Group II Report: Berries

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Abstract

The goal of the present study was to investigate the berries, macroscopic purple or pink aggregates found in selected shallow pools in the Sippewisset Salt Marsh. We aimed to identify the bacterial species present in the berries and to determine how and/or why they are formed. Cloning and sequencing of 16S rDNA extracted from the berries suggested the presence of the following phylogenetic groups : purple sulfur bacteria (most closely related to *Thiorhodococcus minus*), sulfate reducing bacteria, and cytophaga. Enrichments and whole cell fluorescent *in situ* hybridization supported the presence of the sulfate-reducing bacteria and cytophagas, and also indicated the presence of green sulfur bacteria. Field observations in the salt marsh led to the hypothesis that mussels are involved in the formation of the berries or berry precursors. Atlantic ribbed mussels (*Geukensia demissa*) were observed expelling berry-like pink material. The mussels in the salt marsh may ingest the bacterial components of the berries, but reject them as pseudofeces. The slime matrix that contributes to the aggregation of the bacterial species in the berry may be derived from mucus produced by the mussel. The results of laboratory simulations with the mussels strongly suggested that these molluscs play a role in the initial formation of the berries and subsequent incubation in the inlet or pond is necessary for the development of a mature berry.

1.0 Introduction

The focus of our research was the "berries" from the Sippewisset Salt Marsh. The berries are macroscopic (1 - 4 mm diam.) purple or pink aggregates found lying on and in the top layer

of sediments within selected shallow pools in the marsh. The goals of our research were to answer the following questions:

1. What bacterial species are present in the berries?
2. How /why are the berries formed?

In order to address these questions an approach encompassing enrichment cultivation, molecular characterization, microscopy, field observations and laboratory simulations was developed.

Previous students in the Microbial Diversity Course have attempted to characterize the berries. Seitz et al. (1993) reported that the dominant species in the berries were purple sulfur phototrophs, embedded in a slime matrix which appeared to stabilize the aggregate. They also observed many small rod-shaped bacteria, along with some spirochetes and diatoms, within the slime. Attempts to disintegrate the slime with proteolytic enzymes and glucoronidases were unsuccessful; the authors speculated that it was composed of complex polysaccharides. During the 1997 Microbial Diversity Course, Banin again investigated the composition of the berries, using an approach including enrichment cultivation, microscopy, and molecular techniques (Banin, 1997). Using berries as inoculum, he successfully enriched for both purple sulfur phototrophs and sulfate reducing bacteria. Scanning electron microscopy confirmed the presence of rod-shaped bacteria and diatoms in the slime around the coccoid purple sulfur bacteria. Cloning and sequencing of 16S rDNA extracted from the berries suggested the presence of at least the following phylogenetic groups : purple sulfur bacteria, sulfate reducing bacteria, cytophaga, and "symbionts". Banin also compared different berries using restriction fragment length polymorphism (RFLP), DGGE (denaturing gradient gel electrophoresis), and checkerboard hybridization. RFLP and DGGE analyses indicated that the species composition of the berries was highly conserved. The checkerboard hybridization results confirmed the presence of purple sulfur bacteria and cytophaga.

This year our group sought to confirm the findings of the previous investigators, as well as apply additional characterization techniques. In addition, more field observations and laboratory experiments to simulate possible conditions leading to berry formation were employed. The results of our investigations suggest that mussels in the salt marsh may play a major role in the formation of the berries.

1.1 Organization of the Report

The various techniques applied to the investigation of the berries are described in the following sections. Section 2 contains a summary of the results from the various enrichments applied to the berries. Section 3 presents the molecular characterization data generated from the berries and from the berry-derived enrichments. In Section 4 the microscopic techniques applied to the berries are described. The field observations and laboratory simulations are presented in Section 5. Section 6 includes our conclusions and recommendations for future research. Finally, references are listed in Section 7.

2.0 Enrichments

Microscopic analysis and characterizations of the 16S rRNA clones from the berries by Seitz et al. (1993), Banin (1997), and our group suggested that several bacteria are conserved between individual berries from the same and different ponds. These are the sulfate reducing bacteria, the purple sulfur phototrophs, and the cytophagas. We therefore attempted to enrich and obtain pure cultures of these three kinds of bacteria. In addition, we used berries as inocula in three other types of enrichments, using the media provided for the Microbial Diversity course enrichment experiments: for heat-tolerant respiratory and fermenting bacteria; for cyanobacteria; and for iron reducers. No growth was observed in the heat-tolerant and cyanobacteria

enrichments, and they are not discussed further. The other four enrichment types are described below.

2.1 Sulfate Reducing Bacteria

Berries were washed in sterile seawater (80%) several times and then crushed with a microscope slide. Crushed berries were then added to a bicarbonate buffered minimal medium with either ethanol (20 mM) and Na_2SO_4 (20 mM) or H_2/CO_2 and Na_2SO_4 (20 mM). The bicarbonate buffered minimal medium (pH 7.1-7.3) consisted of: 0.2 g/l KH_2PO_4, 0.25 g/l NH_4Cl, 20.0 g/l NaCl, 3.0 g/l $MgCl_2*6H_2O$, 0.5 g/l KCl, 0.15 g/l $CaCl_2*2H_2O$, 30 ml/l of an 84 g/l stock Na_2HCO_3, 2 ml/l Na_2S, 1X concentration of trace element solution SL10, and 1 ml/l of the vitamin stock: 100 ml H_2O, 4 mg 4-aminobenzoic acid, 1 mg D (+) -Biotin, 10 mg nicotinic acid, 5 mg Ca- D(+)-pantothenate, 15 mg pyridoxamine dihydrochloride, 10 mg thiaminium dichloride, and 5 mg cyanocobalamine. Due to problems with the incubator, the primary enrichment bottles were incubated initally at >30 ^oC for one day, then moved to room temperature for 5 days, and then finally moved to 30 ^oC for final incubation. All subsequent enrichments were performed at 30 ^oC.

Both the ethanol and H_2/CO_2 enrichments showed turbidity within the first week of incubation. These primary enrichments were subcultured into secondary liquid enrichments. The ethanol secondary but not the H_2/CO_2 enrichment showed turbidity. Agar shakes were made of the ethanol secondary and the H_2/CO_2 primary enrichments into Hungate tubes and flat flasks with H_2/CO_2 headspace, respectively. Two colony types grew in both enrichments. The first was a discrete brown colony, consisting of motile curved rods, consistent with the morphology of sulfate reducing bacteria. The second was a hazy, undefined colony (in which some rings of growth were observed) consisting of 3 cell types: long thin rods that twitched, phase dark cocci, and phase bright cocci, which could be interpreted as the 3 stages in the life

cycle of cytophaga: vegetative rods, microcysts and spores. Secondary and tertiary shakes were performed. Fluorescence *in situ* hybridization (FISH) and transmission electron microscopy (TEM) analyses of these isolates are described in section 4.

2.2 Purple Sulfur Phototrophs

The protocol and results of the purple sulfur bacteria (PSB) enrichments are described in Erik Zinser's independent project report. The key results obtained are that there are at least two purple sulfur phototrophs present in the berry, a motile bacteria with irregular ovoid cells often in pairs, and the likely predominant PSB, which is a large coccus that forms tight aggregates on agar surfaces. In addition, we enriched for a putative green-sulfur bacterium from the berry, as noted by a positive hybridization with the green sulfur FISH probe (see sect. 4).

2.3 Cytophagas

Enrichments for cytophagas were performed on dilute complex media plates. Washed berries were crushed with a microscope slide and passed through a hypodermic needle, and then spread onto two types of plates: YPG (yeast extract + peptone + glucose in sea water) and TYE (0.05% tryptone, 0.05% yeast extract in sea water (70%)). Incubations were perfomed at room temperature under oxic conditions. Many different colony types arose over the course of two weeks. Table 1 contains data for several of the isolates.

Table 1. Isolates from Cytophaga Enrichments.

Isolate	Medium	Colony Char.	Cell Char.
A	YPG	rough translucent	irregular rods in chains
B	YPG	round opaque irridescent	cocci, some chains
C	YPG	large opaque mucoid	cocci, some chains
D	YPG	large opaque very mucoid	diplo-bacilli phase-bright
E	YPG	round, dark yellow	cocci, some clusters
F	YPG	rough, pale yellow	cocci (?) in clumps of 10+
G	YPG	yellow center, rough translucent border	rods of var. length
H	TYE	pale pink, agar degrader	motile rods
I	TYE	opaque, off-white agar degrader	curved, motile rods
J	TYE	pale yellow spreading morph.	thin rods of var. length, no evid. of gliding

None of the isolates displayed all of the common characteristics of cytophagas: thin rods, non-motile in liquid, and gliding motility on surfaces. Nor did the one candidate (J) show a positive hybridization with the flavo FISH probe (see section 4). Hence we were unable to isolate a cytophaga species from the berry with these enrichments.

2.4 Iron Reducing Bacteria

Seven washed berries were crushed on a slide and the material was divided between two Pfennig bottles containing non-reduced anoxic sea water medium. The medium composition was identical to that described for the sulfate reducing bacteria, except that no sulfide was added as reducing agent and sulfate was not added as an electron acceptor. Instead the medium contained 40 mM ferrihydrite [amorphous $Fe(OH)_3$] as the electron acceptor, and 10 mM acetate as electron donor and carbon source. The bottles were incubated without headspace at 30 °C. After one week a slight darkening of the orange iron solids, indicating reduction, was observed in one of the bottles, and a ferrozine assay indicated the presence of ferrous iron in the medium. Several days later the solids in the other bottle also began to darken. The earlier positive primary enrichment was subcultured into 3 different types of secondary enrichments: (i) 5 mM acetate and 40 mM ferrihydrite; (ii) 40 mM ferrihydrite with a headspace of 80/20 H_2/CO_2 (approximately 30 ml headspace in a 60 ml stoppered bottle); and (iii) 5 mM acetate, 40 mM ferrihydrite, and 10 mM 2,6-anthroquinone disulfonate (AQDS). AQDS is a model compound for humic substances, which may serve as electron acceptors in nature. Reduced humic substances can in turn chemically reduce ferric iron. Five ml aliquots from the primary enrichment were used as inocula, and the bottles were again incubated at 30 °C.

In the secondary enrichments, very little activity was observed, except for a slight darkening of the iron solids in the enrichment with AQDS, observed after one week. It is possible that the inoculum, which was primarily supernatant rather than the iron solids, was insufficient in the secondary enrichments. Another possible reason for the lack of growth was that sulfur was limiting; a few weeks into the incubation it was recognized that a sulfur source had been inadvertently omitted from the medium formulation. No further attempts were made to re-cultivate iron reducing activity from the berries.

3.0 Molecular characterization

3.1 DNA extraction

Berries were washed with filtered sterilized seawater, crushed between two microscope slides, and passed several times through syringe needles with a series of smaller gauges. DNA from the different samples were extracted using the DNA lysis protocol described in the Microbial Diversity course guide (1998). The method is based on cell lysis through the use of a beadbeater in the presence of a mix of phenol/chloroform/isoamyl alcohol, and later precipitation with ethanol. In the cases where PCR amplication was not achieved, the Wizard PCR preparation DNA system (Promega) was used to purify the DNA.

3.2 PCR reaction

PCR reactions were conducted according to the Microbial Diversity course guide (1998). Two set of primers were employed: (1) universal forward (position 8) and universal reverse (position 1492) for amplification of bacterial 16S rDNA and (2) universal forward (position 8) labelled with digoxigenin (DIG) and universal reverse (position 1492) for checkerboard hybridization. PCR products were sized by electrophoresis on an agarose gel.

3.3 Cloning and sequencing

Amplified bacterial 16S rDNA from the berries was cloned into *Escherichia coli* using the TA cloning kit (Invitrogen). Ten clones were sent for automatic sequencing of a 500 base pairs segment. Further analysis of the sequences was conducted by comparing them to existing 16S rDNA sequences in the Genbank database using BLAST. Phylogenetic analysis was conducted using the program RNA1 (Bruce Paster, personal comunication).

Of the ten clones sent for sequencing, six clones were most closely related to *Thiorhodococcus minus* (94% similarity), two clones were related to the chloroplast of *Odontella sinensis* (91% similarity), and two clones were related to a freshwater obligate oligotroph IO95, cytophaga (100% similar for the first 19 base pairs and 88% similar for the last 310 base pairs). The clone similar to *T. minuss* clustered with purple sulfur bacteria and it was very similar (one base pair difference out of 500) to a clone previously sequenced from the berries (Banin 1997; Figure 1).

3.4 Checkerboard Hybridization (CH)

Checkerboard hybridization was conducted using a minislot/blot apparatus using a nylon membrane according to the protocol given in the Microbial Diversity course guide (1998). The PCR products amplified with the DIG forward primer from one pond berry, five pond berries, and pink flocs (from non-berry ponds in the Sippewisset Salt Marsh) were used in the first CH analysis (Figure 2). For the second CH the following samples were used: (1) purple sulfur bacteria (PSB) enrichment, (2) mixed culture of PSB and cytophaga, (3) sulfate reducing bacteria (SRB) enrichment, (4) five pond berries, (5) five berry-like aggregates from the mussel incubation with the PSB enrichment (see section 5.2; DNA dilution 1:10 and 1:100), (6) five brown "berries" (mussel feces or pseudofeces), (7) pink sand (1:10 dilution), and (8) DNA extracted from a mussel crushed by passing through a syringe (Figures 3).

In the first CH (Figure 2), the universal probes, the Flavo/Bacteroides probe and SRB probes hybridized with all the samples. The probe for the SRB species, from the berry sequenced in 1997, hybridized only with the DNA from one and five berries. In the second CH (Figure 3), the signal for the pond berry (lane 35) was lower than in the first CH, but revealed the presence of SRB, spirochetes, and flavobacteria. The DNA extracted from the berry-like aggregates formed by a mussel incubated with PSB (1:10 dil.) was positive for gamma- and

alpha-proteobacteria, SRBs, spirochetes, and flavobacteria (lane 36). No results were obtained for the PSB berry dil. 1:100, the brown berry, the pink sand dil. 1:10, and the mussel dil. 1:10 (lanes 37 to 40). The probes for the berry PSB and the berry cytophaga clones 9 and 10 did not hybridize with any of the DNA samples from the berries. This result was unexpected, because we believe these bacteria to be important components of the berry microbiota.

4.0 Microscopy

4.1 Crystal Violet Staining

In order to visualize the matrix surrounding the purple sulfur bacteria, crushed berries were smeared on a slide and stained using the Anthony staining method for bacterial polysaccharide capsules (Gerhardt et al., 1994). The air-dried film was covered with a 1% (w/v) aqueous solution of crystal violet for 2 minutes. The dye was washed off with a 20% (w/v) aqueous solution of copper sulfate. After partial drying, a cover slip was put on the slide and it was examined under bright field oil immersion (1000X) microscopy. The staining revealed coccoid and rod-shaped bacteria stained dark purple, with a light purple capsule layer surrounding them. Figure 4 includes the crystal violet-stained berries, along with other phase contrast images from the berry.

4.2 Transmission Electron Microscopy (TEM)

Berries were washed in filtered sterile seawater (80%), crushed between two microscope slides, and suspended in sterile seawater (80%). The berry suspension was prepared for TEM on copper grids coated with plastic and carbon. TEM samples were heavy metal stained with 1% phosp otungstic acid (PTA; pH 7). TEM showed the presence of bacterial aggregates (Figure 5), *Caulobacter*- like organisms (Figure 6a), and a long curved flagellated rod (Figure 6b). TEM

was also applied to samples from the SRB enrichments (ethanol as electron donor) with berries. Figure 7 shows one of the morphotypes observed, a short slightly curved rod.

4.3 Fluorescent *In Situ* Hybridization (FISH)

Whole cell fluorescent *in situ* hybridization was performed on the berry and several berry isolates to detect sulfate-reducing bacteria, cytophagas, and green sulfur bacteria. The protocol from the 1998 Microbial Diversity course handout was followed, with the following modifications. Berry samples were prepared by washing several times in sterile sea water (80%), and then crushed with a microscope slide before passage through a hypodermic needle. All samples were fixed in 4% paraformaldehyde for 3.5 hours. The wash buffer contained 0.9 M NaCl, rather than 0.5 M. All samples were counterstained with DAPI (blue signal in all figures). The following probes were used:

Probe Name/ Dye	Phylogen. Group:	Sequence:	Tm (C)	Hyb/Wash Temp
Flavo Rhodamine	CFB division	5'-tcagtrccagtgtggggg	58	37
SRB2 Cy3	sulfate-reducers	5'-cgygcgccrctytact	57	37
GSulf FITC	Green Sulfur	5'-cttttargggatttcctctg	54	37

Probing of the berry was complicated by the autofluorescence of the predominant purple sulfur bacteria (see Figures 8a and 8b; red signal). However, probing of the enrichments/isolates from the berry was more informative. Both of the putative SRB isolates from the anaerobic Na_2SO_4 enrichments with either ethanol or H_2/CO_2 showed good hybridization with the SRB2 probe (Figures 8c and 8d), but no autofluorescence without the probe. Most cells showed fluorescence throughout the cell as noted by the red color, while some cells showed partial or no

fluorescence, which may indicate differences in the amount of target 16S rRNA within the cells. The FISH results support the conclusion that we have isolated an SRB from the berry.

The Gsulf probe showed a positive hybridization with some of the cells from a phototroph enrichment from the berry. As the culture was impure, it makes sense that not all cells from this culture would show a positive signal. Probing the putative cytophagas from the berry with the Flavo probe provided no evidence that these were cytophagas. While the positive control (a known cytophaga) showed a strong positive signal, both the yellow swarmer cells from the TYE enrichment and the hazy brown colony from the anaerobic sulfate-reducer enrichments showed no detectable signal. Hence, positive identification of these two isolates awaits further analysis.

5.0 Field Observations and Laboratory Simulations

5.1 Field Observations

The berry pond and the environment surrounding the pond were observed in order to assess the community at the pond and to attempt to understand why the berries form in the berry pond and do not form in other ponds in the salt marsh. The berry pond is connected to an inlet through which water flows in and out from the adjacent tidal channel as the tide changes (Figure 9). A site characterization of the berry pond, the inlet, and the channel was conducted. Temperature and salinity were measured in the field; pH, oxygen and sulfide were measured in the lab with collected samples. The results of these measurements are presented in Table 2.

Table 2. Site Characterization Data for the Berry Pond.

	Temp. (°C)	pH	O_2 (mg/l)	S^{-2} (mg/l)	Salinity (‰)
Pond	31	~6	13-14	0.5-1.5	30
Inlet	31-33	~6	10-13	0.2	30
Channel	27	~6	10-12	0.01-0.08	20

The inlet feeding into the berry pond is inhabited by Atlantic ribbed mussels (*Geukensia demissa*). While the ribbed mussel is a common inhabitant of the salt marsh, the mussels are not present in the berry pond itself. Mussels in the inlet were observed expelling berry-like pink material (Figure 10). Atlantic ribbed mussels, like all bivalve molluscs, are suspension-feeding organisms which feed on phytoplankton, zooplankton, dissolved organic matter, detritus, and bacteria (Langdon and Newell, 1990). After observing mussels excreting berry-like aggregates, we devised two hypotheses addressing the potential role that mussels play in berry formation.

The first hypothesis states that the mussels do not contribute to berry formation. This hypothesis suggests that the berries form independently of the mussels and that the mussels simply consume the berries as they feed. This scenario further suggests that the berry aggregates are not digested by the mussels, but are rather regurgitated. If this hypothesis is supported, then it is possible that berry formation serves to protect its bacterial components by making them indigestible or unpalatable to the mussels.

The second hypothesis states that mussels form the berries or a precursor to the "mature" berries found in the berry pond. This hypothesis suggests that the bacterial components of the berry are present in the water that the mussels filter. In this scenario, mussels would ingest the bacteria, the berry would form in the mussel, and the berry would be excreted by the mussel. Berries therefore accumulate in the inlet where the mussels reside, and when the tide comes in

are washed into the berry pond. This hypothesis allows for the possibility that the berries excreted by the mussel are not yet "mature"--meaning that they lack components present in the berries in the berry pond. Maturation of the berry may occur after the berries are deposited in the berry pond. The second hypothesis also allows for the possibility that the slime that holds the berries together (Seitz et al., 1993) is produced in the mussel and not by one of the bacterial components of the berries.

The hypothesis that the berries, or berry precursors, are formed in the mussels is intriguing because it is known that a portion of material ingested by filter-feeding molluscs is rejected as pseudofeces. As pseudofeces are produced, molluscs increase their mucus production. This mucus serves to aggregate the particles that are being rejected as pseudofeces (Bayne and Newell, 1983). The mussels in the salt marsh may ingest the components of the berries, but reject them as pseudofeces. This theory suggests that the berries are mussel pseudofeces. The slime matrix that contributes to the aggregation of the bacterial species in the berry may in fact be the mussel mucus that contributes to pseudofeces production.

5.2 Laboratory Simulations

A series of laboratory experiments were conducted to determine if mussels contribute to the formation of the berries. Glass bowls were used as experimental chambers. Five treatments were established with two replicates per treatment. Treatment 1 consisted of a mussel in a chamber containing water collected from the berry pond. Treatment 2 consisted of a mussel in a chamber containing water collected from the berry pond and sediment collected from a nearby pond that did not contain berries. Treatment 3 consisted of a mussel in a chamber containing water collected from the berry pond, sediment from the nearby pond, and pink sand collected from the tidal channel. Treatment 4 consisted of two berries in a chamber containing water collected from the berry pond and sediment collected from the nearby pond. Treatment 4 was

designed to determine if the treatment conditions would facilitate (1) an increase in size of the two berries or (2) the formation of additional berries. Treatment 5 served as the control and consisted of a chamber filled with water collected from the berry pond. Treatments were incubated for three weeks in the laboratory. Pond water was changed every few days in order to ensure that the mussels had sufficient food to filter.

Mussels in Treatment 3 excreted berries during the process of suspension feeding. Treatments 1, 2, 4, and 5 did not result in the formation of berries. The two berries incubated in treatment 4 did not increase in size. The results of the simulation experiments suggest that the presence of pink sand is essential to berry formation in the mussel. The color of the pink sand can likely be attributed to the presence of purple sulfur bacteria, the dominant component of the berries (Seitz et al., 1993).

To further test the abilty of mussels to produce berries under controlled conditions, mussels were fed berry pond water spiked with enrichment cultures of both purple sulfur bacteria (PSB) and green sulfur bacteria (GSB). As the mussels filtered the spiked pond water, they excreted the PSB and the GSB in the form of aggregates. The aggregates differed from the berries from the berry pond and the berries produced by mussels in treatment 3 in that they were looser and less discrete clumps, but they were clearly aggregated bacteria. The mussels very rapidly (< 1 day) filtered and excreted the PSB and GSB, suggesting that these bacteria were not digested by the mussel but rather were excreted as unwanted material or pseudofeces.

6.0 Conclusions and Recommendations for Future Research

The results of the field observations and the laboratory simulations with the mussels strongly suggest that the mussels play an essential role in the formation of the berries. Therefore,

the second hypothesis concerning the mussels was supported by the results of the laboratory simulations.

Berries collected from the berry pond and berries produced by the mussels in the laboratory should be compared to determine if they are indeed the same. It is possible that the berries excreted by the mussels are not yet "mature" and that incubation in the berry pond results in berry maturation via the addition of other bacteria to the aggregates. It should be determined, using molecular techniques, whether berries excreted by the mussels are fully developed in terms of the microbial composition as compared to the pond berries, although they differ in shape and texture.

Another possible topic of future research is the population distribution within the berries, using most probable number (MPN) enumeration. Application of MPN enumeration will require disaggregation of the berries. This may necessitate the characterization of the mucus holding the berries together, in order to determine an appropriate disaggregation technique. Incubation of berries in the dark did not result in berry disaggregation.

Autoinducer activity was detected in the berries (Pat Fidopiastis personal project report, 1998). Future research should further pursue this autoinducer activity.

We observed that while the PSB of light pink berries were full of sulfur granules, most of the PSB's in the dark purple berries lacked sulfur granules. In addition, after a 6-day incubation in sulfide-rich purple sulfur phototroph medium, the purple sulfur bacteria from an originally dark purple berry exhibited sulfur granules. It will be of interest to more extensively study the relationship between berry color and phototroph composition within the berry.

Other suspension-feeding organisms in the salt marsh may also produce berries. Future studies should address the role that these organisms play in berry formation.

Finally, additional enrichment conditions for cytophagas should be conducted. For example, cultivation on TYE and YPG plates under oxic and anoxic conditions and at different temperatures could be tried.

7.0 References

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

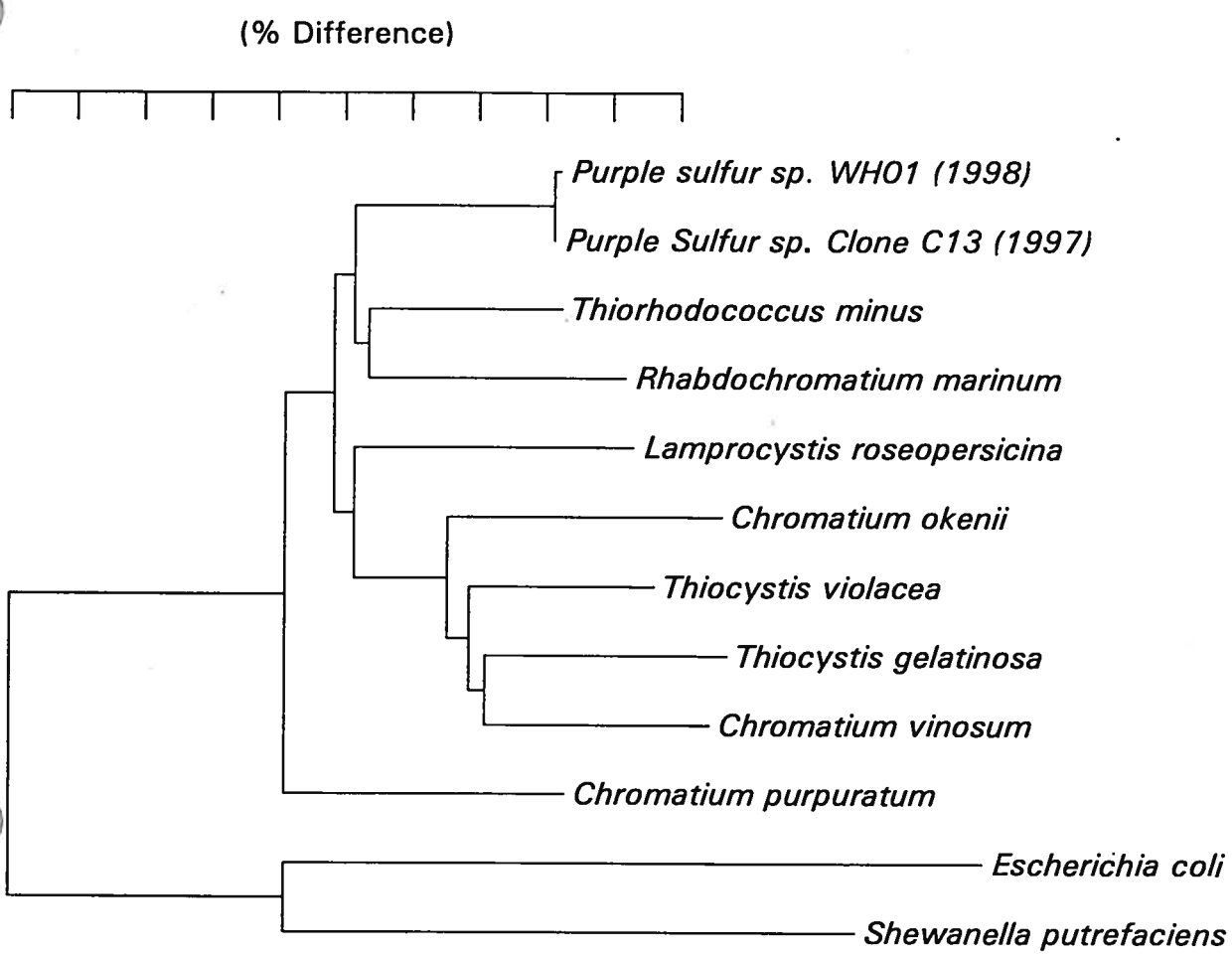
(% Difference)
Purple sulfur sp. WH01 (1998)
Purple Sulfur sp. Clone C13 (1997)
Thiorhodococcus minus
Rhabdochromatium marinum
Lamprocystis roseopersicina
Chromatium okenii
Thiocystis violacea
Thiocystis gelatinosa
Chromatium vinosum
Chromatium purpuratum
Escherichia coli
Shewanella putrefaciens

FIGURE 1

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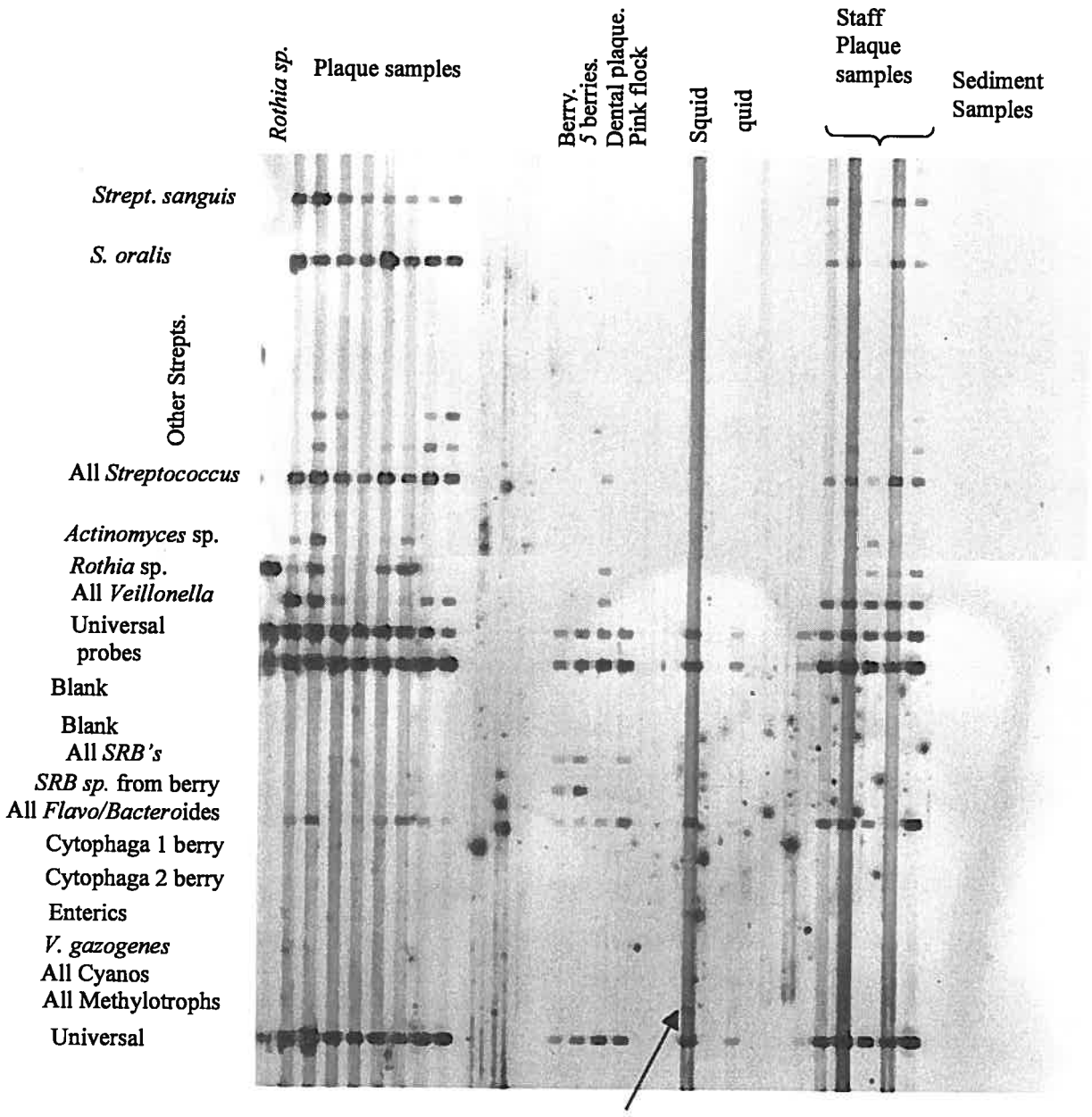
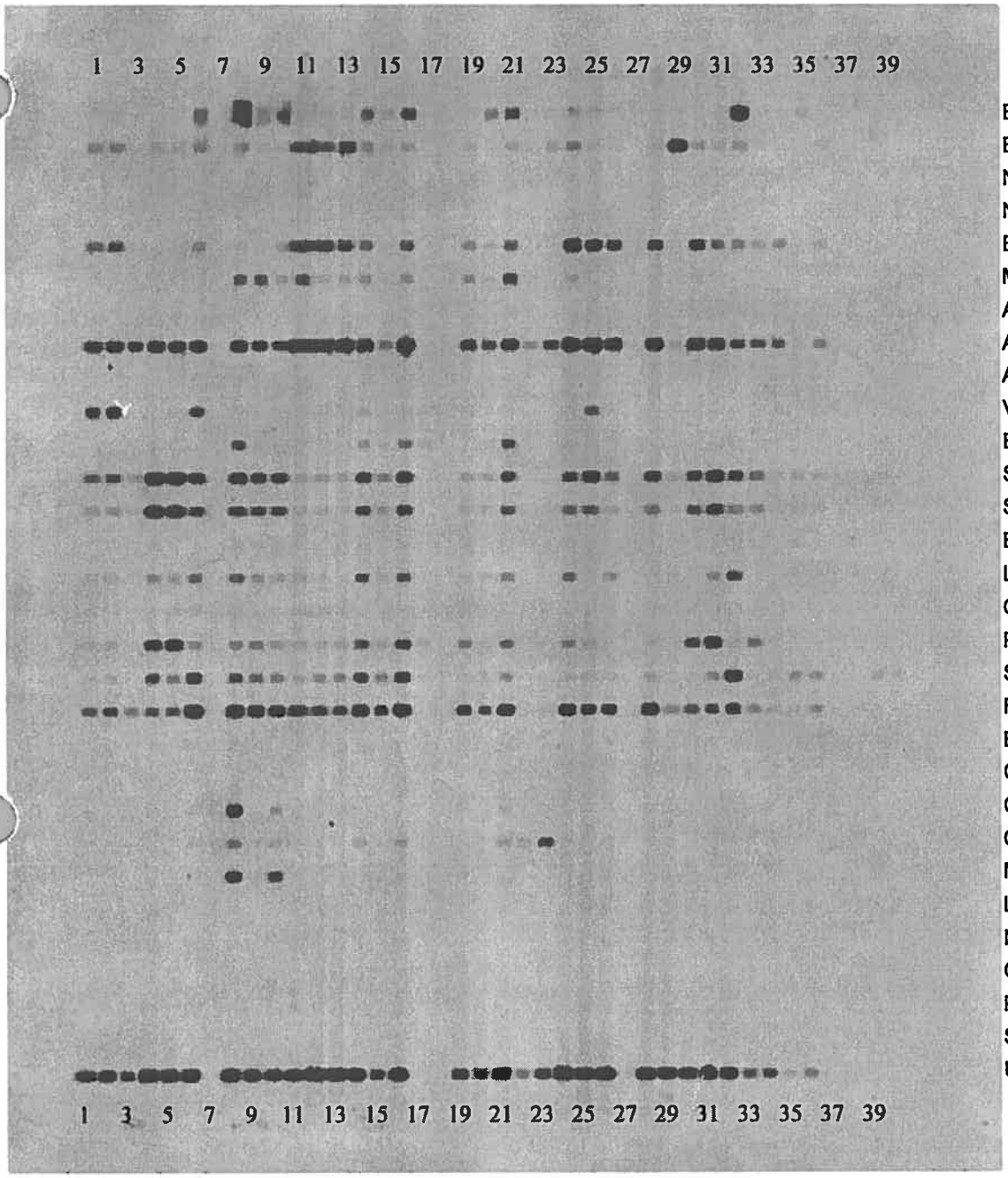
Checkerboard hybridization of environmental samples



Notes: (see Bruce for explanation)

- Berry samples have specific SRB identified last year and flavobacteria, but not the specific species of *Cytophaga* identified last year (last year not all berries had both Cytophaga species)
- Pink flock has SRB's (but not the specific species found in the berry) and flavobacteria
- Squid samples have flavobacteria and 1 sample may have methylotrophs (arrow-- hard to see)
- Dental plaque samples have different distributions of streptococci and other bacteria, including flavobacteria (*Bacteroides* and related bacteria)
- Sediment samples gave no signal—probably DIG primers were not used.

FIGURE 2



Berry "Purple Sulfur "
Betas-all
Nitrosolobus/Nitrosovibrio
Nitrosomonas spp
Enterics / some gammas
Methylotrophic bacteria
ANG Alteromonas sp.
Alphas-all
ANG Shewanella sp.
Vibrio gazogenes
Berry "Plastid"
SRB2, some deltas
SRB, some deltas
Berry SRB clones C16/C18
LoGC Gram Positive
Green Sulfur-all
Planctomyces-all
Spirochetes-
Flavos-all
Bacteroides-
Cytophaga berry C10
Cytophaga berry C9
Cyanobacteria
Microcoleus sp.
Lyngbia sp.
Nostoc sp.
Oscillatoria sp.
Phormidium ectocarpi .
Synechococcus sp.
Universal

Key:

1. Silvana-salt pond 1
2. Silvana-salt pond 2
3. John oyster
4. Group I- ED
5. Group I--LD
6. Group I-MD
7. Group I-G1 [No good]]
8. Group I-P2
9. Group I-G3
10. Group I-B4
11. Milva enrich $t_0 + O_2$
12. Milva enrich $t_2 + O_2$
13. Milva enrich $t_4 + O_2$
14. Milva enrich $t_8 + O_2$
15. Milva enrichment t_6 w/o O_2
16. Grp II sediment
17. Grp II sediment [No good]
18. [No good]
19. Patti Sip. Hi Nitrogen plot
20. Patti Sip. Low Nitrogen plot
21. Patti Sip. Control plot
22. Karin Cyan. Assoc.
23. Karin Cyan. Pure
24. Scott- Begg. Environ
25. Andreas Deep Sea
26. Andreas Deep Sea
27. Andreas [no good]
28. Andreas Deep Sea
29. Yoshiko ThioS
30. Yoshiko-H_2/Fe
31. Grp II--PSB
32. Grp II Cyto/PS
33. Grp II SRB
34. Yoshiko-H_2/Fe
35. Pond Berry
36. Pk sand berry 1/10
37. Pk sand berry 1/100
38. Brown berry
39. Pink sand 1/10
40. Mussel 1/10

FIGURE 3

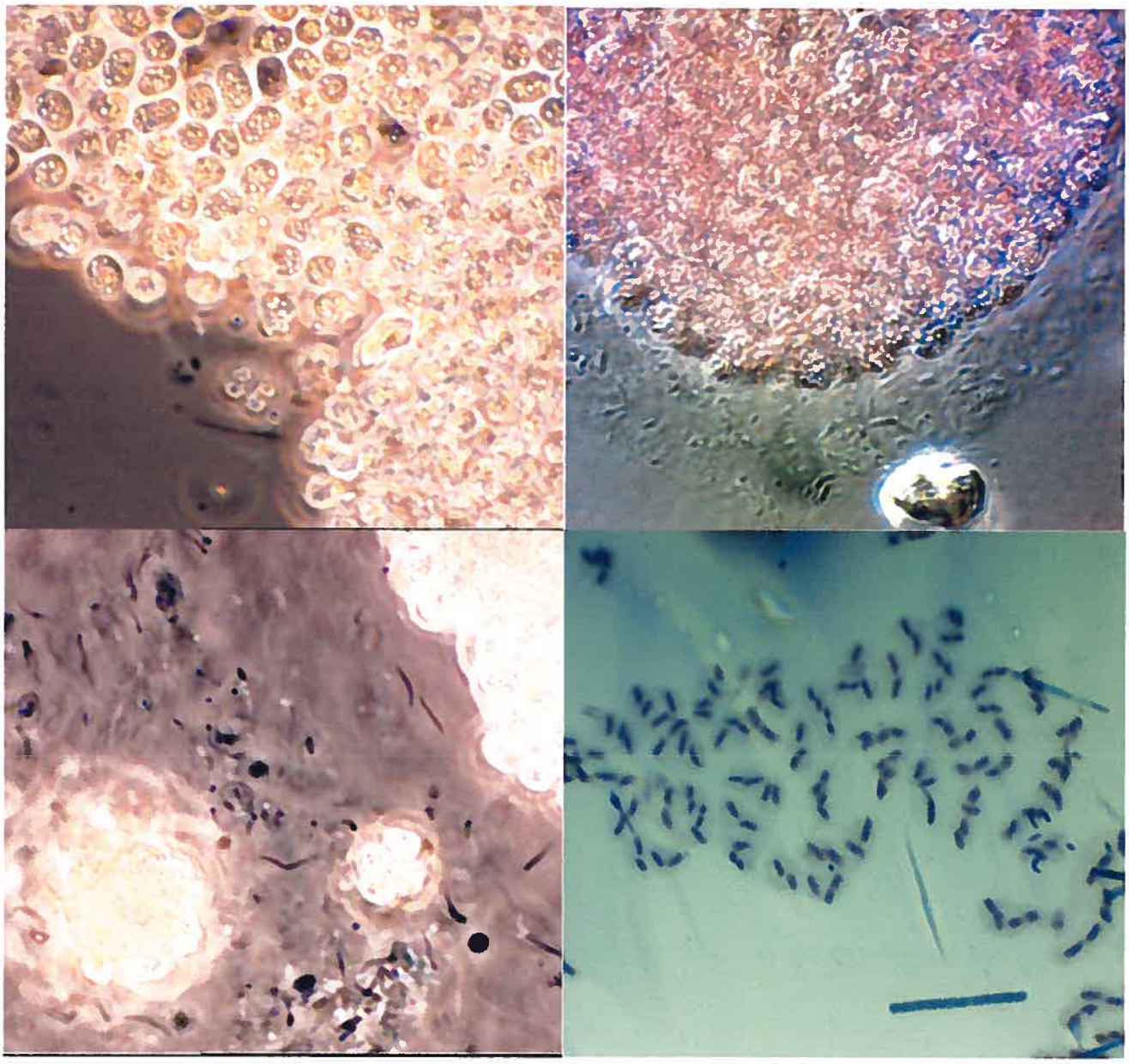


FIGURE 4

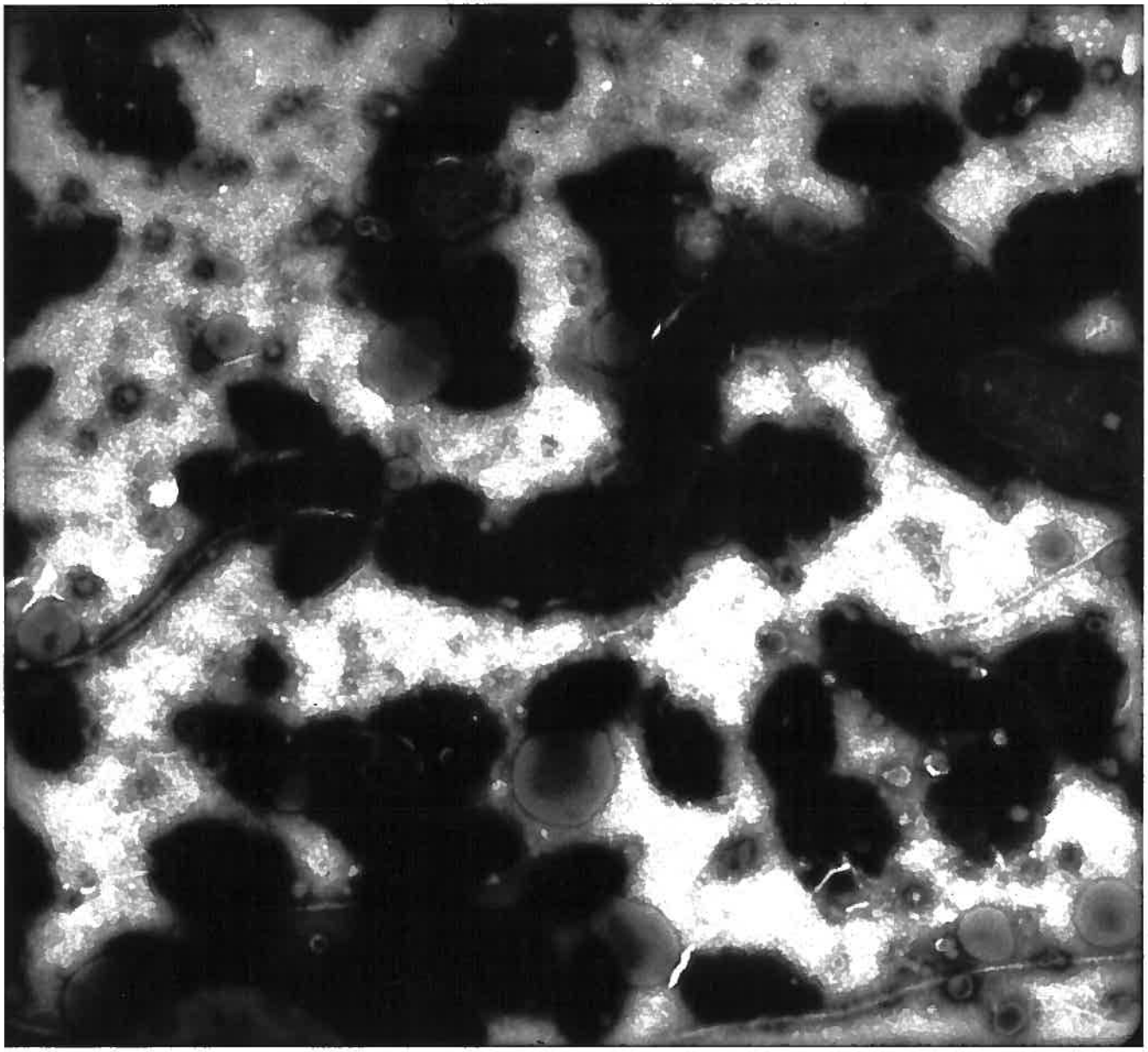


FIGURE 5

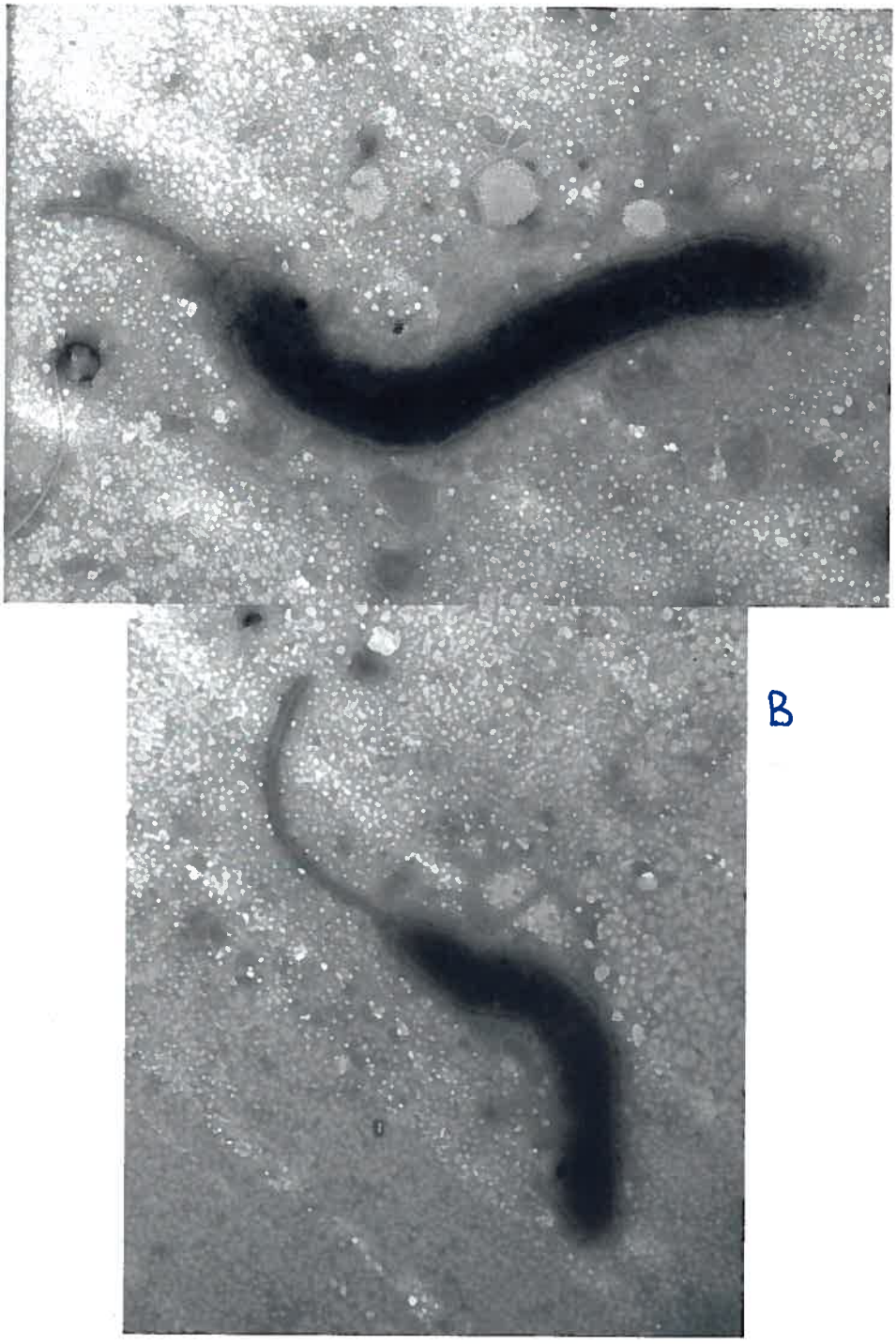


FIGURE 6

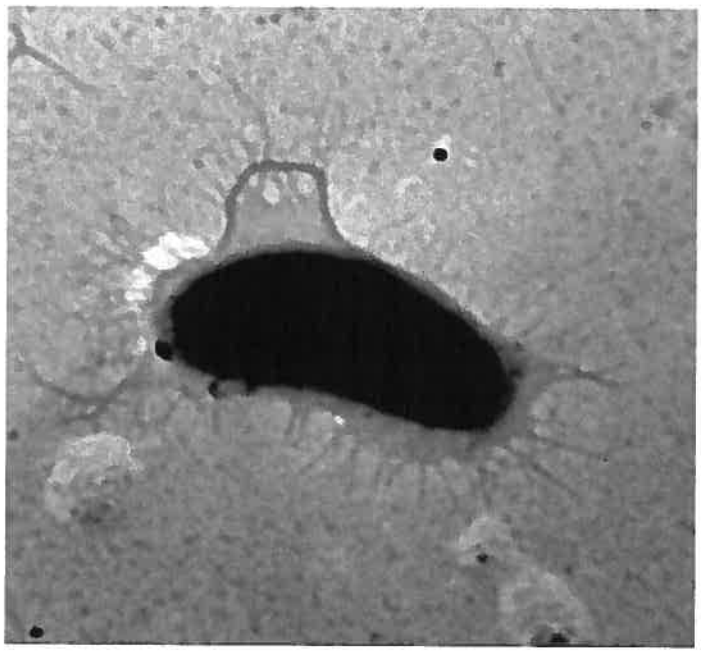


FIGURE 7

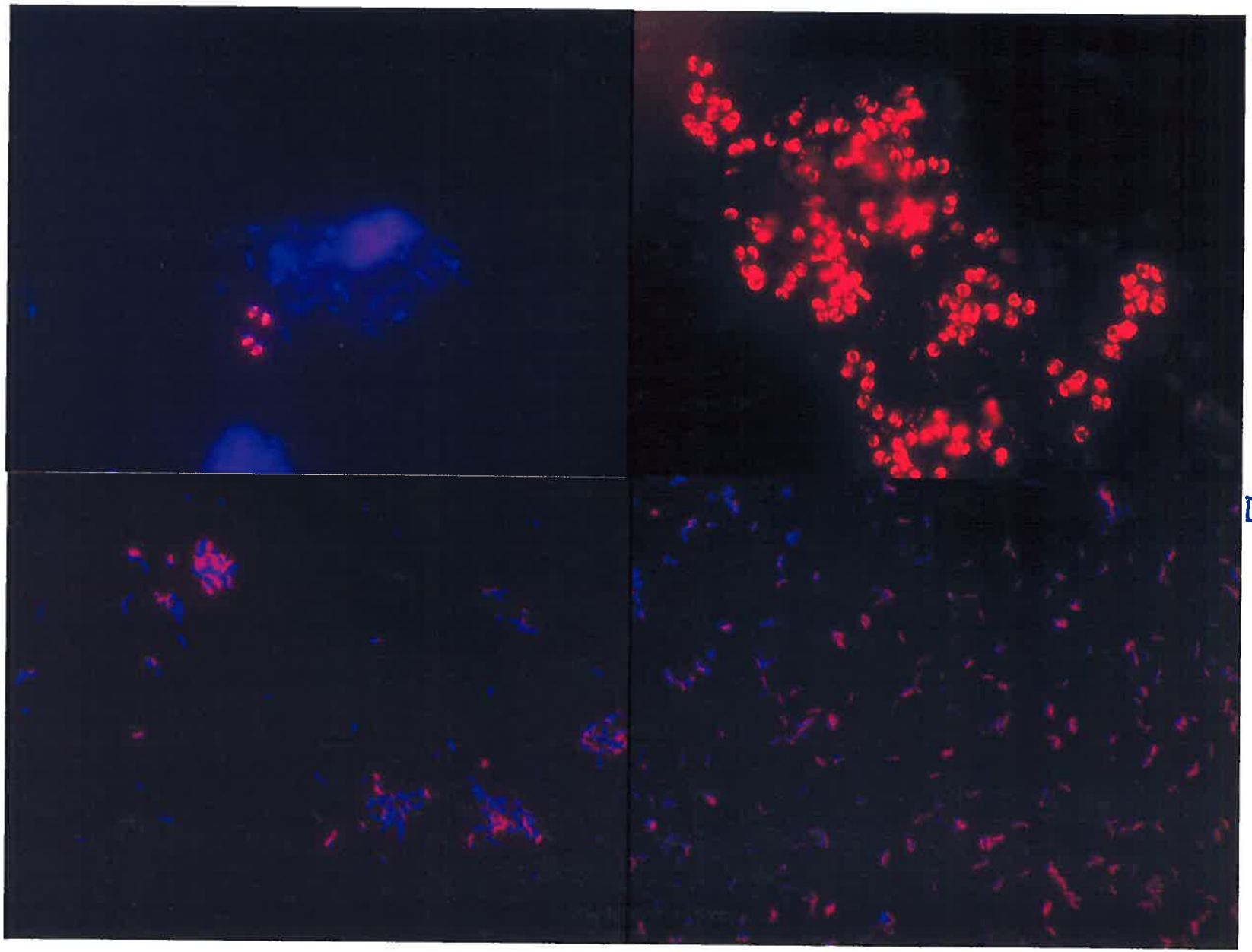


FIGURE 8

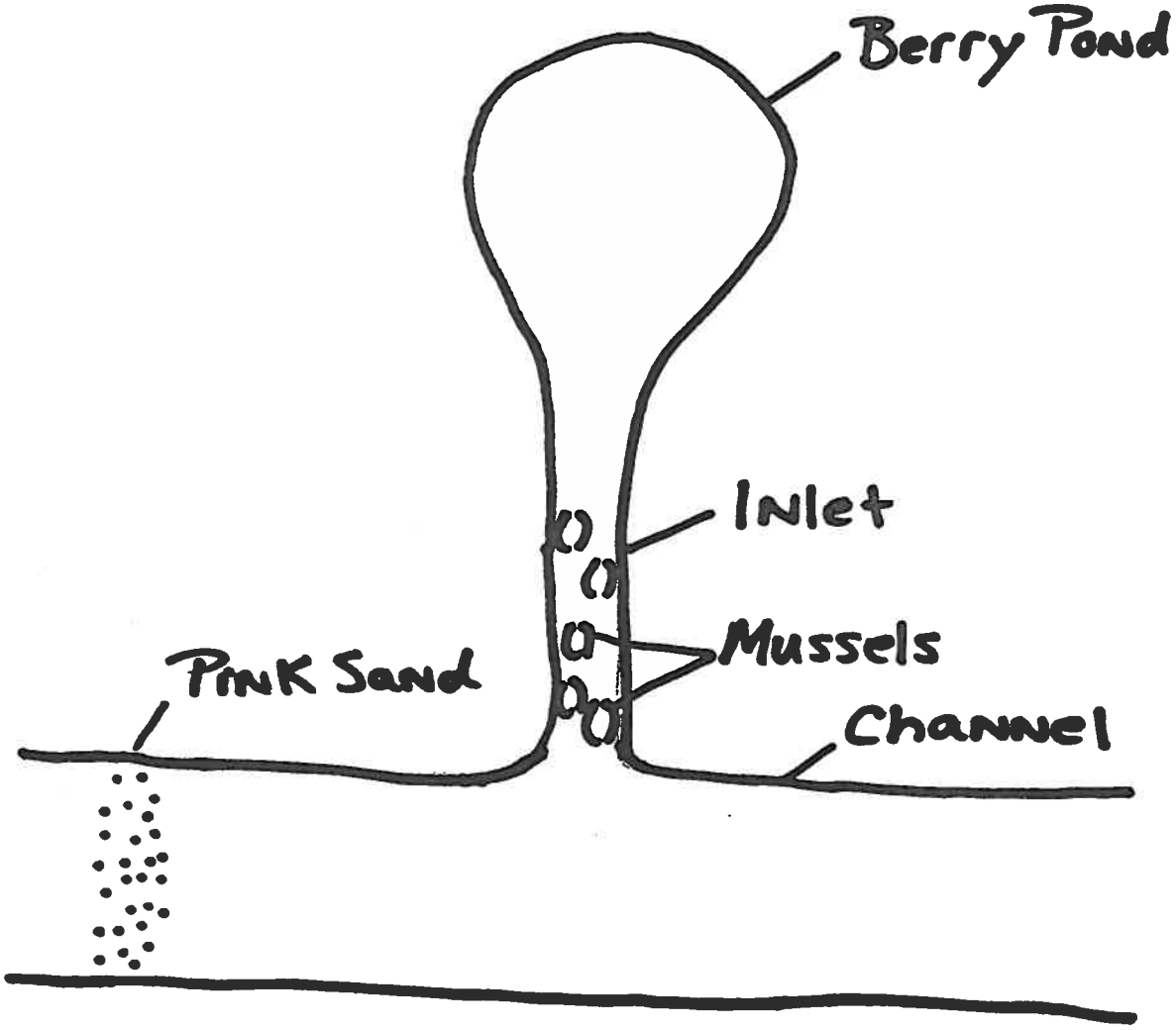


FIGURE 9

FIGURE 10