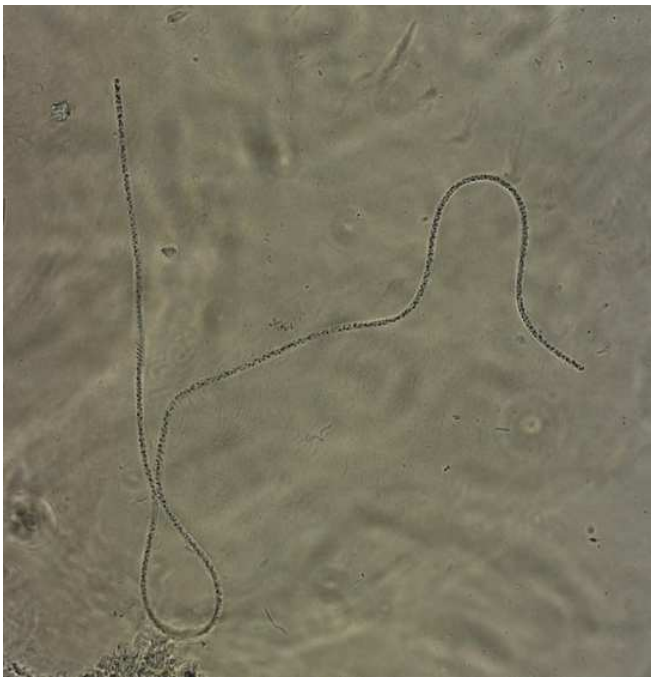
The monitoring of ecosystem succession by community analysis



Geert Marinus van der Kraan

Project
Microbial Diversity Summer course
2007
Marine Biological Institute
Woods Hole

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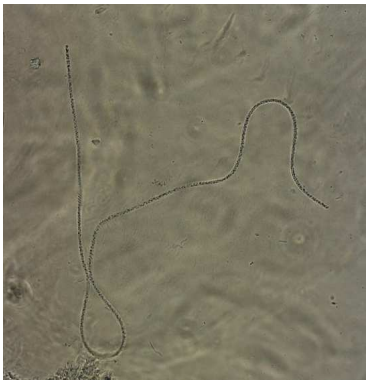

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Microbial Diversity summer course
Project

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Summary

In this project an attempt was made to monitor the increasing species richness of 2 new opened areas in marine environments analyzing the (bacterial) community through by application of multiple techniques. One environment was seawater entering the lab in the Loeb building the other one was the water from Eel pond. A Buoy was dropped with the slides attached to them drifting 80 cm below the water surface (both locations: Woods hole, Massachusetts, USA) These (new areas) slides provided in this case were alcohol cleaned glass slides (7.5 by 2.5 cm). On 50% of the glass slides, dried agarose was deposited to increase bacterial attachment and ease material extraction for DNA analysis.
The agar surfaces displayed a more abundant ecosystem when compared to the glass slides.

Community analysis by clone libraries after 4 days already displayed a wide variation in species richness regarding both environments. Richness kept increasing in later time frames (9 and 13 days). After 9 days already eukaryotes were present as were multi cellular organisms such as nematodes. After 17 the buoy slides displayed visible forms of marine plant life. The clone libraries however were too small to get a good overview of the species richness and the succession over time.

On the ethanol cleaned glass slides a microscope study was performed in order to monitor the succession process. First the glass slides are colonized by filamentous organisms.

CARD Fish analysis displayed that both marine environments regarding the cell counts from the main groups α, β, γ, proteobacteria and cytophaga are the same. β-proteobacteria in both environments were very low in abundance. (data not in report)

Both marine environments were equal regarding their bacterial community but not regarding the deposits on the slides themselves. The water that enters the labs is most probably filtered. If the experiments were performed in a way which is more broadly set-up, then the monitoring of the succession of the ecosystem would have been studied in a realistic way.

The succession process through microscopy was well monitored.

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Introduction

1.1 Ecosystems

Ecosystems progress over time when a new untouched area opens up. The new area is first colonized by a few species, a pioneer ecosystem emerges which will lay the basis for other species that colonize the ecosystem later. The ecosystem is in progression when new species emerge. Finally it will reach a steady state in which all species are in balance *(example figure 1)*.

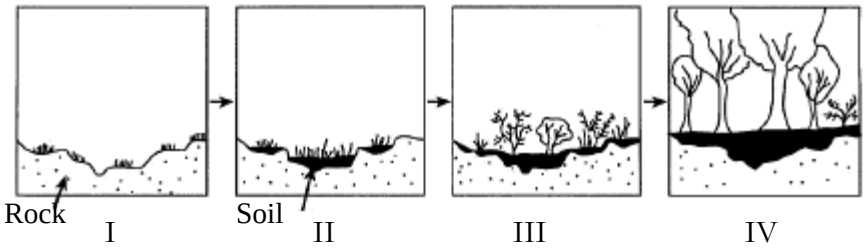


Figure 1: Example of ecosystem succession
I – pioneer ecosystem
II & III – succession (new species emerge)
IV – balanced ecosystem

1.2 Biofilms

The same holds for ecosystems occupied by microorganisms. Also here the emerged ecosystem is first occupied by opportunists who lay the basis for other species after a certain time frame depended on conditions a visible structure emerges, a bio film *(bio film succession figure 2)*

Figure 2: Bio film formation, the process resembles ecosystem succession on a larger scale. Complex microbial communities appear in a biofilm.

Biofilms are complex clusters of microorganisms which can be recognized by the excretion of a matrix which provides protection. Biofilms can be characterized by surface attachment, structural heterogeneity, genetic diversity and complex community interactions. They are usually found on exposed surfaces to water, they can also be found as mats on liquid surfaces, a biofilm will quickly grow and become visible if the right conditions are there. Biofilms can contain many different types of microorganism, e.g. bacteria, archaea, protozoa and algae; each group performing specialized metabolic functions, although biofilms sometimes can also be formed by one single species.

Goal

The Goal was to study the emergence of new species while a bio film is in succession judged by species richness and microscope studies through clone library construction. Due to the limited amount of time only the bacterial population was studied. To study the overall process microscopy was used Judged was the emergence of new species emerge at later time frames and how the two different environments differ from each other.

Research questions:

- Is it possible to detect changes in the bio film ecosystem?
- Can ecosystem progression be monitored?
- Is there is any resemblance between the first pioneers and the fast growers plated on agar plates.
- Compare the different sample times concerning species richness do new species emerge?
- Compare both ecosystems (regarding species richness and cell counts). Are they the same?

Materials & Methods

2.1 Slide preparation

16 glass Microscope slides (7.5 - 2,5 cm) were cleaned with ethanol (96%). On 8 slides 2 ml of a 1% agarose solution was placed. The slides were dried overnight.

2.2 Buoy set-up

An empty carboy was filled for 50% with water. Also rocks were added to the interior of the carboy to increase the weight. A rubber plug was placed on the bottleneck to prevent air from leaking out. On the Buoy an 80 cm piece of rope was attached on which a slide holder was attached. The slide holder's material was metal and therefore it stayed down. The slides were placed in the slide holder. The buoy was dropped off attached to a dock in Eel pond, Woods hole

2.3 Lab set-up

A plastic bin (40 – 20 – 20 cm) was filled with seawater entering the lab. A hose was placed in the bin which provided the bin with fresh seawater entering the lab. A similar slide set-up was placed at the bottom of the bin with similar slides placed in the holder.

2.4 DNA extraction

DNA extraction was performed at different timeframes. An agarose containing slide was collected from both locations. The agarose was scraped off using a sterile razorblade (cleaned with etOH). DNA contained in the cells which were present in the agarose was extracted using the MOBIO Ultraclean soil DNA Isolation KIT.

2.5 PCR amplification

For the PCR amplification, the environmental DNA samples were diluted 5,10 and 15 xs in σ-H_2O. To amplify the bacterial DNA, reactions were performed using the 16S_8F and 16S_1492R bacterial primers.

16S_8F	5'-GTTTGATCMTGG C-3´	(tm 47 °C)
16S_1492R	5'-TACCTTGTTAYGAC T-3´	(tm 41,6 °C)

Typical reaction mixture:
12.5 µl promega master mix
2.5 µl 16S_8F primer
2.5 µl 16S_1492R primer
6.5 µl σ-H_2O
2.0 µl diluted environmental DNA.

PCR program used:

Initial denaturation	95 °C	5 min	
Template denaturation	95 °C	30 sec	Steps repeated 30 xs
Annealing	46 °C	30 sec	
Extension	72 °C	1.5 min	
Final extension	72 °C	5 min	
Hold	4 °C	endless	

2.6 Clone library set-up

After PCR amplification the PCR products were inserted into a plasmid using the Invitrogen TOPO TA cloning kid. This kid contains linear vectors which together with a PCR product combine into a circular plasmid. The TOPO vector contains a DNA ligase which combines the two fragments. As amplification system *E.coli* TOP10 competent cells were used. As selection marker agar plates containing Luria-Bertani broth (LB) and kanamycin were used.

Typical cloning reaction:
2 µl Fresh PCR product
2 µl σ-H_2O
1 µl pCR®4-TOPO vector.

The reaction was prepared on ice and incubated for 5 minutes at room temperature and then placed back on ice. One shot of competent cells was thawed. 2 µl of the reaction mixture was added to the competent cells. The cells were incubated for 30 minutes on ice, after incubation they were heat shocked for 40 seconds at 42 °C. After the heat shock 250 µl SOC medium was added to the cells. The cells in tubes were placed horizontally in a 37 °C incubator for 30 minutes. 2 agar plates were incubated with the cells (40 and 80µl). The plates were incubated overnight at 37 °C. Colonies were picked 18 hours later. A 96 wells plate was filled with media containing LB media with kanamycin, 1,2 ml in each well. Colonies on the plates were numbered and picked with wooden toothpicks under a flame, each well was incubated with a single colony. After 96 colonies were picked the plate was covered with a paper sticker and incubated. The plate was sent of to the sequencing facility.

For a detailed description of the cloning procedure: Chapter 12 of the Microbial Diversity 2007 course manual.

2.7 Microscopy

Glass slides were collected from both locations in a 50 ml falcon tube. A 100 ml 0.1% w/v solution of crystal violet was prepared. The glass slides were cleaned on one side and then placed in the CV solution for 10 minutes. After cell fixation, excess CV was rinsed off with sterile sea water base H_2O. Images from the glass slides were taken at 100, 400 and 1000xs magnification using a Zeiss microscope: Zeiss imager A1. The used camera was an Axiocam mRc. The software used for image processing was Axio VS40 V4.6.3.0.

2.8a Agar plating/colony PCR

Marine Low Tryptone yeast extract (LTY) agar plates were poured. (0.05% tryptone, 0.05% yeast extract), combined with a standard sea water based (SW) basic solution.
400 g NaCl, 60 g $MgCl_2.6H_2O$, 3g $CaCl_2.2H_2O$. 10g KCl added to 20 liters of water.
15 g/l agar was added. The media was autoclaved before use.

100 µl of water from both environments was plated. Appearing colonies were picked with a pipette tip and the transferred material was placed in 20 µl of a 0.05% NP40 solution. The suspension was boiled for 5 minutes, 2 µl of the boiled material was subjected to PCR amplification as described in section 2.5. After the PCR amplification the primer dimmers were removed by an exosap(enzyme) treatment 0.25 µl ExoSAP-IT enzymes were added to 3.25 µl σ-H_2O. 1.5 µl of PCR product was added to 3.5 µl of Exosap master mix. Run a program at which the tubes are held at 37 °C for 30 minutes followed by 15 minutes at 80°C. Products were sent for sequencing.

Results & Discussion

3.1 Microscope study of the succession

Two environments were monitored during the process of succession regarding biofilm formation in a marine environment. Microscope images were taken from EtOH cleaned glass microscope slides stained with crystal violet. Images were also taken from glass slides on which an amount of agarose was deposited (used for DNA extraction). First environment was the water from Eel pond the second environment was marine water entering the lab in the LOEB building. Both environments are located in Woods hole. Both environments were monitored over a time frame of 17 Days.

In the first 7 days little differences could be detected between the two environments when regarding the glass surfaces. Mostly filamentous bacteria and in chain dividing cells were observed. *(figure 3 & 4).*

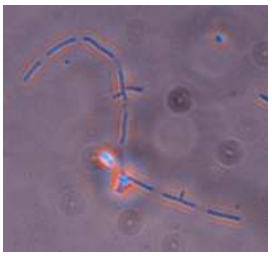


Figure 3: In chain dividing cells (1000xs) **Figure 4:** Filamentous organisms (1000xs)

At day 9 a significant difference between the two environments emerges. More organic material is deposited on the glass slides from eel pond and the presence of diatoms on the slides from eel pond was more dominant *(figure 5, 6 & 7).* On the glass slides for the lab set-up it is clearly visible that the filamentous organisms are more dominant in regard to the glass slides from Eel pond.

Figure 5a: Glass slide from eel pond (day 9)
5b: Glass slide fro lab set-up (day 9)

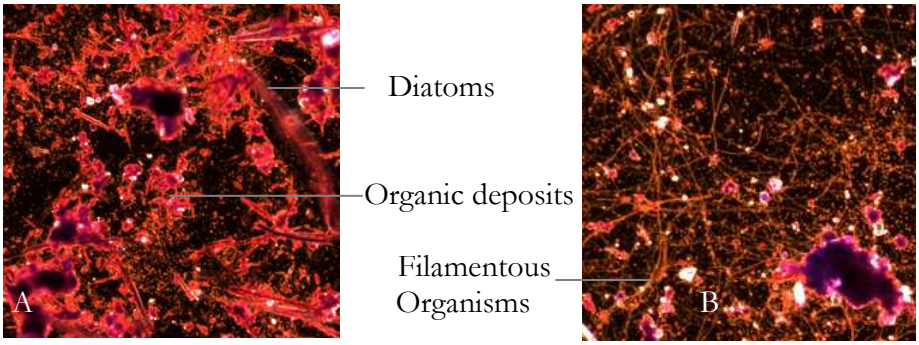


Figure 6a: 100xs dark field image of slide from eel pond stained with CV
6b: 100xs dark field image of slide from lab set-up stained with CV

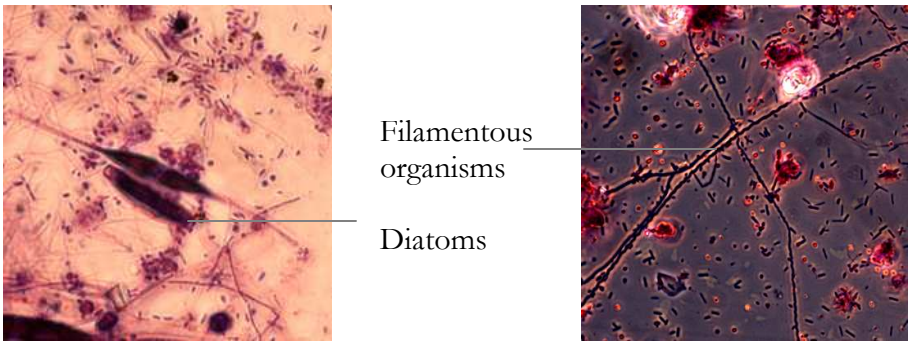


Figure 7a: 1000xs image of slide from eel pond stained with CV
7b: 1000xs image of slide from lab set-up stained with CV

Regarding the glass slides, from day 9 on the surfaces changed very little over time. When the glass slide surfaces were compared with the agar slide surfaces significant changes could be observed. The cell densities in the agar were higher and the different colony morphologies observed were also more diverse in respect to the glass only surfaces *(figure 8)*. In the agar surface, rod shaped bacteria were the most dominant.

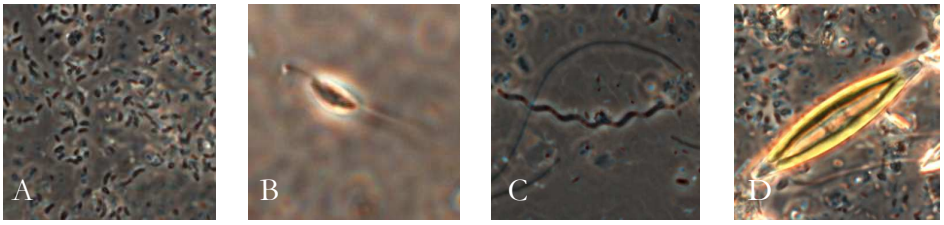


Figure 8: Examples of morphologies in the agar surface after 4 days (Eel pond slide).
(all 1000xs, A: rods, B: undefined, C: spirillum, D: diatom(eukaryote))

On day 13 of the agarose surfaces part of the environment in the agar had become anoxic which is now a completely different environment from the glass slides. This is concluded due to the emergence of *Beggiatoa* species *(figure 9a)* which were observed in slides from both environments. These organisms oxidize H_2S to elemental sulfur S^o. H_2S can be produced by bacteria without oxygen by sulfate reduction. Sulfur globules could be observed in the cellular body *(figure 9b)*.

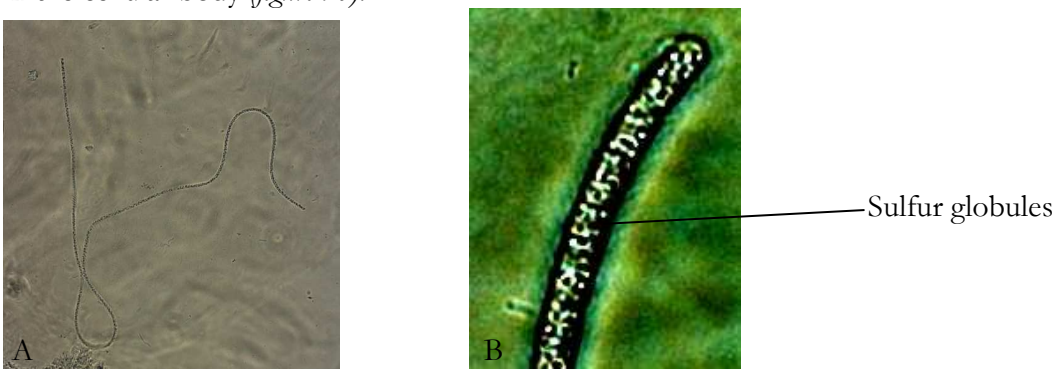


Figure 9a: *Beggiatoa* at 400xs enlargement
9b: Tip of *Beggiatoa* at 1000xs enlargement

On day 13 Also multi cellular life forms appeared in the agar surfaces. Most of them were nematodes, which are small multicellular microorganisms which are commonly found in marine and freshwater environments. Many of them are parasitic. They reproduce sexual; the females release the eggs into the environment. Also unknown multicellular organisms were observed *(figure 10)*.

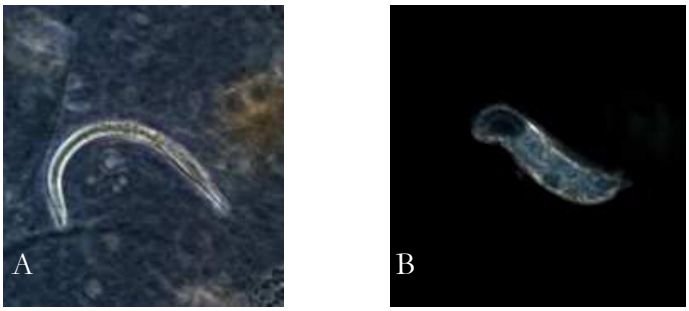


Figure 10a: 100xs dark field image of a nematode, Eel pond slide
10b 100xs dark field image of an unknown multicellular organism, eel pond slide.

On day 17, both set ups were collected. The buoy was reclaimed from the dock in Eel pond and photographed, The lab set-up was broken down. On the buoy an extensive visible bio film had emerged also eggs from an unknown sea animal were deposited on the slide holder. Also the biofilm on the glass slides had grown extensively *(Figure 10 & 11)*. Monitoring of both environments was ended on day 17.

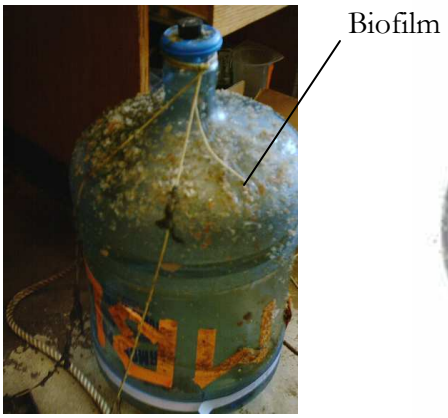


Figure 10: Buoy after 17 Days

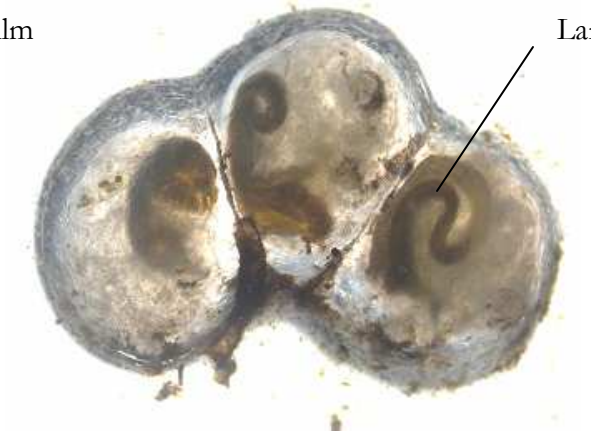


Figure 11: Image of eggs on deposited on slides (eggs were approx 0.7 cm in diameter).

3.2 Community analysis

During the succession of the biofilm in both environments DNA was extracted from the agar surfaces at different time frames using the soil DNA extraction kid, this to assess the species richness *(table 1)*. The DNA amount was measured on a Nanodrop spectrophotometer.

Table 1: DNA extraction events/DNA yield (ng/µl in extracted solution)

Eel pond buoy	Lab set-up.
Day 4 3.7ng/µl	-
-	Day 6 6.6 ng/µl
Day 9 6.8 ng/µl	Day 9 7.1 ng/µl
-	Day 13 7.7 ng/µl

A PCR amplification was performed on dilutions of all environmental samples. The dilution factor was not always consistent with a good amplification. Therefore the PCR amplifications were repeated multiple times using different dilutions *(Table 2)*. (For the used amounts of reagents and DNA see sections 2.4 and 2.5) From the amplified PCR signals clone libraries were constructed (section 2.6)*(table 2)*

Table 2: Dilutions tried PCR amplification signal quality/number of clones submitted

	PCR Signal quality	Number of submitted clones	
Eel pond buoy day 4			
Undiluted	no signal	-	
5xs	good signal	48	
Eel pond buoy day 9			
Undiluted	no signal	-	
5xs	no signal	-	
10xs	faint signal	48	
Lab set-up day 6			
Undiluted	no signal	-	
5xs	good signal	32	
Lab set-up day 9			
Undiluted			
2xs	no signal	-	
4xs	no signal	-	
10xs	good signal	32	
12xs	good signal	-	
Lab set up day 13			
Undiluted	no signal	-	
2xs	no signal	-	
4xs	no signal	-	
12xs	faint signal	32	(192 in total)

Already on day 4, a large number of different organisms were observed on the agarose surface of the eel pond buoy slide (Phylogenetic trees, See appendices 1 to 4). The same holds for the lab set-up agarose surface slides analyzed on day 6. The sequences from both environments were compared in a phylogenetic tree. Sequences from both environments yielded much overlap. The same holds for the sequences obtained on day 9. A conclusion drawn from the clone libraries is that they number of submitted clones was simply too small to obtain a good overview of the real amount of species which was present in the agarose surfaces. However an increase in species richness is observed in the later time frames in respect to the earlier time frames. According to the bacterial population both waters host the same species *(table 3)*.

Table 3: Found groups/bacteria that are known

Name	Found in	Description
Alteromonas macleodii	lab/Eel p.	Marine γ-proteobacteria
Var. Pseudoalteromonas spp.	Lab	Marine proteobacteria
*Various Glaciecola pallidula	Lab/Eel p.	Bacteria ass with diatoms γ-proteobacteria
Reinekia marinesedimenta	Lab	-
Teredinibacter turnerae	Lab	Marine γ-proteobacteria
Oceanicola batsensis	Eel pond	Marine α- proteobacteria
Gyrosigma fasicola	Eel pond	-
Various microbulbifer spp.	Lab/Eel p.	γ-proteobacteria
Eubostrichtus dianae	Lab/Eel p.	-
Thalasomonas ganghwensis	Lab	Bacteria ass with Oysters
Various mucus bacteria	Eel pond.	-
Large unidentified groups	Lab/Eel p.	-
Haslea pseudostrearia	Eel pond	Diatom
Lewinella persicus	Lab	Cytophaga
Cytophaga spp.	Eel pond.	-
Nitrospira marina	Lab	NO_2 oxidizer
Polyangium vitellinum	Lab	Myxobacter
Bacteriovorax stolpii	Lab	Bdellovibrio species
Bdellovibrio	Lab	bacterial parasite
Roseibium denhamense	Lab	Marine α- proteobacteria
Photobacterium damselae	Lab/Eel p.	Bacteria ass with fish
Various spp. from arctic env.	Lab/Eel p.	-

** Many unknown shared groups were observed, most shared ones fell into the "uncultured unknown bacteria group"

3.3 Colony PCR

5 colonies from each marine LTY plate (1 plate for each environment) were picked and isolated. The colonies from the isolates were picked and subjected to DNA extraction and 16S rRNA PCR amplification. The retrieved DNA was sent for sequencing *(figure 12)*.

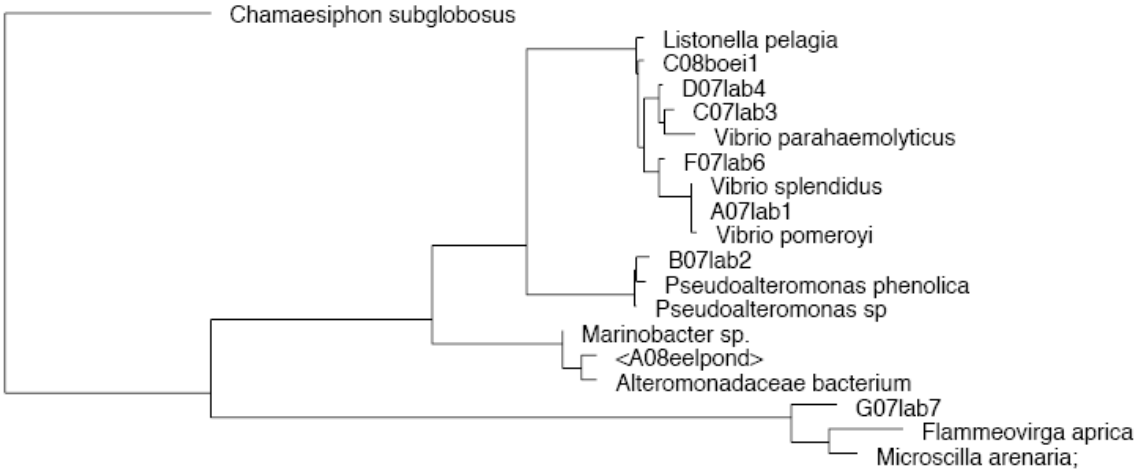


Figure 12: Phylogenetic tree of picked colonies. Each sequence has the extension Boei or Lab which designates the origin (boei=buoy).

From the phylogenetic data we can conclude that most plated bacteria do not play an important role in the biofilm formation. This also implies that plating bacteria automatically means enriching for a certain group. This has to be taken into consideration when studying an environment.

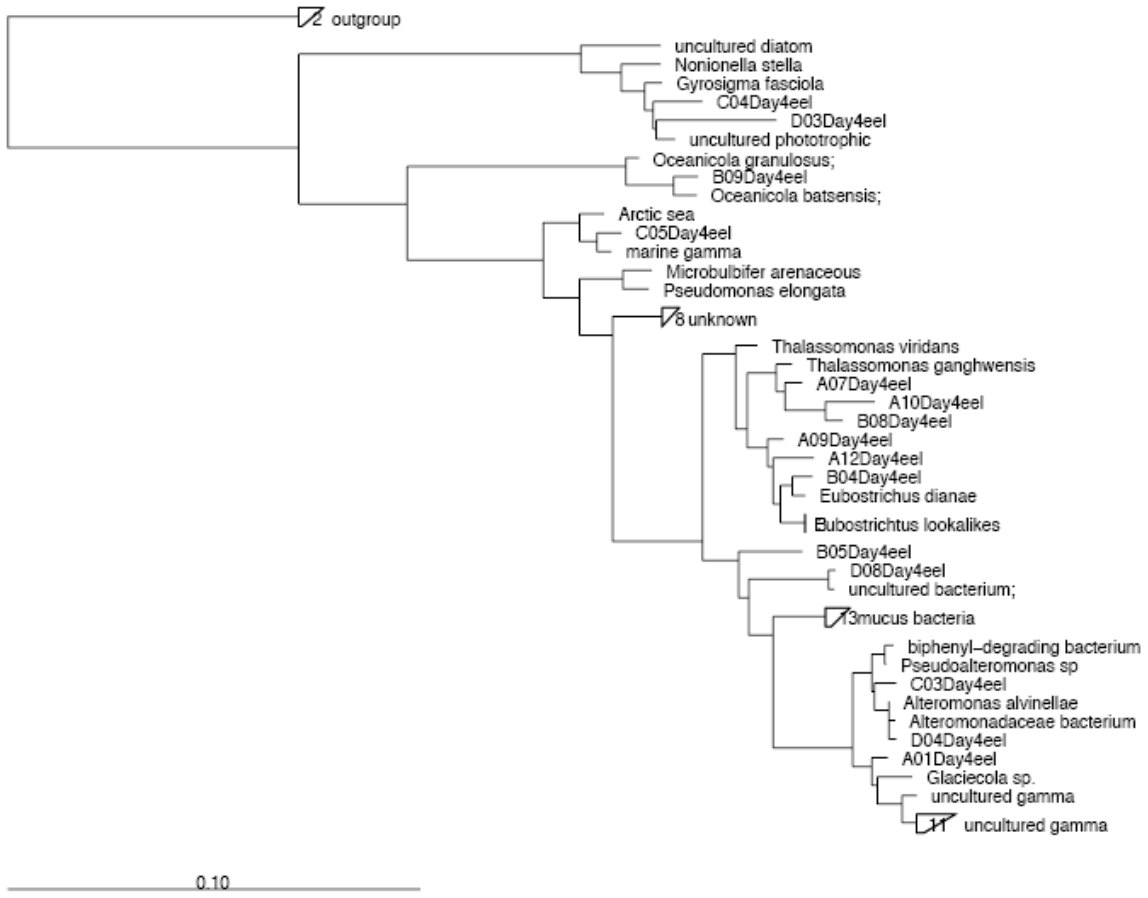
Conclusion

Regarding the bacterial community/Card-FISH results: waters resemble each other very much. The difference lies in the amount of organic material and eukaryotes present in the water from eel pond which leads to the conclusion that lab water is probably filtered when it enters the Loeb building. Unfortunately the clone libraries were too small to get a good overview on the species richness of both environments; it looks like that it was just the tip of the iceberg. Many bacterial species were found.
The succession process is detectable and can be monitored by a microscope study on the environment. First filamentous organisms attach to the surface which are later followed by many others

Before the experiments started, an assumption was made that glass and agar surfaces were similar in their behavior regarding bacterial attachment. This assumption cannot hold after the observed differences in bacterial populations. Therefore water & agarose surfaces differ much from each other and must be studied as separate environments.
Organisms on marine LTY plates do not resemble much with the found organisms present in the biofilm.

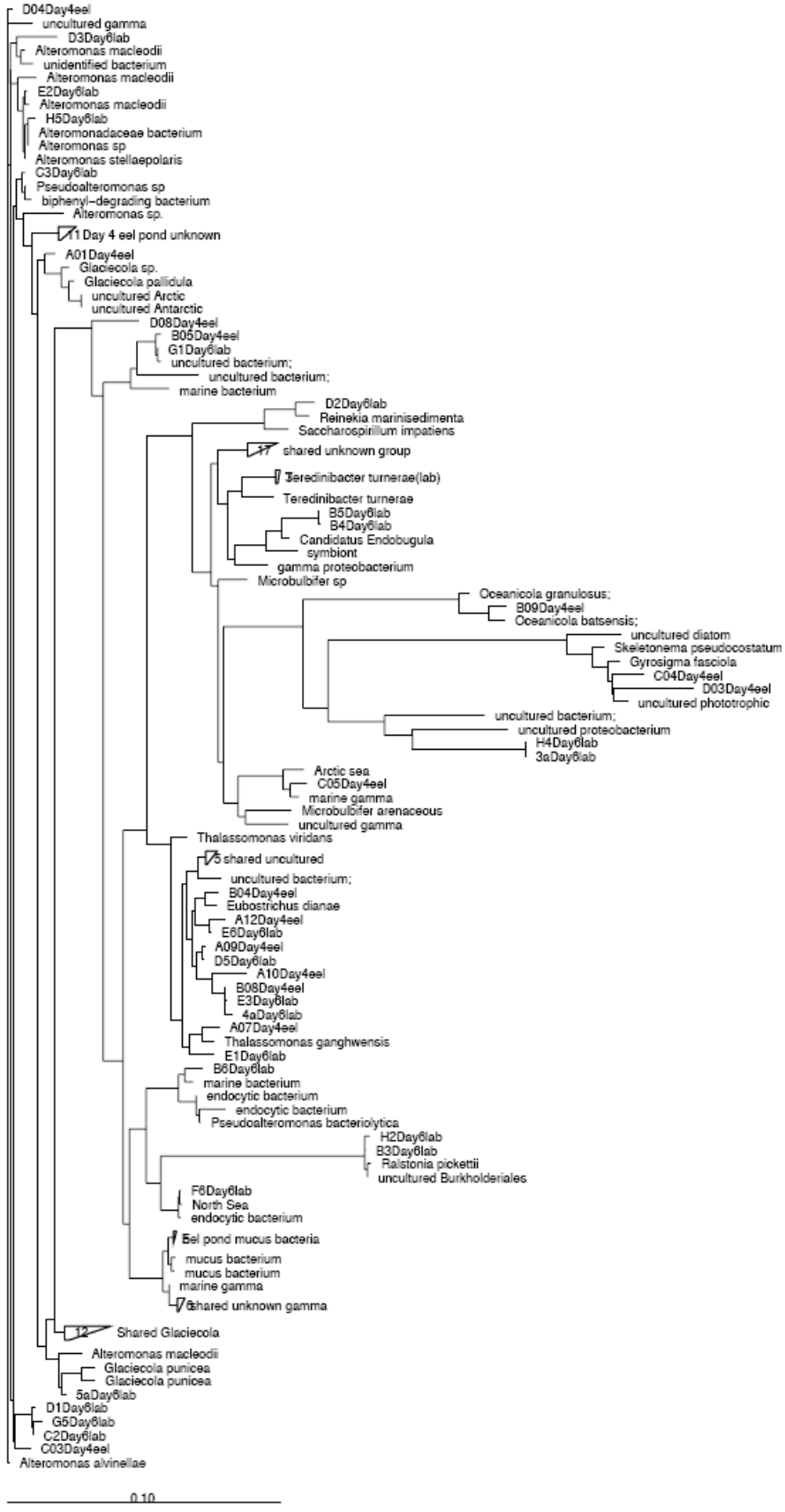
A small portion of the bacterial population was known in the ARB database therefore much information of bacterial species still has to be obtained.

Appendix I: Tree Buoy day 4



Appendix II: Tree Lab set-up day 6

Appendix III: Tree shared Lab/Buoy set-up day 9 and 13



Appendix IV: Images of environments.

Buoy in Eel pond

Lab set-up (aquatic table second floor Loeb building)