Composition and Distribution of Natural Microbial Communities in Biotrickling Filter Biofilms

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Introduction

Bacteria posses several adaptive mechanisms for responding to those physicochemical factors that define their environment, such as nutrient availability, pH, temperature, organic and ionic content and the presence of antagonistic agents. Depending on the types and numbers present, bacteria can effect alterations in their physiology or physical state in response to the environment. Organic and inorganic acid production, heavy metal binding. Transformation of xenobiotics, and extracellular polysaccharide production are important adaptive tools for bacteria in this regard. In most ecosystems, these activities are dependent on the ability of bacteria to attach to surfaces (Mittelman, 1998).

Biofilms play an important role in almost all aspects of microbiology. A biofilm consists of cells immobilized at a substratum surface and frequently embedded in an organic polymer matrix of microbial origin (Characklis and Marshall, 1990). They may appear as either beneficial or potentially harmful populations of microorganisms. Most microbiologists regard suspension culture as the normal state of growth of prokaryotes. This is particularly true for research into physiology and biochemistry of bacteria, whereby homogeneous suspensions of bacteria are readily harvested and manipulated for experimental purposes. The reality of prokaryotic life in natural habitats is that many organisms spend part or all of their life spans attached to surfaces (Marshall, 1976). Adhesion to surfaces in nature is generally considered to be nonspecific. Bacteria adhere to a wide variety of different inanimate and possible animate surfaces with varying degrees of adhesive strength. In most cases bridging polymers are involved in nonspecific adhesion. They are either extracellular polysaccharides, proteins, or glycoproteins. The precise mechanisms whereby such polymers interact with a range of substratum surfaces is so far not known (Marshall, 1992). The combined effects of continuous adhesion and both growth and reproduction at surfaces eventually give rise to a macroscopic slime, or biofilm. The bacteria constituting the biofilms in our intestines, sewage treatment plants, bioremediation plants, etc. are mostly beneficial. Harmful biofilms are also abundant, ranging from relatively harmless dental plaque to *Pseudomonas aeruginosa* bifilms in the alveoli of cystic fibrosis patients, but biofouling of ships and offshore material is also a serious problem in the oil and shipping industries.

In order to improve the performance of some beneficial biofilms and to avoid or remove harmful biofilms, it is important to understand the mechanism of biofilm formation, growth, and maintenance. Natural biofilms consisting of multiple species are structurally organized in patterns, which depend on several factors such as nutrient supply, flow rate, pH, temperature, etc. In such dynamic systems the individual cell experience conditions determined by the outer environment, the already existing structures, and the local microbial activities. Furthermore, subpopulations may form locally which are completely different from the majority of the community.

The central question promoting this study was to investigate who are the primary colonizers of submerged inert surfaces in seawater under laboratory conditions. In order to study the composition and distribution of bacteria in a biofilm forming after inoculation of a biotrickling filter with a natural seawater sample, two independent approaches were chosen. The first experimental setup tempted to stimulate biofilm formation by using an artificial sea water medium supplemented with a short chain fatty acid as carbon source. The second attempt utilized untreated natural seawater and an insoluble sugar polymer to initiate the growth of a biofilm. A third experiment was set up in a flow chamber to document biofilm formation microscopically. The analysis of the bacterial biofilm community was studied by terminal restriction fragment length polymorphism (T-RFLP)(Marsh, 1999, Marsh et al. 2000).

Material and Methods

Laboratory biotrickling filter setup and operating conditions

Two parallel laboratory-scale biotrickling filters were constructed that served for biofilm formation. A schematic of the experimental apparatus is shown in Figure 1. Reactor 1 was supplied with ASW (artificial seawater) supplemented with lactate (Sigma Chemical Co., St. Louis, MO, USA) as a carbon source to a final concentration of 2 mM. Artificial seawater consisted of 23.5 g/l NaCl, 4.9 g/l $MgCl_2$, 3.9 g/l Na_2SO_4, 1.1 g/l $CaCl_2 \cdot 2H_2O$, 0.66 g/l KCl, 0.096 g/l KBr and 0.026 g/l H_3BO_3 adjusted to pH 7.2. Following autoclave sterilization a $NaHCO_3$ stock solution (0.19 g/l) was added separately by filter sterilization through a 0.2-µm-pore-size Millipore syringe filter. Reactor 2 was kept under a constant flow of unsterile seawater. The reactor bed was overlaid with chitin from crab

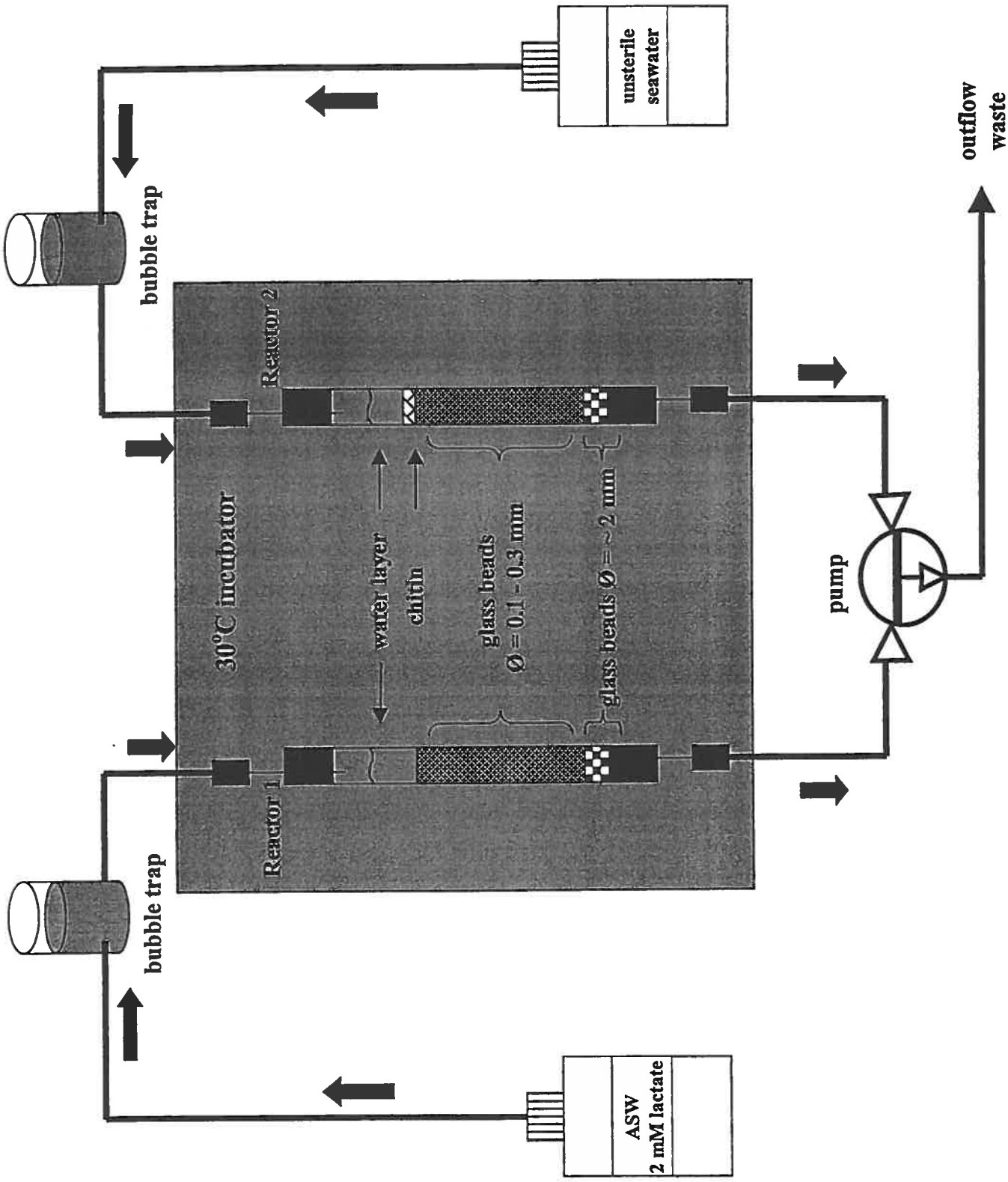


Fig.01: Schematic representation of the experimental design showing bubble traps, trickling filter bioreactors and the peristaltic pump. Reactor 1 was supplied with ASW and 2 mM lactate, reactor 2 with chitin and unsterile seawater.

shells (practical grade, poly-N-acetylglucosamine, Sigma Chemical Co., St. Louis, MO, USA).

Each trickling filter consisted of a glass tube (total reactor height 135 mm, internal diameter 14 mm) with a packed bed height of 85 mm (bed volume: 13.1 cm^3). The reactor bed (or substratum) consisted of acid-washed glass beads. The first 5 mm from the bottom of the glass tube were filled with glass beads of 2 mm in diameter. Over the first beads layer the glass tube was then filled up to a height of 85 with smaller glass beads (diameter 710 – 1.180 microns, Sigma Chemical Co., St. Louis, MO, USA). The biotrickling reactors were assemble and prepared in a 30°C incubator. The bioreactors were inoculated with untreated seawater for two days. After inoculation the medium flow was arrested for 3 h. Medium flow was then started until the reactor bed was covered by 15 mm of medium. Then a constant in- and outflow of medium at a rate of 0.25 ml/h was established using a Dynamax RP-1 peristaltic pump (Rainin, Woburn, MA, USA). Reactor 1 was run for 7 days, reactor 2 for 2 weeks. After the constant flow was stopped the reactor bed volume was subdivided in four horizontal sections each of 20 mm in height. The amount of 1 g of glass beads of each section was utilized for DNA isolation. For reactor 2 also the chitin layer (0.25 g) was used for DNA isolation.

Flow chamber experiment

Biofilms were cultivated in a two-channel flow cell with individual channel dimensions of 1 by 4 by 40 mm supplied with a flow of ASW medium supplemented with lactate as a carbon source to a final concentration of 2 mM.

The flow system was assembled and prepared in a 30°C incubator as shown in Figure 2. The substratum consisted of a microscope glass cover slip (Fisherbrand 12-544E, 24x50x1.5 mm, Fisher Scientific, Pittsburgh, PA, USA). Flow cells were inoculated with untreated seawater for two days. After inoculation, the medium flow was arrested for 3 h. Medium flow was then started, and the substrate was pumped through the flow cells at a constant rate of 0.25 ml/h using a peristaltic pump. The flow chamber was run for 10 days prior to staining and microscopy.

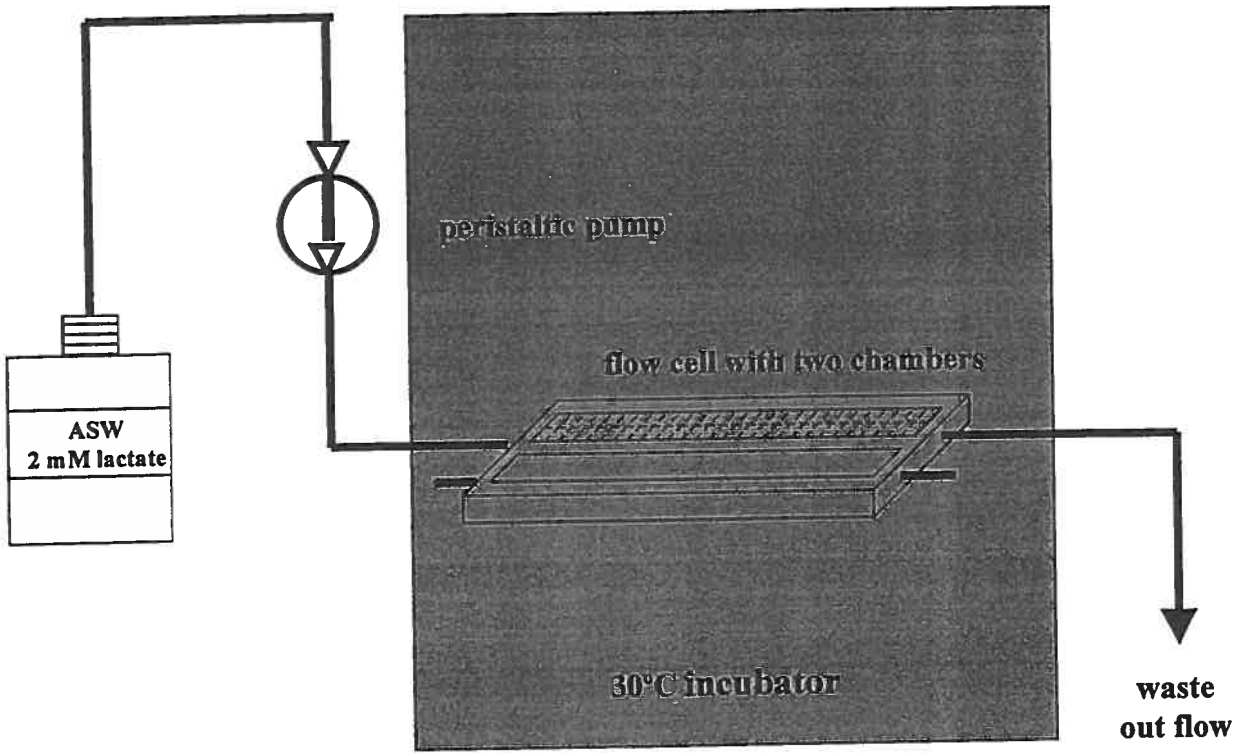


Fig.02: Schematic representation of the experimental design showing peristaltic pump and laminar flow cell. The cell was run with ASW medium and 2 mM lactate.

Microscopy and image analysis

Prior to microscopy the biofilms were incubated with a 1:50 (v/v) SYBR Green I solution (Sigma, St. Louis, MO, USA) for 45 min in the dark. The staining solution was then replaced by an freshly prepared anti-fade solution consisting of equal amounts of 1x phosphate-buffered saline (1× PBS) (130 mM sodium chloride, 10 mM sodium phosphate buffer, pH 7.2) and glycerol (v/v) with 1% phenyldiamine.

All microscopic observation and image acquisition were performed on a scanning confocal laser microscope (SCLM) (LSM 510; Zeiss, Germany) equipped with detectors and filter sets for SYBR Green. Images and 3D animations of the biofilms were generated by using the LSM Software package (Zeiss, Germany).

Genomic DNA extraction

DNA was extracted by using the UltraClean soil DNA isolation kit (Mo Bio Laboratories, Inc., Solana Beach, CA, USA) following the manufacturer instructions. The DNA was quantified and analyzed spectrophotometrically by taking point measurements at 230, 260, and 280 nm (Biophotometer: Eppendorf, Germany). The concentrations of all DNA extracts were adjusted to 100 ng μl^{-1}.

Amplification of the 16S rDNA genes

For PCR amplification of nearly full-length 16S rRNA structural genes (16S rDNA) domain Bacteria-specific oligonucleotide primers described by Eden (1991) were used. The forward-primer SDBact0008F-HEX (5'-AGAGTTTGATCCTGGCTCAG-3') carried a fluorescent tag (hexachlorofluorescein, Operon Inc.) on the 5'-prime end. The reverse primer was SDBact1492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR amplification was performed by using reaction mixtures (final volume, 50 µl) containing 100 ng of sample DNA, 2.5 U of Taq-DNA-Polymerase (Fisher Scientific, Nepean Ontario, Canada), 1x reaction buffer, 2 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 2.5 µl of a 1% BSA solution, and each primer at a concentration of 20 µM. Reaction mixtures that were identical except that they contained no DNA were used as negative controls, and no products were obtained in all cases. The thermal cycling program used was as follows: initial denaturation at 94°C for 5 min; 25 cycles consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s: and a final extension step consisting of 72°C for 5 min. Amplified PCR products were analyzed by

electrophoresis on 1% (wt/vol) SeaKem LE agarose (FMC, Rockland, Maine) gels in 0.5x TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA); the 1 kb DNA ladder (Promega Madison, WI, USA) was used as the molecular size standard.

16S rDNA T-RFLPs

The PCR products obtained from the amplification reactions were purified by using microcon 100 microconcentrators (Amicon, Inc., Berverly, MA, USA). Aliquots (10 µl) of 200 ng DNA were cleaved for 2 h at 37°C with 5 U of restriction endonuclease in the manufacturer's recommended reaction buffer. Hydrolysis was performed with the restriction endonuclease Rsa I [GT'CA] [where the prime shows the site of cleavage] (Promega Madison, WI, USA). The restriction endonuclease was deactivated by heating the reaction mixture to 80°C for 30 min after the reaction was completed. The electrophoresis on a denaturing polyacryamide gel was done by Accugenix (a division of Acculab, Inc., Newark, DE, USA)

Analysis of T-RFLPs

The chromatograms received from Accugenix were visualized with the GeneScan® 3.1 software from Perkin Elmer Corp. (PE Applied Biosystems, USA). The phylogenetic analysis was done with the web-based TAP T-RFLP program from the Ribosomal Database Project II homepage (Maidak et al., 2001).

Results

Microscopy and image analysis

All microscopic observation and image acquisition were performed on a scanning confocal laser microscope. Images were received from the biofilm grown in the flow chamber with ASW medium and lactate as carbon source to a final concentration of 2 mM. Images were recorded after staining of the biofilm with SYBR Green by SCLM. Images shown in Figure 3 represent horizontal cross sections through microcolonies attached to the inner cover slip surface of the flow chamber. The microcolonies shown on the images were located directly beyond the inflow side of the flow chamber. All four images were recorded with the same SCLM settings.

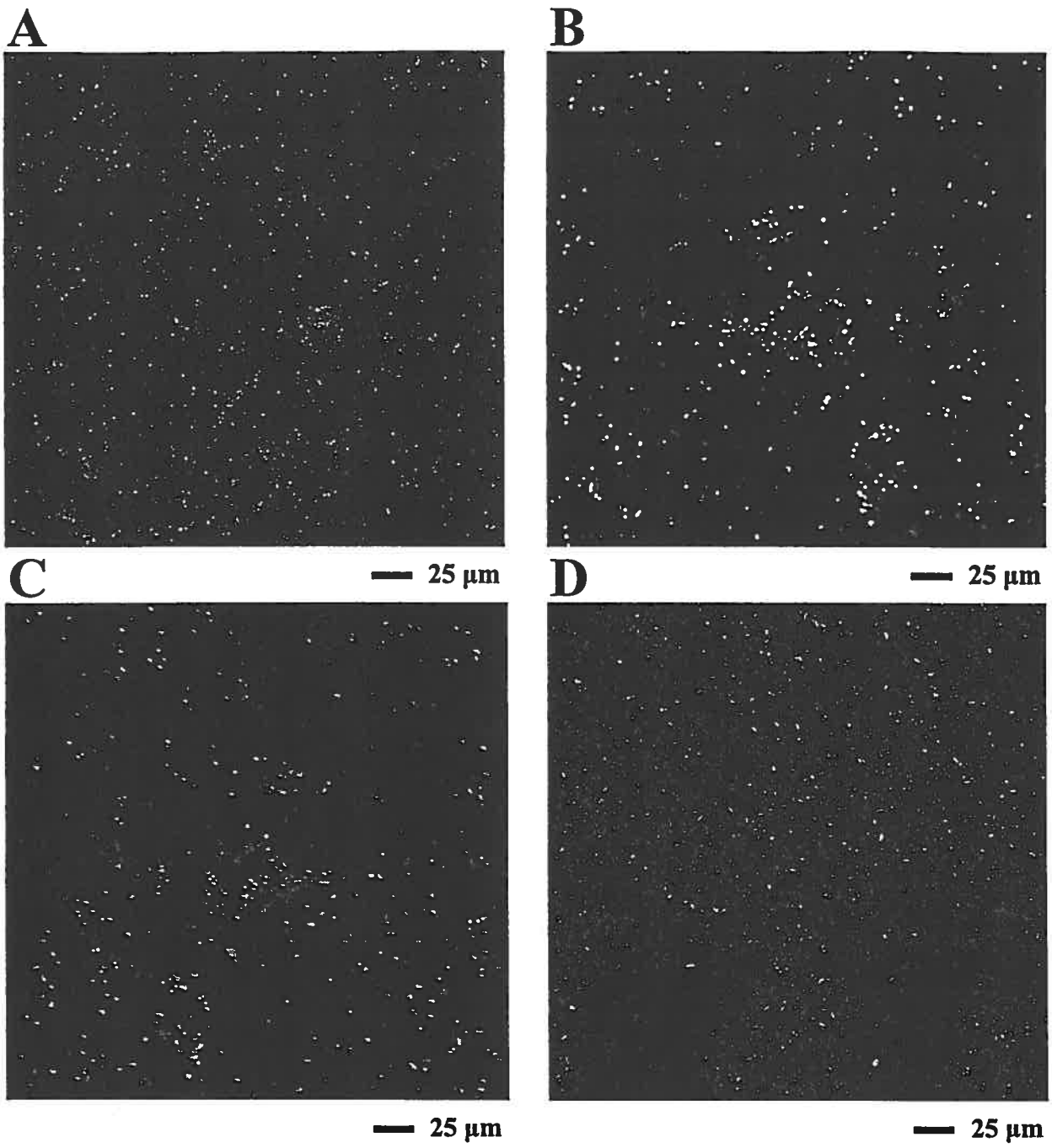


Fig.3: Biofilm as observed in a flow chamber. Biofilm was established and grown in ASW medium supplemented with 2 mM lactete (A-D). Staining with SYBR Green. The fluorescent signals emitted by the cells were visualized with SCLM. Each image is presented as a horizontal cross section through a microcolony of cells located at the glass surface in the flow chamber. All images were recorded with the same SCLM setting.

Amplification of the 16S rDNA genes

PCR amplification of the 16S rDNA genes of the isolated DNA samples was performed using universal Bacteria-specific oligonucleotide primers described by Eden (1991). The PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromid staining (Fig.4). It turned out that all DNA samples from all column sections yielded a PCR product of 1.5 kb, which is due to the expected fragment size for the appointed primers.

16S rDNA T-RFLPs

T-RFLP chromatograms obtained from the different sections of the glass bead beds of either bioreactors were visualized and normalized with the GeneScan® software from PE Applied Biosystems. The T-RFLP chromatograms sustained for reactor 1 are compared in Figure 5. The peak pattern of chromatograms did not significantly change over the vertical profile of the column. Nevertheless a unique peak at a fragment size of 312 bp (data point 3470) could be observed (Table 1). The identification of the peak pattern was done with the web based T-RFLP analysis tool TAP T-RFLP as part of the Ribosomal Database Project II homepage. Thereafter the biofilm that formed in reactor 1 mainly consisted of species belonging to the groups *Caulobacter*, *Rhodobacter* and *Sphingomonas*. The single peak only observed in the upper two-centimeter section could be assigned to mainly account for species belonging to the genus *Cytophaga*. T-RFLP chromatograms obtained for the different sections of reactor 2 are opposed in Figure 6. The digest of the PCR product of the chitin layer gave no analyzable peak pattern. The other chromatograms turned out to be very similar with exception of section 4 (4-6 cm depth). The natural bacterial community that formed a biofilm in the different sections of reactor 2 was dominated by species of the genus *Cytophaga* (Fig.8). The results of the TAP T-RFLP analysis of particular peaks that showed up in comparison of all chromatograms obtained for reactor 2 were listed in Table 2.

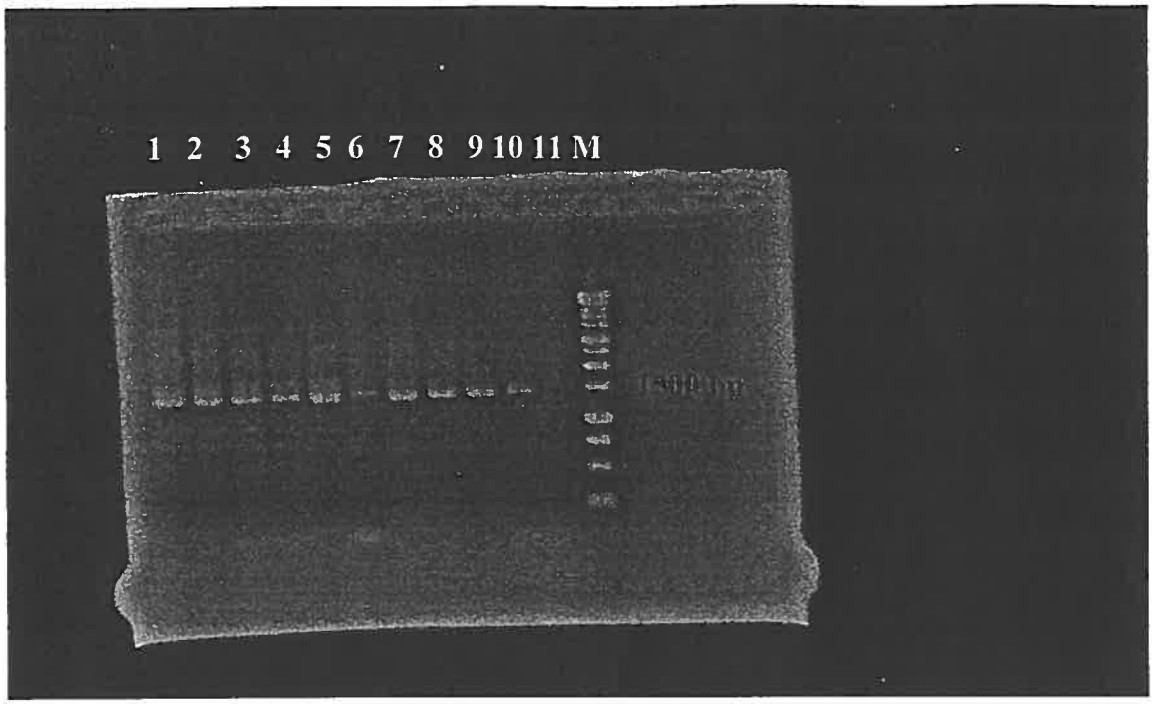


Fig.4: Ethidium bromide stained 1% agarose gel in 0.5x TBE. Separation of PCR-products (1.5 kb). PCR done with primer SDBact0008F-HEX and SDBact1492R. 1-5 sections reactor 2. 7-10 sections reactor 1. 6 positive control. 11 negative control. 1 DNA chitin layer. 2+7 DNA reactor beads of 0-20 mm depth, 3+8 20-40 mm depth, 4+9 40-60 mm depth, and 5+10 60-80 mm depth. M 1 kb molecular size marker (Promega Madison, WI, USA).

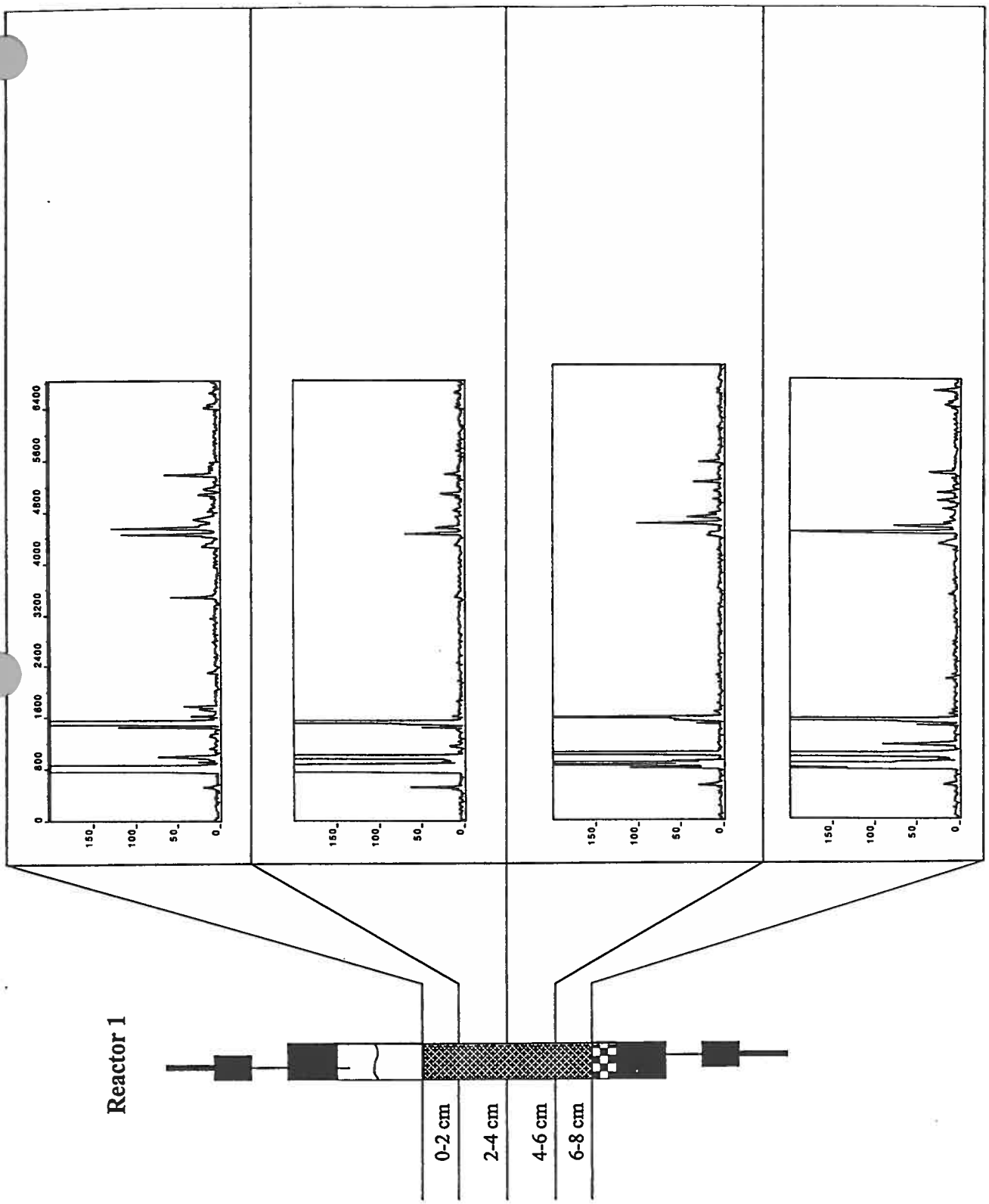


Fig.5: T-RFLP chromatograms obtained from biofilms grown on the different sections of a biotrickling filter reactor bed. The reactor bed consisted of glass beads with 710 – 1.180 microns in size range. The bioreactor was supplied with ASW medium supplemented with lactate as carbon source to a final concentration of 2 mM.

Table 1: Identification of the chromatogram peaks obtained for the different sections of reactor 1 (ASW, 2 mM lactate). The shown results were created with the web site based T-RFLP analysis program TAP-RFLP of the Ribosomal Database Project II (Maidak et al., 2001). Section 1: 0-2 cm; section 2: 2-4 cm; section 3: 4-6 cm and section 4: 6–8 cm. (see also Fig. 5). Numbers in brackets (in column 'Organisms') indicate number of species or strains found.

Fragment size	Data point	Column section	Organisms
40-43	970-990	1,2,3,4	Not in database
98	1440	1,2,3,4	*Methylobacterium extorquens*
103	1490	1,2,3,4	*Bergeyella zoohelcum*
105	1510	1,2,3,4	*Rhizobium sp., Gemmata obscuriglobus*
107	1525	1,2,3,4	*Methylorhabdus multivorans, Rhodospirillum rubrum*
117	1610	1	*Methylosulfonomonas methylovora, Clostridium sordellii, Clostridium ghoni*
135	1765	1	*Unnamed organism*
312	3470	1	*Cytophage sp. (5), Sporocytophaga myxococcoides, Flexibacter maritimus, Microscilla aggregans sub sp. Catalatica, Psychroserpens burtonensis (3)*
412	4450	1,2,3,4	*Campylobacter sp. (3)*
422	4550	1,2,3,4	*Caulobacter sp. (9), Rodobacter sp.(9), Roseobacter sp. (2), Sphingomonas sp. (6)*
437	4680	1	*Clostridium filamentosum*

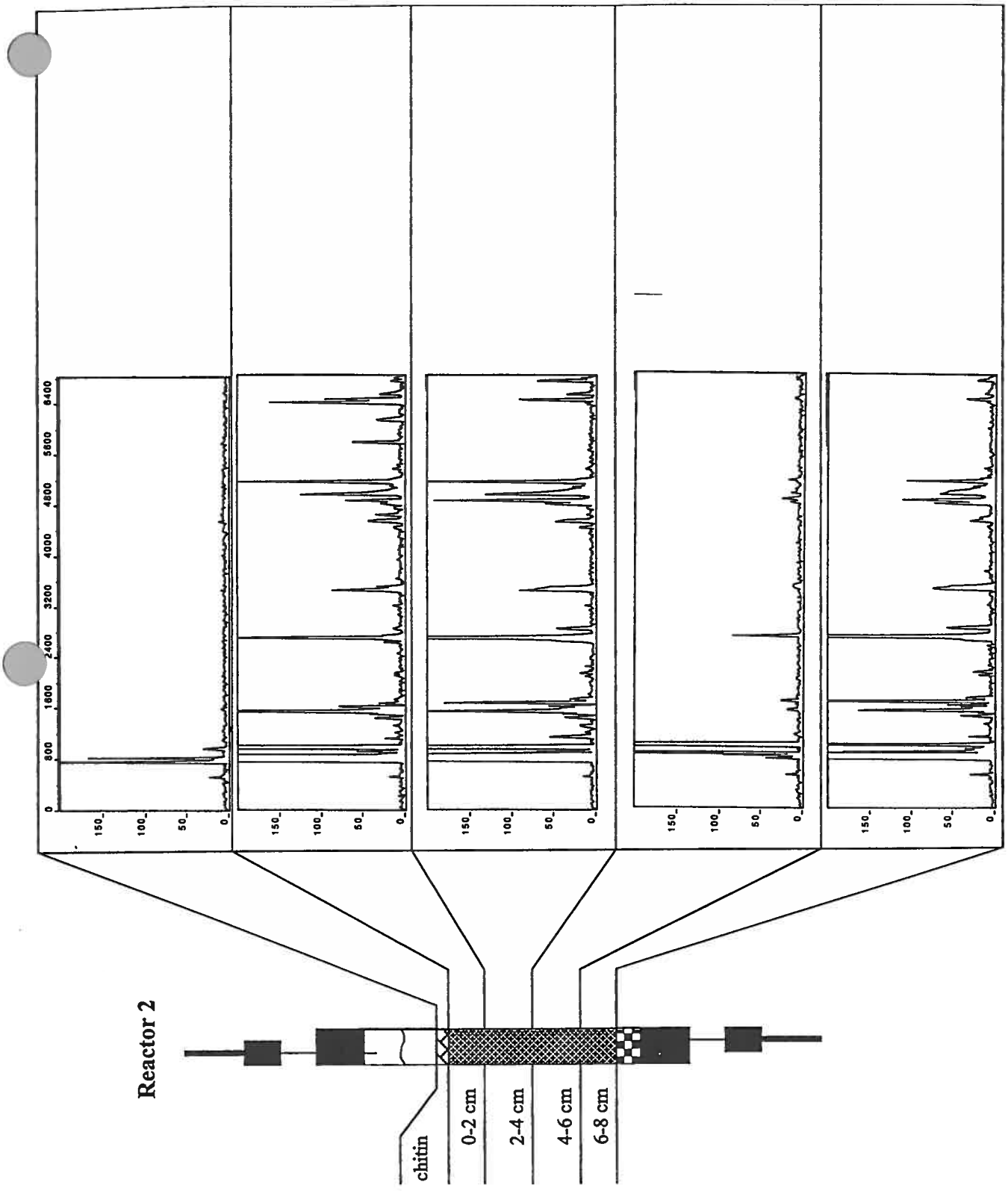
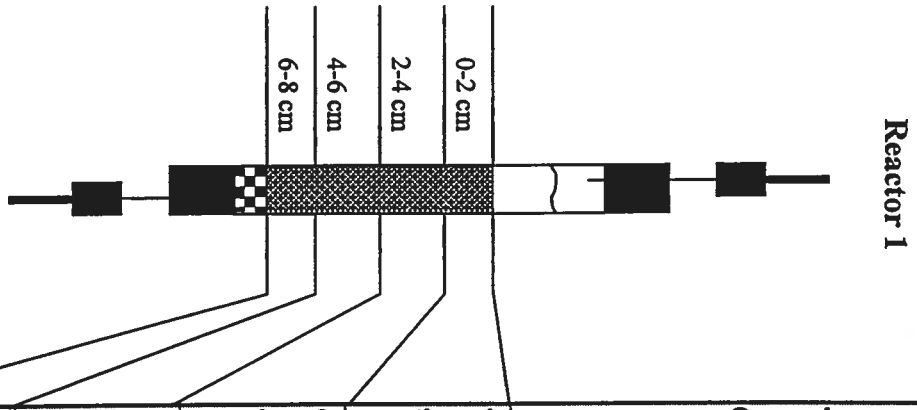


Fig.6: T-RFLP chromatograms obtained from biofilms grown on the different sections of a biotrickling filter reactor bed. The reactor bed consisted of glass beads with 710 – 1.180 microns in size range and was overlaid by chitin flakes. The bioreactor was kept under a constant flow of unsterile seawater.

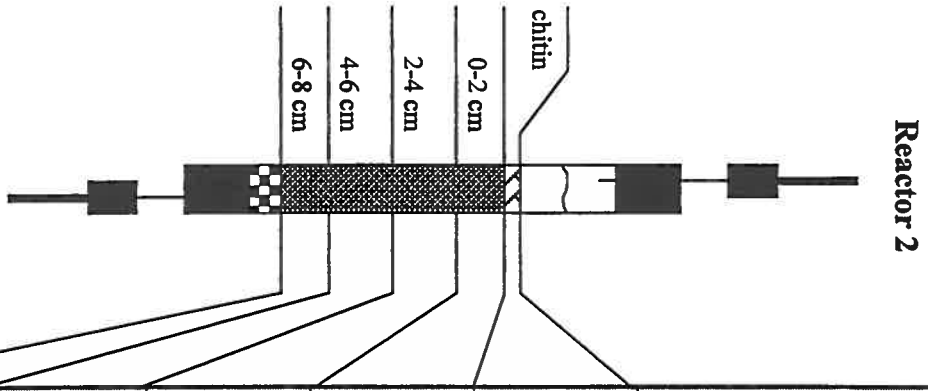
Table 2: Identification of the chromatogram peaks obtained for the different sections of reactor 2 (seawater, chitin). The shown results were created with the web site based T-RFLP analysis program TAP-RFLP of the Ribosomal Database Project II (Maidak et al., 2001). Section 1: 0-2 cm; section 2: 2-4 cm; section 3: 4-6 cm and section 4: 6–8 cm. (see also Fig. 5). Numbers in brackets (in column 'Organisms') indicate number of species or strains found.

Fragment size	Data point	Column section	Organisms
30-43	970-990	1,2,3,4,5	Not in database
98	1440	2,3,5	*Methylobacterium extorquens*
103	1490	2,3,5	*Bergeyella zoohelcum*
105	1510	2,3,5	*Rhizobium sp., Gemmata obscuriglobus*
107	1525	2,3,5	*Methylorhabdus multivorans, Rhodospirillum rubrum*
109	1540	2,3,5	*Bradyrhizobium sp. (2), Methylobacterium sp. (3)*
117	1610	2,3,5	*Methylosulfonomonas methylovora, Clostridium sordellii, Clostridium ghoni*
234-236	2680-2700	2,3,4,5	*Shewanella benthica (2)*
309-311	3440-3460	2,3,5	*Marine snow assoziated clone, Polaribacter sp. (3), Cytophaga sp.(4), Sphingobacterium spiritivorum, Flexibacter sp., Persicobacter diffluens, Pedobacter heparinus, Microscilla sericea*
467	4950	2,3,5	*Cytophaga sp., Sulfobacillus thermosulfidooxidans, Thermobispora bispora (2), Butyrivibrio fibriosolvens, Eubacterium limosum, Clostridium acidiurici*
489	5150	2,3,5	*Paenibacillus sp. (7), Caryophanon latum*



section 4	section 3	section 2	section 1	Organisms
x	x	x	x	*Methylobacterium extorquens*
x	x	x	x	*Bergeyella zoohelcum*
x	x	x	x	*Rhizobium sp.*
x	x	x	x	*Gemmata obscuriglobus*
x	x	x	x	*Methylorhabdus multivorans*
x	x	x	x	*Rhodospirillum rubrum*
x	x	x	x	*Campylobacter sp. (3)*
x	x	x	x	*Caulobacter sp. (9)*
x	x	x	x	*Rodobacter sp.(9)*
x	x	x	x	*Roseobacter sp. (2)*
x	x	x	x	*Sphingomonas sp. (6)*
			x	*Methylosulfonomonas methylovora*
			x	*Clostridium sordellii*
			x	*Clostridium ghoni*
			x	*Unnamed organism*
			x	*Cytophaga sp. (5)*
			x	*Sporocytophaga myxococcoides*
			x	*Flexibacter maritimus*
			x	*Microscilla aggregans sub sp. catalatica*
			x	*Psychroserpens burtonensis (3)*
			x	*Clostridium filamentosum*

Fig.7: Organisms identified by T-RFLP analysis and their location on the column. Shown are the results of the bioreactor (1) that was supplied with ASW medium supplmented with lactate as carbon source to a final concentration on 2 mM. Numbers in brackets indicate numbers of species or strains found.



section 5	section 4	section 3	section 2	section 1	Organisms
x		x	x		*Methylobacterium extorquens*
x		x	x		*Bergeyella zoohelcum*
x		x	x		*Rhizobium sp.*
x		x	x		*Gemmata obscuriglobus*
x		x	x		*Methylorhabdus multivorans*
x		x	x		*Rhodospirillum rubrum*
x		x	x		*Bradyrhizobium sp. (2)*
x		x	x		*Methylobacterium sp. (3)*
x		x	x		*Methylosulfonomonas methylovora*
x		x	x		*Clostridium sordellii*
x		x	x		*Clostridium ghoni*
x		x	x		*Marine snowassoziated clone*
x		x	x		*Polaribacter sp. (3)*
x		x	x		*Cytophaga sp.(10)*
x		x	x		*Sphingobacterium spiritivorum*
x		x	x		*Flexibacter sp.*
x		x	x		*Persicobacter diffluens*
x		x	x		*Pedobacter heparinus*
x		x	x		*Microscilla sericea*
x		x	x		*Sulfobacillus thermosulfidooxidans*
x		x	x		*Thermobispora bispora (2)*
x		x	x		*Butyrivibrio fibriosolvens*
x		x	x		*Eubacterium limosum*
x		x	x		*Clostridium acidiurici*
x		x	x		*Paenibacillus sp. (7),*
x		x	x		*Caryophanon latum*
x	x	x	x		*Shewanella benthica (2)*

Fig.8: Organisms identified by T-RFLP analysis and their location on the column. Shown are the results of the bioreactor (2) that was supplied with unsterile seawater and chitin. Numbers in brackets indicate numbers of species or strains found.

Discussion

The early stages of colonization and the succession of bacterial assemblages on human dental surfaces are well documented. In contrast, little progress has been made in defining the early stages of colonization of surfaces in natural aquatic environments. This is due to the limitations of the traditional cultivation-based methods used to study aquatic microbial communities. It is well known that the majority (≥ 95%) of bacteria in nature cannot be cultivated by using traditional techniques (Amann, 1995). Culture-independent techniques that involve extraction of total community genomic DNA followed by amplification of 16S rDNA by PCR have revealed that the phylogenetic diversity in naturally occurring communities is immense (Giovannoni, 1990). These methods are also very useful for tracking the dynamics of natural bacterial communities, for defining successional stages of biofilm communities, and for identifying key organisms in biofilm development.

The recorded microscopic images showed the growth of a monolayer of single cells mainly persisting of small rod shaped cells or cocci (Fig.3). Primary stages of microcolony formation could be observed. Microcolonies mainly consisted of extracellular polysaccharides (EPS) with only few cells embedded or attached. It was not possible to obtain any microscopic images from biofilms grown on glass beads. The beads act as optical lenses that scattered crosslight. Therefore it was not feasible to focus the microscope on a particular layer. Early reports indicated that very small bacteria were the primary colonizers of surfaces immersed in seawater and were succeded by conventional rod-shaped and, somewhat later, by prosthecate bacteria (Marshall et al. 1992).

The T-RFPL analysis of the different horizontal sections of the reactor beds of either growth conditions revealed no dramatic changes in the community composition (Fig. 5 and 6). The bioreactors were nearly dominated by the same community of bacteria through out their whole vertical profile of the column bed. Only the first section of reactor 1 showed a unique peak that was not observed in the sections underneath (Fig.5). The T-RFLP analysis software assigned the peak to the genus *Cytophaga* (Fig.7 and Table.1). *Cytophaga* are unicellular, Gram-negative bacteria that can adopt to low nutrient levels and usually subsist on biomacromolecules (e.g. cellulose and chitin),

which they are specialized to degrade. They are the most common of all gliding bacteria. In marine environments they are abundant near shores, on living and dead seaweeds, in aerobic and anaerobic bottom sediments, and on decaying sea animals, such as crustaceans with their chitin exoskeleton. Reichenbach (1992) described that the general strategy for their isolation is to provide conditions that promote spreading growth on surfaces. These properties make the species of the genus *Cytophaga* the perfect candidates for early colonization of surfaces exposed to seawater under both growth conditions chosen in this study. For reactor 2 incidence for species of the genus *Cytophaga* were found over the whole vertical profile of the reactor bed (Fig.8 and Table 2). The reduced band pattern of the chromatogram pertained to section 4 (4-6 cm depth) of reactor 2 may be due to an overall reduced fluorescence intensity of the sample (Fig.6). It is unlikely to expect a significant change in the community structure of a section (section 4, reactor 2) that lies among two layers that showed comparable peak pattern in their T-RFPL chromatograms (section 3 and 5, reactor 2).

The biofilm community in reactor 1 which was supplied with a constant flow of ASW supplemented with lactate as carbon source revealed also the occurrence of two other dominant groups of bacteria (see Fig.7). In each case nine species could be assigned to the groups *Caulobacter* and *Rhodobacter*. *Caulobacter* species are highly abundant in aquatic environments especially when nutrient conditions are low. They belong to the dimorphic prosthetic bacteria and have the property to adhere to a wide variety of materials, including glass surfaces. Schmidt (1992) reported that *Caulobacter* species are often seen as members of biofouling communities on submerged surfaces. The second most abundant group of organisms in the biofilms of reactor 1 could be assigned to purple nonsulfur bacteria of the genus *Rhodobacter*. *Rhodobacter* species are widely distributed in nature and found in all kinds of stagnant water bodies. They are usually found in water bodies and sediments of intertidal flats, salt marshes, and polluted harbor basins, but not in the open sea (Imhoff and Trueper, 1992). Their appearance in the biofilm of reactor 1 could be due to the fact that chemoheterotrophic growth (aerobically in the dark) in the presents of oxygen is common among purple nonsulfur bacteria.

Dang et al. (2000) tried to access the diversity of bacterial colonists involved in early succession on a variety of surfaces and to determine the phylogenetic affiliations of the most common early colonists. The highest relative abundance was obtained for the *Roseobacter* subgroup of α-Proteobacteria for several different types of surfaces. The conclusions of Dang et al. could only in parts be confirmed by this study. Evidence for the appearance of *Roseobacter* species could only be found in reactor 1, which was mainly dominated by *Caulobacter* and *Rhodobacter* species. Nevertheless many studies have shown that the Roseobacter group plays an important role in all kinds of ecological niches in coastal marine waters.

In total the whole project worked out fine. The chosen conditions of media and carbon sources proved to promote the formation a natural biofilms on the selected glass substratum. DNA isolation from different vertical sections of the reactor bed was feasible. The PCR amplification of 16S rDNA genes from the isolated DNA samples with universal Bacteria-specific oligonucleotide primers was successful. Also the T-RFLP analysis of the obtained PCR products led in most cases up to interpretable results. In future studies more replicates have to be inducted. Each column should at least be run in parallels. Also a negative control reactor without any substrate should be integrated. The PCR amplification and T-RFLP analysis will conduct to more reliable results if they are run in replicates. To get hold of specific sequences and to make the identification of organisms more precise the obtained PCR products can be used to construct a clone library. The clone library could then be utilized as fundament for a tree based phylogenetic analysis of the primary colonizers of submerged surfaces in marine waters.

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