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Community Structure: From the Environment to the Lab

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Introduction:

The long held perception that microbes are homogeneously distributed in the natural environment has recently begun to change to a perception that microbes are distributed heterogeneously. It is now known that bacterial abundance and species richness varies on a millimeter scale or smaller. (Long, 2001). However, the characterization, tracking, and identification of members of a natural community still pose a large barrier to the understanding microbial interactions and complex community structure. One of the causes of this barrier is problems encountered in cultivating and isolating pure microbial cultures from the environment (Schut, 1993).

A common method for measuring the growth and diversity of sea water bacterial communities are dilution cultures monitored by a variety of molecular techniques (Fuchs, 2000). Most of the molecular techniques used in monitoring these cultures such as Amplified Ribosomal DNA Restriction Analysis (ARDRA), terminal-Restriction Fragment Length Polymorphism (t-RFLP), 16s rDNA sequences, and Denaturing Gradient Gel Electrophoresis (DGGE) do not provide an ideal picture of a complex bacterial community. 16s rDNA clone libraries allow the estimation of the diversity of a community but are rather time consuming for spatial and temporal comparisons, and the frequency of clones cannot be directly correlated to community composition. While ARDRA, t-RFLP, and DGGE can provide rapid insight into a community's structure they do not have the accuracy of specific counts obtained by fluorescent *in situ* hybridization (FISH) (Fuchs, 2000).

FISH is a recently developed staining technique that makes use of rRNA-targeted probes to allow the phylogenetic identification of bacteria in their communities without the need for cultivation by the use of epifluorescence, and confocal laser scanning microscopy, or by means of flow cytometry (Fuchs, 2000). This technique overcomes many of the barriers posed by other molecular techniques, allowing the enumeration and identification of individual microbial cells in a number of different environmental contexts, including sea water.

This study investigated the shifts in the morphological and compositional characteristics of a marine bacterial community under lab conditions. We monitored the

effect of dilution and the addition of a complex carbon source on surface seawater bacterial communities sampled from the pier at Stony Beach and from an offshore site near Elizabeth Island with FISH probes specific for Eubacteria, *α-Proteobacteria*, *β-Proteobacteria*, *γ-Proteobacteria*, *Cytophagal-Flavobacterium*, and DAPI staining. The counting was done with the use of epifluorescence microscopy.

Materials and Methods:

Sampling Sites

Onshore surface sea water samples were collected from the pier off Stony Beach, and offshore surface sea water samples were collected near Elizabeth Island near Woods Hole, MA. All experiments were started within an hour of returning to the lab with the sea water samples.

Dilution Experiments

Four dilution experiments were conducted, two using onshore sea water and two using offshore sea water. For all dilution experiments 200mL of sea water were sequentially filtered through polycarbonate filters of 1.4μm, 0.2μm and 0.02μm pore size. The filtrate was then mixed with 100mL of unfiltered sampled sea water in sterile media jars, to create a final dilution of 1:3 of the original bacterial population. The final filtration through the 0.02μm polycarbonate filter ensured the dilution of the phage population along with the bacterial population.

Non-Diluted Experiments

Four experiments were conducted, half using onshore sea water and the other half using offshore sea water. All experiments were conducted using 300mL of unfiltered sea water from the sampling site in sterile media jars.

Addition of Sea Water Complete Media

One percent sea water complete media was added to one onshore and one offshore sample from the diluted and non diluted experiments described above. Sea

water complete media containing 5% yeast extract (Difco), 3% bactopeptone (Difco) and 3% glycerol (Fisher Chemicals) was added to water from the sea water table and autoclaved for thirty minutes. Experiments without 1% sea water complete media were sampled every twenty four hours by fixing a 20mL sample with 4% formaldehyde (final concentration). When 1% sea water complete media (final concentration) was added cultures were sampled every two hours as previously described.

FISH

Bacteria from fixed samples were collected on a 0.2μm polycarbonate filter (Millipore) placed on top of a 1.2μm nitrocellulose filter (Sartorius) by filtering a 0.25mL-20mL aliquot of the fixed sample under suction using a standard Nalgene filter tower. Fluorescence *in situ* hybridization (FISH) of collected cells counterstained with DAPI was performed according to the protocol used for the class FISH experiment. The oligonucleotide probes used were EUB338 (5'-GCTGCCTCCCGTAGGAGT-3') specific for domain *Eubacteria* (Amann 1990); ALF968 (5'-GGTAAGGTTCTGCGCGTT-3') specific for the alpha-subclass of *Proteobacteria*, (Neef, 1997); BET42a (5'-GCCTTCCCACTTCGTTT-3') specific for the beta-subclass of *Proteobacteria* (Manz, 1992); GAM42a (5'-GCCTTCCCACATCGTTT-3') specific for the gamma-subclass of *Proteobacteria* (Manz, 1992); and CF319a (5'-TGGTCCGTGTCTCAGTAC-3') specific for the *Cytophaga-Flavobacterium* cluster (Manz, 1996). All probes were commercially synthesized and labeled with indocarbocyaninie dye Cy3 at the 5' end of the chain. The beta hybridization was performed using an unlabeled gamma probe as a competitor and the gamma hybridization was performed using an unlabeled beta probe as a competitor. This increased the stringency of the hybridization of this set of probes because they target a 16s rRNA sequence that varies by only one base. The hybridization buffer contained 360μL 5M NaCl, 40μL Tris-HCL pH=7.4, 700μL formamide , 900μL MilliQ H_2O, and 2μL 10% SDS added last to avoid precipitation. For EUB338, ALF968, and CF319a 1μL of probe solution was added to 9μL hybridization buffer and placed on sections of the filter. For BET42a and GAM42a 1μL of the labeled probe and 1μL the unlabeled competitor probe were added to 8μL hybridization buffer and placed on filter sections. Up to three filter sections were placed on a standard glass microscope slide, which was

inserted into the hybridization chamber (50mL Falcon tube w/ tissue and remaining hybridization buffer). The chamber was incubated at 46°C for more than 90 minutes but less than 180 minutes. The slides were then inserted into the washing buffer (1mL 1M Tris-HCl pH 7.4, 700μL 5M NaCl, 500μL 0.5M EDTA pH 8.0, MilliQ H_2O to 50mL and 50μL 10% SDS added last to prevent precipitation) and incubated in a 48°C water bath for 15 minutes. Samples were air dried, then covered with 50μL 1μg/mL DAPI solution and incubated for three minutes at room temperature. Filter sections were rinsed for a minute in MilliQ H_2O and a minute in 80% ethanol and allowed to air dry. The sections were then mounted on standard glass microscope slides using a 1:4 mixture of Vectashield:Citifluor to prevent fading.

Microscopy and Counting

The absolute concentration of each type of cell in the sample was determined by epifluorescence microscopy (Zeiss Axiovert microscope equipped with a 100x Plan Neofluar objective). Ten fields (2800μm^2) of each FISH probe and DAPI stain were averaged to obtain the number of cells per milliliter and their type. The FISH probe fluoresced with the Rhodamine filter set.

Results:

Morphological Transitions

At the beginning of the experiment most of the cells observed under DAPI illumination and all the specific FISH probes were very small cocci with the occasional small rod, after fifteen hours incubation with 1% sea water complete media added to the diluted and undiluted cultures the bacterial population observed were almost exclusively large rods (Fig. 1a,b).

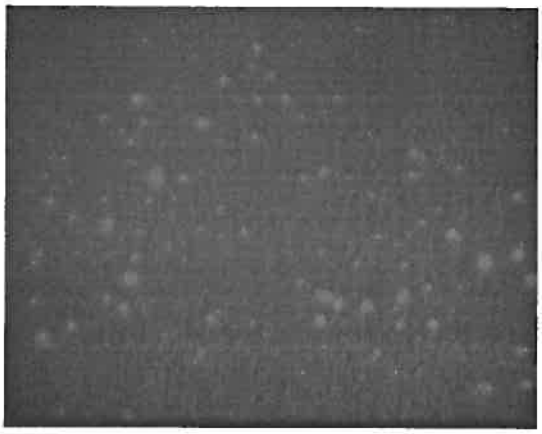


Figure 1a: Eubacteria FISH probe from offshore not diluted T=0 h

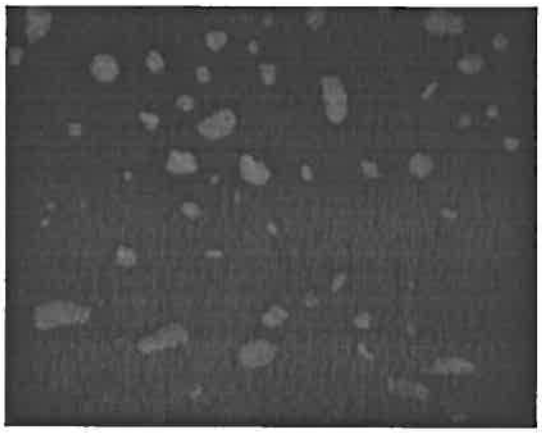


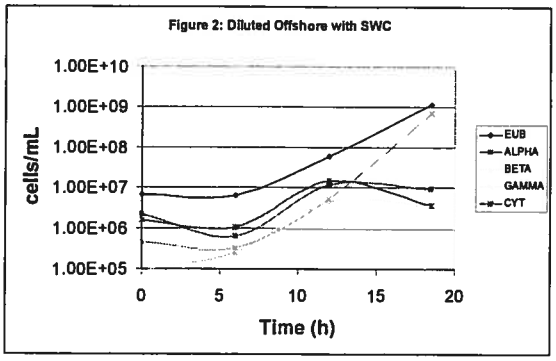
Figure 1b: Eubacteria FISH probe from offshore not diluted T=15 h

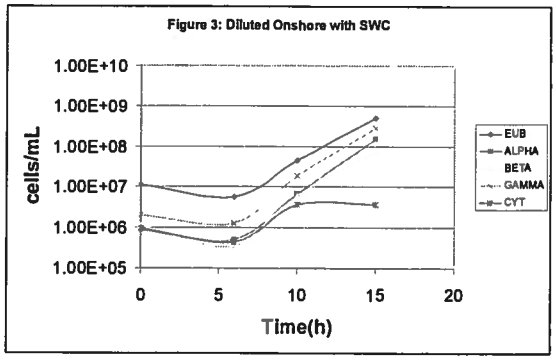
Without the addition of sea water complete media similar results were observed after nearly 96 hours.

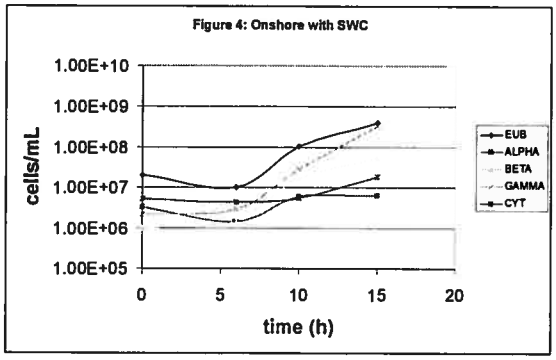
In addition to the morphological changes observed, cells also clustered together after six hours incubation with 1% SWC media was added (Fig. 1b). This phenomenon was not observed without the addition of sea water complete media. The morphological and clustering of cells were observed in the diluted and undiluted samples.

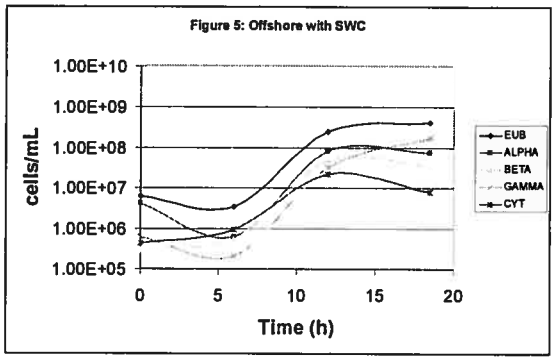
Structural Shifts

The initial bacterial population in all samples with and without sea water complete media started at ~10^7 cells/mL. In all cases with 1% sea water complete media the γ-*proteobacteria* made up more than 90% of the population of bacterial community (Table 1, Fig. 2-5). In three cases the α-*proteobacteria* were the second most abundant group (Table 1, Fig. 2, 3, and 5) in one case the β-*proteobacteria* ended up as the second most abundant gro p (Table 1, Fig. 4). In several cases the *Cytophaga/Flavobacterium* group was the most abundant at zero hours but least abundant at the last time point (fifteen to eighteen and half hours) (Table 1, Fig. 2-5). Generally the β-*proteobacteria* started as the least abundant group and ended as one of the two least abundant groups (Table 1, Fig. 2-5).

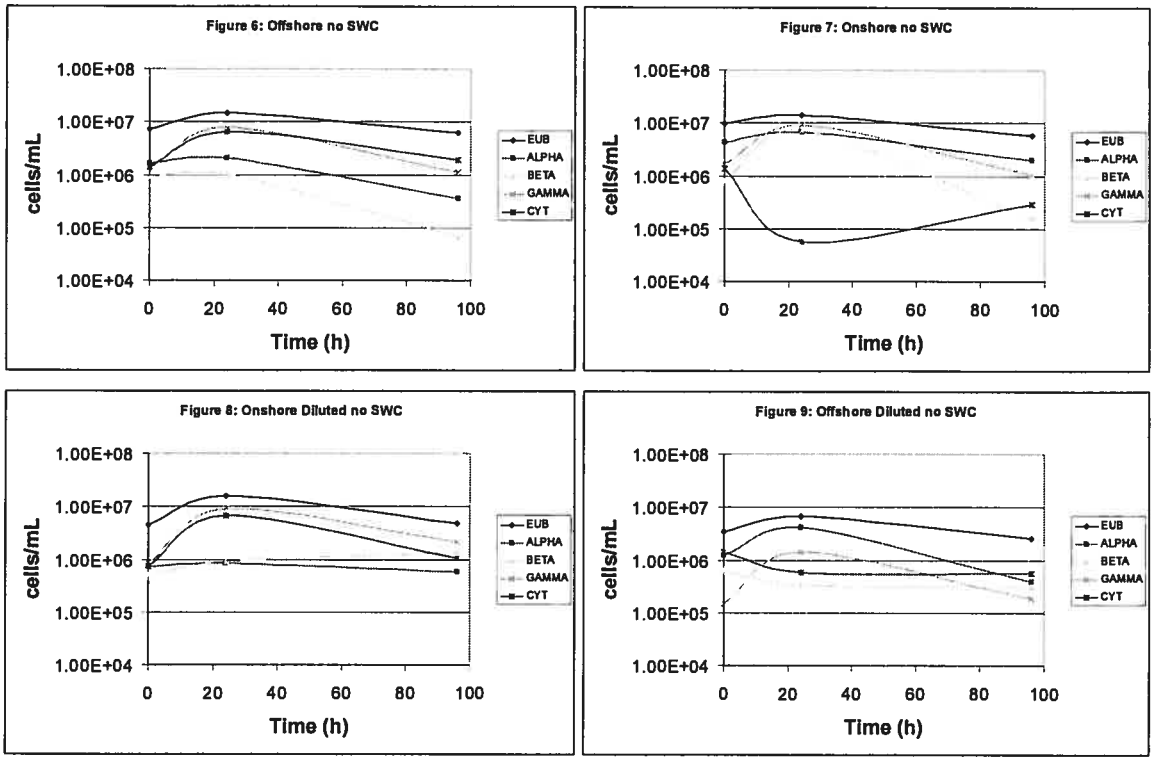








When no sea water complete media was added little overall growth was observed in the cultures, their cell concentration was basically the same after 96 hours. However, in all samples a spike in the population was observed after 24 hours. In all four cases the γ-*Proteobacteria* spiked after 24 hours then declined in numbers, in three of the four cases they did not end up as the dominant community member (Fig. 6-9). In the two offshore samples the *Cytophaga/Flavobacterium* started as the dominant member of the community and was still the dominant member after 96 hours of incubation at room temperature. β-*Proteobacteria* generally ended as the smallest group in the community.



Discussion:

Community Structure Shifts

In all samples enriched with 1% sea water complete (SWC) media, the γ-*Proteobacteria*, which was not dominant at the beginning, consistently became the most abundant bacterial group in the diluted and non diluted cultures. This indicates a succession in the bacterial community during the experiment. It appears that γ-*Proteobacteria* may fill the niche of the typical "R" strategist which rapidly exploit extra nutrients when they become available. The *Cytophaga/Flavobacterium* appear to fulfill the niche of a typical "K" strategist in which long-term survival on limited resources is the selective advantage instead of fast growth. It is also possible that the *Cytophaga-Flavobacterium* are specialist in the degradation of particulate matter that may have been largely absent from our dilution cultures (Fuchs, 2000).

Further evidence that the *Cytophaga-Flavobacterium* may be "K" strategists and γ-*Proteobacteria* "R" strategists comes from the cultures without 1% SWC. In these cultures there was a spike in γ population at 24 hours then a slow decline in the population until 96 hours (Fig. 6-9). Although the *Cytophaga-Flavobacterium*

population did not spike at 24 hours, also they did not experience the decline in population seen in the γ-*Proteobacteria* until 96 hours.

Antagonistic Interaction among Marine Bacteria

It has been suggested that γ-*Proteobacteria* are producers of antibiotic inhibitory compounds (Long, 2001). In their study they found *Vibrionales* and *Altermonadales,* members of the γ-*Proteobacteria* sub-groups, were the least sensitive to inhibition by other groups of bacteria. In our studies when 1% SWC media was added the bacterial community quickly entered into log growth, resulting in a dense culture, possibly with high concentrations of inhibitory compounds making the γ-*Proteobacteria's* resistance to inhibitory compounds a distinct advantage. This is a possible explanation for the γ-*Proteobacteria* being the most abundant group at the

It has been shown that these inhibitory compounds are often involved in quorum sensing and are produced only when a bacterial culture reaches a specific density. Since the culture did not become very dense without the addition SWC it is possible that these molecules were not produced, or did not accumulate in high enough concentrations to affect the growth of the other members of the community.

We believe that the dominance of γ-*Proteobacteria* in the systems with SWC is probably a combination of the "R" strategists such as *Vibrionales* and *Altermonadales* and the γ-*Proteobacteria's* decreased sensitivity to inhibitory compounds.

Conclusions:

FISH was successfully used for the visualization for the changes of a microbial community under lab conditions. The data obtained from this study shows that community structure changes rapidly when a complex carbon source is added, or even when the sample is removed from its natural environment. Further characterization of the cultures by other molecular techniques such as ARDRA, t-RFLP, and 16s rDNA clones libraries could help confirm the theories presented above. In addition the use of specific FISH probes to confirm the presence of *Vibrionales, Altermonadales* and other species would provide a more complete picture of the evolution of the bacterial community when it is brought into the laboratory.

TABLE 1 Offshore Not Diluted with SWC						
Time (h)	Eubacteria (cells/mL)	α-*proteo* (cells/mL)	β-*proteo* (cells/mL)	γ-*proteo* (cells/mL)	*Cytohphaga/ Flavobacterium (cells/mL)*	DAPI (cells/mL)
0	6.40E+06	4.32E+06	3.08E+05	6.38E+05	4.45E+05	1.43E+07
6	3.59E+06	6.27E+05	5.81E+05	2.05E+05	9.69E+05	4.47E+06
12	2.45E+08	8.21E+07	4.93E+07	3.19E+07	2.28E+07	2.65E+08
18.5	4.14E+08	7.30E+07	3.37E+07	1.71E+08	8.21E+06	6.25E+08
Offshore Diluted with SWC						
Time (h)	Eubacteria (cells/mL)	α-*proteo* (cells/mL)	β-*proteo* (cells/mL)	γ-*proteo* (cells/mL)	*Cytohphaga/ Flavobacterium (cells/mL)*	DAPI (cells/mL)
0	6.87E+06	2.26E+06	9.12E+04	4.56E+05	1.57E+06	8.09E+06
6	6.37E+06	6.27E+05	2.74E+05	3.53E+05	1.05E+06	8.92E+06
12	5.91E+07	1.19E+07	4.56E+06	5.47E+06	1.46E+07	6.45E+07
18.5	1.14E+09	9.12E+06	3.65E+06	6.88E+08	3.65E+06	1.27E+09
Onshore Not Diluted with SWC						
Time (h)	Eubacteria (cells/mL)	α-*proteo* (cells/mL)	β-*proteo* (cells/mL)	γ-*proteo* (cells/mL)	*Cytohphaga/ Flavobacterium (cells/mL)*	DAPI (cells/mL)
0	2.03E+07	3.36E+06	9.46E+05	2.19E+06	5.32E+06	2.24E+07
6	1.04E+07	1.44E+06	1.55E+06	2.94E+06	4.38E+06	1.51E+07
10	1.03E+08	6.16E+06	1.71E+07	2.80E+07	5.47E+06	1.05E+08
15	3.97E+08	6.38E+06	5.29E+07	3.27E+08	1.82E+07	4.43E+08
Onshore Diluted SWC						
Time (h)	Eubacteria (cells/mL)	α-*proteo* (cells/mL)	β-*proteo* (cells/mL)	γ-*proteo* (cells/mL)	*Cytohphaga/ Flavobacterium (cells/mL)*	DAPI (cells/mL)
0	2.03E+07	3.36E+06	9.46E+05	2.19E+06	5.32E+06	2.24E+07
6	1.04E+07	1.44E+06	1.55E+06	2.94E+06	4.38E+06	1.51E+07
10	1.03E+08	6.16E+06	1.71E+07	2.80E+07	5.47E+06	1.05E+08
15	3.97E+08	6.38E+06	5.29E+07	3.27E+08	1.82E+07	4.43E+08

TABLE 2 Offshore No SWC						
Time (h)	Eubacteria (cells/mL)	α-*proteo* (cells/mL)	β-*proteo* (cells/mL)	γ-*proteo* (cells/mL)	*Cytohphaga/ Flavobacterium (cells/mL)*	DAPI (cells/mL)
0	7.14E+06	1.62E+06	9.01E+05	1.31E+06	1.43E+06	1.59E+07
24	1.47E+07	2.04E+06	1.00E+06	7.72E+06	6.42E+06	1.77E+07
96	6287619	364830.11	68405.65	1140094.1	1926759.043	6.86E+06
Onshore No SWC						
Time (h)	Eubacteria (cells/mL)	α-*proteo* (cells/mL)	β-*proteo* (cells/mL)	γ-*proteo* (cells/mL)	*Cytohphaga/ Flavobacterium (cells/mL)*	DAPI (cells/mL)
0	9.69E+06	4.38E+06	8.09E+05	1.63E+06	1.35E+06	1.70E+07
24	1.42E+07	6.67E+06	7.31E+06	9.03E+06	5.70E+04	2.25E+07
96	5.85E+06	2.01E+06	1.60E+05	1.06E+06	2.85E+05	7.26E+06
Onshore Diluted No SWC						
Time (h)	Eubacteria (cells/mL)	α-*proteo* (cells/mL)	β-*proteo* (cells/mL)	γ-*proteo* (cells/mL)	*Cytohphaga/ Flavobacterium (cells/mL)*	DAPI (cells/mL)
0	4.50E+06	7.30E+05	5.02E+05	8.44E+05	7.30E+05	6.77E+06
24	1.59E+07	8.55E+05	9.12E+05	9.01E+06	6.82E+06	1.89E+07
96	4.85E+06	5.81E+05	1.37E+06	2.14E+06	1.05E+06	1.03E+07
Offshore Diluted No SWC						
Time (h)	Eubacteria (cells/mL)	α-*proteo* (cells/mL)	β-*proteo* (cells/mL)	γ-*proteo* (cells/mL)	*Cytohphaga/ Flavobacterium (cells/mL)*	DAPI (cells/mL)
0	3.40E+06	1.24E+06	6.38E+05	1.48E+05	1.40E+06	5.47E+06
24	6.74E+06	4.13E+06	3.31E+05	1.43E+06	5.93E+05	6.92E+06
96	2.53E+06	3.88E+05	3.08E+05	1.82E+05	5.70E+05	3.39E+06

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