An Investigation of School Street Marsh's Iridescent Surface Film

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Microbial Diversity
July 28, 2004

Introduction:

The neuston realm is a vast habitat covering over 70% of the earth's surface. By definition, the neuston is the collection of species living at the air-water interface, a microlayer less than ~1000μm thick. The physicochemical conditions of the neuston layer are very different from those of the remaining water body. The conditions are influenced by strong UV irradiance and have likely contributed to the evolution of a highly diverse and abundant assemblage of species. The neuston is likely to be a habitat for specialized microorganisms as well as a habitat for other microorganisms that find their way to or become adhered to the surface. Ultimately, microorganisms living in the neuston contribute to biogeochemical processes occurring at the air-water interface.

Bacteria living at the air-water interface, also known as piconeuston (<2 μm), are being increasingly recognized as important links in the recycling of organic matter in aquatic ecosystems. High densities of metabolically active bacterioneuston are found in the surface microlayer (Sieburth et al. 1976; Carlucci et al. 1985). For example, bacterioneuston off the coast of Baja California accounted for 1.4 to 5.9% of the total microbial carbon biomass and a similar percentage (1.9 to 5.1%) of the microbial carbon production in the surface microlayer. The enrichment of bacteria in the surface microlayer results, at least in part, from the greater degree of hydrophobicity of bacterioneuston and thus, their adhesion to organically enriched surface microlayers (Dahlback, et al., 1981). Bacterioneuston likely play an important role in degrading not only natural organics, but also anthropogenic chemicals collected at the water surface.

Materials & Methods:

Site Description:

All water samples were collected from the School Street Marsh in Woods Hole, Massachusetts. The marsh is a seemingly shallow, slightly acidic (pH 6.0), freshwater ($0^{o}/_{oo}$) environment that is dominated by the perennial cordgrass, *Spartina patens*. A prominent feature of the marsh is an iridescent film that covers a large portion of the air-water interface (Figure 1).

Figure 1: An iridescent film forms on the surface of the water at School Street Marsh, Woods Hole, Massachusetts.

Sample Collection:

The film covering the air-water interface was collected with autoclaved, 10cm x 10cm fiberglass insect screens (Saint-Gobain Technical Fabrics). In the field, screens were laid on the surface of the water and allowed to remain for 10s. Each screen was then carefully lifted and

transferred into a 50mL Falcon tube. Each screen, centrifuged at 500rpm for 30s, yielded 1mL of surface water. All samples (Table 1) were used within one hour with the exceptions of SSM_Old and SSM_New. SSM_Old and SSM_New were sediment and water samples collected in the field and then kept in the wet lab. Periodically, the iridescent film at the surface-water interface was collected with a cover slip and used for microscopy and DNA extraction.

Sample	Date Collected	Screens Used
SSM_Old	6.24.04	0
SSM_New	7.13.04	0
SSM1	7.13.04	5
SSM2	7.13.04	5
SSM3	7.15.04	5
SSM4	7.15.04	5
SSM5	7.16.04	3
SSM6	7.17.04	5
SSM7	7.17.04	5
SSM8	7.21.04	1
SSM9	7.21.04	2
SSM10	7.26.04	3

Table 1: List of the samples collected, date of collection and the number of screens used to collect each sample (therefore mLs collected).

Media Preparation & Plating:

Water from School Street Marsh was filter sterilized through a 0.2µm filter (Nalgene) and used to make water agar plates (15g 3x washed agar/L). Samples SSM1 and SSM2 were used to create a dilution series ($10^0 - 10^{-4}$) and 100µl of each dilution was used to inoculate the water agar plates. The agar plates were kept at 25°C for 4 days. The cells from the 10^{-2} plates were used in the construction of a clone library, whereas several colonies from the 10^{-3} plates were isolated and identified.

Isolation & Identification:

16 colonies were isolated from the SSM1 and SSM2 10^{-3} dilution series plates. The 16 colonies were picked with an inoculating needle and streaked onto LB agar plates. Single colonies were picked with a 10µl pipette tip into 20µl of 0.05% Triton X 100, boiled for 5 minutes and then spun at 10,000rpm for 5 minutes. 2µl of the DNA were used as template for a PCR reaction. The 2µl of template DNA were added to a master mix composed of 16µl sterile water, 2.5µl 10x PCR reaction buffer, 1.0µl dNTPs, 1.0µl $MgCl_2$, 1.0µl forward primer (8F), 1.0µl reverse primer (1492R) and 0.5µl Taq polymerase. The template was amplified with a 16S rDNA program that included 25 cycles of 95°C for 30sec, 55°C for 30sec and 72°C for 1 minute. The PCR products were run on a 1.25% agarose gel at 100V for 30 minutes. An ExoI-Sap protocol was performed on PCR reactions yielding product in order to dephosphorylate residual dNTPs and degrade primers such that they did not interfere with the subsequent sequencing reaction.

Clone Library Construction:

DNA was extracted from several samples (Table 2) by bead beating according to the Ultraclean Fecal DNA Isolation Kit protocol (MoBio Laboratories).

2µl of the DNA was used as template for a PCR reaction. The 2µl of template DNA were added to a master mix composed of 16µl sterile water, 2.5µl 10x PCR reaction buffer, 1.0µl dNTPs, 1.0µl $MgCl_2$, 1.0µl forward

Sample	Extracted DNA From
SSM1	Plate Wash
SSM2	Plate Wash
SSM6	1.5ml Sample
SSM7	1.5ml Sample
SSM_New	Cover Slip Wash
SSM_Old	Cover Slip Wash

Table 2: List of the samples used during the DNA extraction and the location from where the cells of the sample originated

primer (8F), 1.0µl reverse primer (1492R) and 0.5µl Taq polymerase. The template was amplified with a 16S rDNA program that included 30 cycles of 95°C for 30sec, 55°C for 30sec and 72°C for 1 minute. The PCR products were run on a 1.25% agarose gel at 100V for 30 minutes.

PCR products were ligated into pCR 2.1 vectors using a TA cloning kit (Invitrogen). 5µl sterile water, 1µl 10x ligation buffer, 2µl pCR 2.1 vector and 1µl T4 DNA ligase were combined with 1µl of the PCR product and allowed to incubate at 15°C overnight. The vectors were then transformed into chemically competent E. coli cells. 10µl and 25µl of the transformed cells were plated onto LB-Kanamycin plates (coated with 40µl of 40mg/mL X-gal). Following an overnight incubation at 37°C, white colonies from the 10µl plates were picked into a 96-well media block and sent for sequencing.

Scanning Electron Microscopy (SEM):

Cover slips were cut into eight pieces using a diamond tipped blade and carefully laid on the surface of the marsh or previously collected sediment and water sample. The cover slips were fixed in 2% glutaraldehyde in filter sterilized School Street Marsh water for 3 hours. The cover slips were rinsed 3x in filter sterilized School Street Marsh water (20 minutes each rinse) and then incubated on ice in 1% osmium tetroxide in filter sterilized School Street Marsh water. Three additional rinses were performed (10 minutes each rinse) followed by ethanol dehydration on ice (50%, 70%, 85%, 95%, 100% ethanol in water, 10 minutes each rinse).

Following the final ethanol rinse, the cover slips were placed in the Critical Point Dryer to dry without surface tension changes. The samples were then placed onto SEM stubs and sputter coated with gold/palladium. Prepared samples were visualized with the MBL Central Microscopy Facility's SEM.

Ferrozine Assay:

Three samples were collected on 7.26.04. Two of those samples were taken with the traditional screen lift method, one sample lifted from a surface with the film and the second sample lifted from a surface without a film. The third sample was taken with a sterile pipette from ~5cm below the air-water interface.

The presence of HCl-extractable Fe(II) in the surface film was determined by the ferrozine assay (Lovely & Phillips 1987). 1mL from each water sample was transferred to 9mL of 0.5 M HCl in a 50mL Falcon tube. After 1 hour at room temperature, a 100µl sample of the extract was added to 9mL of ferrozine (1g/L) in 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer at pH 7. After being mixed for 15s, the mixture was passed through a 0.2µm filter. The amount of Fe(II) was determined by measuring the A_{562} of the filtrate and comparing the absorbance value to a standard curve.

A second 1mL of each water sample was extracted by the same procedure as described above except that the extractant was 9mL of 0.25 M hydroxylamine hydrochloride in 0.25 M HCl. Under these conditions hydroxylamine reduces Fe(III) to Fe(II).

The amount of hydroxylamine-reducible Fe(III) was calculated as the difference between the Fe(II) measured in the hydroxylamine and HCl extractions (Lovely & Phillips 1987).

Results & Discussion:

Isolation & Identification:

16 colonies were isolated from the SSM1 and SSM2 10^{-3} dilution series plates. Of the 16 colonies picked with an inoculating needle and streaked onto LB agar plates, the DNA of 14 single colonies was PCR amplified. Of the 14 PCR products, 10 PCR amplified 16S rDNA sequences were obtained. The sequences were ~450bp in length and were ultimately used to conduct a BLAST search for a nearest neighbor (Table 3).

Strain ID	# Of Seq	% Match
Acinetobacter sp.	3	99%
Aeromonas sp.	1	99%
Enterobacter sp.	1	99%
Shewanella massilia	2	99%
Shewanella oneidensis	1	98%
Shewanella sp.	2	100%

Table 3: For the 10 16S rDNA sequences amplified from strains cultured on School St. Marsh Water Agar Plates, the nearest neighbor and the percent sequence similarity are listed.

Clone Library Construction:

DNA was extracted from several samples by bead beating and the DNA used as template for the PCR amplification of community 16s rDNA. 30 cycles were required in order to obtain a concentration of 16S rDNA high enough to provide an effective ligation and transformation. The high number of cycles may have resulted in a PCR bias and in future studies, PCR conditions should be optimized.

The ligation and transformation were successful and 159 of the 192 sequences (Tables 4-5) were imported into ARB and used to construct a phylogenetic tree (Appendix A).

Samples Pooled	# Of Clones Sent	# Sequences Rec'd
SSM1_2	72	63
SSM6_7	72	57
SSM_New	24	20
SSM_Old	24	19

Table 4: List of the number of clones sent and the number of sequences received for each of the clone library constructed plates.

Nearest Neighbors	# Of Seq
Enterobacter sp	7
Aeromonas sp.	9
Shewanella sp.	7
Pseudomonas sp.	3
Acidovorax sp.	2
Rhodoferax sp.	11
Comamonas sp.	44
Aquabacterium sp.	5
Matsuebacter sp.	1
Burkholderia sp.	9
Herbaspirillum sp.	1
Methylobacillus sp.	4
Chromobacterium sp.	5
Uncultured Sludge sp.	1
Nevskia sp.	8
Frateuria sp.	1
Desulfobacterium sp.	1
Arcobacter sp.	1
Thiomicrospira sp.	2
Rhodobacter sp.	5
Sphingomonas sp.	1
Flexibacter sp.	2
Verucomicrobium sp.	1

Organelles	28
Total Clones	159

Table 5: A list of the number of clones and their nearest neighbors according to a phylogenetic tree generated in ARB

Scanning Electron Microscopy (SEM):

The samples prepared with the MBL Central Microscopy Facility's SEM were visualized successfully. The Critical Point Drying was a crucial step in the preparation of samples. Without the drying step, the morphology of the cells became distorted and difficult to see. 20 images were captured and 8 are available in Appendix B (SSM_New) and Appendix C (SSM_Old).

Ferrozine Assay:

The presence of HCl-extractable Fe(II) and the amount of hydroxylamine-reducible Fe(III) were determined by the ferrozine assay (Lovely & Phillips 1987). A standard curve was generated from the measured A_{562} of known concentrations of ferrous sulfate (figures 2-3). The function providing the best-fit line was subsequently used to determine the iron concentration, both Fe(II) and Fe(III), of each sample provided the A_{562} (figures 4-5). The method was straightforward and quantitative although there may have been errors generated as a result of the assay only being run a single time.

Abs. 562 nm	Molarity (mM)
0	0
0.1423	0.25
0.2729	0.5
0.5321	1
1.2832	2

Figure 2: The A_{562} of known concentration of ferrous sulfate was measured in order to generate

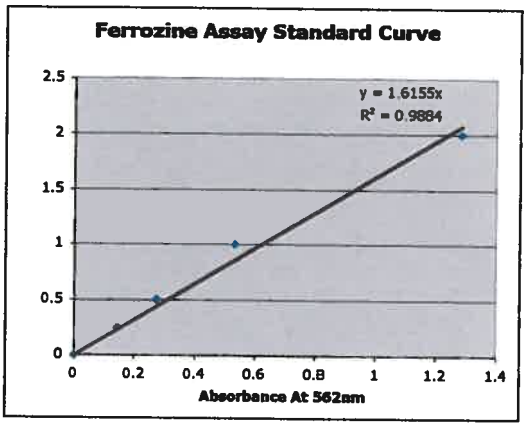

Figure 3: Standard curve generated with the A_{562} of a known concentration of ferrous sulfate.

	Surface Film	Surface w/out Film	Below Surface Film
Fe(II)	45.3	1.8	2.6
Fe(III)	70.2	3.7	0.5
Total Fe	115.5	5.5	3.1

Figure 4: Concentration of iron as determined using measured A_{562} and the standard curve.

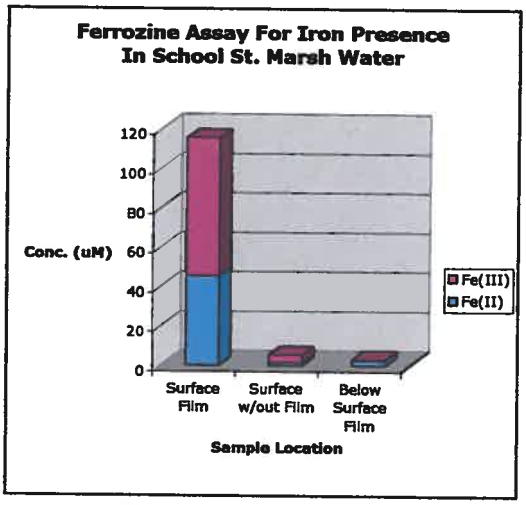

Figure 5: Graphical representation of the Fe(II) and Fe(III) concentrations (in μM) for the three different samples run through the ferrozine assay.

The results of the ferrozine assay indicate that iron is present in the surface film. Although iron may not be the only metal present, the concentration of iron in the surface film as opposed to a

surface without the film or the water beneath the surface film, lends support to the argument that the surface film is a metal oxide.

Additional experiments not explained in detail here also lend support to the surface layer containing metal oxides. Glass cover slips coated with the surface film were extracted with sterile water (pH 7), School Street Marsh water (pH 6), methanol and 0.5 M HCl. Only the 0.5 M HCl extracted any material from the slide. Insolubility in water and methanol supports the argument that the composition of the surface film in not completely organic in nature.

Future studies might use microelectrodes to analyze the oxygen content and pH of the surface layer and the underlying water. Should the surface layer provide a microaerophilic or anaerobic microenvironment, the surface may provide a unique opportunity to enrich for and study a diverse range of microorganisms. Further analysis of the surface film could also be accomplished with either an environmental scanning electron microscope (ESEM) or by atomic adsorption (Lovely & Phillips 1988). Either of these techniques would generate a clearer picture of the elemental composition of the film.

Acknowledgements:
I would like to express my gratitude to William Metcalf, Tom Schmidt, John Breznak, John Waterbury, Derek Lovely, Kelvin Gregory & Brad Postier. I would also like to thank Falk Warnecke, Rachel Whitaker, Amaya Garcia, Benjamin Griffin & Wynne Lewis. I appreciate the mentorship, conversations and friendship extended by every member of the 2004 Microbial Diversity Course. I look forward to continuing to learn with you!

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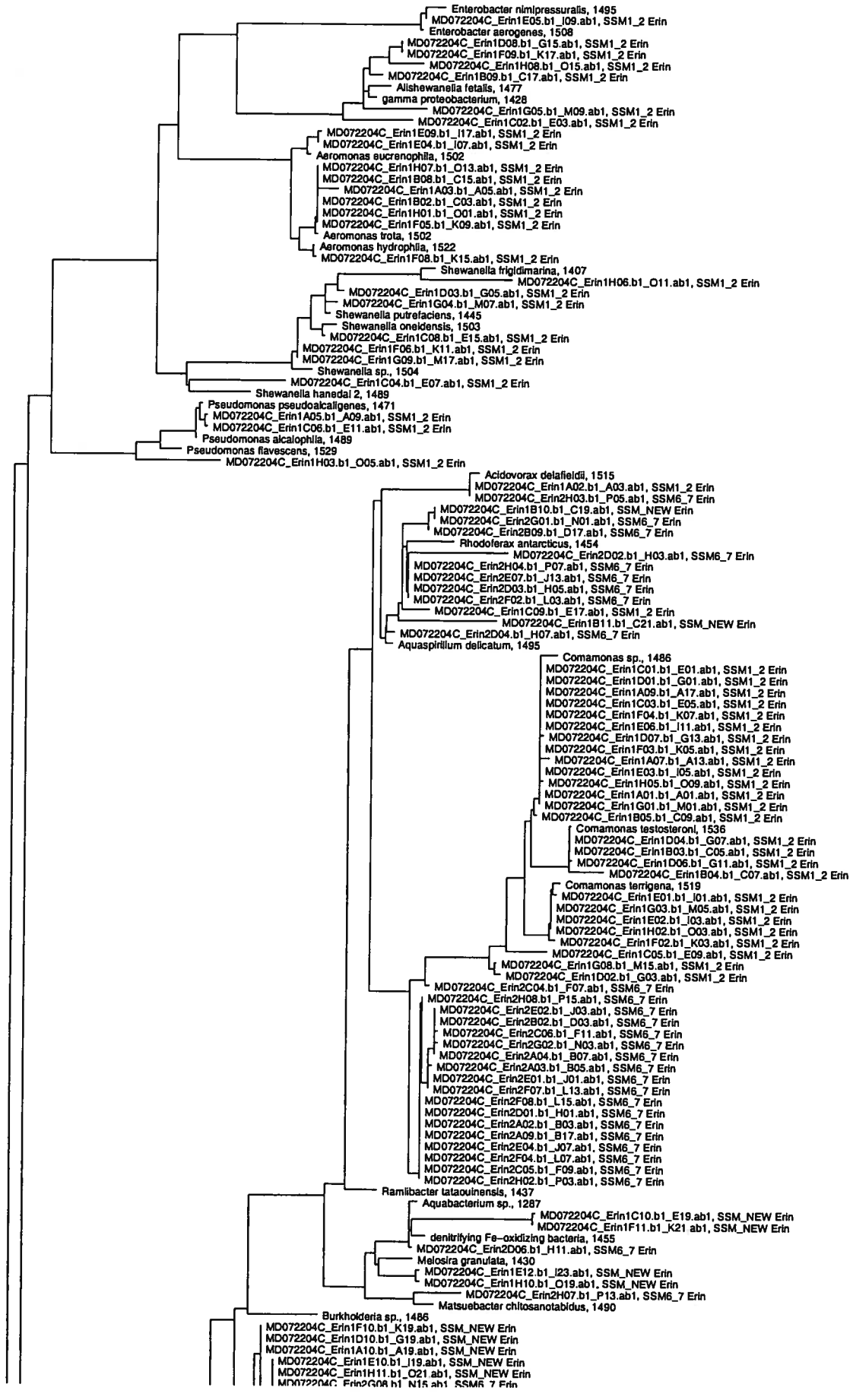
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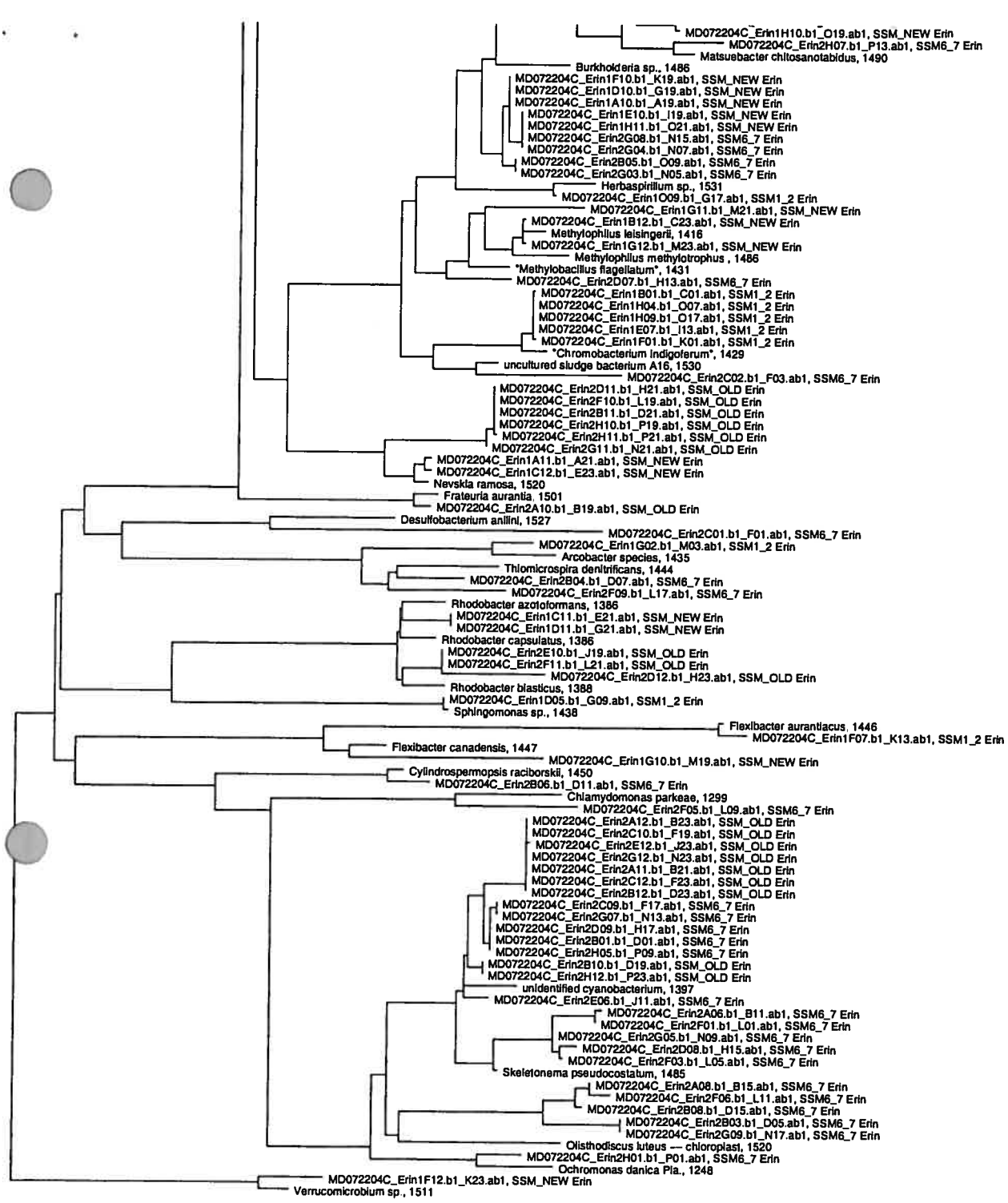
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MD072204C_Erin1G12.b1_M23.ab1, SSM_NEW Erin
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MD072204C_Erin1B01.b1_C01.ab1, SSM1_2 Erin
MD072204C_Erin1H04.b1_O07.ab1, SSM1_2 Erin
MD072204C_Erin1H09.b1_O17.ab1, SSM1_2 Erin
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MD072204C_Erin1F01.b1_K01.ab1, SSM1_2 Erin
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MD072204C_Erin2C02.b1_F03.ab1, SSM6_7 Erin
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MD072204C_Erin2F10.b1_L19.ab1, SSM_OLD Erin
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MD072204C_Erin2H10.b1_P19.ab1, SSM_OLD Erin
MD072204C_Erin2H11.b1_P21.ab1, SSM_OLD Erin
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MD072204C_Erin1C12.b1_E23.ab1, SSM_NEW Erin
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Thiomicrospira denitrificans, 1444
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MD072204C_Erin2F09.b1_L17.ab1, SSM6_7 Erin
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Rhodobacter capsulatus, 1386
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MD072204C_Erin2D12.b1_H23.ab1, SSM_OLD Erin
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MD072204C_Erin1D05.b1_G09.ab1, SSM1_2 Erin
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Skeletonema pseudocostatum, 1485
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MD072204C_Erin2B03.b1_D05.ab1, SSM6_7 Erin
MD072204C_Erin2G09.b1_N17.ab1, SSM6_7 Erin
Olisthodiscus luteus — chloroplast, 1520
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Ochromonas danica Pla., 1248
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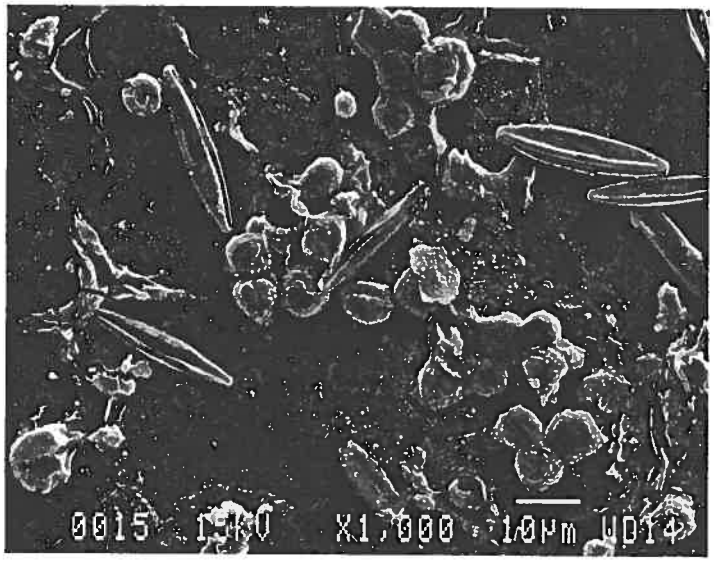

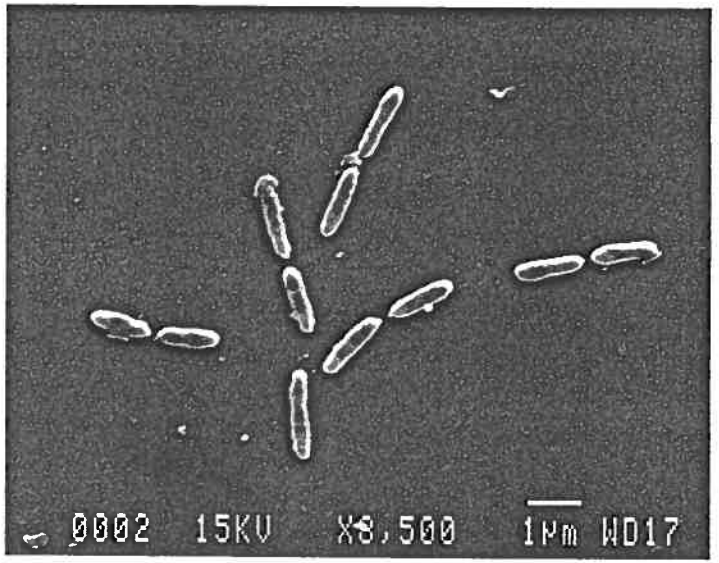

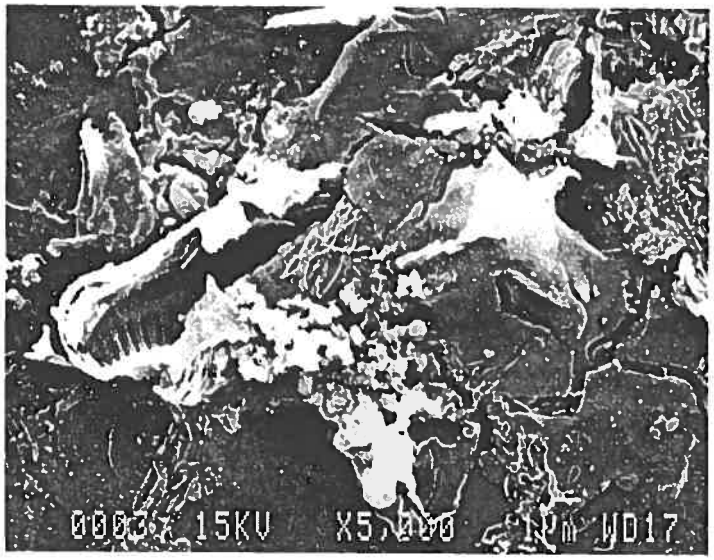

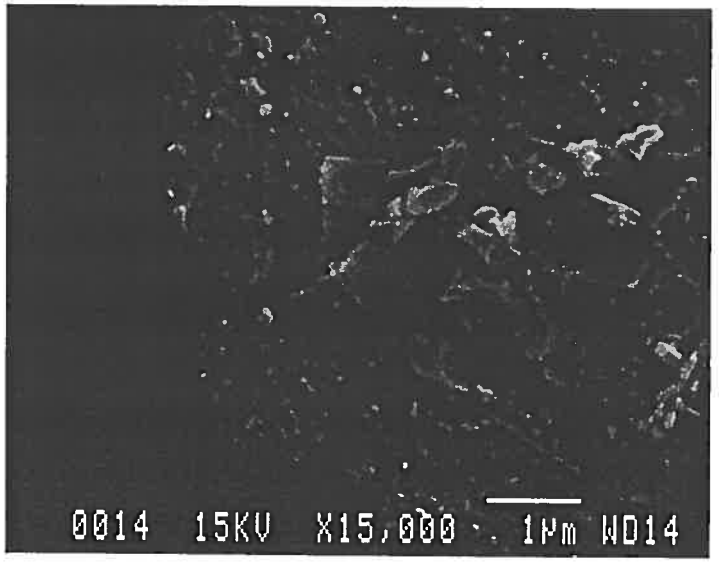

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