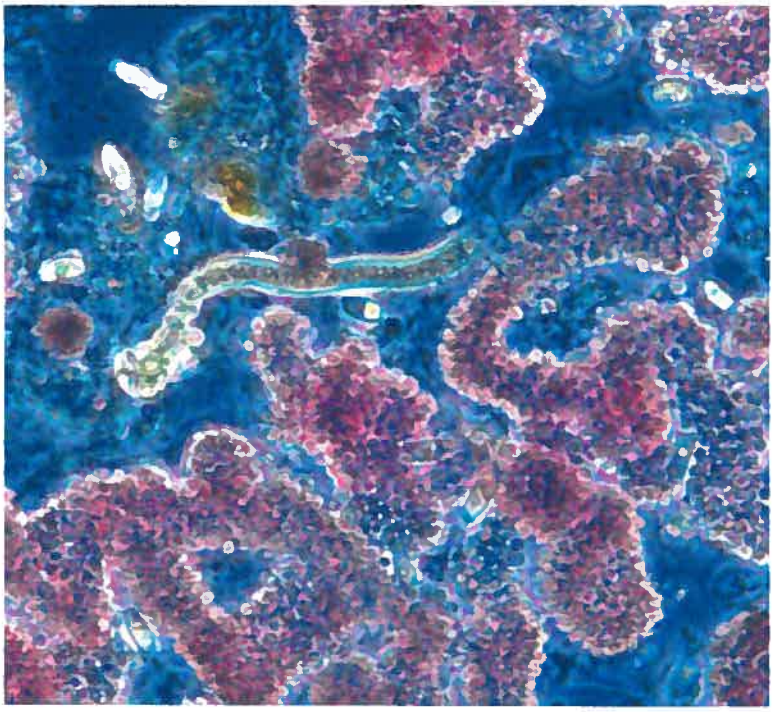

Figure 6: 10x phase contrast of mashed berries

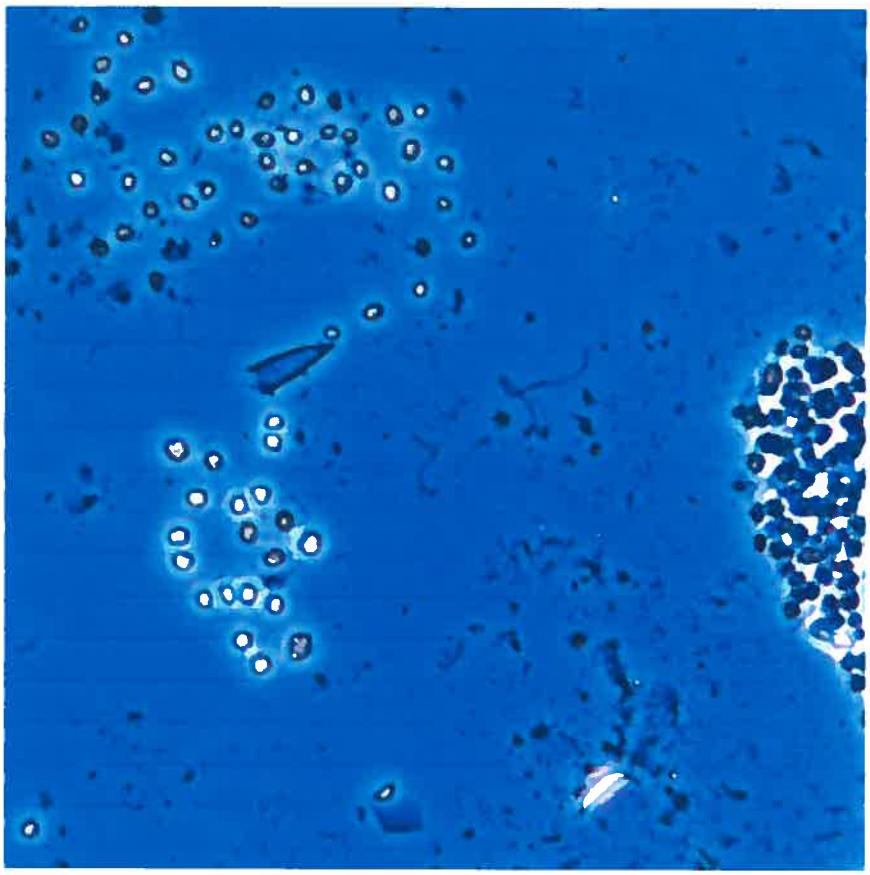

Figure 7: 40x phase contrast of dissociation with pH 5 overnight

Figure 8: ...after two days

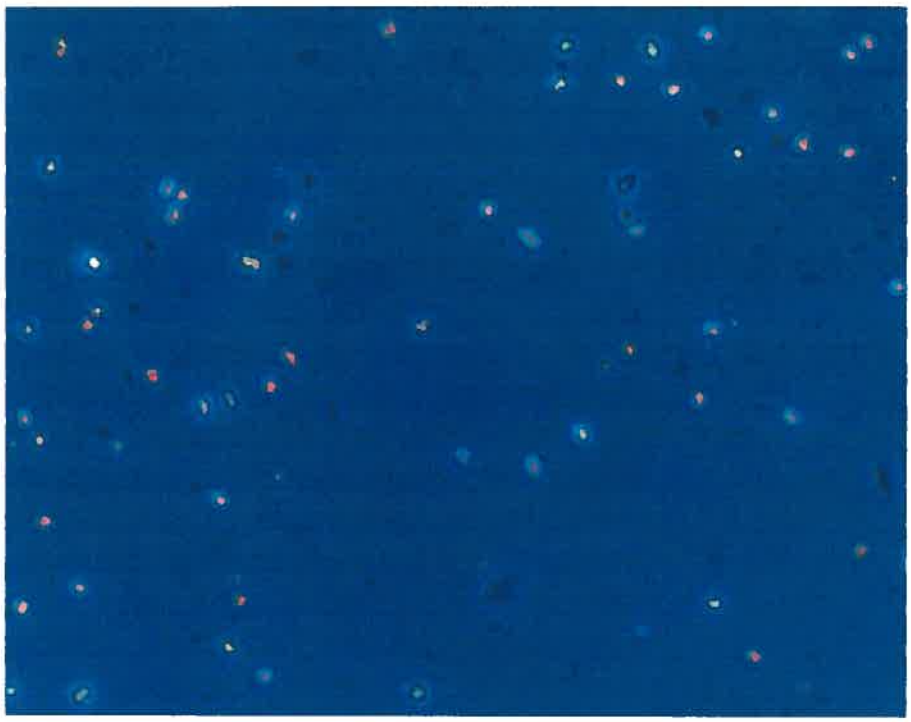

Figure 9: 40x phase contrast of dissociation with 10 mM EGTA overnight

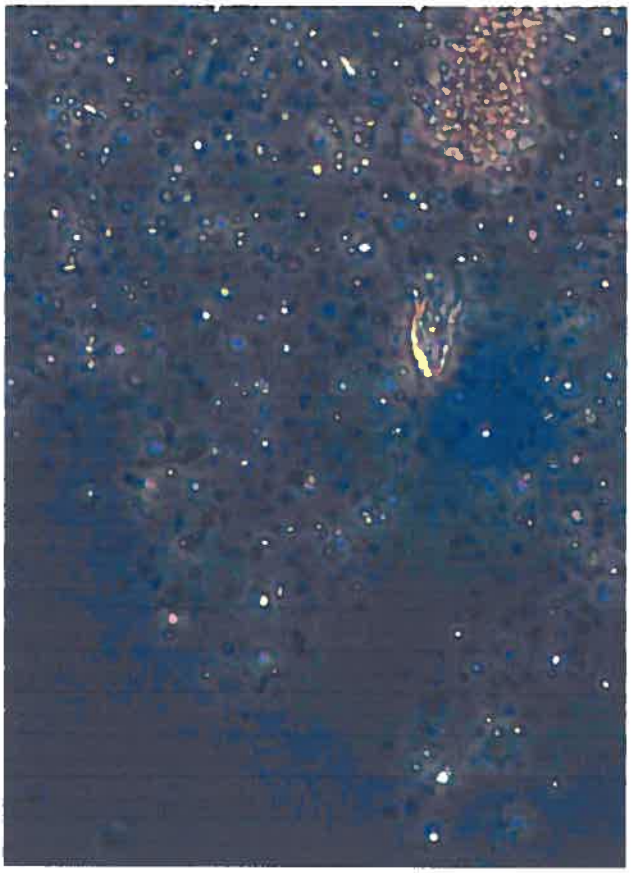

Figure 10: 10x phase contrast of reaggregation after addition of 10mM $CaCl_2$

Figure 11: 10x phase contrast. The India Ink test