# Initial Biofilm Formation

Project Microbial Diversity 2003

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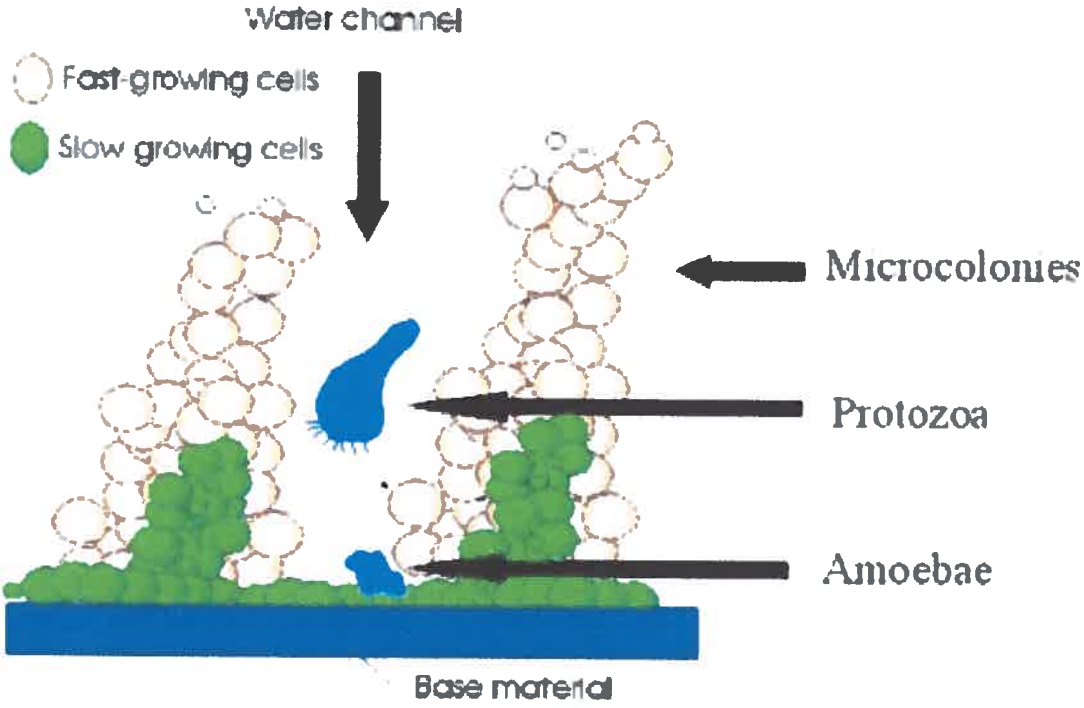


www.sobs.soton.ac.uk/ ehu/pics/biofilm.jpg

# 1. Introduction

Solid surfaces that are in contact with water in natural or artificial environments are rapidly colonized by microorganisms. This phenomenon was first observed in the early 1940 as the "bottle effect" for marine microorganisms (Heukelekian and Heller 1940). Soon, it was recognized that the abundance of bacteria on surfaces are enormously higher than in the surrounding medium (Zobell 1943). But due to lack of better methods, further investigations of microbial biofilm could only be achieved using light microscopy. The development of new methods like electron microscopy and confocal scanning laser microscopy (CSLM) and molecular methods (FISH, DNA array technique) allows a more detailed study of biofilms.

Today a biofilm is defined as an assemblage of microbial cells that is irreversible associated (not removed by gentle rinsing) with a surface and enclosed in a matrix of primarily polysaccharide material (Dolan 2002). Biofilms are formed on a wide variety of surfaces, including industrial water pipelines, living tissues (like tooth surfaces or lungs), or natural water systems (Lappin-Scott and Costeron 1989, Watnick and Kolter 1999) and today, it is often used in batch reactor for the removal of unwanted elements of sewage (Gieseke *et al.*, 2003). The initial biofilm formation is influenced by many different factors. The characteristics of the substratum such as roughness and hydrophobicity affect the composition of the biofilm just like other physico-chemical factors (Bos *et al.*, 1999). In addition, nutrient concentrations in the surrounding environment or substrate in laboratory experiments, pH, temperature and organisms composition of the natural population or inoculums have influence on the biofilm formation (Heydorn *et al.* 2000). Traditional investigations of biofilm growth in the laboratory do not really simulate natural conditions. But they are helpful in understanding the different strategies of single organisms to maintain under different conditions and life stages in biofilms (Klausen *et al.*, 2003). The initial biofilm formation seems to follow a certain sequence of species, but these are poorly understood chemical and biological processes (Cooksey & Wigglesworth-Cooksey, 1995). In the beginning the material exposed to an aqueous medium will be immediately conditioned or coated by polymers of this medium (called conditioning film) (Loeb and Neihof, 1975). After the conditioning film is placed, pioneers species of bacteria begin to attach to the surface, followed by subsequent colonization by secondary microorganisms. The specific and/or unspecific interactions of these species permit new organisms to establish in the biofilm community (Dang & Lovell, 2000). In detail, the roles of primary colonizer and the development of biofilms on marine surfaces are largely unknown.

In coastal areas where nearly 70 % of the human world populations live, the load of sewage and nutrients from land runoff is high, especially of ammonium. The impact of this pollution on marine ecosystem is long recognized for escalate phytoplankton blooms. This causes high carbon flux to the sediments, which ends up in higher productivity and results in the spread out of anoxic areas. But the influence of this higher nutrient load on the initial biofilm formation is not well studied. The goals of this study were (i) to assess the diversity of early bacterial colonizer in natural seawater and (ii) to investigate the influence of high nutrient load on the primary colonization of new exposed surfaces.

## 2. Material and Methods

Experimental set-up

The substrate source of this experiment was natural unfiltered seawater obtained from the seawater pipeline in the laboratory. Silicone tubes (Nalgene 50 Silicone Tubing, 250 ID x 0.375 OD) were used for substratum. Four different treatments were made:

- Natural seawater
- Natural seawater enriched with ammonium (500 µM)
- Natural seawater fixed with formaldehyde (2 % end concentration): control for attachment of death material to the surface
- Natural Seawater: control for even growth of the biofilm at the different ends of the tubes

For exclusion of artifacts on bottle effect and alteration of the needed seawater a flow through system was used (except for the death control, this was a recirculation system). The flow rate was adjusted to 1 ml $min^{-1}$. Air bubble traps were interposed between the pump system and the substratum to minimize disturbance caused by air bubbles.

Sampling

The first sampling time point was at 4pm, 2 days after incubation began and daily henceforth until the 8$^{th}$ day, which is the last day of sampling. Approximately 15 cm of the ending side of the silicone tube was cut with a scalpel and rinsed gently with 0.2 µm filtered seawater to dislodge pelagic organisms clanged to the surface. Then the tubes were cut halved per length. One half of the tube was rubbed with a cotton coated stick to acquire the biomass for DNA extraction. The cotton was transferred in 2 ml Bead Solution tubes (MoBio) and was beaded for15 sec in the bead beater at maximum before frozen by – 80 °C until extraction. The other half of the tube was fixed for 2 h in 4 % formaldehyde and then transferred to 2 % formaldehyde until further analysis.

DNA Extraction and T-RFLP of rRNA genes

The sample appointed for the DNA extraction was added to MoBio Ultra Clean Soil DNA Isolation Kit. The DNA extraction was done as described by the manufacturer (and modified by Brian). 2 µl of DNA extraction was used as template for the standard PCR amplification of 16S rRNA genes. As primers, the universal Eubacteria labeled 8F-Fam Primer (5´-AGAGTTTGATCMTGGCTCAG -3´) and 1492R (5´- GGTTACCTTGTTACGACTT -3´) were used (each 0.5 µl) with 20 µl Taq DNA Polymerase for the PCR amplification under following conditions in an Eppendorf Mastercycler gradient, program EUBAC:

1 x [95 °C 5 min]
30 x [95 °C 1 min (denaturation); 55 °C 1 min (annealing); 72 °C 1:30 min (extension)]
1 x [72 °C 8:30 min]

The PCR 16S rRNA products were purified with Micron as described by manufacturer. The purified PCR products were digested with the restriction enzyme HhaI (GCG^C) and 10 x Buffer No. 4 at 37 °C for 2 h. The restriction enzyme was inactivated by heat treatment at 86 °C for 5 min. The digestion products were submitted to an institute. Data files were analyzed with GeneScan 3.1.

Nutrient measurements

Ammonium was estimated with Ammonium Aquarium Test Strips (Jungle Laboratories corporation). Phosphate was measured with Aquarium Test for Fresh & Salt Water (Hagen).

Microscopy

The Number of bacteria was estimated with DAPI counts. The tube pieces fixed in 20-30 ml 2 % formaldehyde were treated with pulsed sonication at low intensity to disconnect the attached bacteria from the tube surface. Afterwards the sample vials were vigorously shaken

for 2 - 3 min and the supernatant liquid filtered onto a 0.2 µm pore size filter (Millipore, Ø 25 mm or Ø 47 mm, depending on the expected biomass). Thereafter, pieces of dry filters were stained with DAPI (4′, 6′-Diamidino-2-Phenyl-Indol) for 5 min and then washed first in distilled water and then in 80 % ethanol. The stained filter pieces were put on microscope slides and after addition of a trop of CITTI covered with cover slips and frozen at – 20 °C until counting on a Zeiss Axiovert 200 by magnification of 1000x. In all, a minimum of 400 bacteria and 20 fields were counted per filter piece to minimize the counting error (10 % HELCOM 1983).
Fixed tube pieces for the Confocal Laser Scanning Microscope (CLSM) were washed before staining with PSB (130 mM NaCl, 10 mM $NaPO_4$, pH 7.2) to remove the formaldehyde. The samples were stained with 1:100 (v/v) SYBR Green Solution in the dark for 15 min. After the staining, the tubes were washed again with PSB to remove all unfixed SYBR Green and they were then fixed on a microscope slide with wax directly before use on the CLSM. The CLSM was a Zeiss LSM Pascal and the software used to analyze the pictures was LSM 5 Image Browser (free available in www.zeiss.com).

## 3. Results and Discussion

The natural seawater used for the experiment was pumped up from the Buzzards Bay, Woods Hole [41°31′′N 70°40′′W] (fig.1). It is characterized by a relatively low ammonium concentration of 0.25 mg $l^{-1}$ and a phosphate concentration under 0.25 mg $l^{-1}$. Salinity was 31 psu, which is relatively low for North Atlantic Water, probably because of freshwater runoff from the land. Oxygen was not measured, but is reported between 8 – 12 ml $l^{-1}$ (www.mwra.state.ma.us/). The ammonium concentration of the enriched seawater was always between 3 – 6 ml $l^{-1}$.

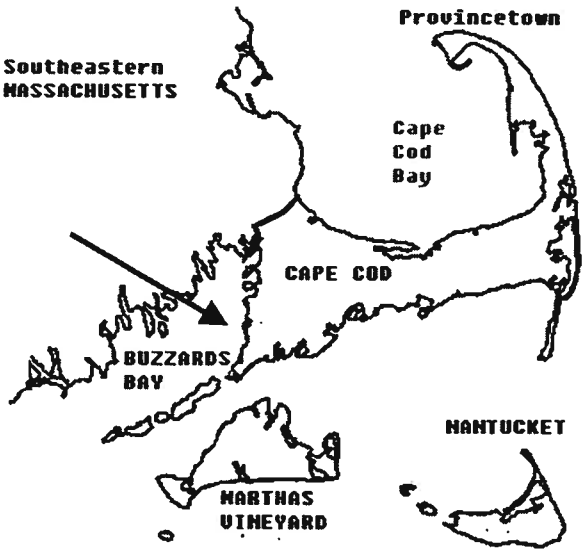


**Fig. 1:** Map of sampling area (modified after www.globalsecurity.org/.../imabuzzards-bay-map1)

### Microscope analysis

The initial bacteria abundance of the natural seawater, which was used as substrate, was $0.72*10^6$ cells $ml^{-1}$. This is in the lower range of observed bacterial abundance in ocean waters (Sanders *et al.*, 1992).
The abundance of the bacteria attached to the substratum after two days was about $0.56*10^6$ cells $cm^{-2}$ in the natural seawater treatment (SW)(fig.2). Until Day 4, the number of cells was stagnant but doubled from Day 4 to Day 5 and goes relatively

linear up to $2.30*10^6$ cells $cm^{-2}$ at Day 8. At Day 5, diatoms as well as heterotrophic nanoflagellates (HNF) appeared for the first time. Their occurrence increased until day 8 (pers. Obs.). The ammonium enriched seawater treatment ($SWNH_4$) showed a totally different pattern. The abundance after two days was nearly the same ($0.39*10^6$ cells $cm^{-2}$), but tripled at Day 3 and remained constant until Day 5. Afterwards the abundance increased up to $2.48*10^6$ cells $cm^{-2}$ and decreased slightly until Day 8. Diatoms HNF and phototrophic nanoflagellates (PNF pers. Obs.) were first present at Day 3 in this treatment. Their numbers increased permanently up to Day 8. At least five different species of diatoms were abundant. *Navicula* spp., *Pseudonitzschia* spp., *Nitzschia* spp., *Rhizosolenia* spp. and a centric unidentified diatom were identified (using web site of the University of Goteborg www.marbot.gu.se/sss/classic/Diatomchecklist.htm). The control treatment (natural seawater fixed with formaldehyde, final concentration 2 %) showed an unexpected increase of bacteria after Day 5. The appearance of all these bacteria was rod-shaped and they were nearly the same size (3 x 1 µm). So it seems that some bacteria were able to maintain a concentration of 2 % or could be even using formaldehyde as substrate.

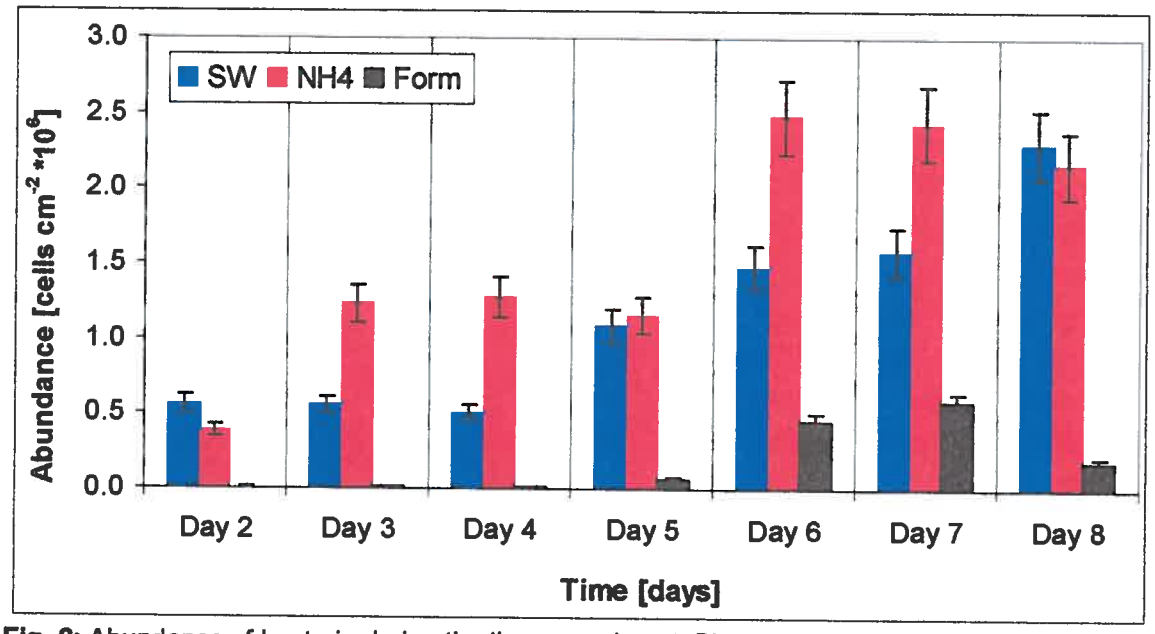


**Fig. 2:** Abundance of bacteria during the time experiment. Blue bars: Natural seawater treatment; red bars: ammonium enriched seawater (500 µM), black bars: control poisoned seawater (2 % formaldehyde). Error bars indicted 10 % counting error.

The observations from the confocal laser scanning microscope (CLSM) confirmed the bacterial count.

To check equal growth of the biofilm in the tubes, which were about 1.5 m long, a treatment with natural seawater was used. At the end of the experiment, I cut samples in both sides of the tube, one near the pump and one at the farther end. The confocal pictures showed no difference of the biofilm formation (fig. 3). But unexpectedly, there were many filamentous bacteria present, which were not present in the SW treatment after 8 days. The negative control (normal seawater fixed with 2 % formaldehyde and recirculated) showed an increase of bacteria between Day 3 and Day 8 (fig. 4) but not as much as supposed by looking on the DAPI counts. The bacteria were relatively big and grouped by 2 to 4 cells. But in all, it remains unexpected that some bacteria can grow under such toxic conditions.

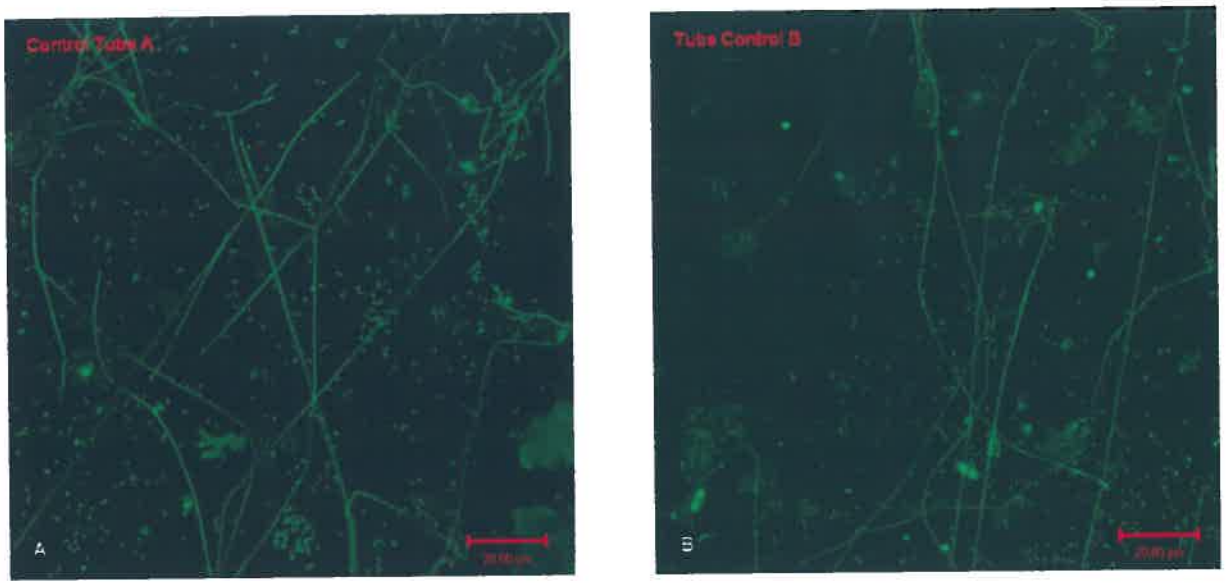


Fig. 3: Control tubes for qual growth on both end of the tubes. Tube A sampled near the pump, Tube B sampled at the end of the tube.

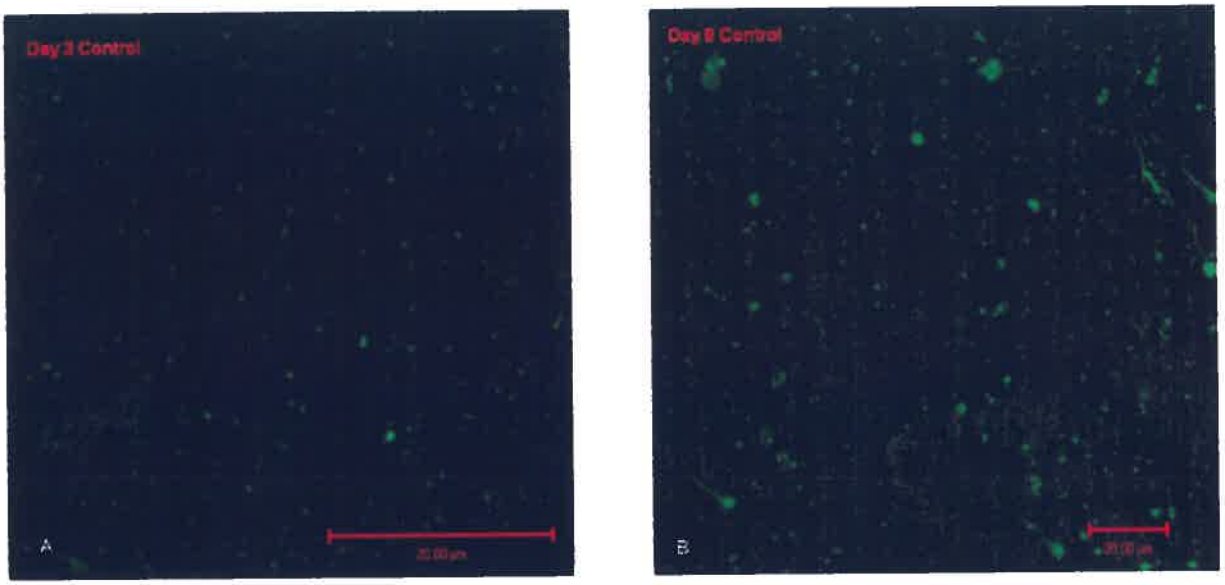


Fig. 4: Toxic control (natural seawater fixed with formaldehyde , 2 % endconcentration). A sample taken at Day 3, B sample taken at the end of the experiment Day 8.

Unfortunately, I wasn't able to obtain pictures from Day 2 because of technical problems. But at Day 3, the biofilm of SW appeared mostly in single cells monolayer with some small colonies of 4 – 6 µm in diameter (fig. 5 A). Also filamentous cyanobacteria, probably *Anabeana* spp., were present. Large areas of the substratum were not colonized by bacteria. In the $SWMH_4$ the substratum was much more occupied by bacterial colonies (fig. 3 B). Two different sizes of colonies were present: smaller colonies (ca. Ø 5 µm) and larger colonies (ca. Ø 9 µm). The larger colonies consisted of an inner red area of unknown substance, which was covered by bacteria. Beside cyanobacteria, diatoms and probably PNF were present (fig. 5 B). At Day 4, SW showed the same picture (note the different size bars) as expected by the DAPI counts (fig. 5 C). The colonies at Day 4 of the $SWNH_4$ were grown; in fact there were also some colonies which were connected to each other (fig. 5 D)

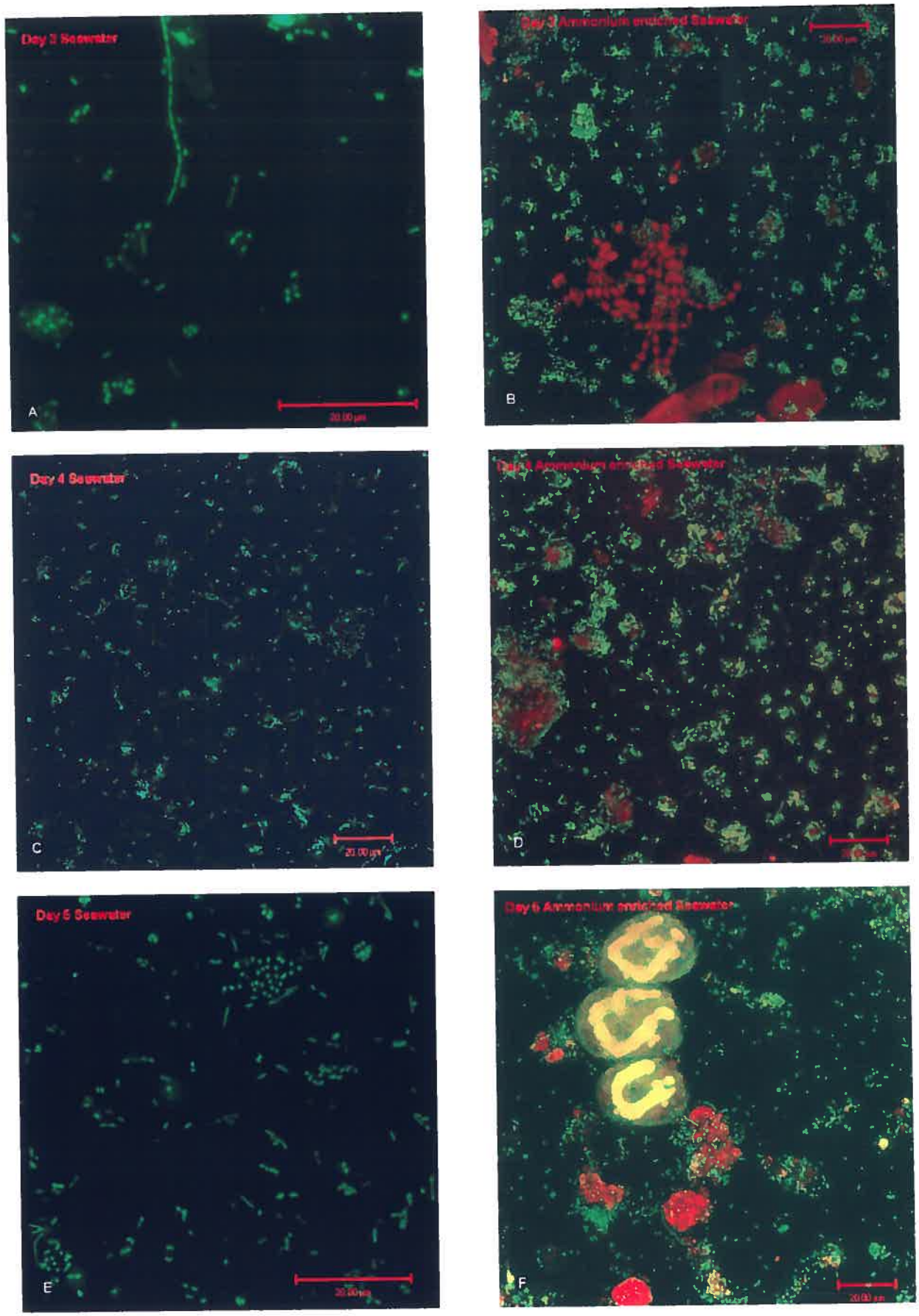


Fig. 5: Initial biofilm formation pictures. A Day 3 SW, B Day 3 $SWNH_4$ , C Day 4 SW, D Day 4 $SWNH_4$, E Day 5 SW, F Day 5 $SWNH_4$. Pictures taken with confocal laser scanning microscope.

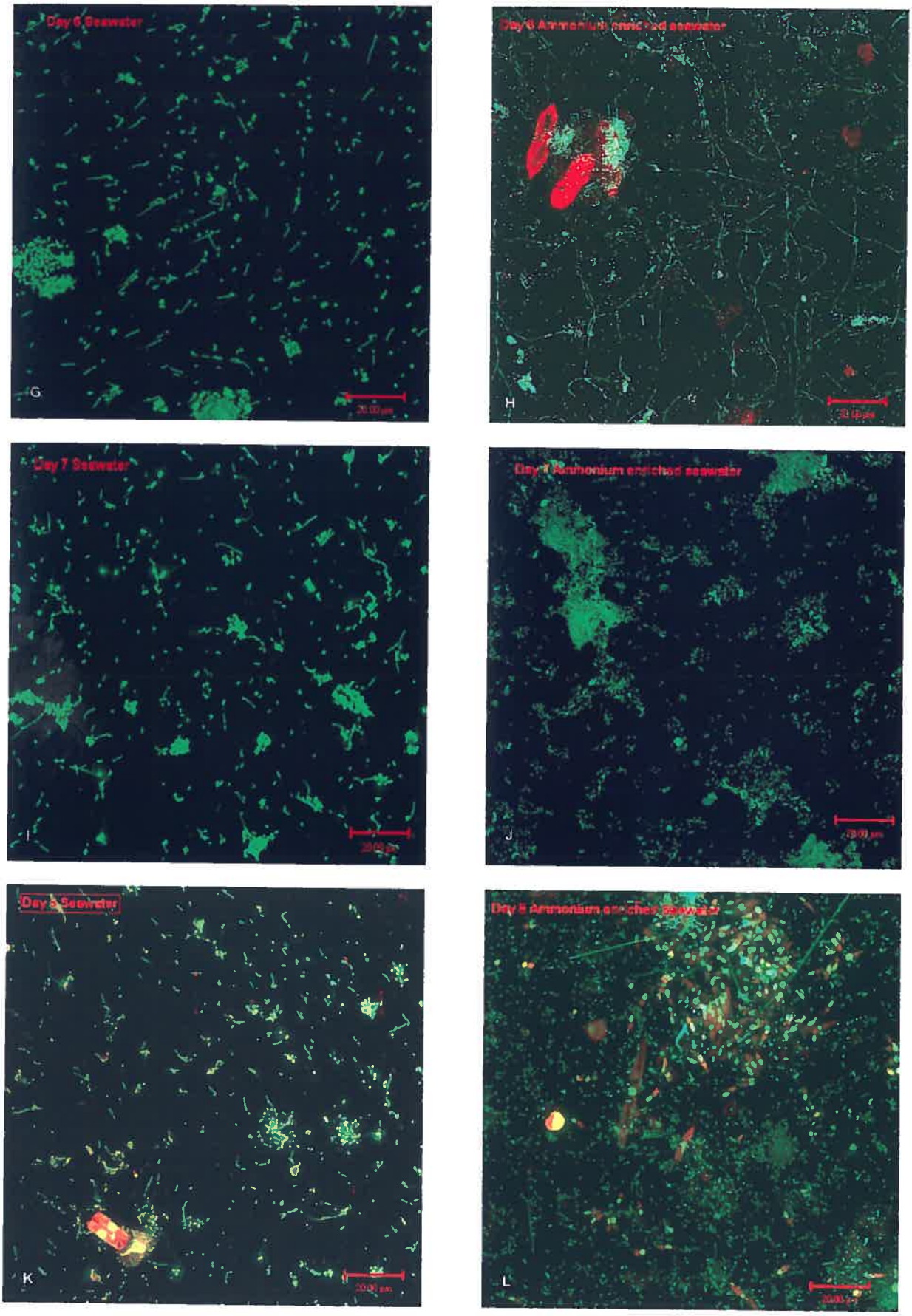


Fig. 5 cont.: Initial biofilm formation pictures. G Day 6 SW, H Day 6 SWNH$_4$, I Day 7 SW, J Day 7 SWNH$_4$. K Day 8 SW. L Day 8 SWNH$_4$.

But not the whole substratum was covered with colonies. The colonies were separated from each other by interstitial voids. These interstitial voids provide a higher influx of substrate and nutrients to the inner parts of the biofilm, especially when the biofilm structure is more complex (Lawrence *et al.* 1991). The voids also allow a better efflux of wastes. Diatoms, PNF and HNF were present and the mysterious red areas were larger. They consisted not of cells, because the DAPI staining showed no present DNA. Maybe these red areas were EPS or degenerated cells which fluorescence under the used wavelength (488 nm) of the laser.

At Day 5, the colonies of the SW were larger (up to 20 µm), which is contrary to the DAPI counts whose abundance seems not to be greater than at Day 4 (fig 5 E). This could be due to some problem with the detector gain at the CLSM at this day, which caused a lower signal of the SYBR Green staining and probably a minor imagination of the actually present number of bacteria. The bacteria in the $SWNH_4$ treatment showed no significance increase, but the phototrophic eukaryotic organisms were more abundant. Interestingly, some relatively big algae with U-shaped chloroplast appeared in higher numbers (fig. 5 F). The red areas disappeared on this day; the red dots observed in Fig. 5 F were living phototrophic flagellates.

Day 6 showed no difference for the bacteria presence to Day 5 for the SW (fig 5 G). But for the first time diatoms were observed. In the $SWNH_4$ a great change could be seen (Fig. 5 H). The number of bacteria increased, also due to the appearance of high numbers of filamentous bacteria, which is consistent with the doubled number of bacteria cells in the DAPI counts. It could be also observed that the size of the bacteria in the $SWNH_4$ treatment were much smaller than the bacteria in the SW. This is as well an evidence for fast growing activity.

The abundance of bacteria at Day 7 was higher than at Day 6 for the SW (fig. 5 I). More colonies were present and wide areas of the substratum were still free of bacteria. Unlike SW, in $SWNH_4$, clusters of colonies were formed and even the abundance was in the same range as the day before (Fig. 5 J). These clusters consist not only of bacteria but also EPS, DNA and Debris are involved (Tolker-Nielsen and Molin 2000). The EPS stabilize the spatial structure in biofilms, but it is not known which role EPS has in shaping the spatial structure in biofilms (Watnick and Kolter 1999). Primarily, occurrence of some holotrichous ciliates was observed in this sample.

In the last day of the experiment the SW treatment showed nearly the same picture. The substratum surface was more covered with a monolayer of bacteria. Also many bacteria were in groups of 4 – 8 bacteria arranged. The $SWNH_4$ was heavily covered with small *Navicula* spp., but some PNF and other diatoms were present. The number of ciliates also increased, nearly 80 ciliates $cm^{-2}$ were present.

As we have seen in this time series experiment, the growth activity in biofilms is heterogeneous in time and space. This is also known from sulfate-reducing bacteria which grow faster in a young multispecies biofilm than in an establish biofilm (Poulsen *et al.* 1993). I suppose that bacteria involved in early stages of succession also show different growth pattern in day changing activity. This pattern could be observed in both initial biofilm treatments. Also the $SWNH_4$ started with a higher activity at the $8^{th}$ day the bacterial numbers seems to be in the same range. But the patterns could not only be explained with differences in growth activity or with substratum occupied by the eukaryotic phototrophs in the $SWNH_4$ because there should be even more enhancing growth because of the release of dissolved organic matter from these organisms (20 – 50 %). So, there must be some other reasons for the relatively low growing abundance of the bacteria in the $SWNH_4$ treatment. The DAPI counts indicate a slightly reduce of bacterial number from Day 6 of the

experiment. This coincides with the first appearance of holotrious ciliates (fig. 6 A). Small ciliates are known to feed on bacteria. The calculated predator/prey correlation for ciliates is investigated to be 8 : 1 (Hansen 1994). From this it follows that these ciliates are able to eat prey up to 2 µm. Beside ciliates, HNF are the main predator on bacteria, their ingestions rates range from 0.1 – 120 bacteria $HNF^{-1}$ $h^{-1}$ (Carlough and Meyer 1991). So, I suppose that the growth activity of the $SWNH_4$ treatment is not lower than the SW treatment at the end of the experiment. The grazing pressure is higher and this results in lower cell numbers.

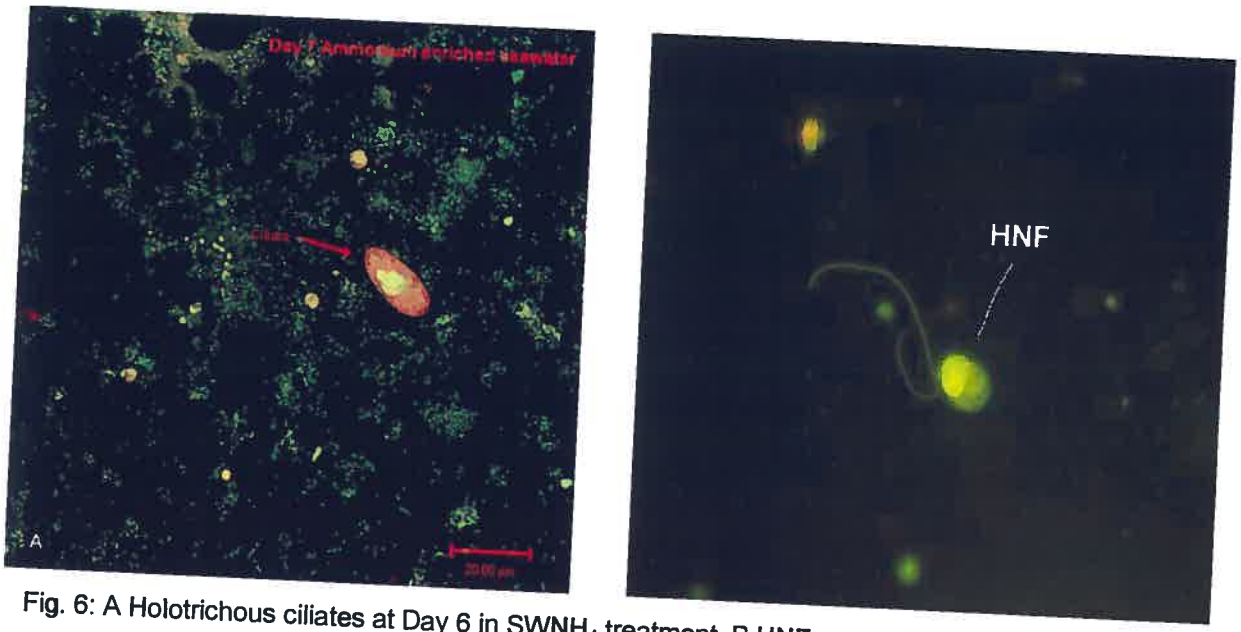


Fig. 6: A Holotrichous ciliates at Day 6 in $SWNH_4$ treatment, B HNF seen since Day 4 in $SWNH_4$

The whole development of the initial biofilm was totally different for the two approaches. It is known that cultures grown on different substrates have different architecture in their biofilms (Talk Alfred Spormann). It seems that biofilms of natural media under different nutrients conditions follows the same strategy, at least in the first 8 days. A shift in the bacterial community could not be detected microscopically because cell morphology of bacteria differs with nutrient supply, growth stage etc. An unexpected result was the discovery of diatom right in the early stages in the ammonium enriched treatment and not in the natural seawater treatment. This indicates that the natural seawater system was limited by nitrogen. This is very unusual in coastal areas where normally the system is loaded with high nutrients concentrations from wastewater runoff. Often nutrient overloads cause blooms of phytoplankton in the water column, this again cause higher release of DON (dissolved organic nutrients) in the water, which can be used by bacteria. So both the higher detritus sedimentation to the sediments and the higher DON supply can change the community living on and in the sediments.
At least 60 – 80 % of the nitrogen entering into the Buzzards Bay originated from wastewater (fig. 7 left side). Other sources of nitrogen are from watersheds including fertilizer, storm water runoff and deposition of the atmosphere (fig. 7 right side). So, one possible explanation for the nitrogen limitation could be that the nitrogen is being used up during the passageway in the seawater supply pipeline. Anyway, the ammonium enriched treatment points out what huge impact a higher nutrient load would have on the initial biolfilm formation. I suppose that there will be at least a shift in the species composition of the bacterial community.

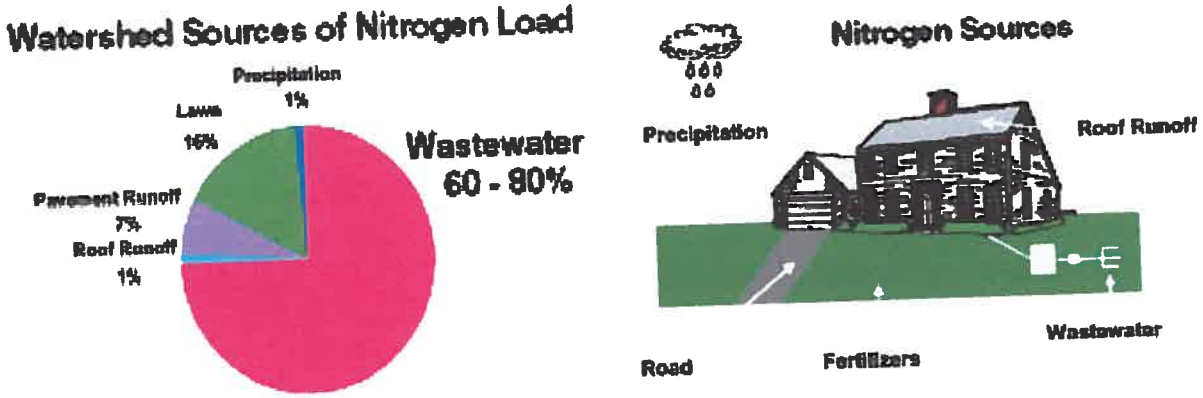


Fig. 7: Left side: Nitrogen load estimated for Buzzards Bay. Right side: Possible nitrogen sources for the nitrogen load. Pictures from www.savebuzzardsbay.org

**Molecular analysis**

The investigation of a possible changing bacterial community during the initial biofilm formation was approached with TRFLP. I got PCR product of most of my samples, but the DNA content seems to be very low. Also I got some TRFLP results, they were not reliable because of different DNA amount loaded to the vials.

## 4. Conclusions

Nutrient enrichment, especially ammonium, seems to increase initial attachment and biomass development on a biofilm, at least in the first 8 days. Higher growth activity is also indicated by smaller cell size of the ammonium enriched cells. But growth activity estimated was not continuous and could changed from day to day in both treatments. The accumulation of biomass can be due to growth of cells in the biofilm but also due to new attached organisms. The architecture of the develop biofilm differs highly. The enriched treatment colonies were formed from the beginning and were connected to each other after a few days. This provides a better protection from possible predators, which appears to be more abundant in the ammonium enriched treatment. The predators may induce this development of clusters since quorum sensing, which allows for communication between bacteria, is reported in biofilms in culture. Early occurrence of diatoms in the enriched treatment is an evidence for nitrogen limitation in the natural seawater. This is very unusual for coastal environments, but could be. Another possible explanation is that the nitrogen is used up while passing the natural seawater supply pipeline. Moreover, one of the most unexpected results is the occurrence of bacteria in natural seawater fixed with formaldehyde (end concentration 2 %). These bacteria could maintain in toxic environment or maybe even use this compound as substrate.

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